A THESIS

FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

Development of loop-mediated isothermal amplification technique and trivalent vaccine for olive flounder against streptococcusis and edwardsiellosis

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2012. 02

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

2012. 02.

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2012.02.

Date

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CONTENT

국문초록

우리나라의 넙치양식은 해산어류양식에서 가장 큰 비중을 차지하고 있으며 가장 널리 보급되어 경제적 가치가 증가하고 있으며 특히 제주도의 경우 전국의 양식넙치 생산량의 약 50%를 생산하고 있다. 현재 넙치양식을 포함한 어류양식산업은 고밀도의 사육으로 인한 바이러스, 세균, 진균, 기생충성 질병이 만연하여 있으며 이는 어류양식산업의 가장 큰 문제점 중 하나이다. 특히 넙치양식에서 이러한 질병 중 세균성 질병에 인한 대량폐사가 끊임없이 일어나 많은 경제적인 손실을 초래한 바 있다. 이러한 세균성 질병에 대처하기 위해 많은 양식어민들은 유사한 질병증후가 발생하거나 예방차원에서 과도한 화학요법제를 사용하고 있다. 화학요법제의 과도한 의존은 지난 수년 동안 지속적으로 행하여져 왔으며 이에 따른 항생제 내성 균주 발생, 양식장 주변의 환경오염등의 사회적 문제가 대두되고 있으며 이를 해결하고자 하는 학계, 기관, 양식어민들의 자성의 목소리가 커지고 있는 상황이다. 따라서 이러한 문제점의 효과적인 해결책으로는 정확한 어류질병의 진단, 항생제를 대처할 수 있는 예방적 방안 중 최선책이라고 할 수 있는 효과적인 백신의 개발이라고 생각된다. 따라서 본 연구에서는 이 두가지 측면으로 연구를 진행하여 양식어민의 화학요법제에 대한 의존도를 낮추고자 하였다. 따라서 양식넙치의 주요 세균성 질병인 연쇄구균증 및 에드워드증의 원인균인 *S. iniae*, *S. parauberis*, *E. tarda* 를 제주도의 넙치양식장의 위의 세균성 질병에 감염된 증상을 보이는 감염어에서 61 개의 wild-type 균주를 분리하여 실험에 사용하였다. 분리된 균주들 중 연쇄구균의 경우 비운동성의 그람양성구균임을 확인하였으며 catalase 및 oxidase 활성의 경우 음성임을 확인하였다. 에드워드 균주의 경우 운동성을 보였으며 그람음성 단간균임을 확인하였고 내염성특성 및 indole,

H_2S 의 생산성을 보였다. 특히 *S. iniae* 균주들은 모두 β-용혈성을 보인 반면 *S. parauberis* 의 경우 α-용혈성을 보였다. 분리균주들은 API 20 strep 및 API 20 E 를 통해 생화학적 특성을 확인하였으며 그 결과 표준균주와 매우 유사한 특성을 보였으며 차 후 백신제조를 위한 Phenotyping 의 기반으로 이용하였으며 *S. iniae* 의 경우 6 가지의 phenotype, *S. parauberis* 의 경우 7 가지의 phenotype, *E. tarda* 의 경우 3 가지의 phenotype 으로 나눌 수 있었다. 모든 분리균주들은 기존에 연구발표된 multiplex PCR 을 통하여 동정하였으며 사용된 프라이머는 Spa-2152, Spa-2870, LOX-1, LOX-2, EDtT, EDtA 를 사용하였다. Multiplex PCR 결과 양성반응의 경우 선명한 단일 밴드를 확인할 수 있었고 *S. iniae, S. parauberis, E. tarda* 의 경우 각각 870, 718, 268 bp 의 증폭산물을 얻을 수 있었다. 추가적으로 분리균주들은 16S rRNA 염기서열을 기반으로 BLAST 분석을 실시한 결과 각각의 보고된 type strain 의 염기서열과 99%이상의 상동성을 보였으며 최종적으로 *S. iniae* 8 균주, *S. parauberis* 48 균주, *E. tarda* 5 균주를 본 연구에 사용하였다. 이들의 백신개발 및 효율적인 분자진단법을 개발하기 위해 RAPD 를 이용하여 genotyping 을 하였으며 6 가지의 random primer 를 사용하였다. 분리균주들의 RAPD fingerprint 결과 *S. iniae* 의 경우 3 가지의 genotype, *S. parauberis* 의 경우 8 가지의 genotype, *E. tarda* 의 경우 2 가지의 genotype 으로 genotyping 하였으며 이러한 결과를 위에서 조사한 phenotype 패턴과 종합하여 백신개발을 위한 후보균주를 각각 3 균주씩 선발하였다. 선발된 균주의 넙치에 대한 병원성을 검증하기 위하여 복강주사 및 침지를 통해 감염실험을 실시하였으며 그 결과 가장 병원성이 높았던 *S. iniae* JJU-019, *S. parauberis* JJU-045, *E. tarda* JJU-054 를 백신제조에 사용하였다. 특히 *S. iniae* 와 *E. tarda* 의 경우 균주별로 병원성의 차이가 크지 않았던 반면 *S. parauberis* 의 경우 균주별

병원성의 차이가 매우 크게 나타났다. 또한 복강주사를 통한 감염실험에 비해 침지감염의 경우 *E. tarda* 를 제외하고 만족할 만한 폐사를 확인할 수 없어 본 연구에서는 복강주사를 통한 방법이 보다 더 적합하다고 사료된다.

앞서 언급한 두가지 측면 중 효율적인 분자진단 기법의 개발의 경우 일반적인 PCR 기법이 가지고 있는 근본적인 문제점인 높은 소요비용을 해결하는 것이 가장 중요하다고 생각한다. 비록 전통적인 진단법에 비해 PCR 기반 진단법이 매우 정확한 진단을 할 수 있는 장점을 지녔지만 고가의 PCR 증폭기기가 필수적이기 때문에 양식어민이 직접 시행하기 불가능 한 실정이며, 비록 국가기관 연구소에서 어류질병진단 서비스를 시행하고 있다고 하더라도 수많은 양식장의 민원을 신속하게 처리하기는 어려워 병목현상을 피할 수 없는 처지이다. 따라서 본 연구에서는 양식어민들이 직접 정확한 분자진단을 저렴하게 시행할 수 있을 것으로 보이는 loop-mediated isothermal amplification (LAMP)이라는 등온증폭법을 적용하고자 하였다. 이는 target gene 의 6 부위의 서열을 설정하여 2 쌍의 inner primer 와 2 쌍의 outer primer 를 이용하여 bst polymerase 로 증폭시키는 기법으로서 특히 bst polymerase 의 최적 활성온도인 65 °C 에서 증폭시키기 때문에 고가의 PCR 증폭기기 없이 항온수조만으로도 시행할 수 있어 진단키트화 하였을 때 양식어민들이 직접 시행할 수 있을 것으로 사료되며 일본에서는 이미 상용화 단계까지 연구되었다는 보고가 있다. 본 연구에서는 실험에 사용된 병원균별로 genbank 에 보고된 유전자 중 species 에 따라 보존적이며 다른 species 에 상동성이 낮은 서열을 BLAST 를 이용하여 선별하였으며 이는 *S. iniae* 의 경우 LctO gene, *S. parauberis* 의 경우 AroK gene, *E.tarda* 의 경우 AroB gene 을 이용하여 species-specific 한 LAMP primer 를 디자인하였다. 사용된 primer 들이 species-specific 한 증폭 여부를 확인하기 위해 RAPD 를 통해 조사한

genotype 별로 5 가지의 균주를 각각 선발하여 각각의 species-related 한 균주들과 주요 어류병원균의 LAMP 증폭여부를 비교하였다. 그 결과 모든 LAMP primer 가 species-specific 한 증폭을 하는 것을 확인하였으며 증폭산물의 전기영동 결과 LAMP 특이적인 사다리 형태의 증폭산물을 확인할 수 있어 사용된 LAMP primer 들이 분자진단용 primer 로서 이용 가능하다고 사료된다. LAMP 의 감도를 측정하기 위해 각각의 균주를 농도별로 단계 희석하여 양식어민이 시행하기 수월할 것으로 사료되는 boiling protocol 을 통해 DNA 를 추출하여 LAMP 반응을 시켰다. 그 결과 *S. iniae* 의 경우 1.0×10^{-6} CFU/ml 의 검출한계를 보였으며 이는 기존의 PCR 기법과 동등한 수준이었다. 하지만 *S. parauberis*, *E. tarda* 의 경우 각각 1.0×10^{-3} CFU/ml, 1.0×10^{-5} CFU/ml 의 검출한계를 보였으며 기존 PCR 기법의 각각 100 배 및 10 배의 비교적 높은 검출 한계치를 보여 매우 유용성 있는 진단기법이라 사료된다. 또한 각각의 LAMP primer 들은 반응온도, 반응시간, Mg^{++}, betaine, dNTP, bst polymerase 농도에 따른 LAMP 반응의 최적조건을 확인하였다. 이러한 결과를 통해 본 연구에서 개발한 LAMP primer 는 기존의 분자진단 기법을 개선할 수 있는 방안으로 의의가 있다고 사료된다.

위에서 언급하였던 방안 중 어류질병의 예방적 측면에서 가장 궁극적인 방안이라 할 수 있는 백신의 개발은 복합백신의 측면으로 접근하였다. 어류양식의 병원체에 대한 백신의 경우 국내에서도 많은 연구가 진행되고 있으나 단일백신의 경우 특정병원체에 대한 방어효과만을 얻을 수 있기 때문에 양식어민들이 어류의 스트레스를 우려하여 적극적인 적용을 아직까지 꺼려하고 있어 상용화가 가능한 복합백신 개발이 시급하다. 특히 본 연구에서는 국내에서는 주로 다루지 않았던 oil adjuvant 백신을 개발하여 효과를 검증하였으며 이는 양식어류에 대한 보호효과를 보다 지속적으로 유지할 수

있는 장점이 있다고 알려져 있다. 따라서 양식넙치의 주요 세균성 질병에 대한 복합백신의 유효성을 측정하기 위해 각각의 단일백신 (FO-3, FO-4, FO-5)과 복합백신 (FO-1)의 보호효과 및 혈액분석, 항체응집가, 비특이적 면역반응을 확인하였다. 사용된 백신의 충분한 면역화를 위해 백신 주사 후 28 일간 면역화 시켰으며 이는 accumulated temperature 를 기반으로 하였고 최소 450 dd 에서 충분히 어류를 면역화 시킬 수 있다는 기존의 노하우를 바탕으로 하였고 감염실험을 실시한 시점의 accumulated temperature 는 약 660 dd 로 충분히 면역화되었다고 사료된다. 백신에 의한 면역화 기간 동안 FO-1 의 접종구의 경우 미미한 폐사가 발생하였으며 이는 폐사율이 0.008%였으며 폐사어의 감염증후가 보이지 않아 백신에 의한 폐사가 아닌 것으로 사료되며 나머지 실험구의 경우 면역화 기간동안 폐사가 일어나지 않아 안전성 측면에서 문제점이 없다고 사료된다. 또한 백신접종 후 즉시 사료섭이가 회복되는 것을 확인할 수 있었으며 이를 통해 사용된 백신이 어체에 큰 스트레스를 미치지 않는다고 판단된다. 특히 면역화 기간 동안 넙치의 성장도를 조사한 결과 대조구에 비해 유사하거나 10% 가량 성장도가 증가하는 것으로 조사되었다. 백신처리구의 혈액분석을 통해 어체의 건강도를 유추해 보았으며 관련 parameter 가 대조구에 비해 유의적인 변화를 보이지 않아 백신접종에 대한 부작용이 없는 것으로 판단된다. 어류의 비특이적 면역기작 중 respiratory burst activity 에 관련하여 NBT reduction activity 및 MPO release activity 에 대한 실험 결과 대조구에 비해 모든 백신처리구에서 유의적으로 높은 결과치를 보였다. 또한 lysozyme activity 의 경우 단일백신인 FO-4 실험구를 제외하고 모든 실험구에서 대조구보다 유의적으로 높은 lysozyme activity 을 보였다. 특히 FO-5 처리구에서 높은 lysozyme activity 를 보였으며 복합백신 처리구의 경우 백신처리 2 주까지 유의적으로 증가되는 양상을 보였으며 백신처리

4 주까지 높은 lysozyme activity 를 유지하는 것으로 확인되었다. cell-mediated immunity 지표인 phagocytosis activity 의 경우 역시 면역화 기간동안 대조구에 비해 높은 활성을 보였다. 특히 단일백신은 FO-3 의 경우 실험구중 가장 높은 활성을 보였으며 복합백신 처리구의 경우 역시 백신처리 2 주까지 유의적으로 증가되는 양상을 보였으며 백신처리 4 주까지 높은 활성을 유지하는 것을 확인할 수 있었다. 백신에 의한 충분한 항체가 생성되었는지 확인하기 위해 응집항체가를 측정하였으며 *S. iniae*, *S. parauberis* 의 경우 비교적 낮은 응집항체가를 보였지만 대조구에 비해 높은 응집가를 확인할 수 있었다. 반면에 *E. tarda* 에 대한 응집항체가의 경우 모든 실험구에서 비교적 높은 응집항체가를 보였으며 단일백신 및 복합백신 모두 대조구에 비해 유의적으로 높은 응집항체가를 보였으며 백신접종 2 주까지 증가되고 유지되는 양상을 보였다. 따라서 실험에 사용된 단일 백신 및 복합백신 모두 충분한 면역화를 유도한다고 사료된다. 최종적으로 직접적인 병원체에 대한 백신의 어체 보호효과를 확인하기 위해 감염실험을 실시하였으며 병원체의 인위감염농도는 위에서 언급한 병원성 실험을 토대로 설정하였다. 감염실험 결과 *S. iniae* 에 대한 최종적인 폐사율은 대조구가 82.5% 임에 반하여 단일백신 FO-3 의 경우 7.5%, 복합백신 FO-1 의 경우 2.5%의 폐사율을 보였으며 상대생존율이 각각 96.97%, 90.31%로 나타나 강력한 보호효과를 확인할 수 있었다. *S. parauberis* 에 대한 최종적인 폐사율은 대조구가 65 % 임에 반하여 단일백신 FO-4 의 경우 10 %, 복합백신 FO-1 의 경우 20 %의 폐사율을 보였으며 상대생존율이 각각 84.62%, 69.23%로 나타났다. 이러한 결과를 통해 사용된 복합백신의 경우 상대생존율이 60% 이상으로 상용화 기준을 상회하는 수준임을 확인할 수 있었다. 반면에 *E. tarda* 에 대한 최종적인 폐사율은 대조구가 85 % 였으며 단일백신 FO-5 의 경우 32.5 %, 복합백신 FO-1 의 경우 47.5 %의

폐사율을 보였으며 상대생존율이 각각 61.76 %, 44.12 %로 나타났다. 이는 복합백신의 경우 상대생존율이 상용화 기준에 미치지 못하였지만 유의적인 방어효과를 얻었으며 차 후의 항원의 농도를 증가시키는 등의 조치를 통해 충분히 상용화 수준까지 끌어 올릴 수 있을 것으로 사료된다. 따라서 이러한 결과를 통해 본 연구에서 사용된 복합백신 FO-1 의 경우 양식넙치의 주요 세균성 질병인 *S. iniae*, *S. parauberis*, *E. tarda* 에 대한 충분한 보호효과 및 넙치의 비특이적인 면역반응을 유도하는 것을 알 수 있었으며 차 후에 field test 를 거친다면 상용화가 가능하다고 사료된다.

본 연구에서는 양식넙치의 세균성 질병의 효과적인 제어를 위해 두가지 측면으로 접근하였으며 양식어민이 직접 사용 가능한 간편하고 효과적이고 정확성을 갖춘 LAMP primer 를 제작하여 충분한 적용가능성을 확인할 수 있었고 양식넙치의 oil adjuvant 기반의 복합백신을 개발하여 지속적이고 다수의 병원체에 대한 보호효과를 얻을 수 있음을 확인 할 수 있었다. 따라서 이러한 결과물이 양식어민의 근심을 덜어 줄 수 있는 방안이 될 수 있을 것이라고 사료된다.

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(GenBank accession number CP002471.1) used for the LAMP primer design. Nucleotide sequences used for primer design are indicated by boxes and arrows.

Figure 21. The partial nucleotide sequence of 3-dehydroquinate synthase AroB gene of *E. tarda* (GenBank accession number CP002154.1) used for the LAMP primer design. Nucleotide sequences used for primer design are indicated by boxes and arrows.

Figure 22. Specificity of LctO gene for the LAMP detection of *S. iniae*. Samples were electrophoresed on 1.5% agarose gels. LAMP was carried out with the different sources of DNA template from the 27 strains. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); 1 and 16, *S. iniae* ATCC 29175^{T}; 2, *S. iniae* JJU-019; 3, *S. iniae* JJU-073; 4, *S. iniae* JJU-075; 5, *S. iniae* JJU-076; 6, *S. iniae* JJU-087; 7, S. parauberis DSM 6631^{T}; 8, S. parauberis JJU-008; 9, S. parauberis JJU-045; 10, *S. parauberis* JJU-055; 11, *Lactococcus garvieae* ATCC 43921^{T}; 12, *L. garvieae* ATCC 49156^{T}; 13, *S. difficilis* CIP 103768^{T}; 14, *S. dysgalactiae* ATCC 12449^{T}; 17, *S. pyogenes* ATCC 12344^{T}; 18, *S. suis* ATCC 43765; 19, *E. tarda* ATCC 15947^{T}; 20, *E. tarda* JJU-032; 21, *E. tarda* JJU-052; 22, *E. tarda* JJU-054; 23, *Flavobacterium columnare* ATCC 43622; 24, *Pseudomonas anguilliseptica* ATCC 33660; 25, *Tenacibaculum maritimum* ATCC 43398; 26, *Vibrio anguillarum* ATCC 19264^{T}; 27, *V. harveyi* ATCC 14126^{T}; 28, *V. ichthyoenteri* IFO 15847; 29, *Yersinia ruckeri* ATCC 22908; 15 and 30 distilled water.

Figure 23. Specificity of AroK gene for the LAMP detection of *S. parauberis*. Samples were electrophoresed on 1.5% agarose gels. LAMP was carried out with the different sources of DNA template from the 27 strains. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); 1 and 16, *S. parauberis* DSM 6631^{T}; 2, *S. parauberis* JJU-003; 3, *S. parauberis* JJU-008; 4, *S. parauberis* JJU-045; 5, *S. parauberis* JJU-055; 6, *S. parauberis* JJU-064; 7, *S. iniae* ATCC 29175^{T}; 8, *S. iniae* JJU-019; 9, *S. iniae* JJU-073; 10, *S. iniae* JJU-076; 11, *Lactococcus garvieae* ATCC 43921^{T}; 12, *L. garvieae* ATCC 49156^{T}; 13, *S. difficilis* CIP 103768^{T}; 14, *S. dysgalactiae* ATCC 12449^{T}; 17, *S. pyogenes* ATCC 12344^{T}; 18, *S. suis* ATCC

43765; 19, *E. tarda* ATCC 15947^{T}; 20, *E. tarda* JJU-032; 21, *E. tarda* JJU-052; 22, *E. tarda* JJU-054; 23, *Flavobacterium columnare* ATCC 43622; 24, *Pseudomonas anguilliseptica* ATCC 33660; 25, *Tenacibaculum maritimum* ATCC 43398; 26, *Vibrio anguillarum* ATCC 19264^{T}; 27, *V. harveyi* ATCC 14126^{T}; 28, *V. ichthyoenteri* IFO 15847; 29, *Yersinia ruckeri* ATCC 22908; 15 and 30 distilled water.

Figure 24. Specificity of AroB gene for the LAMP detection of *E. tarda*. Samples were electrophoresed on 1.5% agarose gels. LAMP was carried out with the different sources of DNA template from the 27 strains. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); 1 and 16, *E. tarda* ATCC 15947^{T}; 2, *E. tarda* JJU-032; 3, *E. tarda* JJU-033; 4, *E. tarda* JJU-051; 5, *E. tarda* JJU-052; 6, *E. tarda* JJU-054; 7, *E. ictaluri* ATCC 33202^{T}; 8, *E. hoshinae* JCM 1679; 9, *S. iniae* ATCC 29178^{T}; 10, *S. iniae* JJU-019; 11, *S. iniae* JJU-073; 12, *S. iniae* JJU-076; 13, *S. parauberis* DSM 6631^{T}; 14, *S. parauberise* JJU-008; 17, *S. parauberise* JJU-045; 18, *S. parauberise* JJU-055; 19, *Flavobacterium branchiophilum* ATCC 35035^{T}; 20, *F. columnare* ATCC 43622; 21, *Pseudomonas anguilliseptica* ATCC 33660; 22, *Tenacibaculum maritimum* ATCC 43398; 23, *Vibrio anguillarum* ATCC 19264^{T}; 24, *V. alginolyticus* ATCC 17749^{T}; 25, *V. harveyi* ATCC 14126^{T}; 26, *V. ichthyoenteri* IFO 15847; 27, *V. ordalii* ATCC 33509; 28, *V. vulnificus* ATCC 27562^{T}; 29, *Yersinia ruckeri* ATCC 22908; 15 and 30 distilled water.

Figure 25. Sensitivity of *S. iniae* identification by LAMP and conventional PCR. Each sample was electrophoresed on a 1.5% agarose gel. (A) LAMP products. A product was seen typical ladder-like pattern on gel electrophoresis; (B) conventional PCR products using primers LOX-1 and LOX-2. A band of 870 bp was seen with positive samples. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); Lanes 1–8, amplification products using 10-fold serial dilutions of template DNA, which extracted variation of cell concentrations (1.0×10^{8} CFU/ml, 1.0×10^{7} CFU/ml, 1.0×10^{6} CFU/ml, 1.0×10^{5} CFU/ml, 1.0×10^{4} CFU/ml, $1.0 \times$

amplification at 70 °C. (B) Effect of reaction length with SiLctO primer set, (D) Effect of reaction length with SpAroK primer set, (F) Effect of reaction length with EtFimB primer set, Lane 1, amplification for 0 min; Lane 2, amplification for 20 min; Lane 3, amplification for 40 min; Lane 4, amplification for 60 min; Lane 5, amplification for 80 min;; Lane 6, amplification for 100 min; Lanes M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea).

Figure 29. Optimum MgSO4 concentrations of the LAMP reaction of three primer sets for detection of *S. iniae, S. parauberis*, and *E. tarda*. (A) Effect of $MgSO_4$ concentrations with SiLctO primer set, (B) Effect of $MgSO_4$ concentrations with SpAroK primer set, (C) Effect of $MgSO_4$ concentrations with EtFimB primer set, Lane 1, 2 mM $MgSO_4$; Lane 2, 4 mM $MgSO_4$; Lane 3, 6 mM $MgSO_4$; Lane 4, 8 mM $MgSO_4$; Lane 5, 10 mM $MgSO_4$; Lane 6, without DNA template in the reaction, amplification with 4mM $MgSO_4$.

Figure 30. Optimum betaine concentrations of the LAMP reaction of three primer sets for detection of *S. iniae, S. parauberis*, and *E. tarda*. (A) Effect of betaine concentrations with SiLctO primer set, (B) Effect of betaine concentrations with SpAroK primer set, (C) Effect of betaine concentrations with EtFimB primer set, Lane 1, 0 M betaine; Lane 2, 0.2 M betaine; Lane 3, 0.4 M betaine; Lane 4, 0.6 M betaine ; Lane 5, 0.8 M betaine; Lane 6, 1.0 M betaine.

Figure 31. Optimum deoxynucleotide triphosphate (dNTP) concentrations of the LAMP reaction of three primer sets for detection of *S. iniae, S. parauberis*, and *E. tarda*. (A) Effect of dNTP concentrations with SiLctO primer set, (B) Effect of dNTP concentrations with SpAroK primer set, (C) Effect of dNTP concentrations with EtFimB primer set, Lane 1, without DNA template in the reaction, amplification with 400 μM dNTP; Lane 2, 0 mM dNTP; Lane 3, 0.2 mM dNTP; Lane 4, 0.4 mM dNTP ; Lane 5, 0.8 mM dNTP; Lane 6, 1.0 mM dNTP.

Figure 32. Optimum bst polymerase concentrations of the LAMP reaction of three primer sets for detection of *S. iniae*, *S. parauberis*, and *E. tarda*. (A) Effect of bst

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INTRODUCTION

Aquaculture is the farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. (FAO, 1995).

In recent years, aquaculture is one of the fastest growing food producing sector in the world, with an average annual growth rate was 8.9% since 1970, including 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production over the same period. The most recent FAO statistics data indicate that the sector reached the aquaculture production was 9.4% of annual percentage growth rate (APR) compared with meat production of farmed terrestrial animals such as pigs (APR of 3.1%), poultry (APR 5.1%), beef and meal (APR 1.2%), and mutton and lamb (APR 1.0%) (FAO, 2004). The aquaculture production was increased from 0.7 kg in 1970 to 6.4 kg in 2002; an annual growth rate was 7.2%. In 2002, the total world aquaculture production (including aquatic plants) was reported to be 51.4 million tons by volume and US$ 60.0 billion by value. This represents the annual production increase 6.1% in global aquaculture production volume and 2.9% in value, respectively. Among the Asia produced 91.2% (by volume) and 82.0% (by value) (FAO, 2004).

The olive flounder is one of the most commercially important marine fish culture in Korea. It was disseminated large-scale aquaculture techniques to increasing economic value since 1989, when developing artificial reproduction techniques. In recent year, the olive flounder industry was increase in Korea, and Korea aquaculture industry was produced 32,141, 40,075 and 43852 tons in the years of 2004, 2005 and 2006, respectively (NFRDI, 2007). The total aquaculture production was reached 48,329 tons in 2008, of which 25,027 tons were produced in Jeju Island. The current trend of aquaculture development is towards increased intensification and commercialization of aquatic production. Compared to other farming sectors, the likelihood in a major disease problems occurring and increases the aquaculture activities intensify and expand. Thus, the aquaculture industry has been

overwhelmed with its share of diseases and its problems due to viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. In olive flounder aquaculture industry in Korea also has these problems such as bacterial, viruses and parasites disease. Antibiotic treatment of to prevent and control of these disease using in aquaculture has been utilized last few decades. However, the current problem and/or issue in the culture of the species are the fact that fish farmers use a large quantity of antibiotics to prevent the species from bacterial diseases (Bondad-Reantaso, 2005).

Recently, mass mortality of flounder showing ascites occurred in many aquaculture farms in southern Korea. While we investigated the cause of the mortality by various bacteria were isolated from diseased fish. The major outbreak of bacterial diseases in cultured olive flounder have been reported such as Streptococcosis, edwardsiellosis, and Vibriosis (Bang et al., 1992; Choi et al., 2009; Lee et al., 1991).

Among, Streptococcal infection of fish is considered one of the importance diseases affecting a variety of wild and cultured fish throughout the world (Kitao 1993; Bercovier et al., 1997; Romalde and Toranzo 1999, 2002). Which is Gram positive cocci based on DNA–DNA hybridization coupled with 16S sequencing has shown that at least five different species are considered of significance in aquaculture namely: *Lactococcus garvieae*, *Lactococccus piscium*, *Streptococcus iniae*, *Streptococcus agalactiae*, *Streptococcus parauberis,* and *Vagococcus salmoninarum*. Therefore, streptococcosis of fish should be regarded as a complex of similar diseases caused by different genera and species capable of inducing a central nervous system damage characterised by suppurative exophthalmia and meningoencephalitis (Toranzo et al., 2005). The “warm water” streptococcosis (causing mortalities at temperatures above 15.8℃) typically involves *L. garvieae*, *S. iniae*, *S. agalactiae,* and *S. parauberis*, whereas “cold water” streptococcosis (occurring at temperatures below 15.8℃) caused by *L. piscium* and *V. salmoninarum*. It is important to report that the aetiological agents of “warm water” streptococcosis are considered as potential zoonotic agents that capable to cause disease in humans (Toranzo et al., 2005).

Among these fish streptococci, *S. iniae* and *S. parauberis* can be regarded as the main aetiological agents causing diseases in marine aquaculture world wide (Toranzo et al., 2005). The disease was first reported in cultured rainbow trout, *Oncorhynchus mykiss* (Hoshina et al., 1958), and later in salmon *Salmo salar*, mullet *Mugil cephalus*, golden shiner *Notemigonus crysoleucas*, sea bass *Dicentrarchus labrax*, and olive flounder *Paralichthys olivaceus* (Inglis et al., 1993). Streptococci can cause acute infections in fish, resulting a greater than 50% mortality rate over a period of 3–7 days (Park et al., 2009). Alternatively, these bacteria can induce chronic infections, resulting only a few mortalities in each in day over a period of several weeks (Roy and Ruth, 2002). Gram positive cocci can be isolated on general purpose media but growth was enhanced on blood agar. Biochemical characterization can be accomplished by traditional tube and plate procedures as well as using commercial miniaturized systems (Eldar et al., 1997; Vela et al., 2000; Ravelo et al., 2001). However, misidentification of *L. garvieae* with *L. lactis* subsp. *lactis* or *S. iniae* with *S. uberis* can occur (Weinstein et al., 1997; Ravelo et al., 2001). Besides, the identification of some bacterial species remains difficult, based only on phenotypic traits. Therefore, serological confirmation and effective molecular diagnosis must be performed by a slide agglutination test or randomly amplified polymorphic DNA (RAPD), and PCR-based protocols. In the case of *S. iniae*, two serotypes (I and II) with different capsule composition were described (Bachrach et al., 2001). However, there was no serogroups detected among the *S. parauberis* strains. In Korea, *L. garviae* and *Streptococcus* sp. were isolated in cultured olive flounder, (Heo et al. 2001) had been reported two or three species which aetiological agents of streptococcosis in olive flounder. However, investigation of Jeju Island, most active culturing of olive flounder had been reported two main aetiological agents such as *S. iniae* and *S. parauberis*, and the occurrence ratio of these pathogens had been appeared transformation that isolated ratio of *S. parauberis* had been increased continually compared to isolated ratio of *S. iniae* (Jeong et al., 2006).

Pathogens, *Edwardsiella tarda*, a Gram negative bacterium belong to the family Enterobacteriaceae, is the causative agent of edwardsiella septicaemia leads to extensive

losses in a diverse array of commercially important fish, including eels (Wakabayashi and Egusa, 1973), chinook salmon (Amandi et al., 1982), olive flounder (Nakatsugawa, 1983), tilapia (Kubota et al., 1981), carp (Sae-Oui et al., 1984), channel catfish (Meyer and Bullock, 1973), and mullet (Kusuda et al., 1976). In fish, this bacterium is associated with acute to chronic diseases of fry, fingerlings, and adults resulting in severe economic loss. In Korea, edwardsiellosis is one of the most important bacterial diseases in farmed olive flounder. The mortality associated with *E. tarda* infection has been persisted despite extensive use of antibiotics. Disease is now a primary constraint in culture of many aquatic species, both economic and social development. Therefore control of aquatic disease has been important point in order to development of sustainable aquaculture. Disease control with chemotherapy is one of the common fish disease control technology. Various chemotherapeutants are available for treatment of bacterial and parasitic disease, however there was no effective treatments are available currently for viral diseases. Antibiotics can be very effective but long term treatment will often lead to resistance and can be environment concern. Therefore, recently advise to limit the use of chemotherapy in aquaculture. The alternatives of chemotherapy consider two main aspects that are rapid diagnosis and effective preventive therapies. Because we should be approach two aspects such as to construct the rapid molecular diagnosis and multivalent vaccine for effective control of fish diseases.

The first aspect is development of rapid molecular diagnosis of fish pathogens. Diagnosis is important not only for the prescribing of effective drugs which appropriate host in adequate doses but also for preventing the evolution of resistant microorganisms, that occurs by treating non infected host showed similar symptoms (Urdea *et al*., 2006.). Therefore, the development of rapid, accurate, and sensitive diagnostic methods for the identification of pathogens is the fundamental for treating and controlling, or even eradicating, infectious disease. Classical pathogen detection is based on culture methodology and microscopy. The culture method is still an basic technology because the method can also provide important information such as the viability of the pathogen and its susceptibility to antibiotics (Poxton, 2005). However, the slow growth and difficulty of selective cultivation

often limits the culture-based diagnosis (Gwendolyn, 2002). The direct observation of microbes or parasites by microscopy is frequently using a rapid and simple diagnostic method. The handiness and cost-efficiency of microscopic tests make them acceptable to use even in developing countries. However, the poor sensitivity of smear tests often causes fatal delays in treatment and the methodology is dependent upon the skill of the microscopist (Hanscheid and Grobusch, 2002). Therefore, the setting up of more rapid, sensitive, and accurate diagnostic methods has long been desired.

In the past few decades, In order to mend the disadvantages of the traditional diagnostic methods, overcome numerous molecular methods have been developed (Alex, 2003) such as in vitro amplification of a specific nucleic acid sequence. Such methodologies may allow rapid diagnosis with a degree of sensitivity and specificity comparable than that of classical culture methods. Many amplification methods, including polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA) have already been developed for nucleic acid amplification tests (NATs) (Versalovic and Lupski, 2002). NATs can suggest which the additional advantages over traditional methods such as by the facilitation of standardization, automation coupled with ability to type species and detects drug resistance (Dong et al., 2008). These PCR-based methods require either high precision instruments for amplification or complicated methods for detection of the amplified products. In addition, PCR has several disadvantages, such as the requirement for thermal cycling, and time spending post-PCR analysis, thereby potentially leading to laboratory contamination. The development of real-time PCR has brought true quantitation of target nucleic acids out of the pure research laboratory and into the diagnostic laboratory, by combining PCR amplification with fluorescent-labelled virus specific probes able to detect amplified DNA during the amplification reaction (Manmohan et al., 2008). The fluorescent chemistry coupled with advanced optical detectors makes it more sensitive than conventional gel-based PCR. The real-time assays have many advantages over conventional PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity,

and easy standardization (Mackay et al., 2002). However, all these nucleic acid amplification methods have several intrinsic disadvantages of requiring either a high precision instrument for amplification or an elaborate complicated method for detection of amplified products. Real-time PCR machines are expensive and thus are not within purchasing reach of laboratories in developing countries and farmors. Therefore, more cost-effective and sensitive are needs to complement the existing PCR-based assay systems (Manmohan et al., 2008).

In aquaculture industries, the rapid diagnosis and prevention of fish diseases in culture systems is extremely important. Efficient management of disease usually begins by preventing to the spread of disease. Disease diagnosis has been mainly based on clinical signs supported by isolation and identification of the pathogens. This requires a rapid and sensitive method to detect the pathogens. Aqua-farming laboratories are not usually equipped with diagnostic equipments, thus small-scale aquaculture operations depend on specialist fish disease laboratories for their diagnostic needs. However, laboratories routinely handling diagnostic cases are often very busy and take such a time to carry out diagnoses. In order for laboratories to handle large number of samples sensitive and rapid diagnostic kits are needed (Savan et al., 2005).

Since loop-mediated isothermal amplification (LAMP) was originally reported by Notomi et al. in 2000, LAMP has attracted a lot of attention as a potentially rapid, accurate, and cost-effective novel molecular diagnosis method. The LAMP method has now been developed as commercial kits and some of them have been adopted as the officially recommended methods for the routine diagnosis and detect of pathogens in Japan.

Loop-mediated isothermal amplification (LAMP) has been suggested as a sensitive strand displacement technique (Notomi et al., 2000). This method amplifies target DNA from a few copies to 10^9 copies in less than an hour under isothermal conditions. It is an offshoot of the basic strand displacement techniques which have been described (Notomi et al., 2000).

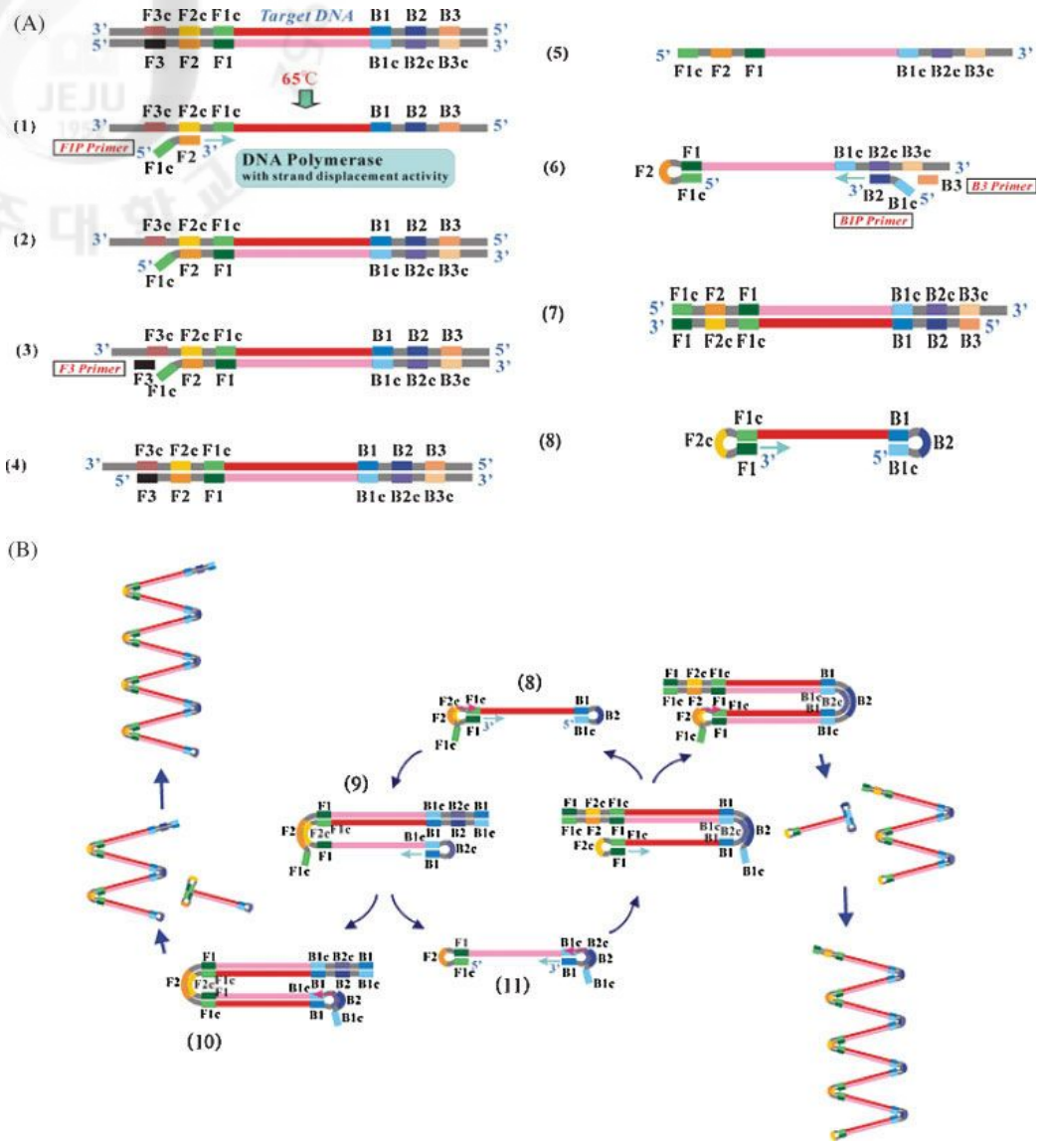

Figure 1. Principles of LAMP amplification. Non-Cyclic Step [1-8]: generation of stem loop DNA with dumbbell-shaped structure at both ends that is ready to enter into cyclic amplification step. Initially, the strand displacement activity of Bst DNA polymerase helps in synthesis of a complementary DNA strand, starting with FIP. The outer primer (F3) then displaces the FIP-linked complementary strand, which forms a stem-loop structure at the 50 end. This serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The final product is a structure with stem-loops at each end. (B) Cyclic Amplification Step [9-11]: exponential amplification of original dumbbellshaped stem-loop DNA employing internal primers. The product is the differently sized structures consisting of alternately inverted repeats of the target sequence on the same strand, giving a cauliflower-like structure.

Briefly, four highly specific primers are constructed from the target DNA, one set of primers anneal to the target region one after the other on the same strand and the primer that anneals at the later stage displaces the strand formed by the first primer with the help of *Bst* DNA polymerase. The *Bst* polymerase has a strand displacement activity. This takes place on both strands and the primers are designed such that loops are formed. The reaction is carried out under isothermal conditions as denaturation of the strand takes place by strand displacement. The reactions produce a series of stem-loop DNAs with various lengths (Figure 1.). The four primers hybridize against six distinct sequences in the target DNA making for highly specific (Savan et al., 2005.).

In this study, we developed a novel approach method of rapid molecular diagnosis known as loop-mediated isothermal amplification (LAMP) that gaining popularity among researchers due to its simple operation, rapid reaction, and easy detection in order to detect major fish pathogens.

The second aspect is development of multivalent vaccine for major bacterial pathogens in olive flounder.

Successful vaccination against infectious diseases has been practiced for over 200 years. Indeed, it has been stated that vaccination is the most cost-effective method of animal suffering infectious diseases and economic losses. Even thought with these successes, infectious diseases continue to be of economic significance to society in reduced productivity and mortality. The adventage of genomics, proteomics, and biotechnology, combined with our understanding of pathogenesis and immune responses to various pathogens provides us with an unprecedented opportunity to develop safer and more effective vaccines for many pathogens.

In aqua-farm of intensive culture, where are reared at high densities, infectious disease pathogens are easily transmitted between individuals. Therefore independent of technology farming, good environmental conditions are important to control and prevent of various fish diseases. On the other hand fish reared in an open aquatic environment, fish suffer many pathogens, which is impossible to escape. Due to the effectiveness of pathogen transportation

in aquatic condition and the high density of fish used in commercial large-scale farming, pathogens quickly spread within a population of aqua-cultured fish. During the 1980s to early 1990s, salmon farming in Norway experienced huge losses due to bacterial diseases (mostly *Vibrio* spp. and *Aeromonas salmonicida*) and a total crash in many aquaculture industry was only prevented by the use of vast amounts of antibiotics (Sommerset et al., 2005). In this regard to control fish disease using, immersion vaccine and oil-adjuvant vaccines was developed against bacterial pathogens such as *Vibrio* and furunculosis (*Aeromonas salmonicida*). The excellent efficacy of these vaccines immediately resulted in a decline in the use of antibiotics, and their extensive use concurrent with a threefold increase in fish production (Grave et al., 1990; Lillehaug et al., 2003). Today, vaccination is an integrated part of most salmon farms and the application of antibiotics is very limited, at least in Northern Europe and North America. The development of fish vaccines is an ongoing interaction between academia, the pharmaceutical industry and regulatory authorities. Until the early 1990s, most fish vaccines were developed and commercialized by small local companies. Till date only, five multinational animal health companies have acquired or formed, joint venture companies with the smaller companies specializing in the field of aquaculture. The major companies of producing fish vaccines are now such as Intervet International (The Netherlands), Novartis Animal Health (Switzerland), Schering-Plough Animal Health (USA), Pharmaq (Norway), and Bayer Animal Health (Bayotek)/Microtek, Inc. (Germany/Canada). In addition Vaccines are mostly developed and distributed by Japanese companies (Sommerset et al., 2005). Ultimately effective vaccines are only way to resolve disease problems and a number of highly successful vaccines have been developed for aquaculture recently. In contrast, the approaches of controlled fish disease have been still dependent to high proportion of chemotherapy in Korea recently. Therefore aquaculture industry of Korea is important to efficient development of fish vaccine in order to be competitive internationally.

Developing and researching of fish vaccine in Korea mostly have begun by National Fisheries Research and Development Institute (NFRDI). There are some researches Kwon

and Bang, (2004) reported that is immersion vaccination against *E. tarda*, and Cho et al., (2006) reported β-hemolytic *S. iniae* vaccine to olive flounder. In 2005, the commercial immersion vaccine and injection vaccine developed against edwardsiellosis to prevent and spread of *S. iniae* in olive flounder aquaculture, recently. In addition there were only a few reports about multivalent fish vaccine, which was reported and patent multivalent vaccine against *E. tarda* and *S. iniae*. However development of multivalent vaccine is very insufficient in olive flounder aquaculture industry in Korea.

The medical definition of multivalent vaccine is that prepared from cultures of two or more strains of the same species of microorganism or virus, also called polyvalent vaccine. Many of these new multivalent, multi-organism vaccines will also induce immune responses which are quantitatively and qualitatively different from those engendered by single antigen or single organism products. It is now well established that simultaneous administration of antigens A + B can alter magnitude and pattern of immune response to both A and B (Gizurarson, 1998; Insel, 1995). Despite uncertain difference of immune response by multivalent vaccination that should be applied to aquaculture in order to preventing fish disease. Because injection vaccines are stressful extremely for fish compared to other farmed animals, fisheries people are reluctant to multiple injections by monovalent vaccine. In addition multivalent vaccine can have great advantages such as reduced administration costs, increased coverage, and decreased exposure to vaccine excipients (De Jong, 1999; Pifferi and Restani, 2003). Therefore development of multivalent vaccine is extremely needed in Korean aquaculture. Recently, distribution of outbreak disease due to mixed infection in olive flounder compared to that was mostly appeared single infection at 1990s in Korea (Kim et al., 2006).

This trend is assumed that affected two major factors such as overuse of chemotherapeutics and developing of several diagnosis techniques. In order to response of this trend, we should be trying two approached ways. The first is simple and effective molecular diagnosis method of LAMP that will be help investigating and understanding to infectious pathogens distribution in olive flounder, furthermore if we set up commercial kit

of LAMP for fisheries people can diagnose successfully major pathogens in olive flounder. The second is developing multivalent that could be replace the using chemotherapeutics as mentioned above.

In this study, we isolate and investigate the characterization of three major pathogens of olive flounder in Jeju Island, Korea such as *S. iniae*, *S. parauberis,* and *E. tarda*, and then we apply LAMP method for detection each pathogens that does not require specialized equipment (e.g. thermo cycler, restriction enzyme digestion, and dot-blot hybridization). These major bacterial pathogens of detection methods provide user friendly operation and quickely analysis, and it has potential application for fish disease diagnosis, which include many olive flounder-farming areas. We also compared the efficacy of the vaccine when given as a monovalent formulation or as a multivalent formulation. Therefore, we used oil base adjuvant base approach to evaluate the multivalent vaccine formulation in order to give effective immunoprotection of major bacterial pathogens in olive flounder.

Part Ⅰ. Phenotyping and Genotyping Studies of Major Bacterial Pathogens of Olive Flounder, *Paralichthys olivaceus* Isolated from aquaculture farm in Jeju Island, Korea.

ABSTRACT

The olive flounder is a most representative marine fish culture in Korea. Recently, mass mortality and economic losses of flounder showing ascites occurred at many aquaculture farms in southern Korea. We investigated the cause of the mortality, by various bacteria were isolated from diseased fish. The major outbreak of bacterial diseases in cultured olive flounder have been reported such as streptococcosis and edwardsiellosis. The current problem and/or issue in fish culture are the fact that fish farmers use a large quantity of antibiotics to prevent the bacterial diseases. To solve these problems, the alternatives of chemotherapy consider two main aspects that are rapid diagnosis and effective preventive therapies. Because we should be approach two aspects such as to construct of rapid molecular diagnosis and multivalent vaccine for effective control of fish disease. In order to perform two kinds of aspects, we isolate and investigate the characterization of three major pathogens of olive flounder in Jeju in Korea such as *S. iniae*, *S. parauberis,* and *E. tarda.*

Bacterial isolates were 61 wild-type strains collected from olive flounder exhibiting signs, including darkened surface, ascitic fluid in the peritoneal cavity, distended abdomen, protruded anus, exophthalmia, haemorrhaging in the eye, and haemorrhaging on the surface. General microbiological examinations revealed that the Streptococcal isolates were nonmotile, Gram-positive cocci, with no catalase or oxidase activities, whereas isolates of *E. tarda* were motile, Gram-negative short rod, salt tolerant, with indole, and H_2S production on triple sugar iron agar. Hemolytic analysis showed that eight isolates of *S. iniae* were β-hemolytic, whereas forty-eight isolates of *S. parauberis* were α-hemolytic. Biochemical

properties and phenotyping of isolates were used the API system. In these results, the phenotyping results show that *S. iniae*, *S. parauberis*, and *E. tarda* were separated into six, seven, and three phenotypes, respectively. All isolates were identified as *S. iniae*, *S. parauberis*, and *E. tarda* via multiplex PCR array using the primers Spa-2152 and Spa-2870, LOX-1 and LOX-2, and EDtT and EDtA, respectively. Positive PCR amplification of DNA templates from *S. iniae*, *S. parauberis,* and *E. tarda* produced a single fragment, of the expected, for each pathogen as 870, 718, and 268 bp, respectively. In addition, the 16S rRNA sequences showed 99% similarity to known bacteria identified in Genbank by BLAST search and the EMBL Nucleotide Sequence Database with *S. iniae*, *S. parauberis*, and *E. tarda*. As a result, eight isolates of *S. iniae*, forty-eight isolates of *S. parauberis*, and five isolates of *E. tarda* were identified, respectively. Streptococci and *E. tarda*, 16s rRNA gene sequences of isolates were used in the phylogenetic analysis. The results of the phylogenetic array show that the isolates were closely related to the type strains *S. iniae* ATCC 29178^{T} and *S. parauberis* DSM 6631^{T}, and *E. tarda* ATCC 15947^{T}, respectively. Fingerprints of isolated bacterial strains were obtained with six primer, respectively. The RAPD analysis of the 7 strains of *S. iniae* revealed different DNA profiles were investigated six patterns with the primer p1, two patterns with the primer p3 and p5, monomorphic patterns with p2, p4, and p14, respectively. The case of 46 strains of *S. parauberis* revealed different DNA profiles were investigated nineteen patterns with the primer p2, nine patterns with the primer p1, five patterns with the primer p4, four patterns with the primer p3 and p5, three patterns with the primer p14, and 5 strains of *E. tarda* revealed conservative patterns, except for primer p3 that were investigated monomorphic DNA profiles. The primers in bacterial strains of *S. iniae*, *S. parauberis*, and *E. tarda* used generated a total of 42, 73, and 57 reproducible bands respectively. The number of bands for each primer in bacterial strains of *S. iniae*, *S. parauberis*, and *E. tarda* varied from 8, 62, and 2 with averages of 1.3, 10.3, and 0.3 bands per primer respectively. The relationships of each bacterial species between the seven, forty six, and five isolates were estimated using p-distance index of similarity and the dendrogram of isolated bacterial strains based on RAPD polymorphism by UPGMA. According to this

matrix, genetic Pairwise distance (p-distance) values of seven strains *S. iniae* were found to be between 0.049 and 0.182. The p-distance values of forty six strains *S. parauberis* and five strains *E. tarda* were found to be between 0.000 to 1.199 , and 0.000 to 0.038, respectively. The dendrogram shows the differences among the *S. iniae* strains, which were characterized three genotype groups. However the dendrogram of *E. tarda* strains shows almost no variation among the *E. tarda* strains, which could be divided only two genotype groups. The dendrogram of *S. parauberis* strains shows the differences among the *S. parauberis* strains, which were characterized eight genotype groups. In order to progress LAMP and trivalent vaccine study, we had been confirmed nine candidate strains as three species strains based these results of RAPD profiles and biochemical properties. For the virulence test, three strains of each species pathogens post-challenge by injection, including *S. iniae*, *S. parauberis*, and *E. tarda* were used to confirm virulence of the pathogens in olive flounder. The cumulative mortality rates of *S. iniae* strains JJU-019, JJU-073, and JJU-076 were 95%, 60%, and 75%, respectively, at 1.0×10^5 CFU/fish group of termination, and *E. tarda* strains JJU-032, JJU-052, and JJU-054 were 85%, 45%, and 100% at 1.0×10^4 CFU/ml group of termination. According to these results, the strains of *S. iniae* and *E. tarda* did not show a difference in virulence each other or they had very strong virulence. However, *S. parauberis* strains were demonstrated high variation each other strains such as JJU-008, JJU-045, and JJU-055. In the case of JJU-8, there was observed almost no mortality in challenged fish. Whereas the other strains such as JJU-045 and JJU-055 were demonstrated certain level of cumulative mortality that were demonstrated 100% and 55% in 1×10^7 CFU/fish dose group at termination, respectively. Among these strains to perform vaccine trials, we had been selected high virulence pathogens strains such as *S. iniae* JJU-019, *S. parauberis* JJU-045, and *E. tarda* JJU-054, respectively. For comparison between intra-peritoneal injection and immersion challenge, we investigated an immersion test model of olive flounder using three strains. Based on these results, we concluded that the intra-peritoneal injection model is more suitable than that of immersion model.

MATERIALS AND METHODS

1. Isolation and cultural condition of bacterial strains isolated from infected olive flounder

The bacterial infected moribund or dead flounders were collected from an aquaculture farms in Jeju Island, Korea from July 2010 until December 2010 and transported to the laboratory in a sterile container. The infected fish showed ocular lesions such as proptosis, rupture, abdominal distension, and black discoloration, which are all main symptoms of streptococcosis and edwardsiellosis (Lee et al., 2007). The kidney were isolated in each fish under aseptic conditions and the bacteria were cultivated separately in Brain Heart Infusion Agar (BHIA; Difco, USA) with 1.5% NaCl and cultured at 25 °C for 48 h. Inoculant of single colonies from plates with dense, virtually-pure culture growth were re-streaked fresh media to obtain pure isolates. The pure bacterial cultures were stored at −70 °C in 15% glycerol. The standard streptococcosis and edwardsiellosis bacterial strain such as *S. iniae* ATCC 29178, *S. parauberis* DSM 6631, and *E. tarda* ATCC 15947 were purchased and used for comparative analysis in the present study.

2. Morphology and physiological properties of isolates

The phenotypic features of the isolates, i.e., colony morphology, hemolytic features, and cell morphology, were determined by cultivating the isolates on sheep blood agar (Korea Media, Korea) at 25 °C and SS agar (Difco, USA). General cell morphology was studied using phase contrast micrography and scanning electron micrography (SEM). Biochemical tests were carried out using two microbial identification products, the API 20–Strep kit and API 20–E kit (bioMérieux, Etoile, France), and the results of each were compared with those of reference strains. We selected three strains each bacterial pathogens such as *S. iniae*, *S. parauberis*, and *E. tarda* that were depended on the phenotype. Phenotype grouping was carried out according to biochemical characteristics of each isolate.

3. Extraction of bacterial DNAs

Bacterial genomic DNA used in multiplex PCR and RAPD analysis was extracted according to the following three methods such as boiling methods, Chelex extraction method and commercial DNA extraction kit. The isolates were grown in BHIA supplemented with 1.5% NaCl. The colonies were picked and re-suspended in 500 μl of sterilized double distilled water; bacterial DNA was then extracted by boiling bacterial cells for 5 min and centrifuged at 6,000 g for 5min. Bacterial DNA was collected on the upper aqueous phase of the supernatant. In other methods, Genomic DNA of isolates was prepared using commercial DNA extraction kit (Bioneer, USA) and Chelex DNA extraction which is fast, cheap, and effective method of DNA extraction following the instructions of the manufacturer. Purified DNA was dissolved in 100 μl of distilled water and then stored at −20°C until used.

4. Primers and multiplex PCR amplification

The isolated strains were identified based molecular characteristics by multiplex PCR for the simultaneous detection of *S. iniae*, *S. parauberis*, and *E. tarda* from pure cultures. The target region and oligonucleotide primer set used for the detection of the three fish pathogens of streptococcosis and edwardsiellosis. The species-specific primers Spa-2152, Spa-2870, LOX-1, LOX-2, EDtT-F, and EDtT-R (Mata, 2004; Sakai, 2009) were used for the species identification of *S. iniae*, *S. parauberis*, and *E. tarda*. The primer sequence and the size of the amplicons are shown in Table 1.

The multiplex PCR was performed in 20 μl reaction mixtures containing DNA template, a 0.05 μM concentration each primer (Bioneer, USA) and AccuPower PCR Premix (1 U Taq DNA polymerase, 250 μM dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye; Bioneer, USA). The amplifications were carried out in a thermocyclers (My gein 32; MJ Research, USA) with the following parameters: an initial denaturation step of 94 °C, 5 min; 30 serial cycles of a denaturation step of 94 °C, 30 sec, annealing at 50 °C, 30 sec, and extension at 72 °C, 1 min; and a final extension step of 72 °C, 5 min. A negative control (no template DNA) and a positive control of reference strains obtained from Korean

collection for type culture (KCTC) were including in the PCR. The PCR products were analyzed by 1.5% agarose gel electrophoresis in 1x TAE (Tris-acetate-EDTA) buffer (Bioneer, USA). Gels were stained with ethidium bromide (0.5 μg/ml), visualized and photographed under UV illumination.

5. Phylogenetic analysis of isolates

The isolated strains were identified based on homology searches by basic local alignment search tool (BLAST) using bacterial 16S rRNA sequences. The almost complete 16S rRNA genes were amplified by PCR with universal primers (Weisburg, 1991) (Table 1). The conditions consisted of 30 cycles of 95°C (2 min), 50°C (30 s), and 72°C (1 min), plus one additional cycle with a final 5 min chain elongation. The amplified DNA products were purified using a PCR purification KIT (Bioneer, Korea) and sequenced by a commercial provider (Macrogen, Korea). The 16S rRNA sequences were aligned online using the BLAST program with those of organisms in GenBank, and percentage similarities of sequences were determined. Alignment was performed using the CLUSTAL W program ver. 1.8. The evolutionary distance matrix was calculated using the Kimura two-parameter method. The phylogenetic tree was constructed by the neighbor-joining method. The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1000 sample replications.

Table 1. Primer sequences used for species-specific PCR assay and the expected amplicon sizes.

Primer	Sequence (5' to 3')	Target gene	PCR amplicon size (bp)	Pathogen
Spa 2152	TTTCGTCTGAGGCAATGTTG	23S rRNA	718	*S. parauberis*
Spa 2870	GCTTCATATATCGCTATACT			
LOX-1	AAGGGGAAATCGCAAGTGCC	Lactate oxidase (lctO)	870	*S. iniae*
LOX-2	ATATCTGATTGGGCCGTCTAA			
EDtT-F	TTCCGCAACCATGATCAAAG	fimA gene	268	*E. tarda*
EDtT-R	AGGGCATATATCCACTCACTG			
27F	AGAGTTTGATCCTGGCTCAG	16S rRNA	1465	Bacterial 16s rRNA universal primer
1492R	GGTTACCTTGTTACGACTT			

6. RAPD fingerprinting and gel electrophoresis for genotyping of isolates

To evaluate bacterial DNA for genotyping, and RAPD fingerprinting were performed as described by Jayarao et al., (1996). The random amplified polymorphic DNA (RAPD) technique was used to distinguish early from recent isolates.

The random primers p1, p2, p3, p4, p5, and p14 were used for genotyping in each species (*S. iniae*, *S. parauberis*, and *E. tarda*), which primer sequences are p1-(5'-[GGTGCGGGAA]-3'), p2- (5'-[GTTTCGCTCC]-3'), p3- (5'-[GTAGACCCGT]-3'), p4-(5'-[AAGAGCCCGT]-3'), p5-(5'-[AACGCGCAAC]-3'), and p14 (5'-[GATCAAGTCC]-3') (Beaza, 2004; Neeman et al., 1998). In addition Primer p14, previously proven useful for discrimination among group a streptococcol strains (Neeman et al., 1998).

The RAPD fingerprinting was performed in 20 µl reaction mixtures containing DNA template, a 0.05 µM concentration each primer (Bioneer, USA) and AccuPower PCR Premix (1 U Taq DNA polymerase, 250 µM dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye; Bioneer, USA). The amplifications were carried out in a thermocyclers (My gein 32; MJ Research, USA) with the following parameters: an initial denaturation step of 95 °C, 5 min; 30 serial cycles of a denaturation step of 95 °C, 1 min, annealing at 32 °C, 2 min, and extension at 72 °C, 2 min; and a final extension step of 72 °C, 5 min. A negative control (no template DNA) and a positive control of reference strains obtained from Korean collection for type culture (KCTC) were including in the RAPD fingerprinting. Amplified products were electrophoresed in 1.5% agarose gel in 1x TAE (Tris-acetate-EDTA) buffer (Bioneer, USA). Gels were stained with ethidium bromide (0.5 µg/ml), visualized and photographed under UV illumination.

7. RAPD fingerprinting analyses and test reproducibility of isolates

The length of each lane finally tracked was within the length of the lane for the DNA molecular weight marker. The RAPD bands (markers) scored as 1 if present and 0 if absence. PCR analyses were duplicated. Only clear and reproducible bands were used for binary data matrix. The RAPD data was used to compute the genetic distances of cultivated isolates

according to Nei's distance index and MEGA ver. 4.1 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2007) used to construct a UPGMA (Unweighted Pair-Group Arithmetic Average Clustering) dendrogram with P-distance model and with similarity (Saitou and Nei, 1987).

8. Preparation of Virulence test by isolates

The virulence studies were a comparative analysis of each bacterial strain. The study period lasted for approximately 3 weeks post-exposure to *S. iniae*, *S. parauberis*, and *E. tarda*. The virulence of pathogens was demonstrated in a controlled laboratory study involving 3~4 groups of olive flounder challenged by intra-peritoneal injection or immersion route with different challenge doses of virulent *S. iniae*, *S. parauberis*, and *E. tarda* strains. Mortality was registered daily along with clinical symptoms of the fish.

The bacterial suspensions for challenge were inoculated into BHIB with 1.5% NaCl and incubated in a shaking incubator for 24 h at 25 °C until late exponential/early stationary phase was reached. To make the challenge stock, the bacterial suspension was centrifuged at 3,500 g for 15 min and re-suspended with sterilized saline until a satisfactory optical density (O.D.) had been reached. Plate count of the final challenge stock solution was measured, and the stocks were kept on ice (0-4 degrees) until used for challenge.

9. Virulence test by intra-peritoneal injection challenge

The culture was diluted to the pre-determined doses with saline. Twenty fish in each group were injected intra-peritoneally with 0.1 ml of diluted challenge stock containing the selected dose of bacterial cells. The sterile saline-injected group was used as a control. Fish rearing temperature was 20 ± 1 °C and 50% of water exchanged an every day. The fish were monitored a minimum of three times daily, and the numbers of dead/moribund fish were recorded daily. We conducted four challenge tests to measure virulence by intra-peritoneal injection, as shown in table 2.

10. Virulence test by immersion challenge

Serial dilution of the challenge inoculum was prepared in challenge buckets (5 L). The fish were removed from the holding/stock tank and placed into buckets containing the challenge solutions at the pre-determined concentrations for 15 min, after which the fish were removed from the challenge solution and placed into freshly prepared holding tanks containing clean rearing seawater. Fish were reared at 20 ± 1 °C, and 50% of water exchanged daily. Fish were monitored a three times daily, and the numbers of dead/moribund fish in each tank were recorded daily. The setup of the immersion challenge test was shown in table 2.

11. Virulence test by immersion challenge

Dead/moribund fish were collected from each tank and recorded the clinical signs and bacterial re-isolation. The challenge isolate was recovered by aseptically removing a bacterial loopful of the kidney, placing it on appropriate agar, and incubating for 48 h at 25 °C. Dead fish from each tank were collected in separate plastic bags marked with the study number, fish markings, tank number, and date. Postmodern examination was performed immediately the fish after died and recorded the clinical signs. The re-isolated bacteria were identified by multiplex PCR array as previously described.

Table 2. Challenge setup of virulence test by intra-peritoneal injection and immersion.

Challenge model	Pathogen	Challenge strain	No. of fish / tank	Challenge dose (CFU/fish)	Tank volume
Intra-peritoneal injection	*S. iniae*	JJU-019	20	1×10^3	250 L
		JJU-073		1×10^5	
		JJU-076		1×10^7	
	S. parauberis	JJU-008		1×10^3	
		JJU-045		1×10^5	
		JJU-076		1×10^7	
	E. tarda	JJU-032		1×10^3	
		JJU-052		1×10^4	
		JJU-054		1×10^5	
Immersion	*S. iniae*	JJU-019	20	1×10^5	250 L
				1×10^6	
				1×10^7	
				1×10^8	
	S. parauberis	JJU-045		1×10^5	
				1×10^6	
				1×10^7	
				1×10^8	
	E. tarda	JJU-054		1×10^5	
				1×10^6	
				1×10^7	
				1×10^8	

RESULTS

1. Isolation of bacterial strains from infected olive flounder

Bacterial isolates were 61 wild-type strains collected from infected olive flounder exhibiting signs, including darkened surface, ascitic fluid in the peritoneal cavity, distended abdomen, protruded anus, exophthalmia, haemorrhaging in the eye, and haemorrhaging on the surface (Table 3). General microbiological examinations revealed that the Streptococcal isolates were nonmotile, Gram-positive cocci, with no catalase or oxidase activities, whereas isolates of *E. tarda* were motile, Gram-negative short rod, salt tolerant, with indole and H_2S production on triple sugar iron agar (Fig. 2, 3). Hemolytic analysis showed that eight isolates of *S. iniae* were β-hemolytic, whereas forty-eight isolates of *S. parauberis* were α-hemolytic.

2. Biochemical properties and phenotyping of bacterial isolates

Regarding the investigation of biochemical characteristics using the API 20 strep system, eight strains of *S. iniae* were positive for pyrrolidonyl arylamidase, alkaline phosphatase, L-leucine arylamidase, arginine di hydrolase, ribose acidification, mannitol acidification, trehalose acidification, and β-hemolysis, but were negative for hippurate hydrolysis, α-galactosidase, β-galactosidase, L-alabinose acidification, inulin acidification, and raffinose acidification, which was very similar with that of *S. iniae* ATCC 29178 (Table 4). The forty-eight strains of *S. parauberis* were confirmed as positive for acetoin production, hippurate hydrolysis, and trehalose acidification, but were negative for α-galactosidase, β-glucuronidase, raffinose acidification, and glycogen acidification, which very similar to that of *S. parauberis* DSM 6631. More than 90% strains were showed same results with positive or negative which excepted mannitol acidification, sorbitol acidification, and lactose acidification (Table 5). Five strains of *E. tarda* were positive for lysine decarboxylase, ornithine decarboxylase, H_2S production, and glucose fermentation/oxidation, but were negative for beta-galactosidase and arginine dihydrolase (Table 6).

Bacterial isolates were examined for grouping based on the biochemical properties of the bacterial strains. The phenotyping results show that *S. iniae*, *S. parauberis*, and *E. tarda* were separated into six, seven, and three phenotypes, respectively (Table 7).

3. Identification by multiplex PCR amplification and phylogenetic analysis

All isolates were identified as *S. iniae*, *S. parauberis*, and *E. tarda* via multiplex PCR array using the primers Spa-2152 and Spa-2870, LOX-1 and LOX-2, and EDtT, and EDtA, respectively (Fig. 4). Positive PCR amplification of DNA templates from *S. iniae*, *S. parauberis* and *E. tarda* produced a single fragment, of the expected, for each pathogen (870, 718, and 268 bp, respectively), as shown in Figure 4.

The 16S rRNA sequences showed 99% similarity to known bacteria identified in Genbank by BLAST search and the EMBL Nucleotide Sequence Database with *S. iniae*, *S. parauberis*, and *E. tarda* respectively. As a result, eight isolates of *S. iniae*, forty-eight isolates of *S. parauberis*, and five isolates of *E. tarda* were identified, respectively. Isolates of the three bacterial species were genotyping analysis in accordance with their 16s rRNA sequences as described previously. Whereas these sequences of each species could not established genotyping, because it did not appear a sufficient variation between each 16s rRNA sequences.

Streptococci and *E. tarda*, 16s rRNA gene sequences of eight *S. iniae*, forty eight *S. parauberis*, and five *E. tarda* isolates were used in the phylogenetic analysis. These sequences were compared to that of a type strain downloaded from the NCBI database. The results of the phylogenetic array show that the isolates of *S. iniae* and *S. parauberis* were closely related to the type strains *S. iniae* ATCC 29178^{T} and *S. parauberis* DSM 6631^{T}, respectively (Figure 5, 6). Further, the phylogenic array of the *E. tarda* isolates showed a close relationship with the type strain *E. tarda* ATCC 15947^{T} (Figure 7).

Table 3. Clinical symptoms, Gram staining, and haemolysis pattern of the isolates in this study

Strain No.	Species	Major symptoms	Gram test	Hemolysis
JJU-001	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-002	*Streptococcus parauberis*	Darkened surface, Distended abdomen, Protruded anus	+	γ-hemolytic
JJU-003	*Streptococcus parauberis*	Haemorrhaging in the inner surface of abdomen,	+	γ-hemolytic
JJU-004	*Streptococcus parauberis*	Darkened surface, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-005	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the inner surface of abdomen,	+	γ-hemolytic
JJU-007	*Streptococcus parauberis*	Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-008	*Streptococcus parauberis*	Darkened surface, Exophthalmia, Haemorrhaging in the eye	+	γ-hemolytic
JJU-009	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the non-ocular side	+	γ-hemolytic
JJU-010	*Streptococcus parauberis*	Exophthalmia, Haemorrhaging in the eye	+	γ-hemolytic
JJU-012	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-013	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-014	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the opercular region	+	γ-hemolytic
JJU-015	*Streptococcus parauberis*	Darkened surface, Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-016	*Streptococcus parauberis*	Darkened surface, Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-017	*Streptococcus parauberis*	Haemorrhaging in the inner surface of abdomen,	+	γ-hemolytic
JJU-018	*Streptococcus parauberis*	Darkened surface, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-040	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-041	*Streptococcus parauberis*	Distended abdomen, Protruded anus, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-042	*Streptococcus parauberis*	Ascitic fluid in the peritoneal cavity, Haemorrhaging in the opercular region	+	γ-hemolytic
JJU-043	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-045	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-046	*Streptococcus parauberis*	Darkened surface, Exophthalmia, Haemorrhaging in the opercular region	+	γ-hemolytic
JJU-047	*Streptococcus parauberis*	Ascitic fluid in the peritoneal cavity, Haemorrhaging in the opercular region	+	γ-hemolytic
JJU-048	*Streptococcus parauberis*	Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-050	*Streptococcus parauberis*	Darkened surface, Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-055	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the inner surface of abdomen	+	γ-hemolytic
JJU-056	*Streptococcus parauberis*	Exophthalmia, Haemorrhaging in the eye	+	γ-hemolytic
JJU-057	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-058	*Streptococcus parauberis*	Exophthalmia, Haemorrhaging in the opercular and the non-ocular side region,	+	γ-hemolytic
JJU-060	*Streptococcus parauberis*	Darkened surface, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-061	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the eye	+	γ-hemolytic
JJU-063	*Streptococcus parauberis*	Darkened surface, Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-064	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the opercular region	+	γ-hemolytic
JJU-065	*Streptococcus parauberis*	Exophthalmia	+	γ-hemolytic
JJU-066	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the opercular region	+	γ-hemolytic
JJU-067	*Streptococcus parauberis*	Darkened surface, Distended abdomen, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-068	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the inner surface of abdomen,	+	γ-hemolytic
JJU-069	*Streptococcus parauberis*	Darkened surface, Ascitic fluid in the peritoneal cavity	+	γ-hemolytic
JJU-070	*Streptococcus parauberis*	Exophthalmia, Haemorrhaging in non-ocular side and inner surface of abdomen	+	γ-hemolytic
JJU-071	*Streptococcus parauberis*	Protruded anus	+	γ-hemolytic

Table 3. Continued.

Strain No.	Species	Major symptoms	Gram test	Hemolysis
JJU-072	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the inner surface of abdomen	+	γ-hemolytic
JJU-090	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the non-ocular side	+	γ-hemolytic
JJU-091	*Streptococcus parauberis*	Darkened surface, Haemorrhaging in the non-ocular side	+	γ-hemolytic
JJU-093	*Streptococcus parauberis*	Darkened surface, Haemorrhaging, Distended abdomen,	+	γ-hemolytic
JJU-095	*Streptococcus parauberis*	Exophthalmia	+	γ-hemolytic
JJU-098	*Streptococcus parauberis*	Darkened surface, Exophthalmia, Haemorrhaging in the non-ocular side	+	γ-hemolytic
JJU-099	*Streptococcus parauberis*	Distended abdomen, Haemorrhaging in the inner surface of abdomen	+	γ-hemolytic
JJU-100	*Streptococcus parauberis*	Darkened surface	+	γ-hemolytic
JJU-019	*Streptococcus iniae*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	+	β-hemolytic
JJU-073	*Streptococcus iniae*	Protruded anus, Ascitic fluid in the peritoneal cavity	+	β-hemolytic
JJU-075	*Streptococcus iniae*	Darkened surface, Protruded anus, Ascitic fluid in the peritoneal cavity,	+	β-hemolytic
JJU-076	*Streptococcus iniae*	Ascitic fluid in the peritoneal cavity, Distended abdomen,	+	β-hemolytic
JJU-077	*Streptococcus iniae*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	+	β-hemolytic
JJU-078	*Streptococcus iniae*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	+	β-hemolytic
JJU-087	*Streptococcus iniae*	Ascitic fluid in the peritoneal cavity, Distended abdomen,	+	β-hemolytic
JJU-102	*Streptococcus iniae*	Distended abdomen	+	β-hemolytic
JJU-032	*Edwardsiella tarda*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	-	γ-hemolytic
JJU-033	*Edwardsiella tarda*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	-	γ-hemolytic
JJU-051	*Edwardsiella tarda*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	-	γ-hemolytic
JJU-052	*Edwardsiella tarda*	Protruded anus, Ascitic fluid in the peritoneal cavity, Distended abdomen	-	γ-hemolytic
JJU-054	*Edwardsiella tarda*	Ascitic fluid in the peritoneal cavity, Distended abdomen	-	γ-hemolytic

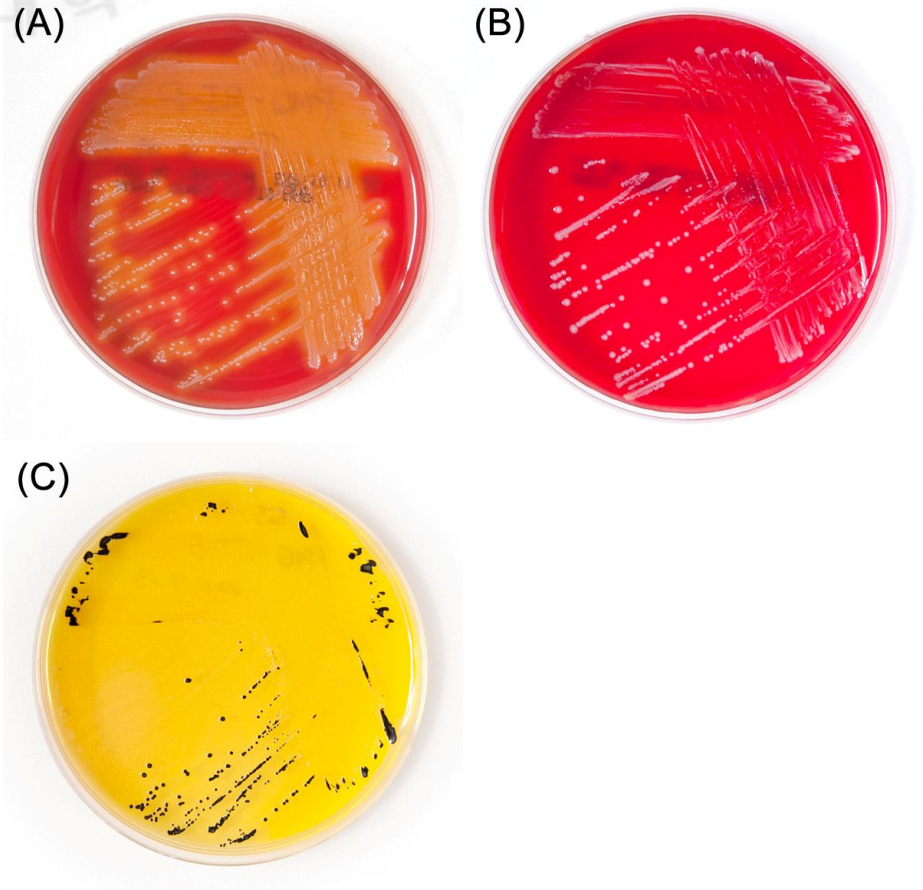

Figure 2. Cultural characterization of strains isolated from infected olive flounder in Korea. A, *S. iniae*; B, *S. parauberis*; C, *E. tarda*

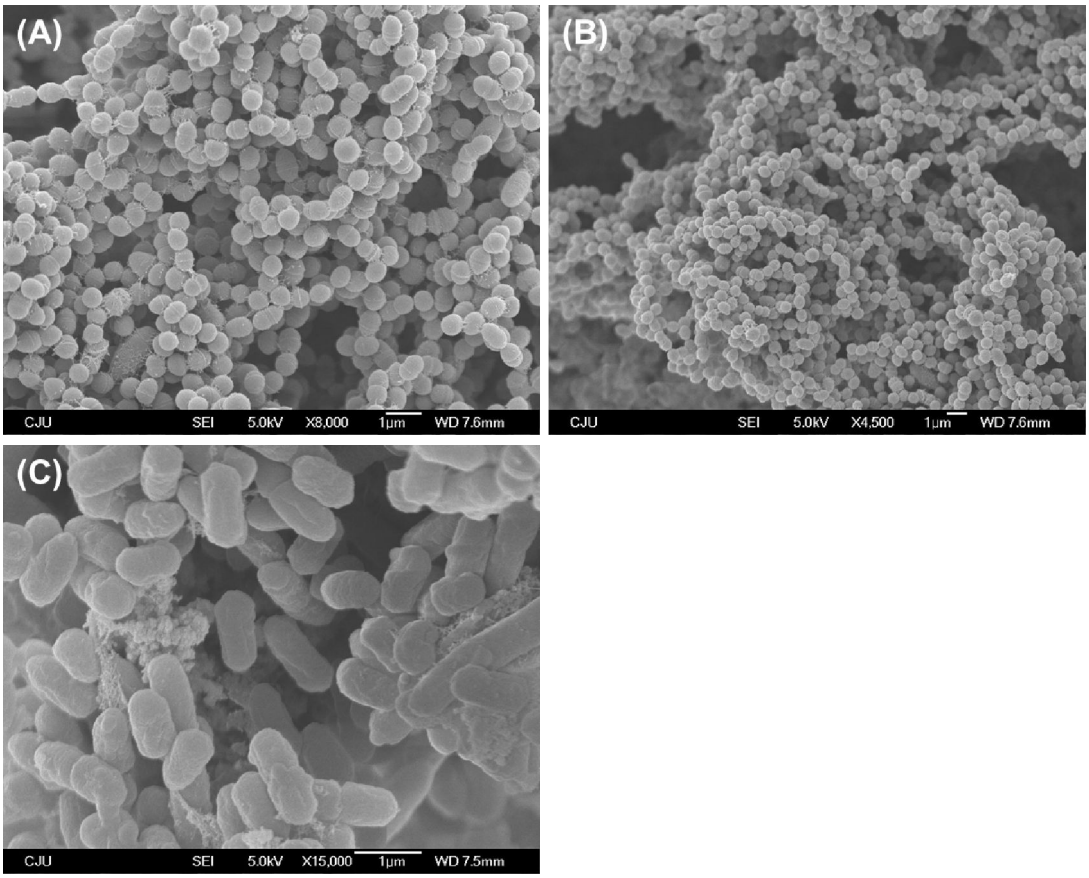

Figure 3. Scanning electron micrograph (SEM) of strains isolated from infected olive flounder in Korea. A, *S. iniae*; B, *S. parauberis*; C, *E. tarda*

Table 4. Biochemical profile of type strain and *S. iniae* strains isolated from infected olive flounder.

API 20 strep system	JJU-019	JJU-073	JJU-075	JJU-076	JJU-077	JJU-078	JJU-087	JJU-102	*S. iniae* ATCC 29178
Acetoin production	-□	-	-	+	-	-	-	-	-
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-
Esculin β-glucosidase	-	+	+	+	+	+	+	+	+
PYRrolidonyl Arylamidase	+	+	+	+	+	+	+	+	+
α-GALactosidase	-	-	-	-	-	-	-	-	-
β-GLUcoRonidase	-	-	-	-	-	-	-	-	+
β-GALactosidase	-	-	-	-	-	-	-	-	-
Alkaline Phosphatase	+	+	+	+	+	+	+	+	+
Leucine Amino Peptidase	+	+	+	+	+	+	+	+	+
Arginine DiHydrolase	+	+	+	+	+	+	+	+	+
D-ribose acidification	+	+	+	+	+	+	+	+	+
L-alabinose acidification	-	-	-	-	-	-	-	-	-
D-mannitol acidification	+	+	+	+	+	+	+	+	+
D-sorbitol acidification	+	+	-	-	-	+	-	-	-
D-lactose acidification	+	-	-	-	-	+	-	-	-
D-trehalose acidification	+	+	+	+	+	+	+	+	+
Inulin acidification	-	-	-	-	-	-	-	-	-
D-raffinose acidification	-	-	-	-	-	-	-	-	-
Starch acidification	+	+	+	+	+	+	-	+	+
Glycogen acidification	-	+	+	+	+	+	+	+	+
β-Hemolysis	+	+	+	+	+	+	+	+	+

Table 5. Biochemical profile of type strain and *S. parauberis* strains isolated from infected olive flounder.

API 20 strep system	JJU-008	JJU-045	JJU-055	*S.parauberis* DSM 6631	*S.parauberis* isolates (No. of positive strains/ No. of total strains)
Acetoin production	+	+	+	+	48/48*
Hippurate hydrolysis	+	+	+	+	48/48
Esculin β-glucosidase	+	+	+	+	42/48
PYRrolidonyl Arylamidase	+	+	+	+	41/48
α-GALactosidase	-	-	-	-	0/48
β-GLUcoRonidase	-	-	-	-	0/48
β-GALactosidase	-	-	-	-	2/48
AlkalinePhosphatase	+	-	+	-	5/48
Leucine Amino Peptidase	+	+	+	+	44/48
Arginine DiHydrolase	+	+	+	+	47/48
D-ribose acidification	-	-	-	-	5/48
L-alabinose acidification	-	-	-	-	1/48
D-mannitol acidification	-	+	-	-	26/48
D-sorbitol acidification	-	+	-	-	27/48
D-lactose acidification	-	+	-	-	21/48
D-trehalose acidification	+	+	+	+	48/48
Inulin acidification	-	-	-	-	3/48
D-raffinose acidification	-	-	-	-	0/48
Starch acidification	-	-	-	-	2/48
Glycogen acidification	-	-	-	-	0/48
β-Hemolysis	-	-	-	-	0/48

Table 6. Biochemical profile of *E. tarda* strains isolated from infected olive flounder.

API 20E system	JJU-032	JJU-033	JJU-051	JJU-052	JJU-054
beta-galactosidase	-	-	-	-	-
arginine dihydrolase	-	-	-	-	-
lysine decarboxylase	+	+	+	+	+
ornithine decarboxylase	+	+	+	+	+
citrate utilization	+	+	+	+	+
H_2S production	+	+	+	+	+
urease	-	-	-	-	-
tryptophane deaminase	-	-	-	-	-
indole production	-	-	-	-	-
acetoin production	-	-	-	-	-
gelatinase	-	-	-	-	-
glucose fermentation/oxidation	+	+	+	+	+
mannitol fermentation/oxidation	-	-	+	+	-
inositol fermentation/oxidation	-	-	-	-	-
sorbitol fermentation/oxidation	-	-	-	-	-
rhamnose fermentation/oxidation	-	-	-	-	-
sucrose fermentation/oxidation	-	-	-	+	-
melibiose fermentation/oxidation	-	-	-	-	-
Amygdalin fermentation/oxidation	-	-	-	-	-
arabinose fermentation/oxidation	-	-	-	-	-
cytochrome oxidase	-	-	-	-	-

Table 7. Biochemical profile grouping of strains isolated from infected olive flounder in Korea.

Species	API group	Number of strains	No. of Isolates
Streptococcus iniae	Ⅰ	3	75, 77, 102
	Ⅱ	1	**19**
	Ⅲ	1	**76**
	Ⅳ	1	**73**
	Ⅴ	1	78
	Ⅵ	1	87
Streptococcus parauberis	Ⅰ	18	1, 2, 7, 10, 12, 17, 41, 42, 43, **45**, 48, 61, 63, 90, 91, 95, 98, 100
	Ⅱ	2	13, 68
	Ⅲ	3	**8**, 40, 67
	Ⅳ	3	65, 66, 93
	Ⅴ	6	14, 16, 46, 47, 63, 99
	Ⅵ	6	50, **55**, 56, 57, 58, 69
	etc.	11	3, 4, 5, 9, 15, 18, 60, 64 70, 71, 72
Edwardsiella tarda	Ⅰ	3	**32**, 33, **54**
	Ⅱ	1	51
	Ⅲ	1	**52**

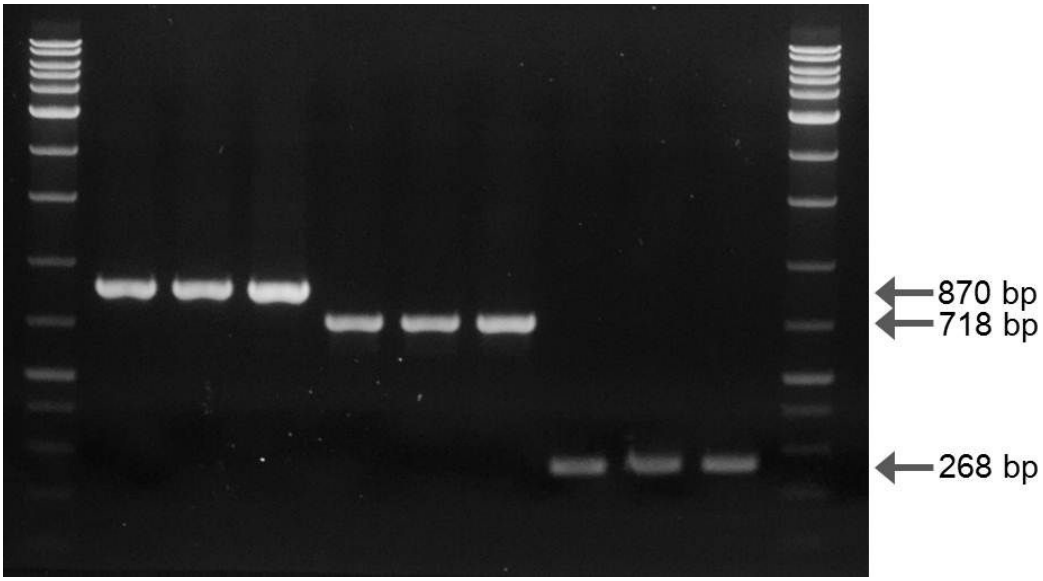

Figure 4. . Amplification products obtained by multiplex PCR assay and developed for the simultaneous detection of *S. iniae* (870 bp), *S. parauberis* (718 bp), and typical *E. tarda* (268 bp). Lanes: 1 and 11, 1000 bp plus DNA Marker (Intron biotechnology, Inc., Korea); 2, *S.iniae* JJU-019; 3, *S.iniae* JJU-073; 4, *S.iniae* JJU-076; 5, *S. parauberis* JJU-008; 6, *S. parauberis* JJU-045; 7, *S. parauberis* JJU-055; 8, *E. tarda* JJU-032; 9, *E. tarda* JJU-052; 10, *E. tarda* JJU-054.

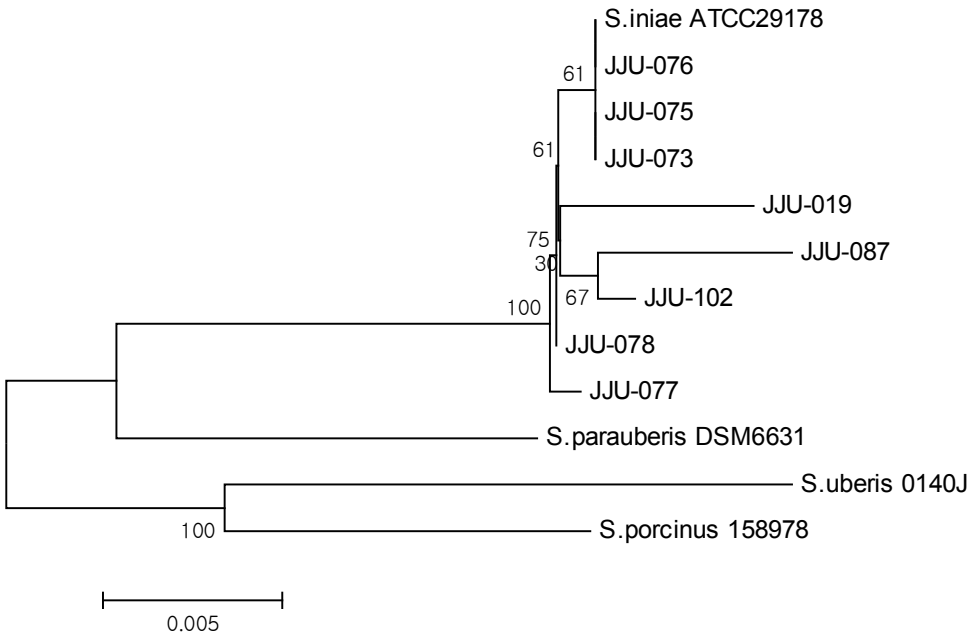

Figure 5. Neighbour-joining tree based relationship of complete 16S rDNA sequences between *S. iniae* strains and member of the *Streptococcus* genus. Numbers at the nodes are levels of bootstrap support (%), based on neighbour-joining analyses of 1,000 resampled datasets. Bar, 0.1 nucleotide substitutions per position.

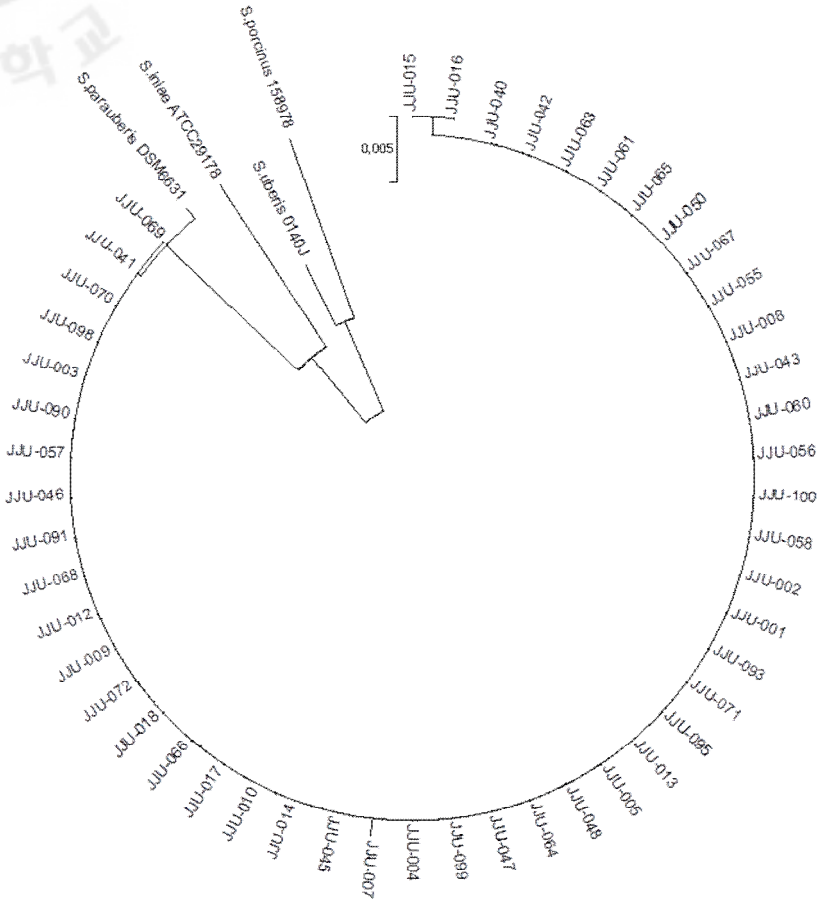

Figure 6. Neighbour-joining tree based relationship of complete 16S rDNA sequences between *S. parauberis* strains and member of the *Streptococcus* genus. Numbers at the nodes are levels of bootstrap support (%), based on neighbour-joining analyses of 1,000 resampled datasets. Bar, 0.1 nucleotide substitutions per position.

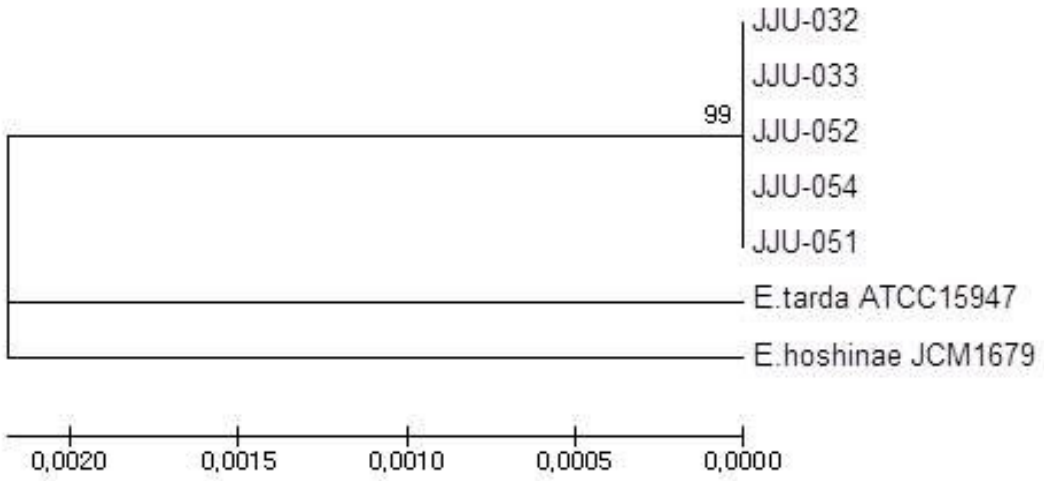

Figure 7. Neighbour-joining tree based on relationship of complete 16S rDNA sequences between *E. tarda* strains and member of the *Edwardsiella* genus. Numbers at the nodes are levels of bootstrap support (%), based on neighbour-joining analyses of 1,000 resampled datasets. Bar, 0.1 nucleotide substitutions per position.

4. RAPD fingerprinting for genotyping of isolates

The genomic DNA of each strain used in this study was amplified using 6 different primers that were shown type-ability to three species isolates and GC content of each primer (Table 8). Fingerprints of isolated bacterial strains such as *S. iniae*, *S. parauberis*, and *E. tarda* obtained with six primer are shown in Fig.8, 9, and 10, respectively. The RAPD analysis of the 7 strains of *S. iniae* revealed different DNA profiles were investigated six patterns with the primer p1, two patterns with the primer p3 and p5, monomorphic patterns with p2, p4, and p14, respectively. The case of 46 strains of *S. parauberis* revealed different DNA profiles were investigated nineteen patterns with the primer p2, nine patterns with the primer p1, five patterns with the primer p4, four patterns with the primer p3 and p5, three patterns with the primer p14, and 5 strains of *E. tarda* revealed conservative patterns, except for primer p3 that were investigated monomorphic DNA profiles. (Table 8). A large number of reproducible bands were produced for most of the primers. However, some primers produced several non-reproducible bands. Six primers tested produced specific band profiles revealing polymorphisms. The negative control tubes (in which the genomic DNA was replaced with double distilled water) did not show any DNA amplification (data not shown). The primers in bacterial strains of *S. iniae*, *S. parauberis*, and *E. tarda* used generated a total of 42, 73, and 57 reproducible bands respectively. The number of bands for each primer in bacterial strains of *S. iniae*, *S. parauberis*, and *E. tarda* varied from 8, 62, and 2 with averages of 1.3, 10.3, and 0.3 bands per primer respectively. When the experiments were replicated, the number and location of all bands were found to be identical, and only minor variations in the intensities of some bands could be seen. Amplified DNA fragments that were reproducible were scored as 0 and 1 in a data matrix and the relationships of each bacterial species between the seven, forty six, and five isolates were estimated using the p-distance index (Table 9 and 10; Nei et al. 1972) of similarity and UPGMA was done with Mega version 4.1 (Figure 11, 12, and 13), that dendrogram of isolated bacterial strains based on RAPD polymorphism. According to this matrix, genetic Pairwise distance (p-distance) values of seven strains *S. iniae* were found to be between 0.049 and 0.182. Hereunder, it was

determined that the samples closest to each other based on their genetic p-distance values are JJU-019 to JJU-073, JJU-073 to JJU-075, JJU-075 to JJU-076, JJU-076 to JJU-077, JJU-073 to JJU-078, JJU-073 to JJU-077, JJU-075 to JJU-078, and JJU-073 to JJU-075, respectively. Whereas the samples was most distant from each other are JJU-077 to JJU-087 (Table 9). The p-distance values of forty six strains *S. parauberis* and five strains *E. tarda* were found to be between 0.000 to 1.199 (Table 10), and 0.000 to 0.038 (Data not shown) respectively.

The resultant dendrogram of three bacterial species isolates obtained by UPGMA (Unweighted Pair-Group Arithmetic Average Clustering) cluster analysis is shown in Fig. 11, 12, and 13. The dendrogram shows the differences among the *S. iniae* strains. Three genotype groups were characterized: the first group formed by *S. iniae* strain JJU-075, JJU-076, JJU-077, and JJU-078; the second group formed by *S. iniae* strain JJU-019 and JJU-073; the third group formed *S. iniae* strain JJU-087, respectively (Figure 11). However the dendrogram of *E. tarda* strains shows almost no variation among the *E. tarda* strains that were the first group formed by *E. tarda* strain JJU-033, JJU-051, JJU-052, and JJU-054, the order group only formed by *E. tarda* strain JJU-032 (Figure 12). The dendrogram of *S. parauberis* strains shows the differences among the *S. parauberis* strains, that eight genotype groups were characterized: the first group formed by *S. parauberis* strain JJU-001, JJU-002, JJU-004, JJU-012, JJU-015, JJU-016, JJU-040, JJU-041, JJU-043, JJU-046, JJU-047, JJU-048, JJU-050, JJU-055, JJU-057, JJU-061, JJU-063, and JJU-090; the second group formed by *S. parauberis* strain JJU-042, JJU-060, and JJU-067; the third group formed *S. parauberis* strain JJU-010, JJU-072, and JJU-093; the fourth group formed JJU-003, JJU-005, and JJU-091; the fifth group formed JJU-007, JJU-013, JJU-014, JJU-018, JJU-045, JJU-056, JJU-058, and JJU-071; the sixth group formed JJU-070, JJU-095, and JJU-098; the seventh group formed JJU-064, JJU-065, JJU-066, and JJU-069; the eighth group formed JJU-008, JJU-009, and JJU-017, respectively (Figure 13). In order to progress LAMP and trivalent vaccine study, we had been confirmed nine candidate strains as three species strains based these results of RAPD profiles and biochemical properties.

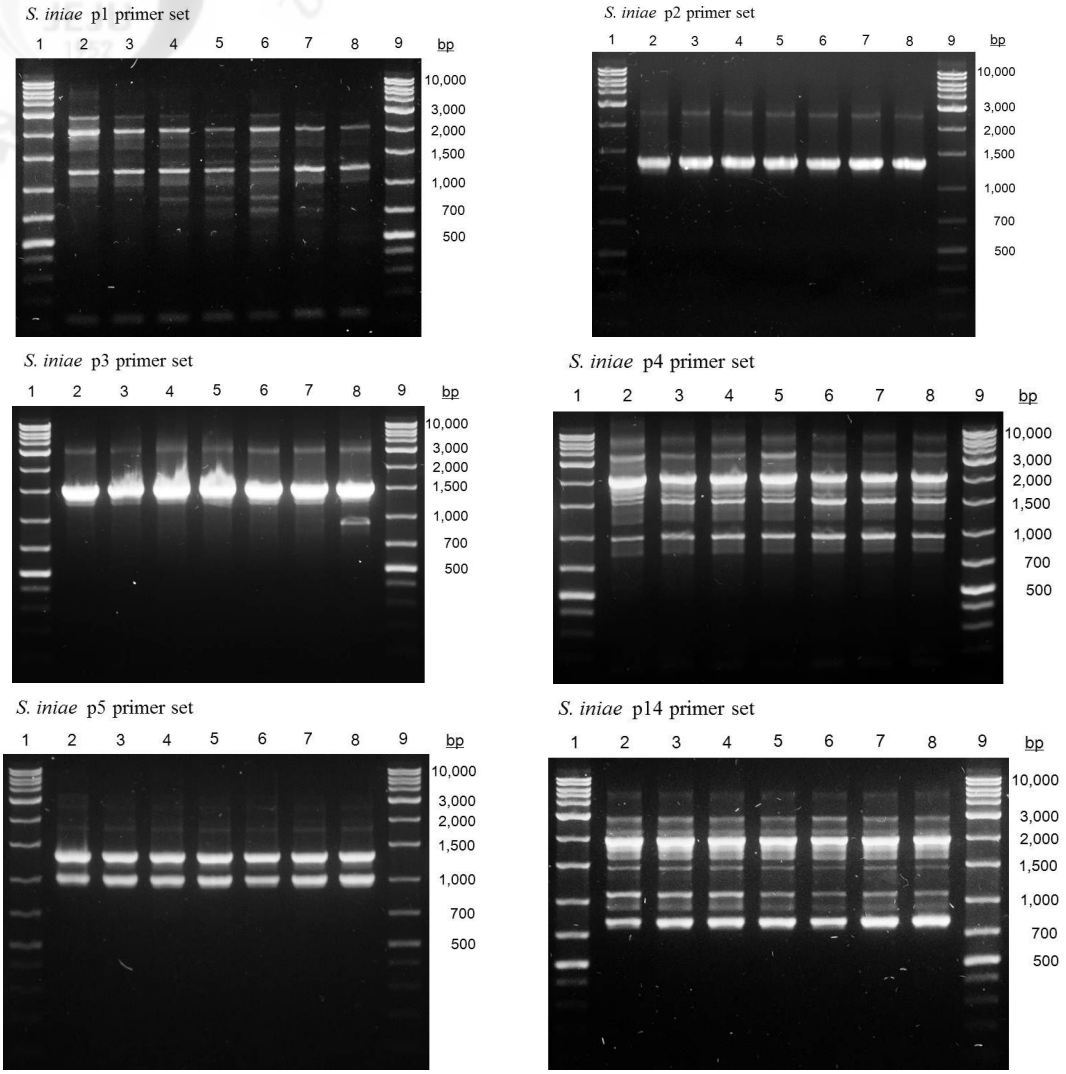

Figure 8. Amplified RAPD products from *S. iniae* strains isolated from infected olive flounder (lanes 2 to 8). Amplification with primer p1, p2, p3, p4, p5, and p14 are shown, respectively.

Lanes 1 and 9, 1000 bp plus DNA Marker (Intron Biotechnology, Korea); 2, *S.iniae* JJU-019; 3, *S.iniae* JJU-073; 4, *S.iniae* JJU-075; 5, *S.iniae* JJU-076; 6, *S.iniae* JJU-077; 7, *S.iniae* JJU-078; 8, *S.iniae* JJU-087.

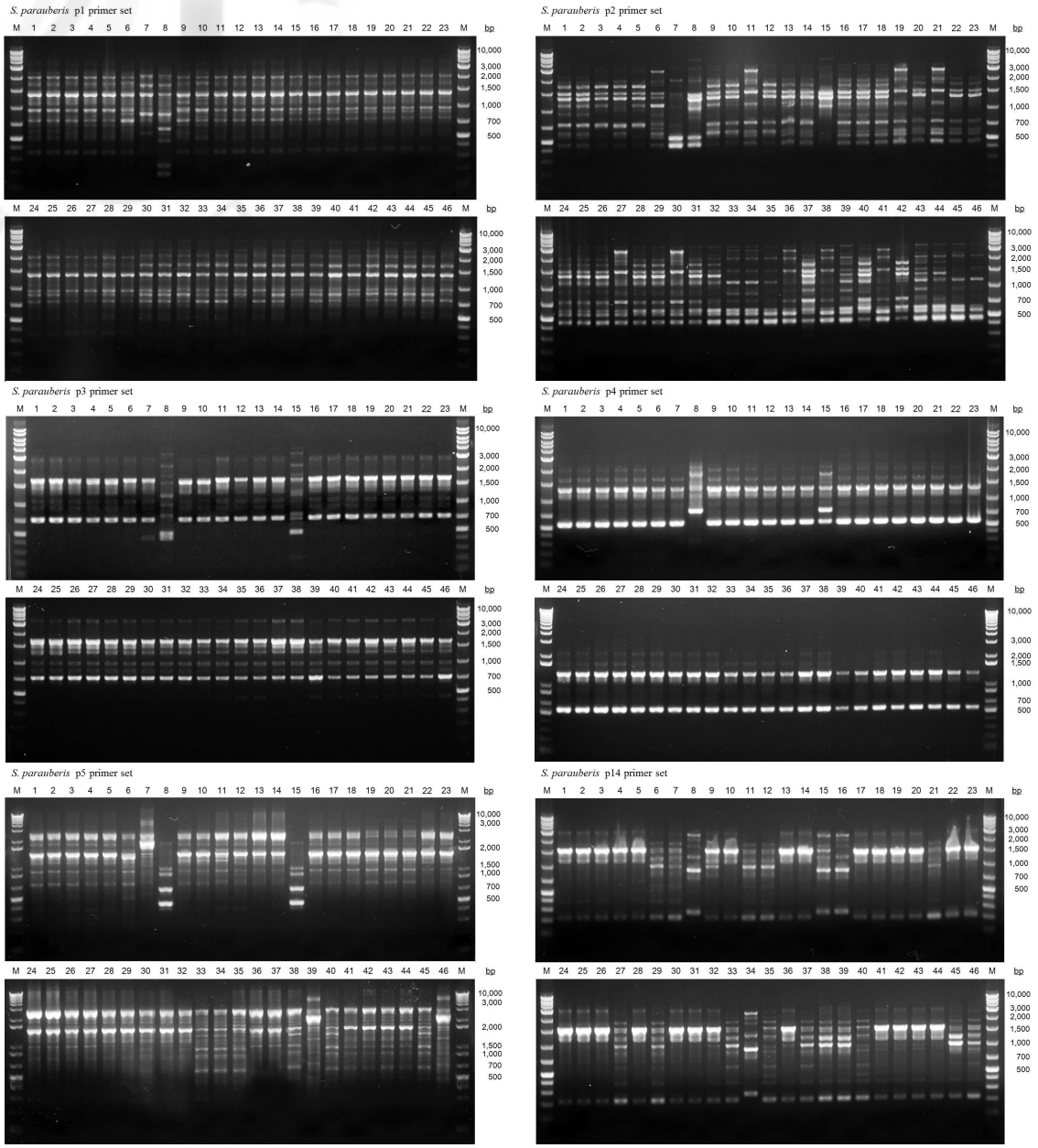

Figure 9. Amplified RAPD products from *S. parauberis* strains isolated from infected olive flounder (lanes 1 to 46). Amplification with primer p1, p2, p3, p4, p5, and p14 are shown, respectively.

Lanes M, 1000 bp plus DNA Marker (Intron Biotechnology, Korea); 1, *S. parauberis* JJU-001; 2, JJU-002; 3, JJU-003; 4, JJU-004; 5, JJU-005; 7, JJU-007; 8, JJU-008; 9, JJU-009; 10, JJU-010; 11, JJU-012; 12, JJU-013; 13, JJU-014; 14, JJU-015; 15, JJU-016; 16, JJU-017; 17, JJU-018; 18, JJU-040; 19, JJU-041; 20, JJU-042; 21, JJU-043; 22, JJU-045; 23, JJU-046; 24, JJU-047; 25, JJU-048; 26, JJU-050; 27, JJU-055; 28, JJU-056; 29, JJU-057; 30, JJU-058; 31, JJU-060; 32, JJU-064; 33, JJU-064; 34, JJU-065; 35, JJU-066; 36, JJU-067; 37, JJU-068; 38, JJU-069; 39, JJU-070; 40, JJU-071; 41, JJU-072; 42, JJU-090; 43, JJU-091; 44, JJU-093; 45, JJU-095; 46, JJU-098.

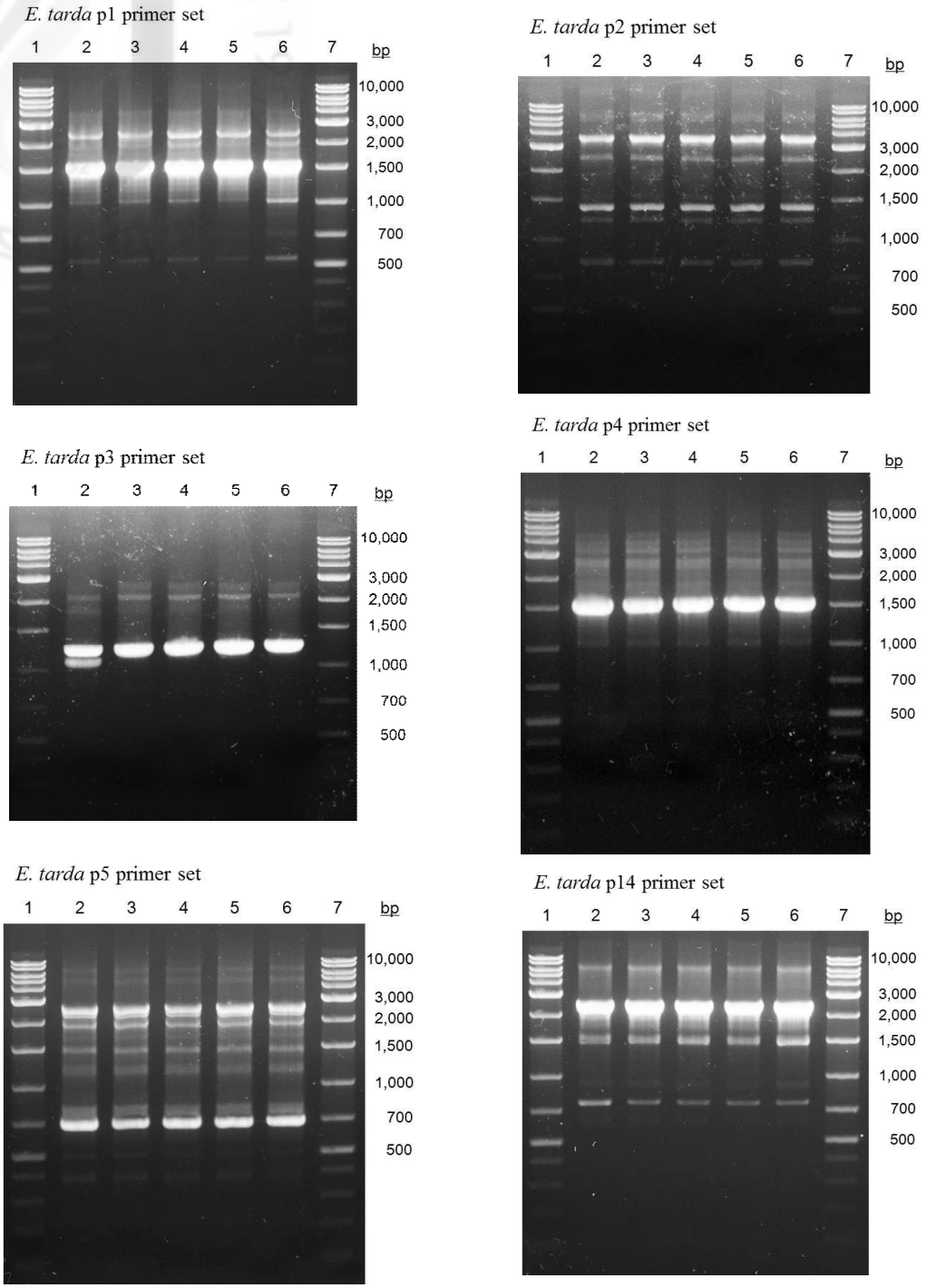

Figure 10. Amplified RAPD products from *E. tarda* strains isolated from infected olive flounder (lanes 2 to 6). Amplification with primer p1, p2, p3, p4, p5, and p14 are shown, respectively.

Lanes 1and 7, 1000 bp plus DNA Marker (Intron Biotechnology, Korea); 2, *E.tarda* JJU-032; 3, *E.tarda* JJU-033; 4, *E.tarda* JJU-051; *E.tarda* JJU-052; 6, *E.tarda* JJU-054.

Table 8. Type-ability and G+C content of RAPD primer in this study.

RAPD Primer	Sequence	G+C content (%)	Type-ability No. of types/Total No. strains		
			S. iniae	*S. parauberis*	*E. tarda*
P1	GGTGCGGGAA	70	6 / 7	9 / 46	1 / 5
P2	GTTTCGCTCC	60	1 / 7	19 / 46	1 / 5
P3	GTAGACCCGT	60	2/ 7	4 /46	2 / 5
P4	AAGAGCCCGT	60	1 / 7	5 / 46	1 / 5
P5	AACGCGCAAC	60	2 / 7	4 / 46	1 / 5
P14	GATCAAGTCC	50	1 / 7	3 / 46	1 / 5

Table 9. Pairwise distance matrix (Nei, 1972) of seven *S. iniae* strains in this study.

	JJU-019	JJU-073	JJU-075	JJU-076	JJU-077	JJU-078	JJU-087
JJU-019	****						
JJU-073	0.049	****					
JJU-075	0.100	0.049	****				
JJU-076	0.154	0.100	0.049	****			
JJU-077	0.100	0.100	0.049	0.049	****		
JJU-078	0.100	0.049	0.049	0.049	0.100	****	
JJU-087	0.127	0.074	0.127	0.127	0.182	0.074	****

The p-distances were calculated from the RAPD data of 42 polymorphic bands.

Table 10. Pairwise distance matrix (Nei, 1972) of seven *S. parauberis* strains in this study.

	001	002	003	004	005	007	008	009	010	012	013	014	015	016	017	018	040	041	042	043	045	046	047	048	050	055	056	057	058	060	061	063	064	065	066	067	068	069	070	071	072	090	091	093	095	098
001																																															
002	0.00																																														
003	0.06	0.06																																												
004	0.04	0.04	0.07																																											
005	0.06	0.06	0.00	0.07																																										
007	0.16	0.16	0.23	0.21	0.23																																								
008	0.46	0.46	0.42	0.44	0.42	0.32																																							
009	1.03	1.03	1.03	1.16	1.03	0.92	0.92																																						
010	0.10	0.10	0.13	0.09	0.13	0.25	0.36	1.00																																					
012	0.07	0.07	0.10	0.06	0.10	0.18	0.48	1.07	0.09																																				
013	0.15	0.15	0.21	0.16	0.21	0.10	0.30	1.11	0.23	0.20																																			
014	0.15	0.15	0.15	0.10	0.15	0.13	0.30	1.20	0.20	0.16	0.09																																		
015	0.04	0.04	0.07	0.03	0.07	0.21	0.44	1.07	0.09	0.06	0.16	0.13																																	
016	0.04	0.04	0.07	0.03	0.07	0.21	0.44	1.07	0.09	0.06	0.16	0.13	0.00																																
017	0.60	0.60	0.51	0.63	0.51	0.58	0.74	0.32	0.74	0.68	0.65	0.60	0.63	0.63																															
018	0.15	0.15	0.18	0.16	0.18	0.16	0.46	0.76	0.20	0.13	0.21	0.18	0.13	0.13	0.51																														
040	0.06	0.06	0.09	0.07	0.09	0.16	0.46	0.96	0.10	0.04	0.21	0.18	0.04	0.04	0.65	0.09																													
041	0.06	0.06	0.09	0.07	0.09	0.16	0.46	0.96	0.10	0.04	0.21	0.18	0.04	0.04	0.65	0.09	0.00																												
042	0.09	0.09	0.15	0.10	0.15	0.16	0.42	1.03	0.13	0.07	0.12	0.21	0.10	0.10	0.71	0.18	0.09	0.09																											
043	0.09	0.09	0.12	0.04	0.12	0.20	0.42	1.03	0.10	0.04	0.21	0.15	0.04	0.04	0.71	0.12	0.03	0.03	0.09																										
045	0.18	0.18	0.25	0.20	0.25	0.16	0.42	0.83	0.23	0.16	0.12	0.21	0.20	0.20	0.55	0.09	0.18	0.18	0.09	0.18																									
046	0.09	0.09	0.12	0.04	0.12	0.20	0.42	1.03	0.10	0.04	0.21	0.15	0.04	0.04	0.71	0.12	0.03	0.03	0.09	0.00	0.18																								
047	0.09	0.09	0.12	0.04	0.12	0.20	0.42	1.03	0.10	0.04	0.21	0.15	0.04	0.04	0.71	0.12	0.03	0.03	0.09	0.00	0.18	0.00																							
048	0.07	0.07	0.10	0.06	0.10	0.18	0.44	1.00	0.09	0.03	0.20	0.16	0.03	0.03	0.68	0.10	0.01	0.01	0.07	0.01	0.16	0.01	0.01																						
050	0.07	0.07	0.10	0.06	0.10	0.18	0.44	1.00	0.09	0.03	0.20	0.16	0.03	0.03	0.68	0.10	0.01	0.01	0.07	0.01	0.16	0.01	0.01	0.00																					
055	0.07	0.07	0.10	0.06	0.10	0.18	0.44	1.00	0.09	0.03	0.20	0.16	0.03	0.03	0.68	0.10	0.01	0.01	0.07	0.01	0.16	0.01	0.01	0.00	0.00																				
056	0.18	0.18	0.25	0.20	0.25	0.07	0.30	1.03	0.23	0.16	0.03	0.12	0.20	0.20	0.71	0.18	0.18	0.18	0.09	0.18	0.09	0.18	0.18	0.16	0.16	0.16																			
057	0.07	0.07	0.10	0.06	0.10	0.18	0.44	1.00	0.09	0.03	0.20	0.16	0.03	0.03	0.68	0.10	0.01	0.01	0.07	0.01	0.16	0.01	0.01	0.00	0.00	0.00	0.16																		
058	0.16	0.16	0.20	0.15	0.20	0.09	0.32	1.00	0.18	0.12	0.10	0.07	0.12	0.12	0.68	0.10	0.10	0.10	0.16	0.10	0.16	0.10	0.10	0.09	0.09	0.09	0.07	0.09																
060	0.10	0.10	0.16	0.12	0.16	0.18	0.44	1.00	0.15	0.09	0.13	0.23	0.12	0.12	0.74	0.20	0.10	0.10	0.01	0.10	0.10	0.10	0.10	0.09	0.09	0.09	0.10	0.09	0.18															
061	0.09	0.09	0.12	0.07	0.12	0.20	0.46	0.96	0.10	0.04	0.21	0.18	0.04	0.04	0.71	0.12	0.03	0.03	0.09	0.03	0.18	0.03	0.03	0.01	0.01	0.01	0.18	0.01	0.10	0.07														
063	0.09	0.09	0.12	0.07	0.12	0.20	0.46	0.96	0.10	0.04	0.21	0.18	0.04	0.04	0.71	0.12	0.03	0.03	0.09	0.03	0.18	0.03	0.03	0.01	0.01	0.01	0.18	0.01	0.10	0.07	0.00													
064	0.30	0.30	0.34	0.32	0.34	0.15	0.36	0.86	0.36	0.28	0.16	0.23	0.28	0.28	0.63	0.23	0.23	0.23	0.23	0.27	0.23	0.27	0.27	0.25	0.25	0.25	0.13	0.25	0.15	0.21	0.23	0.23												
065	0.30	0.30	0.34	0.32	0.34	0.25	0.48	0.68	0.36	0.28	0.27	0.34	0.28	0.28	0.48	0.13	0.23	0.23	0.23	0.27	0.13	0.27	0.27	0.25	0.25	0.25	0.23	0.25	0.25	0.21	0.23	0.23	0.09											
066	0.34	0.34	0.38	0.36	0.38	0.18	0.36	0.86	0.36	0.28	0.20	0.27	0.32	0.32	0.68	0.27	0.27	0.27	0.27	0.30	0.27	0.30	0.30	0.28	0.28	0.28	0.16	0.28	0.18	0.25	0.27	0.27	0.03	0.12										
067	0.10	0.10	0.13	0.06	0.13	0.25	0.40	1.16	0.15	0.12	0.13	0.16	0.06	0.06	0.68	0.20	0.10	0.10	0.07	0.07	0.16	0.07	0.07	0.09	0.09	0.09	0.16	0.09	0.18	0.09	0.10	0.10	0.25	0.25	0.28									
068	0.15	0.15	0.18	0.16	0.18	0.07	0.34	0.96	0.20	0.13	0.12	0.09	0.13	0.13	0.65	0.09	0.09	0.09	0.18	0.12	0.18	0.12	0.12	0.10	0.10	0.10	0.09	0.10	0.01	0.20	0.12	0.12	0.13	0.23	0.16	0.20								
069	0.34	0.34	0.38	0.28	0.38	0.21	0.32	0.92	0.32	0.25	0.16	0.20	0.28	0.28	0.74	0.27	0.27	0.27	0.23	0.23	0.23	0.23	0.23	0.25	0.25	0.25	0.13	0.25	0.15	0.21	0.23	0.23	0.09	0.18	0.06	0.21	0.16							
070	0.32	0.32	0.32	0.34	0.32	0.13	0.23	0.89	0.38	0.30	0.25	0.21	0.30	0.30	0.51	0.25	0.25	0.25	0.25	0.32	0.28	0.32	0.28	0.28	0.27	0.27	0.27	0.21	0.27	0.16	0.34	0.28	0.28	0.20	0.30	0.23	0.34	0.15	0.27					
071	0.20	0.20	0.23	0.21	0.23	0.12	0.36	0.86	0.25	0.18	0.16	0.13	0.18	0.18	0.58	0.13	0.13	0.13	0.23	0.16	0.23	0.16	0.16	0.15	0.15	0.15	0.13	0.15	0.06	0.25	0.16	0.16	0.09	0.18	0.12	0.25	0.04	0.12	0.16					
072	0.18	0.18	0.21	0.13	0.21	0.27	0.42	1.03	0.16	0.10	0.21	0.25	0.13	0.13	0.83	0.21	0.12	0.12	0.09	0.09	0.18	0.09	0.09	0.10	0.10	0.10	0.18	0.10	0.20	0.07	0.09	0.09	0.23	0.23	0.20	0.07	0.21	0.13	0.36	0.27				
090	0.03	0.03	0.06	0.04	0.06	0.20	0.46	1.03	0.10	0.07	0.18	0.15	0.01	0.01	0.60	0.12	0.03	0.03	0.12	0.06	0.21	0.06	0.06	0.04	0.04	0.04	0.21	0.04	0.13	0.13	0.06	0.06	0.27	0.27	0.30	0.07	0.12	0.30	0.28	0.16	0.15			
091	0.10	0.10	0.10	0.12	0.10	0.21	0.40	0.92	0.18	0.15	0.23	0.20	0.09	0.09	0.48	0.20	0.10	0.10	0.16	0.13	0.27	0.13	0.13	0.12	0.12	0.12	0.27	0.12	0.21	0.18	0.13	0.13	0.28	0.28	0.32	0.12	0.20	0.36	0.20	0.25	0.20	0.07		
093	0.15	0.15	0.18	0.10	0.18	0.23	0.42	0.96	0.16	0.07	0.25	0.21	0.10	0.10	0.71	0.18	0.09	0.09	0.12	0.06	0.21	0.06	0.06	0.07	0.07	0.07	0.21	0.07	0.16	0.10	0.06	0.06	0.27	0.27	0.27	0.10	0.18	0.20	0.28	0.23	0.06	0.12	0.13	
095	0.36	0.36	0.40	0.34	0.40	0.20	0.30	0.89	0.38	0.27	0.21	0.25	0.30	0.30	0.60	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.27	0.27	0.27	0.18	0.27	0.16	0.27	0.25	0.25	0.10	0.20	0.10	0.30	0.18	0.10	0.18	0.13	0.25	0.32	0.34	0.21
098	0.34	0.34	0.38	0.32	0.38	0.21	0.25	1.07	0.40	0.28	0.20	0.23	0.28	0.28	0.68	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.28	0.28	0.28	0.20	0.28	0.18	0.28	0.27	0.27	0.18	0.28	0.18	0.28	0.20	0.18	0.13	0.21	0.27	0.30	0.32	0.23	0.07

The p-distances were calculated from the RAPD data of 73 polymorphic bands.

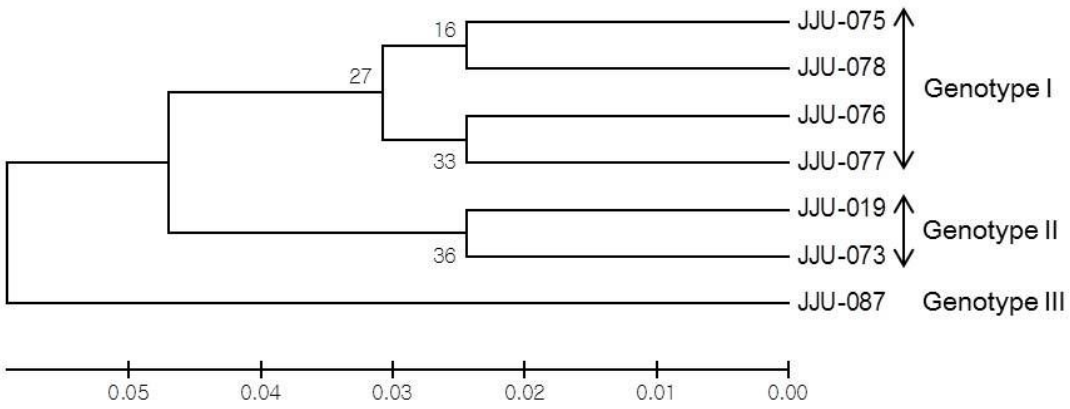

Figure 11. Dendrogram representing the relationships between seven *S. iniae* strains based on UPGMA cluster analysis of the RAPD profiles derived from six primers using p-distance model.

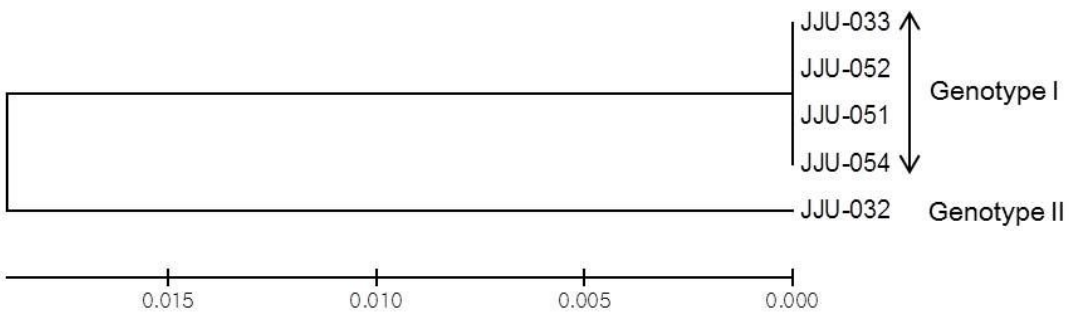

Figure 12. Dendrogram representing the relationships between five *E. tarda* strains based on UPGMA cluster analysis of the RAPD profiles derived from six primers using p-distance model.

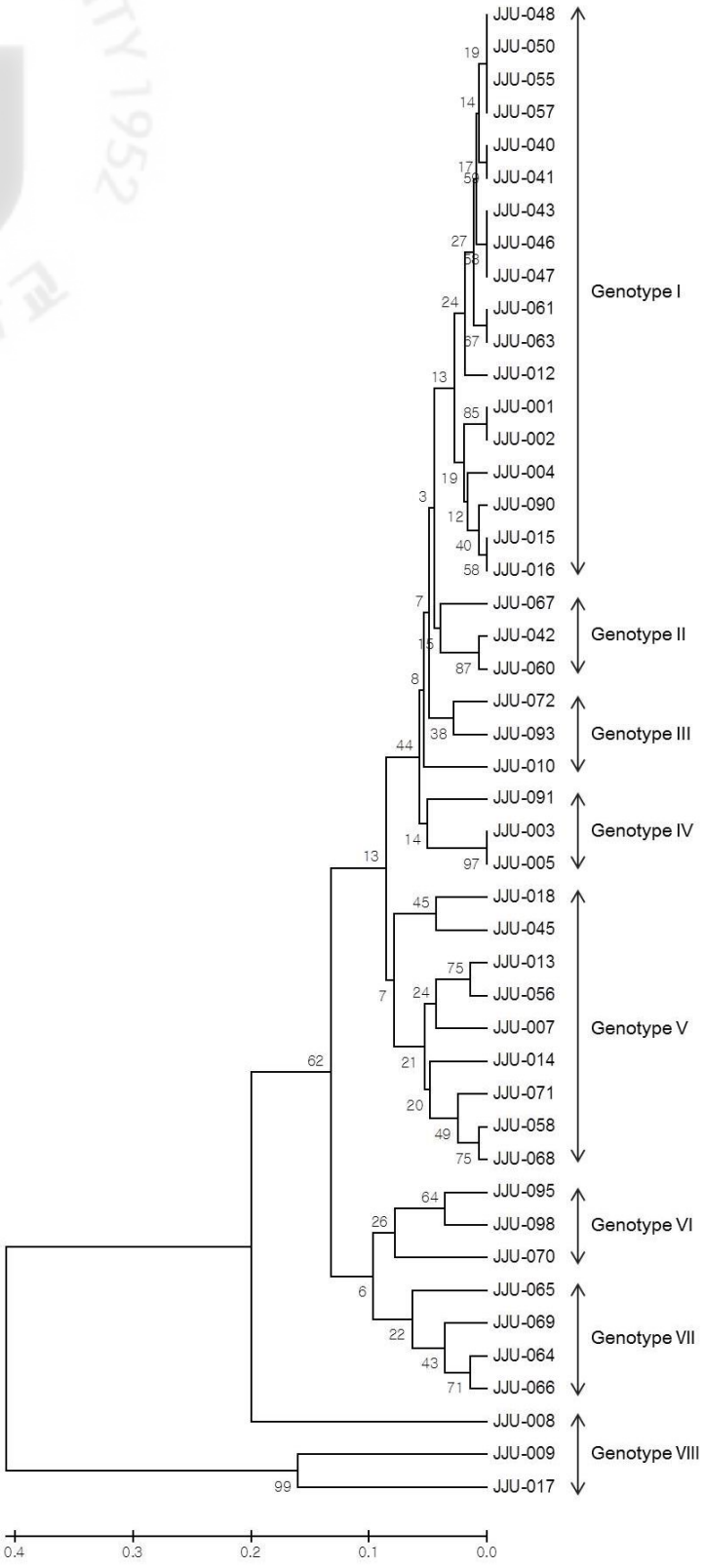

Figure 13. Dendrogram representing the relationships between forty eight *S. parauberis* strains based on UPGMA cluster analysis of the RAPD profiles derived from six primers using p-distance model.

5. Virulence test by intra-peritoneal injection challenge

For the virulence test, three strains of each species pathogens post-challenge by injection, including *S. iniae*, *S. parauberis*, and *E. tarda* were used to confirm virulence of the pathogens in olive flounder. These strains were selected according to RAPD profiles and biochemical properties. The cumulative mortality rates of *S. iniae* strains JJU-019, JJU-073, and JJU-076 were 95%, 60%, and 75%, respectively, at 1.0×10^5 CFU/fish group of termination, and *E. tarda* strains JJU-032, JJU-052, and JJU-054 were 85%, 45%, and 100% at 1.0×10^4 CFU/ml group of termination (Figure 14 and 16). According to these results, the strains of *S. iniae* and *E. tarda* did not show a difference in virulence each other or they had very strong virulence. However, *S. parauberis* strains were demonstrated high variation each other strains such as JJU-008, JJU-045, and JJU-055. In the case of JJU-8, there was observed almost no mortality in challenged fish. Whereas the other strains such as JJU-045 and JJU-055 were demonstrated certain level of cumulative mortality that were demonstrated 100% and 55% in 1×10^7 CFU/fish dose group at termination, respectively (Figure 15). Among these strains to perform vaccine trials, we had been selected high virulence pathogens strains such as *S. iniae* JJU-019, *S. parauberis* JJU-045, and *E. tarda* JJU-054, respectively.

6. Virulence test by immersion challenge

For comparison between intra-peritoneal injection and immersion challenge, we investigated an immersion test model of olive flounder using three strains, *S. iniae* JJU-019, *S. parauberis* JJU-045, and *E. tarda* JJU-054. The cumulative mortality rates of *S. iniae* JJU-019 and *E. tarda* JJU-054 were 35% and 100% at 1×10^7 CFU/ml group for 15 days post-challenge (Figure 17a and 17c). However, there was no mortality in the *S. parauberis* JJU-045 group during the challenge period (Figure 17b). The challenge model of immersion with *E. tarda* JJU-054 was suitable, whereas the immersion models of other bacterial species did not effective compared to intra-peritoneal injection. Based on these results, we concluded that the intra-peritoneal injection model is more suitable than that of immersion model.

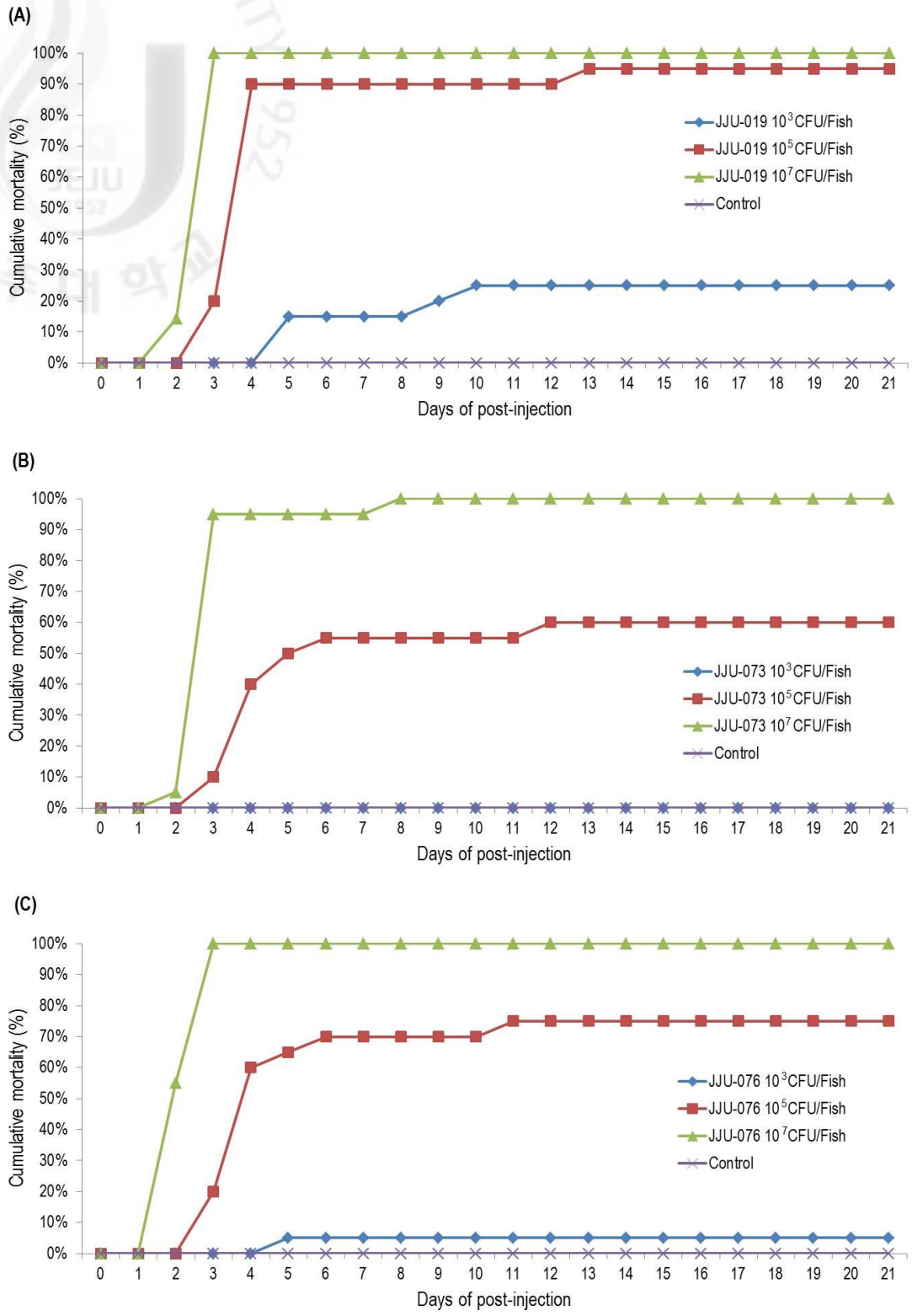

Figure 14. Cumulative mortalities of *S. iniae* strains in olive flounder by intra-peritoneal injection.

A , *S. iniae* JJU-019; B, *S. iniae* JJU-073; C, *S. iniae* JJU-076.

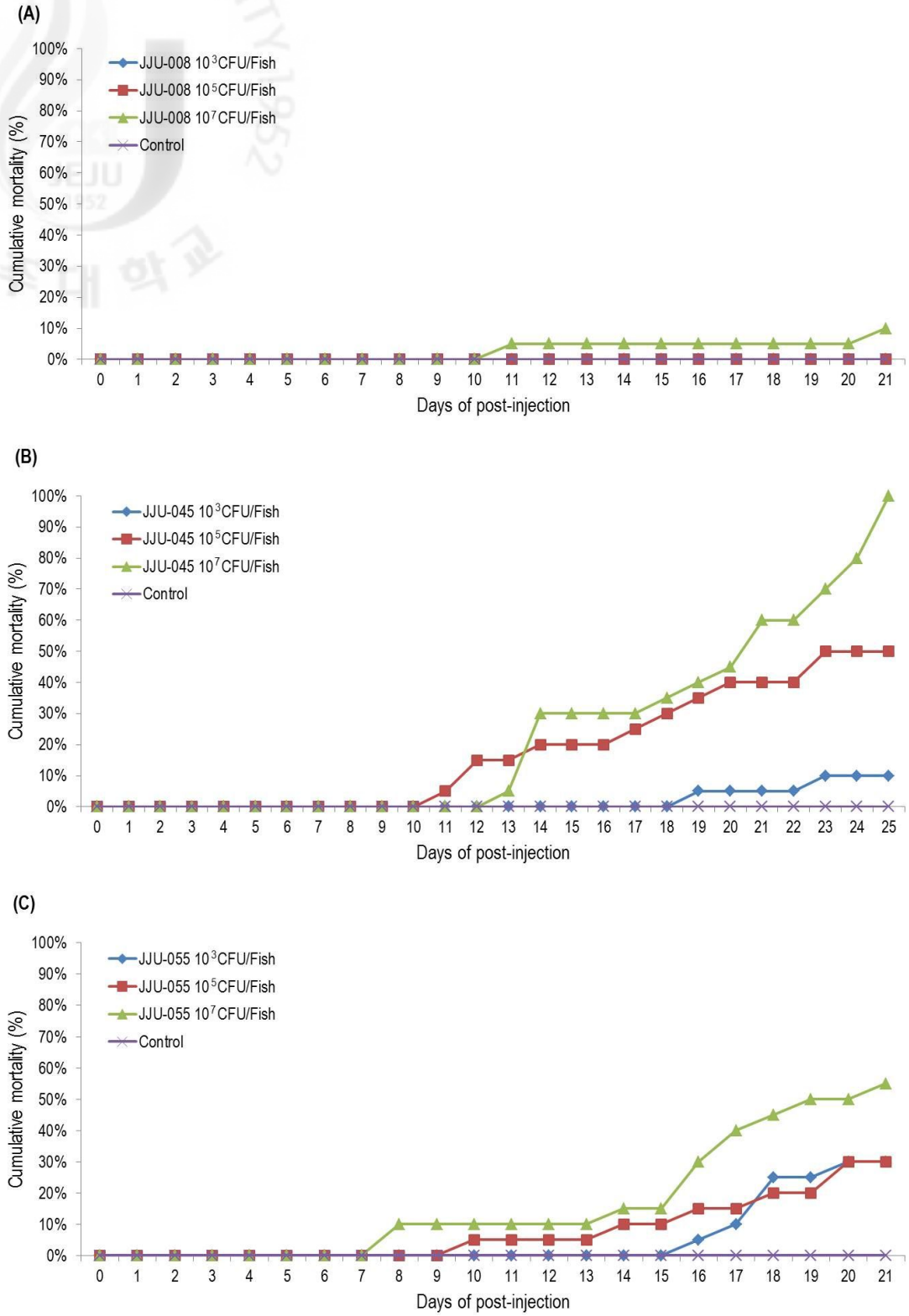

Figure 15. Cumulative mortalities of *S. parauberis* strains in olive flounder by intra-peritoneal injection. A, *S. parauberis* JJU-008; B, *S. parauberis* JJU-045; C, *S. parauberis* JJU-055.

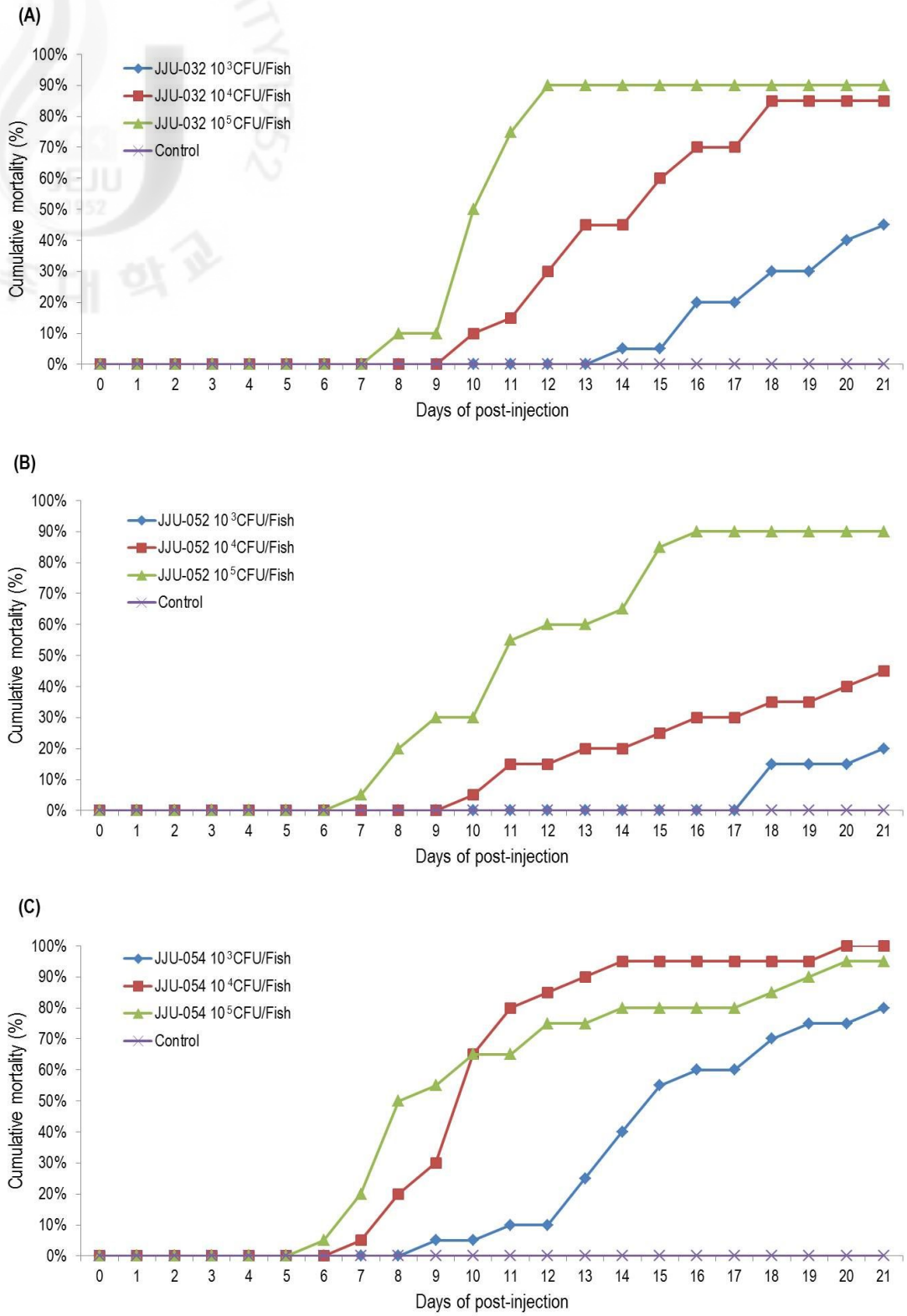

Figure 16. Cumulative mortalities of *E. tarda* strains in olive flounder by intra-peritoneal injection.

A, *E. tarda* JJU-032; B, *E. tarda* JJU-052; C, *E. tarda* JJU-054.

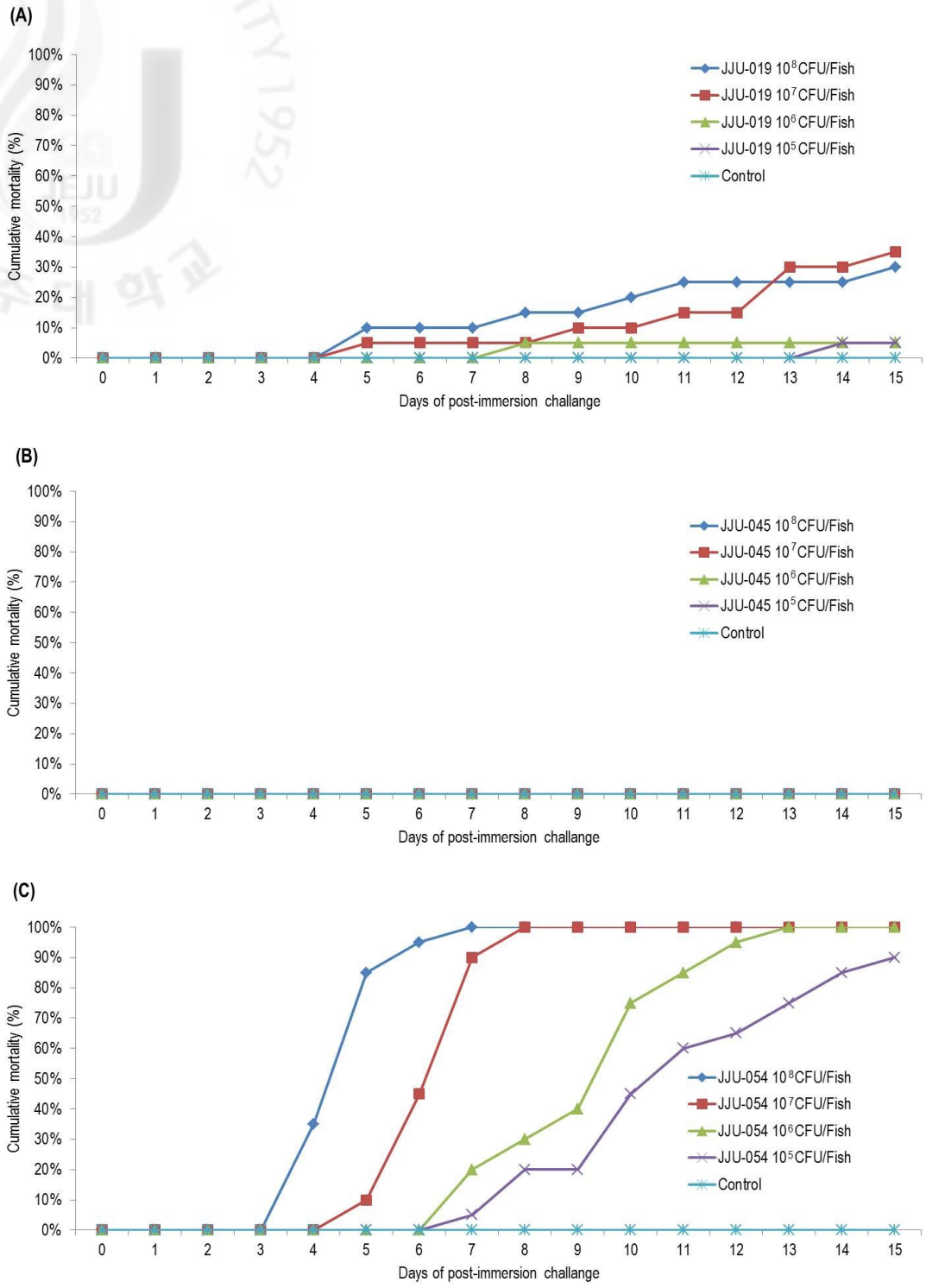

Figure 17. Cumulative mortalities of three bacterial strains in olive flounder by immersion challenge. A, *S. iniae* JJU-019; B, *S. parauberis* JJU-045; C. *E. tarda* JJU-054.

DISCUSSION

In present study, we isolated 61 wild-type bacterial strains in naturally infected olive flounder that were on the fish farm in Jeju Island, Korea. The infected olive flounder were observed several symptoms such as darkened surface, ascitic fluid in the peritoneal cavity, distended abdomen, protruded anus, exophthalmia, haemorrhaging in the eye, and haemorrhaging on the surface which were major symptoms of streptococcusis and edwardsiellosis. These bacterial strains were identified three pathogens such as *S. iniae*, *S. parauberis*, and *E. tarda* by phonotypical and molecular characteristics. According to these results, we have been collected and identified eight isolates of *S. iniae*, forty-eight isolates of *S. parauberis*, and five isolates of *E. tarda*, finally. *S. iniae* is a Gram-positive bacterial pathogen, which can infected both humans and wide range of fish species (Agnew and Barnes, 2007; Shoemaker et al., 2001; Lahav et al., 2004; Bromage and Owens, 2002; Yuasa et al., 1999; Nho et al., 2009; Nguyen et al., 2002; Zhou et al., 2008; Du, 2001), including olive flounder. Disease outbreaks can occur under unfavorable environmental conditions, such as those caused by high temperature and high rearing density, leading to heavy economic losses (Austin, 2008; Low et al., 1999). Although the pathogenic mechanisms of *S. iniae* remain mostly unclear, it observed that some disease-associated *S. iniae* are able to survive within host immune responses (Zlotkin et al., 2003). In the case of *S. parauberis* that another pathogen of streptococcusis, which is caused by *S. parauberis*, formerly *S. uberis* type II (Williams and Collins, 1990). It is mainly known for causing mastitis in cows (Bentley et al., 1993; McDonald et al., 2005). This species has also been reported to produce streptococcosis, an infectious disease in fish caused by Gram-positive cocci in certain farmed fish species, such as olive flounder (Nho et al., 2009) and turbot (Domenech et al., 1996), resultant fish mortality rates over 70%. The edwardsiellosis is also causing huge economic losses in olive flounder farms compare to streptococcusis. *E. tarda* is a Gram-negative bacteria *E. tarda*, the causative agent of edwardsiellosis, which is responsible for mass

mortality and severe morbidity in a variety of freshwater and marine fish species including flounder, turbot, carp, tilapia, eel, and catfish. In flounder, *E. tarda* infection typically induces symptoms such as hemorrhagic septicemia, which can lead to mass mortality especially under stressful environmental conditions (Matsuyama et al., 2005; Thune et al., 1993; Castro et al., 2006; Joh et al., 2011). As mentioned above, it is very important an aspect of fish pathology that understanding characteristics of various bacterial pathogens from farmed olive flounder in Korea such as *S. iniae*, *S. parauberis*, and *E. tarda*. General microbiological examinations revealed that the Streptococcal isolates were non-motile, Gram-positive cocci, with no catalase or oxidase activities, whereas isolates of *E. tarda* were motile, Gram-negative short rod, salt tolerant, with indole and H_2S production on triple sugar iron agar. These results were similar to type strains of each species such as *S. iniae* ATCC 29178, *S. parauberis* DSM 6631, and *E. tarda* ATCC 15947. In addition, there were reports that pathogen of streptococcusis is characterized typical hemolytic properties depend on each streptococcal species. Therefore we investigated hemolytic properties as results; eight isolates of *S. iniae* were β-hemolytic, whereas forty-eight isolates of *S. parauberis* were α-hemolytic. For diagnostic work and developing of vaccine work, the choice of typing methods should be determined by a number of considerations, such as the goal of isolate identification, speed, ease of use, cost, availability of equipment and trained personnel, etc (Ruth, and Jeffrey, 2009). The phenotyping of bacterial isolates were used the biochemical properties by API system. In these results, the phenotyping results show that *S. iniae*, *S. parauberis*, and *E. tarda* were separated into six, seven, and three phenotypes, respectively. Phenotypic identification methods are based on evaluation of the expression of genetically encoded characteristics by bacterial isolates, including morphology, growth characteristics, ability to metabolize substrates, and antimicrobial resistance (Bannerman et al., 1993; Devriese et al., 1994; Watts and Yancey, 1994; Ieven et al., 1995). Over the years, many phenotypic methods have been developed for the identification of streptococci in diagnostic laboratories. Biochemical characterization can be accomplished by traditional tube and plate

procedures as well as using commercial miniaturized systems (Eldar et al., 1999a; Vela et al., 2000; Ravelo et al., 2001). The API 32 Strep (bioMérieux), API 20 Strep system (bioMérieux), API zym system (bioMérieux), and other combinations of biochemical tests proved to be useful for a fast presumptive identification and phenotyping of some of the aetiological agents of streptococcosis (Weinstein et al., 1997; Ravelo et al., 2001). An inherent weakness of phenotypic methods is that there is variability in expression of phenotypic characteristics by isolates belonging to the same species (Bannerman et al., 1993; Ieven et al., 1995; Heikens et al., 2005). Furthermore, the interpretation of phenotypic tests can be subjective (Carretto et al., 2005). Variability in the expression and interpretation of phenotypic characteristics limits the reproducibility of tests, i.e. the ability to generate the same results every time the tests are used. In addition to reproducibility, the typeability and accuracy of phenotypic testing are imperfect. Typeability is the proportion of isolates that are assigned a type by a typing system (Struelens, 1996). An increase in the number of tests that is included in a system generally improves typeability. Phenotypic methods are usually considered less expensive than genotypic methods. Whether or not this is true depends in part on turnover, which affects overhead costs and opportunities and needs for automation. In some clinical laboratories, phenotypic tests are used with such high frequency that an investment in automation of reading and interpretation of tests is profitable (Ieven et al., 1995). In other laboratories, test frequency may be so low that expiration of reagents and the costs of replacing them are a concern. Regardless of test volume, additional testing may be needed to obtain final results from phenotypic methods. This increases cost and turn-around-time of phenotyping testing, thereby narrowing or eliminating the cost and time differences between phenotypic and genotypic identification methods (Ieven et al., 1995; Thorberg and Brändström, 2000). When comparing the cost of phenotypic and genotypic methods, the costs of obtaining inaccurate results must also be considered. Recently, molecular techniques such as ribotyping, RAPD and PFGE, have been usefully applied in epidemiological studies to study the heterogeneity within some of the aetiological agents of fish streptococcosis, and

eswardsiellosis. The simplicity and applicability of the RAPD technique have captivated many scientists interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question (Bardakci, 2001). Therefore we were selected RAPD fingerprints that one of the molecular techniques for genotyping to improve typeability. In this study, RAPD fingerprints of isolated bacterial strains were obtained with six primer, respectively. The RAPD analysis of the 7 strains of *S. iniae* revealed different DNA profiles were investigated six patterns with the primer p1, two patterns with the primer p3 and p5, monomorphic patterns with p2, p4, and p14, respectively. The case of 46 strains of *S. parauberis* revealed different DNA profiles were investigated nineteen patterns with the primer p2, nine patterns with the primer p1, five patterns with the primer p4, four patterns with the primer p3 and p5, three patterns with the primer p14, and 5 strains of *E. tarda* revealed conservative patterns, except for primer p3 that were investigated monomorphic DNA profiles. The primers in bacterial strains of *S. iniae*, *S. parauberis*, and *E. tarda* used generated a total of 42, 73, and 57 reproducible bands respectively. The relationships of each bacterial species between the each isolates were estimated using p-distance index of similarity and the dendrogram of isolated bacterial strains based on RAPD polymorphism by UPGMA. The dendrogram shows the differences among the *S. iniae* strains, which were characterized three genotype groups. However the dendrogram of *E. tarda* strains shows almost no variation among the *E. tard*a strains, which could be divided only two genotype groups. The dendrogram of *S. parauberis* strains shows the differences among the *S. parauberis* strains, which were characterized eight genotype groups. In addition to this study, there have been various genotyping studies of these pathogens and it was described above. With regard to *S. iniae*, the ribotyping allowed to differentiate the American and Israeli fish strains regardless of the host, demonstrating a lack of epidemiological links between infections in the two countries (Eldar et al., 1997). In the case of *S. paruberis* isolated from

turbot in Spain exhibited the same ribopattern; however, the RAPD analysis showed a higher discrimination power allowing differentiation of the isolates on the basis of their farm of origin (Romalde et al., 1999). In a report of *E. tarda*, the biochemical, serological and molecular characteristics of a group of 21 *E tarda* strains isolated from turbot, *Psetta maxima*, in two different areas of Europe were analyzed and compared with a total of 13 strains of this bacterial species with different geographical and host origins. In addition, RAPD analysis demonstrated that although the *E. tarda* strains from turbot were compiled in a unique group, two clonal lineages could be detected (Castro et al., 2006; Castro et al., 2010). For the virulence test, three strains of each species pathogens post-challenge by injection, including *S. iniae*, *S. parauberis*, and *E. tarda* were used to investigate virulence of the pathogens in olive flounder. The cumulative mortality rates of *S. iniae* strains JJU-019, JJU-073, and JJU-076 were 95%, 60%, and 75%, respectively, at 1.0×10^5 CFU/fish group of termination, and *E. tarda* strains JJU-032, JJU-052, and JJU-054 were 85%, 45%, and 100% at 1.0×10^4 CFU/ml group of termination. According to these results, the strains of *S. iniae* and *E. tarda* did not show a difference of virulence each other and they had very strong virulence. However, *S. parauberis* strains were demonstrated high variation each other strains such as JJU-008, JJU-045, and JJU-055. In the case of JJU-8, there was observed almost no mortality in challenged fish. Whereas the other strains such as JJU-045 and JJU-055 were demonstrated certain level of cumulative mortality that were demonstrated 100% and 55% in 1×10^7 CFU/fish dose group at termination, respectively. Among these strains to perform vaccine trials, we had been selected high virulence pathogens strains such as *S. iniae* JJU-019, *S. parauberis* JJU-045, and *E. tarda* JJU-054, respectively.

In conclusion, mentioned above results that general microbial, biochemical, phenotypic, and genotypic properties of three pathogenic species are very important in terms of fish pathological information and based research covered in part 2 and 3 studies such as loop-mediated amplification techniques and developing tri-valent vaccine.

Part Ⅱ. Evaluation of a loop-mediated isothermal amplification (LAMP) method for rapid detection of important bacterial pathogens *Streptococcus iniae*, *S. parauberis*, and *Edwardsiella tarda* in Olive Flounder, *Paralichthys olivaceus*

ABSTRACT

The development of rapid, accurate, and sensitive diagnostic methods for the identification of pathogens is fundamental for treating and controlling, or even eradicating of infectious disease. Classical pathogen detection is based on culture methodology and microscopy, which has the disadvantage such as slow growth and difficulty of selective cultivation. Therefore, the setting up of more rapid, sensitive, and accurate diagnostic methods has long been desired molecular diagnosis. However, PCR has several disadvantages, such as the requirement for expensive thermal cycling because it is not within purchasing reach of laboratories in developing countries and farming people. Aqua-farming laboratories are not usually equipped with diagnostic equipment, thus small-scale aquaculture operations depend on specialist fish disease laboratories for their diagnostic needs. However, laboratories routinely handling diagnostic cases are often very busy and take time to carry out diagnoses. In order for laboratories to handle large number of samples sensitive and rapid diagnostic kits are needed. The loop-mediated isothermal amplification (LAMP) has attracted method a lot of attention as a potentially rapid, accurate, and cost-effective novel molecular diagnosis tools. In this study, we develop a novel approach method of rapid molecular diagnosis known as LAMP that is gaining popularity among researchers due to its simple operation, rapid reaction, and easy detection in order to detect major fish pathogens efficiently. All the *S. iniae*, *S. parauberis*, and *E. tarda* isolates were selected five strains, respectively that were according to genotyped results on above by RAPD profiles. In order to design the species-

specific LAMP DNA oligonucleotide primers, we first searched the nucleic acid sequences of each three bacterial species such as *S. iniae*, *S. parauberis*, and *E. tarda* deposited in the GenBank database, and used the BLAST program to choose the species-specific gene sequences. Based on these results, we choose specific gene in each bacterial species which were LctO, AroK, and AroB gene used for species-specific LAMP reation in three bacterial species such as *S. iniae S. parauberis,* and *E. tarda*, respectively. These primers set of four primers composed of two outer primers which initiate strand displacement and two inner primers which structure "the loop" through the reaction. To determine the specificity of the primers of the LctO, AroK, and AroB gene, species-related strains and other fish bacterial pathogens were tested. We observed that the each primer only amplified the DNA of target species bacterial strains. These LAMP products were shown a typical ladder-like pattern on gel electrophoresis which indicated that stem-loop DNA with inverted repeats was formed. This result was indicated that the LAMP reaction was highly specific to *S. iniae, S. parauberis,* and *E. tarda* strains, respectively. To determine the sensitivity of three primer sets in LAMP detection of *S. iniae*, *S. parauberis*, and *E. tarda* respectively, which tested serial 10-fold dilutions. The detection limit of LAMP for *S. iniae* could be reached at 1.0×10^{-6} CFU/ml dilution level. With previous primers for detecting *S. iniae*, the detection limit of PCR could be also reached at 1.0×10^{-6} CFU/ml dilution level. Whereas two LAMP arrays for detecting *S. parauberis* and *E. tarda* were indicated detection limit that was higher than conventional PCR. First of these results, the detection limit of LAMP for *S. parauberis* could be reached at 1.0×10^{-3} CFU/ml dilution level, and conventional PCR primers for detecting *S. parauberis*, which could be reached at 1.0×10^{-5} CFU/ml dilution level. In case of the detection limit of LAMP for *E. tarda* that could be reached at 1.0×10^{-5} CFU/ml dilution level, and conventional PCR primers for detecting *E. tarda*, which could be also reached at 1.0×10^{-6} CFU/ml dilution level. These results revealed that the LAMP assay was 10-100 fold sensitive than the PCR assay in *E. tarda* and *S. parauberis*, respectively. Especially the results of LAMP for detecting *S. parauberis* was extremely higher than

conventional PCR consequently, the SpAroK primer set could be effectively use than other PCR-based diagnosis protocols in the fields of olive flounder aquaculture. The effect of temperature on the LAMP reaction in three primer sets such as SiLct, SpAroK, and EtFimB for species-specific detection of each bacterial species were determined that amplification of *S. iniae* was optimum at 60 °C and 65 °C with SiLctO primer set. In the case of *S. parauberis,* the amplification temperatures were optimum at from 55, 60, and 65 °C with SpAroK primer set that was indicated wide amplification temperature range. Finally, the amplification temperatures of LAMP reaction for *E. tarda* were optimum at 60 °C and 65 °C with EtAroB primer. We investigated the several LAMP reaction lengths from 0 to 100 min with three primer sets such as SiLct, SpAroK, and EtFimB for species-specific detection of each species bacteria. We observed that increasing the length of the LAMP reaction with SiLctO primer set increased, Amplification was faintly detected at 40 min, and initially detected typical pattern product clearly at 60 min to 100 min. The case of *S. parauberis* and *E. tarda* increasing the length of the LAMP reactions increased the target yield, and amplification of was initially detected at 40 min, and reached maximal at 60 min to 100 min. The effect of Mg^{++} concentrations ranging from 2 to 12 mM on the LAMP reaction was determined in LAMP reaction with three primer set such as SiLctO, SpAroK, and EtAroB respectively. LAMP reaction with SiLctO was observed at 4mM and 8mM. Whereas LAMP reaction with other primer sets such as SpAroK and EtAroB were indicated typical amplicons in the wide range of Mg^{++} concentrations, compared to amplified with SiLctO primers. The LAMP reactions detection range with SpAroK was between 4 mM and 12 mM and EtAroB was between 4 mM and 8mM, respectively. Consequently, Optimum $MgSO_4$ concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 4mM, 6mM, and 4mM, respectively. The LAMP reaction in the presence of different concentrations of betaine was tested with three primer sets such as SiLctO, SpAroK, and EtAroB. For these results, Optimum betaine concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 0.4 M, 0.8 M, and 0.2 M, respectively. The LAMP

reactions in the presence of different concentrations of deoxynucleotide triphosphate were amplified with three primer sets such as SiLctO, SpAroK, and EtAroB. In these results, LAMP reaction with SiLctO, and SpAroK primers, Optimum deoxynucleotide triphosphate concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 0.8 mM, 0.2 mM, and 0.2 mM, respectively. This chapter to investigate that the LAMP reactions in the presence of different concentrations of bst polymerase were amplified with three primer sets such as SiLctO, SpAroK, and EtAroB. In these results, Optimum bst polymerase concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 4 U, 8 U, and 4 U in 25 μl reaction volume, respectively.

MATERIALS AND METHODS

1. Bacterial strains and DNA preparation

The source of the isolation and the geographic origin of the *S. iniae*, *S. parauberis*, and *E. tarda* isolated strains, three reference strains, other *Streptococcus* species, *Edwardsiella* species, and other major fish pathogenic bacteria used in this study are indicated in Table 11. All bacterial strains were grown on optimal culture media, and incubate for 24~48h, at each optimal culture temperature, according to the bacterial characteristics. In addition, all the *S. iniae*, *S. parauberis*, and *E. tarda* isolates were selected five strains in each such as *S. iniae* strains JJU-019, JJU-073, JJU-075, JJU-076, and JJU-087; *S. parauberis* strains JJU-003, JJU-008, JJU-045, JJU-055, and JJU-064; *E. tarda* strains JJU-032, JJU-033, JJU-051, JJU-052, and JJU-054, respectively, which were according to genotyped results on above by RAPD profiles.

Bacterial genomic DNA used in LAMP analysis was extracted according to the following three methods such as boiling methods, Chelex extraction method, and commercial DNA extraction kit. The isolates were grown in BHIA supplemented with 1.5% NaCl. The colonies were picked and re-suspended in 500 µl of sterilized double distilled water; bacterial DNA was then extracted by boiling bacterial cells for 5 min and centrifuged at 6,000 g for 5 min. Bacterial DNA was collected on the upper aqueous phase of the supernatant. In other methods, Genomic DNA of isolates was prepared using commercial DNA extraction kit (Bioneer, USA) and Chelex DNA extraction which is fast, cheap, and effective method of DNA extraction following the instructions of the manufacturer. Purified DNA was dissolved in 100 µl of distilled water and then stored at −20°C until used for the experiment.

Table 11. Description of *S. iniae*, *S. parauberis*, *E. tarda*, three reference strains, and other bacterial strains used in this study. (ATCC = American Type Culture Collection; CIP, Collection of Institute Pasteur, Paris, France; DSM = Deutsche Sammlung von Microorganigmen; IFO = Institute for Fermentation, Osaka, Japan JCM = Japan Collection of Microorganisms)

Species	Strain	Source
***Streptococcus* spp.**		
Streptococcus iniae	ATCC 29178^T	Amazon dolphin, North America
	JJU-019	Olive flounder, Jeju
	JJU-073	Olive flounder, Jeju
	JJU-075	Olive flounder, Jeju
	JJU-076	Olive flounder, Jeju
	JJU-087	Olive flounder, Jeju
Streptococcus parauberis	DSM 6631^T	From mastitis sample milk
	JJU-003	Olive flounder, Jeju
	JJU-008	Olive flounder, Jeju
	JJU-045	Olive flounder, Jeju
	JJU-055	Olive flounder, Jeju
	JJU-064	Olive flounder, Jeju
Lactococcus garvieae	ATCC 43921^T	Bovine mastitis, United Kingdom
	ATCC 49156^T	From kidney of yellowtail, Japan
Streptococcus difficilis	CIP 103768^T	Tilapia brain, Israel
Streptococcus dysgalactiae	ATCC 12449	From vagina, United Kingdom
Streptococcus pyogenes	ATCC 12344^T	Scarlet fever
Streptococcus suis	ATCC 43765	Meningitis in piglet
***Edwardsiella* spp.**		
Edwardsiella tarda	ATCC 15947^T	Human, faeces
	JJU-032	Olive flounder, Jeju
	JJU-033	Olive flounder, Jeju
	JJU-051	Olive flounder, Jeju
	JJU-052	Olive flounder, Jeju
	JJU-054	Olive flounder, Jeju
Edwardsiella ictaluri	ATCC 33202^T	Channel catfish, USA
Edwardsiella hoshinae	JCM 1679	Atlantic puffin, *Fratercula arctica*
Other fish pathogen		
Flavobacterium branchiophilum	ATCC 35035^T	Yamame, *Oncorhynchus masou*, Japan
Flavobacterium columnare	ATCC 43622	Salmonid fish
Pseudomonas anguilliseptica	ATCC 33660	Japanese eel, Japan
Tenacibaculum maritimum	ATCC 43398	Red sea bream, Pagrus major, Japan
Vibrio anguillarum	ATCC 19264^T	From cod, *Gadus morhua*, Norway
Vibrio alginolyticus	ATCC 17749^T	Spoiled horse mackerel, causing food poisoning
Vibrio harveyi	ATCC 14126^T	luminescing amphipod, *Talorchestia* sp., USA
Vibrio ichthyoenteri	IFO 15847	Flounder, *Paralichthys olivaceus*, Japan
Vibrio ordalii	ATCC 33509	Coho salmon
Vibrio vulnificus	ATCC 27562^T	Human blood, USA
Yersinia ruckeri	ATCC 22908	Rainbow trout

2. Design of LAMP oligonucleotide primer

In order to design the species-specific LAMP DNA oligonucleotide primers, we first searched the nucleic acid sequences of each three bacterial species such as *S. iniae*, *S. parauberis*, and *E. tarda* deposited in the GenBank database, and used the BLAST program (Altschul et al., 1997) to choose the species-specific gene sequences. Based on these results, we choose specific gene in each bacterial species which were L-lactate oxidase gene, Shikimate kinase AroK gene, and 3-dehydroquinate synthase AroB gene used for species-specific LAMP reation in three bacterial species such as *S. iniae S. parauberis,* and *E. tarda*, respectively. The accession numbers of these sequences were JF795258.1, CP002471.1, and CP002154.1. These sequences were further analyzed by the Primer Explorer version3 (https://primerexplorer.jp/lamp3.0.0/index.html) to have the LAMP primer sets that were labeled SiLctO, SpAroK, and EtFimB respectively, these primers set of four primers composed of two outer primers which initiate strand displacement and two inner primers which structure “the loop” through the reaction: F3 (Forward outer primer), B3 (Backward outer primer), FIP (Forward inner primer) and BIP (Backward inner primer) (Table 12, See Ref. Notomi et al., 2000 for nomenclature of primers). In addition, each internal primer that recognizes both sense and anti-sense strands of the target DNA was connected by a TTTT spacer (Notomi et al., 2000). The FIP consisted of the complementary sequence of F1, a TTTT spacer and F2. The BIP consisted of B1c, a TTTT spacer and the complementary sequence of B2c. The outer primers consisted of the F3 and the B3 (the complementary sequence of B3c) (Figure 18). The positions of the LAMP primers used in this study are shown in Figure 19, 20, and 21. Depending on these design, the LAMP primers were synthesized commercially by oligo DNA synthesis services (Marcrogen, Korea).

Table 12. Sequence of LAMP primers used for specific amplification of three bacteria, *S. iniae*, *S. parauberis*, and *E. tarda*.

Bacterial specie	Primer set	Primer[a]	Target gene (GenBank accession no.)	Length	Sequence
S. iniae	SiLctO	F3	L-lactate oxidase (LctO) (JF795258.1)	22-mer	5'-CCTTTGGTTATATAGCAAGTGG-3'
		B3		18-mer	5'-ACTTGCGATTTCCCCTTG-3'
		FIP (F1C+TTTT+F2)		47-mer (F1C: 21-mer, F2: 22-mer)	5'-CGCCTTTTAATCCGTGAGGAA-TTTT-GAGATACCTTCACCTTACATGA-3'
		BIP (B1C+TTTT+B2)		44-mer (B1C: 22-mer, B2: 18-mer)	5'-ATGGCGATAAATTAGCGTCACC-TTTT-ATTGGCTAATTTGTGCGC-3'
S. parauberis	SpAroK	F3	Shikimate kinase AroK (CP002471.1)	23-mer	5'-GCTTTTAAAGATTGAGGACCATT-3'
		B3		22-mer	5'-TTGTTTATTGAGGAAGTAGCAG-3'
		FIP (F1C+TTTT+F2)		48-mer (F1C: 24-mer, F2: 20-mer)	5'- ACCACACAGAAGAAATGATTCGTC-TTTT-GGTTATTTCCTTACCCTGCA-3'
		BIP (B1C+TTTT+B2)		48-mer (B1C: 20-mer, B2: 24-mer)	5'- TGTCCCTTGGCCTGAATAGC-TTTT-CGATTACTTATCATCTTCCGATAG-3'
E.tarda	EtAroB	F3	3-dehydroquinate synthase AroB (CP002154.1)	19-mer	5'-ACGCACCGATCATGTTCTT-3'
		B3		16-mer	5'-GCGCGCTGCTGGAGAA-3'
		FIP (F1C+TTTT+F2)		42-mer (F1C: 19-mer, F2: 19-mer)	5'-CGACAACGCTGTTGGCGCA-TTTT-ATGATTGACCGCGGTCTTC-3'
		BIP (B1C+TTTT+B2)		43-mer (B1C: 21-mer, B2: 18-mer)	5'-TGATAGCATGCGGCGGCGAAA-TTTT-CCACGGGCGAGATACCAC-3'

[a] For nomenclature see Ref. Notomi et al., 2000.

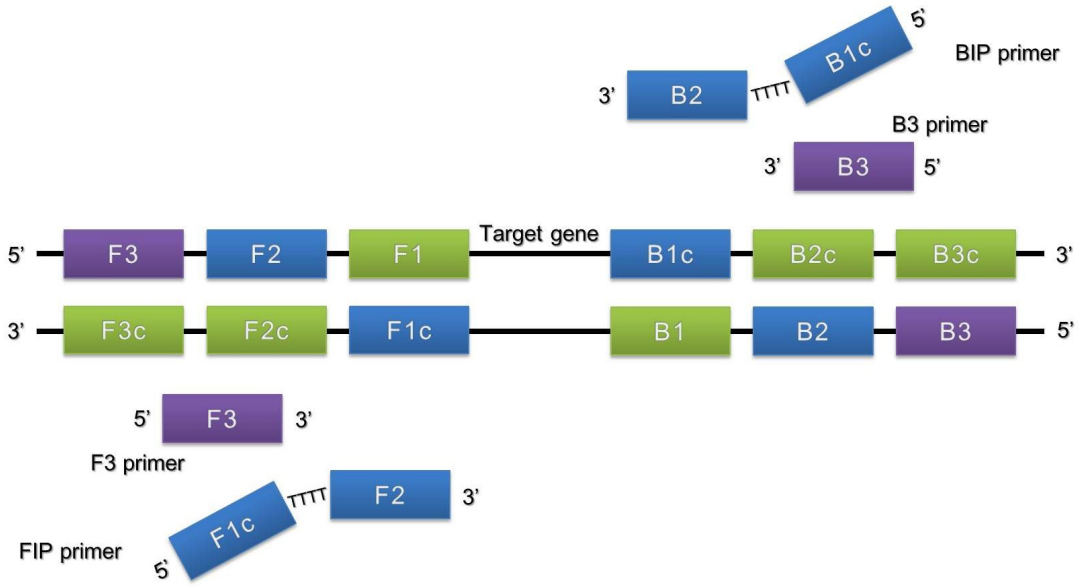

Figure 18. Schematic representation of primers used for loop-mediated isothermal amplification. Two inner primers [forward inner primer (FIP) and backward inner primer (BIP)] and two outer primers (F3 and B3) were designed to amplify six regions of target gene.

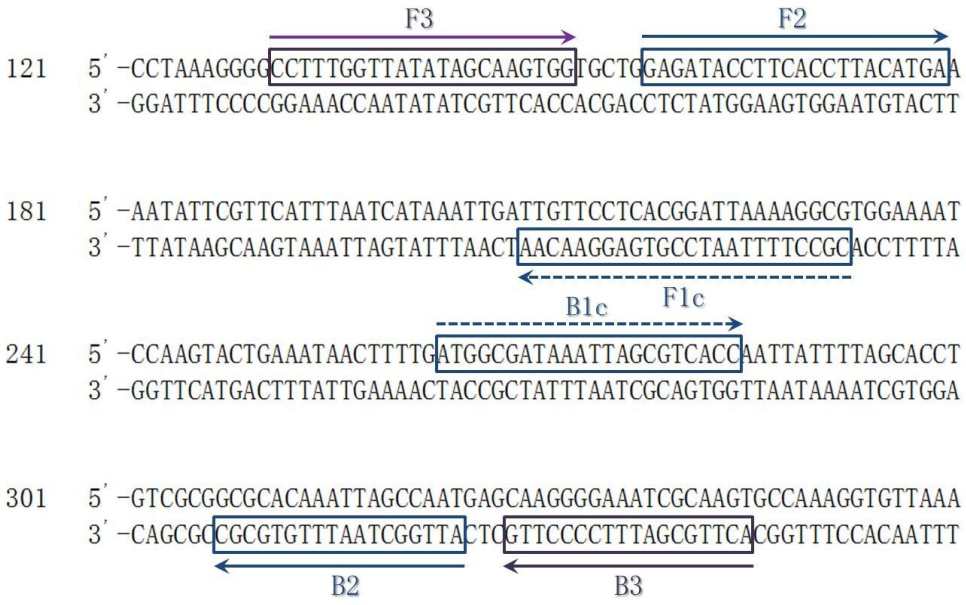

Figure 19. The partial nucleotide sequence of L-lactate oxidase (LctO) gene of *S. iniae* (GenBank accession number JF795258.1) used for the LAMP primer design. Nucleotide sequences used for primer design are indicated by boxes and arrows.

Figure 20. The partial nucleotide sequence of Shikimate kinase AroK gene of *S. parauberis* (GenBank accession number CP002471.1) used for the LAMP primer design. Nucleotide sequences used for primer design are indicated by boxes and arrows.

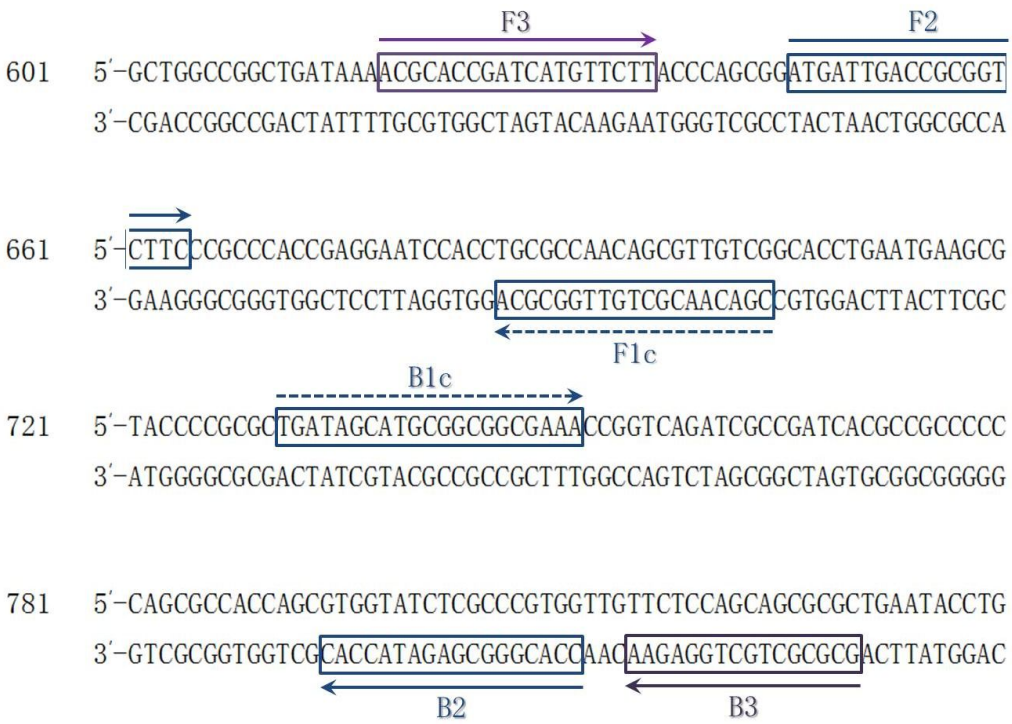

Figure 21. The partial nucleotide sequence of 3-dehydroquinate synthase AroB gene of *E. tarda* (GenBank accession number CP002154.1) used for the LAMP primer design. Nucleotide sequences used for primer design are indicated by boxes and arrows.

3. LAMP reaction

The LAMP reaction was carried out according to the method described by Notomi et al. (2000), in a 25 μl reaction mixture containing the following reagents with final concentrations: 1 × reaction mix (20mM Tris-HCl (pH 8.8), 10mM $(NH_4)_2SO_4$, 10mM KCl, 2mM $MgSO_4$, 0.4% Triton X-100) with 6 mM $MgSO_4$ (New England Biolabs, Beverly, MA), 0.8 M betaine (Sigma-Aldrich Corporation, USA), 400 μM dNTP (TaKaRa Biotechnology Co., Ltd), 0.2 μM each of F3 and B3 primers, 0.8 μM each of FIP and BIP, and appropriate amount of template genomic DNA or distilled water (negative control). In a heat block, the reaction mixture was heated at 95 ℃ for 5min, then cooled on ice. Finally, 8U Bst DNA polymerase large fragment (New England Biolabs, Beverly, MA) was added. Subsequently, the mixture was incubated at 65 ℃ for 60 min and then heated at 80 ℃ for 10 min to terminate the reaction (Notomi et al., 2000). A negative control (no template DNA) and a positive control of reference strains obtained from Korean collection for type culture (KCTC) were including in the LAMP reaction. After the reaction, 1 ml of 10-fold diluted original SYBR Green I (Molecular Probes Inc.) was added to 25 μl LAMP products. The solution turned green in the presence of LAMP amplification products, while it remained orange in the absence of amplicon. For further confirming the presence of amplicons, Amplified LAMP products (3 μl /well) were electrophoresed in 1.5% agarose gel in 1x TAE (Tris-acetate-EDTA) buffer (Bioneer, USA). Gels were stained with ethidium bromide (0.5 μg/ml), visualized and photographed under UV illumination.

4. Specificity of LAMP reaction

To determine the specificity of LAMP method to *S. iniae*, LAMP was carried out with the different DNA templates from the total 26 bacterial strains: *S. iniae* ATCC 29178^T, 5 isolated strains of *S. iniae*, 4 strains of *S. parauberis*, 2 strains of *L. garvieae*, *S. difficilis*, *S dysgalactiae*, *S. pyogenes*, *S. suis*, and other fish pathogens such as 4 strain of *E. tarda*, *F. columnare*, *P. anguilliseptica*, *T. maritimum*, *V. anguillarum*, *V. harveyi*, *V. ichthyoenteri*, and

Y. ruckeri (Table 11). In case of *S. parauberis*, LAMP was carried out with the different DNA templates from the total 26 bacterial strains: *S. parauberis* DSM 6631^T, 5 isolated strains of *S. parauberis*, 4 strains of *S. iniae*, 2 strains of *L. garvieae*, *S. difficilis*, *S dysgalactiae*, *S. pyogenes*, *S. suis*, and other fish pathogens such as 4 strain of *E. tarda*, *F. columnare*, *P. anguilliseptica*, *T. maritimum*, *V. anguillarum*, *V. harveyi*, *V. ichthyoenteri*, and *Y. ruckeri* (Table 11) to determine the specificity of LAMP method. For *E. tarda*, LAMP was carried out with the different DNA templates from the total 26 bacterial strains: 8 strains in the *Edwardsiella* family such as *E. tarda* ATCC 15947^T, 5 isolated strains of *E. tarda*, *E. ictaluri*, *E. hoshinae* and other fish pathogens such as 4 strains of *S. iniae*, 4 strains of *S. parauberis*, *F. branchiophilum*, *F. columnare*, *P. anguilliseptica*, *T. maritimum*, *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. ichthyoenteri, V. ordalii*, *V. vulnificus*, and *Y. ruckeri* (Table 11) to determine the specificity of LAMP method, under the experimental conditions described above. Each strain was examined at least twice.

5. Sensitivity of LAMP reaction

In order to test the sensitivity of each primer sets in LAMP identification of three bacterial species *S. iniae*, *S. parauberis*, and *E. tarda*, DNA templates were respectively extracted from *S. iniae* ATCC 29178^T, *S. parauberis* DSM 6631, and *E. tarda* ATCC 15947^T. In order to prepare of DNA extraction, each bacteria were adjusted to 1×10^8 CFU/ml bacterial cell concentration, and the bacterial cell suspensions were used to make eight 10-fold serial standard dilutions. These serial dilutions were extracted DNA templates according to boiling method that mentioned above. To assess the detection sensitivity of the LAMP protocol and conventional PCR, eight DNA templates as 10-fold serial dilution samples were used. A 10-fold diluted DNA templates were used for LAMP using pre-determined conditions according to mentioned above. After the reaction, LAMP products were electrophoresed on 1.5% agarose gel in 1x TAE buffer (Bioneer, USA), that were stained with ethidium bromide (0.5 μg/ml), visualized and photographed under UV illumination.

6. Sensitivity of conventional PCR

Three pair of primers (Table 1) was used to amplify 870, 718, and 268 bp amplicons of the each bacterial genome such as *S. iniae*, *S. parauberis*, and *E. tarda*, respectively (Mata et al., 2004; Sakai et al., 2009).

The Conventional PCR was performed in 20 µl reaction mixtures containing DNA template that mentioned above, a 0.05 µM concentration each primer (Bioneer, USA) and AccuPower PCR Premix (1 U Taq DNA polymerase, 250 µM dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye; Bioneer, USA). The amplifications were carried out in a thermocyclers (My gein 32; MJ Research, USA) with the following parameters: an initial denaturation step of 94 °C, 5 min; 30 serial cycles of a denaturation step of 94 °C, 30 sec, annealing at 50 °C, 30 sec, and extension at 72 °C, 1 min; and a final extension step of 72 °C, 5 min. The PCR amplicons were analyzed by 1.5% agarose gel electrophoresis in 1x TAE (Tris-acetate-EDTA) buffer (Bioneer, USA). Gels were stained with ethidium bromide (0.5 µg/ml), visualized and photographed under UV illumination.

7. Optimization of LAMP reaction conditions

Optimization of LAMP conditions for three bacterial species were determined by amplifying DNA that DNA were respectively extracted from *S. iniae* ATCC 29178^T, *S. parauberis* DSM 6631, and *E. tarda* ATCC 15947^T. The LAMP reaction was carried out in a 25- µl reaction mixture containing the following reagents with final concentrations: 1 × reaction mix (20mM Tris-HCl (pH 8.8), 10mM $(NH_4)_2SO_4$, 10mM KCl, 2mM $MgSO_4$, 0.4% Triton X-100) with 6 mM $MgSO_4$ (New England Biolabs, Beverly, MA), 0.8 M betaine (Sigma-Aldrich Corporation, USA), 400 µM dNTP (TaKaRa Biotechnology Co., Ltd), 0.2 µM each of F3 and B3 primers, 0.8 µM each of FIP and BIP, and appropriate amount of template genomic DNA or distilled water (negative control), and different concentrations of MgSO4 (2, 4, 6 and 10 mM). In a heat block, the reaction mixture was heated at 95 ℃ for

5min, then cooled on ice. Finally, 8U Bst DNA polymerase large fragment (New England Biolabs, Beverly, MA) was added. Subsequently, the mixture was incubated at 65℃ for 60 min and then heated at 80℃ for 10min to terminate the reaction (Notomi et al., 2000).

The reaction temperature and time were optimized to facilitate specific and rapid amplification of three major bacterial pathogens in olive flounder. The reaction temperature was optimized at 45, 55, 60, 65, and 70 °C for 60 min in a block heater and then heated at 80 °C for 10 min to terminate the reaction. The reaction mixture of these LAMP assay with the reaction mixture were amplified for different periods of times 20, 40, 60, 80, and 100 min to determine the shortest required amplification time at a predetermined temperature (65 °C).

Optimum quantity of each ingredient to be added was also standardized. The following changes were attempted in the reaction to optimize the quantity of whitish precipitate obtained. Optimization of (i) $MgSO_4$ concentration-different concentrations of $MgSO_4$, viz., 0, 2, 4, 6, and 10 mM (including the 2 mM $MgSO_4$ in the buffer) were tried; (ii) betaine concentration-different concentrations of betaine viz., 0, 0.2, 0.4, 0.6, 0.8, and 1.0 M were tried; (iii) dNTP concentration-different concentrations of dNTP viz., 0, 0.2, 0.4, 0.8, and 1.0 mM were tried; (iv) concentration of the Bst DNA polymerase-three concentrations, viz., 4 U, 8U, and 12 U were attempted.

RESULTS

1. Specificity of LctO gene in LAMP detection of *S. iniae*

To determine the specificity of the primers for *S. iniae* L-lactate oxidase (LctO) gene, *Streptococcus* spp. 7 species and other fish bacterial pathogens 8 species of bacteria such as, *S. iniae*, *S. parauberis*, *L. garvieae*, *S. difficilis*, *S. dysgalactiae*, *S. pyogenes*, *S. suis*, *E. tarda*, *F. columnare*, *P. anguilliseptica*, *T. maritimum*, *V. anguillarum*, *V. harveyi*, *V. ichthyoenteri*, and *Y. ruckeri*, which are probably associated with olive flounder were tested. As shown in Figure 22, we observed that the primers only amplified the *S. iniae* LctO gene (Figure 22, lanes 1–6), showing a typical ladder-like pattern on gel electrophoresis which indicated that stem-loop DNA with inverted repeats was formed (Notomi et al., 2000). Whereas LAMP reaction was negative for all of other bacterial species (Figure 22, lanes 7–14 and 17-29), and distilled water that was used as negative controls (Figure 22, lanes 15 and 30). This result was indicated that the LAMP reaction was highly specific to *S. iniae* strains.

2. Specificity of AroK gene in LAMP detection of *S. parauberis*

To determine the specificity of the primers of the *S. parauberis* Shikimate kinase (AroK) gene, *Streptococcus* spp. 7 species and other fish bacterial pathogens 8 species of bacteria such as, *S. parauberis*, *S. iniae*, *L. garvieae*, *L. garvieae*, *S. difficilis*, *S. dysgalactiae*, *S. pyogenes*, *S. suis*, *E. tarda*, *F. columnare*, *P. anguilliseptica*, *T. maritimum*, *V. anguillarum*, *V. harveyi*, *V. ichthyoenteri*, and *Y. ruckeri*, which are probably associated with olive flounder were tested. As shown in Figure 23, we observed that the primers only amplified the *S. parauberis* AroK gene (Figure 23, lanes 1–6), showing a typical ladder-like pattern on gel electrophoresis which indicated that stem-loop DNA with inverted repeats was formed (Notomi et al., 2000). Whereas LAMP reaction was negative for all of other bacterial species (Figure 23, lanes 7–14 and 17-29), and distilled water that was used as negative controls

(Figure 23, lanes 15 and 30). This result was indicated that the LAMP reaction was highly specific to *S. parauberis* strains.

3. Specificity of AroB gene in LAMP detection of *E. tarda*

To determine the specificity of the primers of the *E. tarda* 3-dehydroquinate synthase AroB gene, *Edwardsiella* spp. 3 species and other fish bacterial pathogens 13 species of bacteria such as, *E. tarda, E. ictaluri, E. hoshinae, S. iniae*, *S. parauberis*, *F. branchiophilum*, *F. columnare*, *P. anguilliseptica*, *T. maritimum*, *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. ichthyoenteri*, *V. ordalii*, *V. vulnificus*, and *Y. ruckeri*, which are probably associated with olive flounder were tested. As shown in Figure 23, we observed that the primers only amplified the *E. tarda* AroB gene (Figure 24, lanes 1–6), showing a typical ladder-like pattern on gel electrophoresis which indicated that stem-loop DNA with inverted repeats was formed (Notomi et al., 2000). Whereas LAMP reaction was negative for all of other bacterial species (Figure 24, lanes 7–14 and 17-29), and distilled water that was used as negative controls (Figure 24, lanes 15 and 30). This result was indicated that the LAMP reaction was highly specific to *S. parauberis* strains.

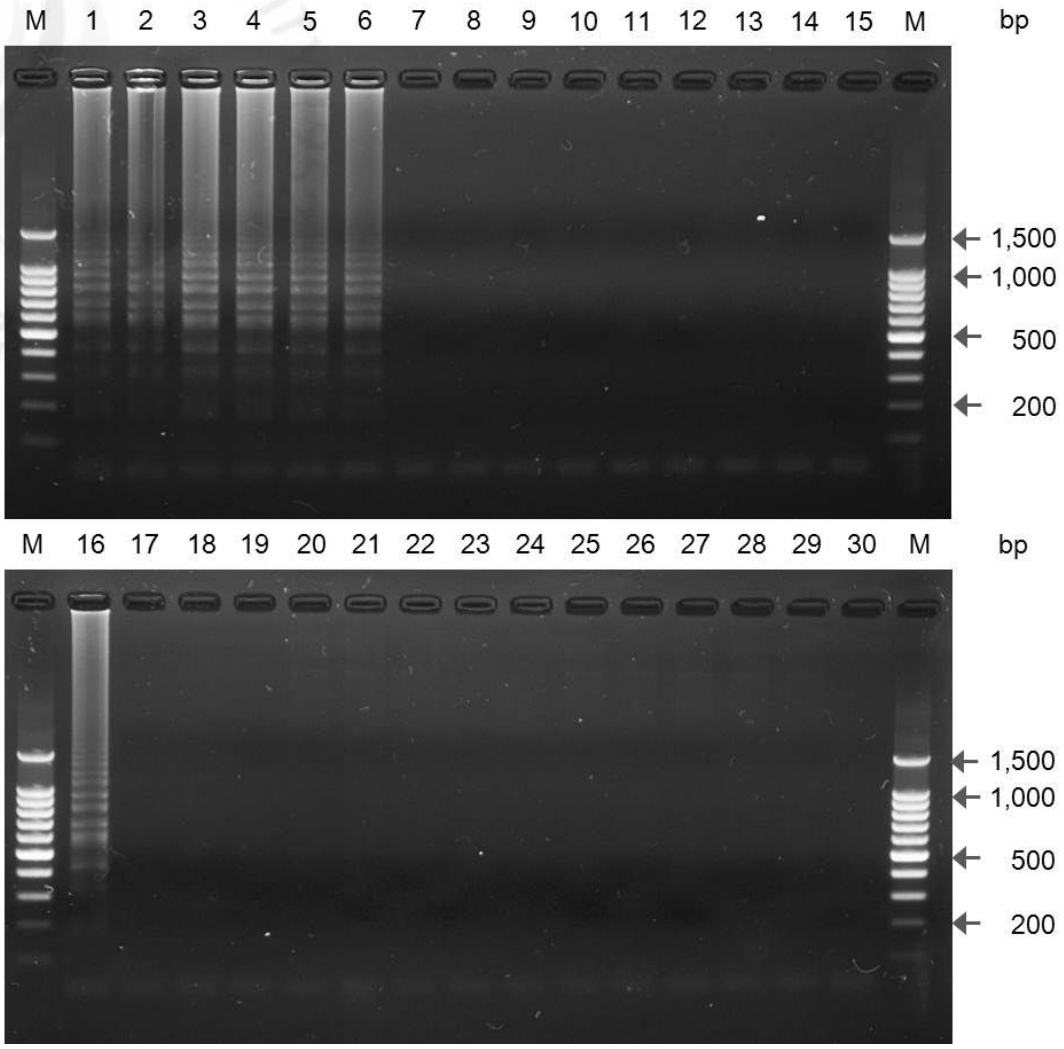

Figure 22. Specificity of LctO gene for the LAMP detection of *S. iniae*. Samples were electrophoresed on 1.5% agarose gels. LAMP was carried out with the different sources of DNA template from the 27 strains. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); 1 and 16, *S. iniae* ATCC 29175^{T}; 2, *S. iniae* JJU-019; 3, *S. iniae* JJU-073; 4, *S. iniae* JJU-075; 5, *S. iniae* JJU-076; 6, *S. iniae* JJU-087; 7, *S. parauberis* DSM 6631 ^{T}; 8, *S. parauberis* JJU-008; 9, *S. parauberis* JJU-045; 10, *S. parauberis* JJU-055; 11, *Lactococcus garvieae* ATCC 43921 ^{T}; 12, *L. garvieae* ATCC 49156 ^{T}; 13, *S. difficilis* CIP 103768 ^{T}; 14, *S. dysgalactiae* ATCC 12449 ^{T}; 17, *S. pyogenes* ATCC 12344 ^{T}; 18, *S. suis* ATCC 43765; 19, *E. tarda* ATCC 15947 ^{T}; 20, *E. tarda* JJU-032; 21, *E. tarda* JJU-052; 22, *E. tarda* JJU-054; 23, *Flavobacterium columnare* ATCC 43622; 24, *Pseudomonas anguilliseptica* ATCC 33660; 25, *Tenacibaculum maritimum* ATCC 43398; 26, *Vibrio anguillarum* ATCC 19264 ^{T}; 27, *V. harveyi* ATCC 14126 ^{T}; 28, *V. ichthyoenteri* IFO 15847; 29, *Yersinia ruckeri* ATCC 22908; 15 and 30 distilled water.

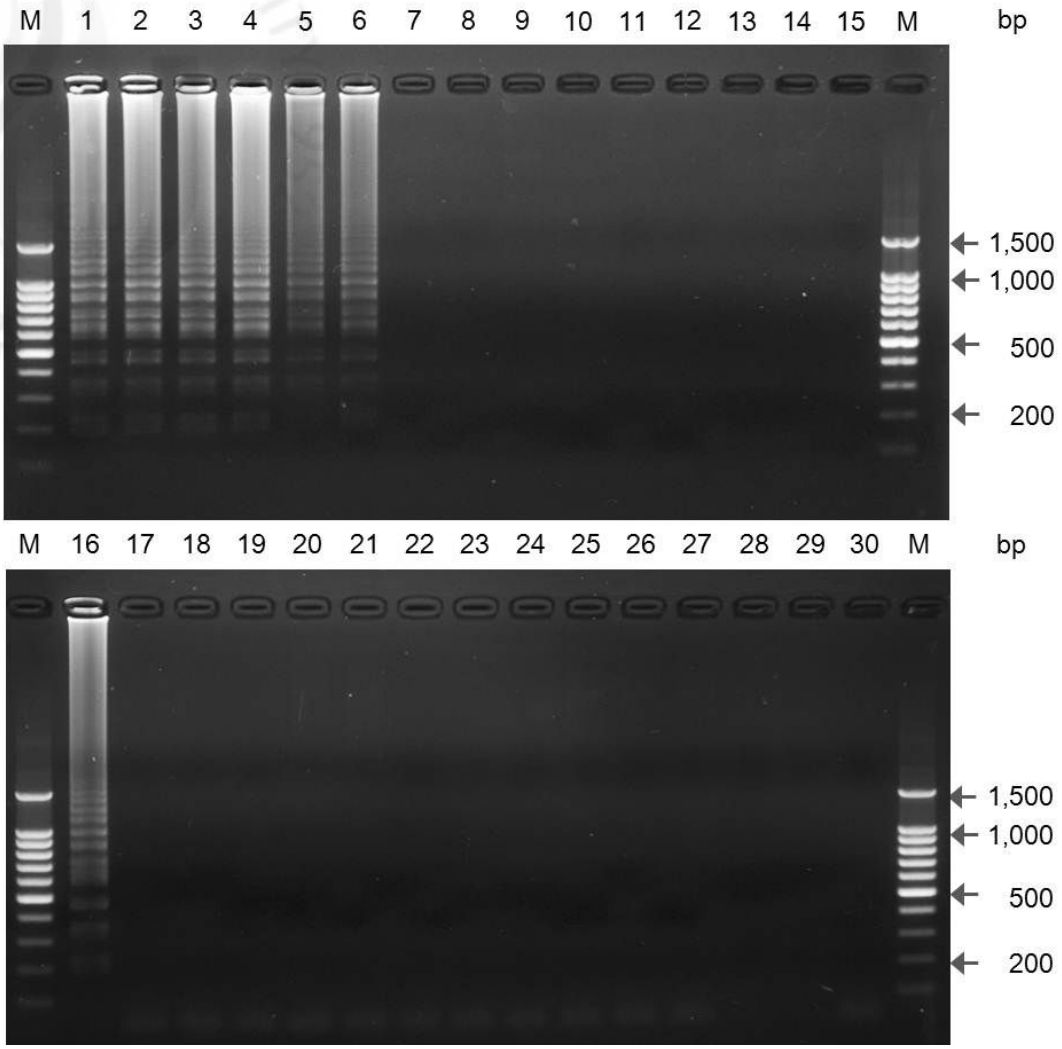

Figure 23. Specificity of AroK gene for the LAMP detection of *S. parauberis*. Samples were electrophoresed on 1.5% agarose gels. LAMP was carried out with the different sources of DNA template from the 27 strains. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); 1 and 16, *S. parauberis* DSM 6631^{T}; 2, *S. parauberis* JJU-003; 3, *S. parauberis* JJU-008; 4, *S. parauberis* JJU-045; 5, *S. parauberis* JJU-055; 6, *S. parauberis* JJU-064; 7, *S. iniae* ATCC 29175^{T}; 8, *S. iniae* JJU-019; 9, *S. iniae* JJU-073; 10, *S. iniae* JJU-076; 11, *Lactococcus garvieae* ATCC 43921^{T}; 12, *L. garvieae* ATCC 49156^{T}; 13, *S. difficilis* CIP 103768^{T}; 14, *S. dysgalactiae* ATCC 12449^{T}; 17, *S. pyogenes* ATCC 12344^{T}; 18, *S. suis* ATCC 43765; 19, *E. tarda* ATCC 15947^{T}; 20, *E. tarda* JJU-032; 21, *E. tarda* JJU-052; 22, *E. tarda* JJU-054; 23, *Flavobacterium columnare* ATCC 43622; 24, *Pseudomonas anguilliseptica* ATCC 33660; 25, *Tenacibaculum maritimum* ATCC 43398; 26, *Vibrio anguillarum* ATCC 19264^{T}; 27, *V. harveyi* ATCC 14126^{T}; 28, *V. ichthyoenteri* IFO 15847; 29, *Yersinia ruckeri* ATCC 22908; 15 and 30 distilled water.

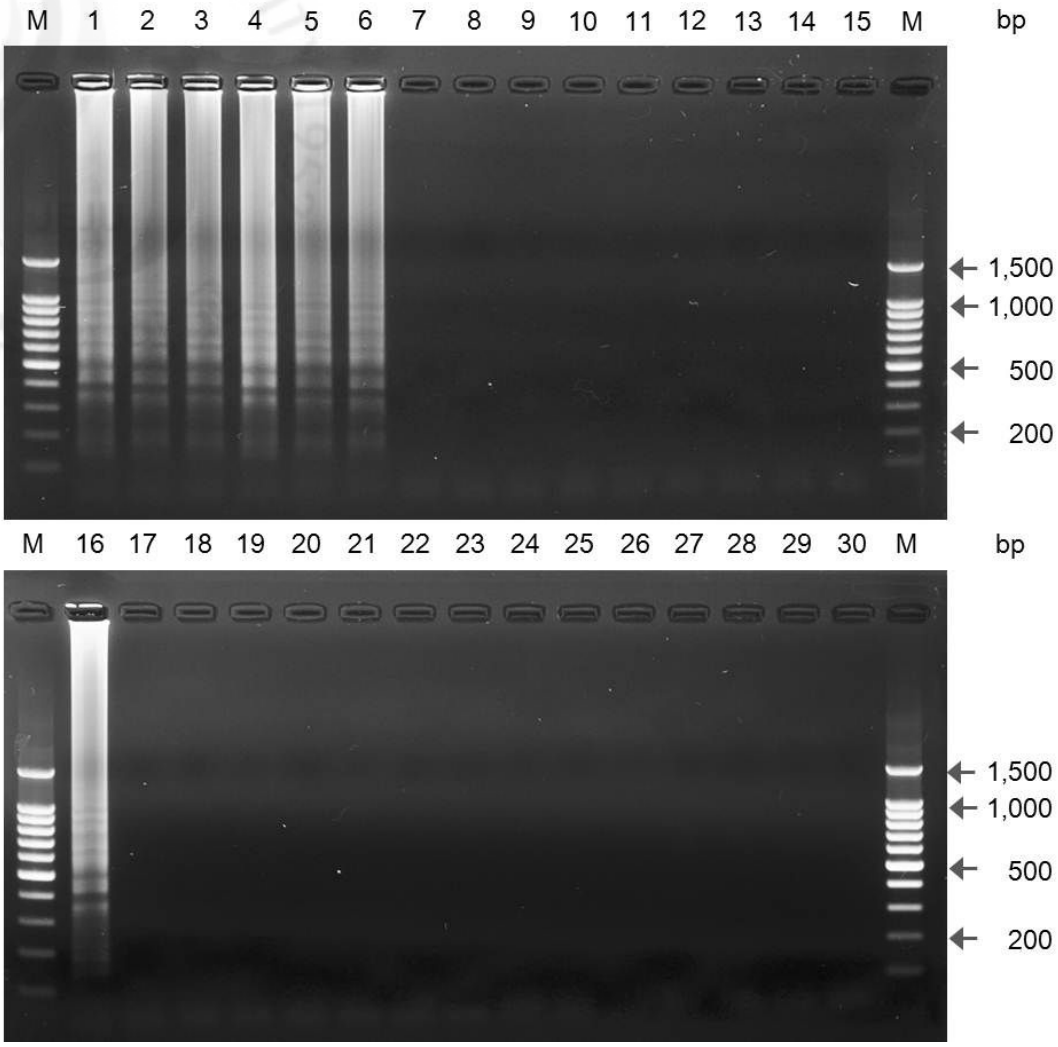

Figure 24. Specificity of AroB gene for the LAMP detection of *E. tarda*. Samples were electrophoresed on 1.5% agarose gels. LAMP was carried out with the different sources of DNA template from the 27 strains. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); 1 and 16, *E. tarda* ATCC 15947^{T}; 2, *E. tarda* JJU-032; 3, *E. tarda* JJU-033; 4, *E. tarda* JJU-051; 5, *E. tarda* JJU-052; 6, *E. tarda* JJU-054; 7, *E. ictaluri* ATCC 33202^{T}; 8, *E. hoshinae* JCM 1679; 9, *S. iniae* ATCC 29178 ^{T}; 10, *S. iniae* JJU-019; 11, *S. iniae* JJU-073; 12, *S. iniae* JJU-076; 13, *S. parauberis* DSM 6631^{T}; 14, *S. parauberise* JJU-008; 17, *S. parauberise* JJU-045; 18, *S. parauberise* JJU-055; 19, *Flavobacterium branchiophilum* ATCC 35035^{T}; 20, *F. columnare* ATCC 43622; 21, *Pseudomonas anguilliseptica* ATCC 33660; 22, *Tenacibaculum maritimum* ATCC 43398; 23, *Vibrio anguillarum* ATCC 19264^{T}; 24, V. alginolyticus ATCC 17749^{T}; 25, *V. harveyi* ATCC 14126^{T}; 26, *V. ichthyoenteri* IFO 15847; 27, *V. ordalii* ATCC 33509; 28, *V. vulnificus* ATCC 27562 ^{T}; 29, *Yersinia ruckeri* ATCC 22908; 15 and 30 distilled water.

4. Sensitivity of LAMP identification

To determine the sensitivity of three primer sets in LAMP detection of *S. iniae*, *S. parauberis*, and *E. tarda* respectively, we tested serial 10-fold dilutions of the pathogen that were previously quantitated by direct plating. As shown in Figure 25A, the detection limit of LAMP for *S. iniae* could be reached at 1.0×10^{-6} CFU/ml dilution level. With previous primers for detecting *S. iniae* (LOX-1 / LOX-2 Ref. Mata et al., 2004), the detection limit of PCR could be also reached at 1.0×10^{-6} CFU/ml dilution level (Figure 25B). Whereas two LAMP arrays for detecting *S. parauberis* and *E. tarda* were indicated detection limit that was higher than conventional PCR. First of these results, the detection limit of LAMP for *S. parauberis* could be reached at 1.0×10^{-3} CFU/ml dilution level as shown in Figure 26A. Whereas with conventional PCR primers for detecting *S. parauberis* (Spa2152 / Spa2870 Ref. Mata et al., 2004), the detection limit of PCR could be reached at 1.0×10^{-5} CFU/ml dilution level (Figure 26B). In case of the detection limit of LAMP for *E. tarda* that could be reached at 1.0×10^{-5} CFU/ml dilution level as shown in Figure 27A. Whereas with conventional PCR primers for detecting *E. tarda* (EDtT-F / EDtT-R Ref. Sakai et al., 2009), the detection limit of PCR could be also reached at 1.0×10^{-6} CFU/ml dilution level (Figure 27B). These results revealed that the LAMP assay was 10-100 fold sensitive than the PCR assay in *E. tarda* and *S. parauberis*, respectively. Especially the results of LAMP for detecting *S. parauberis* was extremely higher than conventional PCR consequently, the SpAroK primer set could be effectively use than other PCR-based diagnosis protocols in the fields of olive flounder aquaculture.

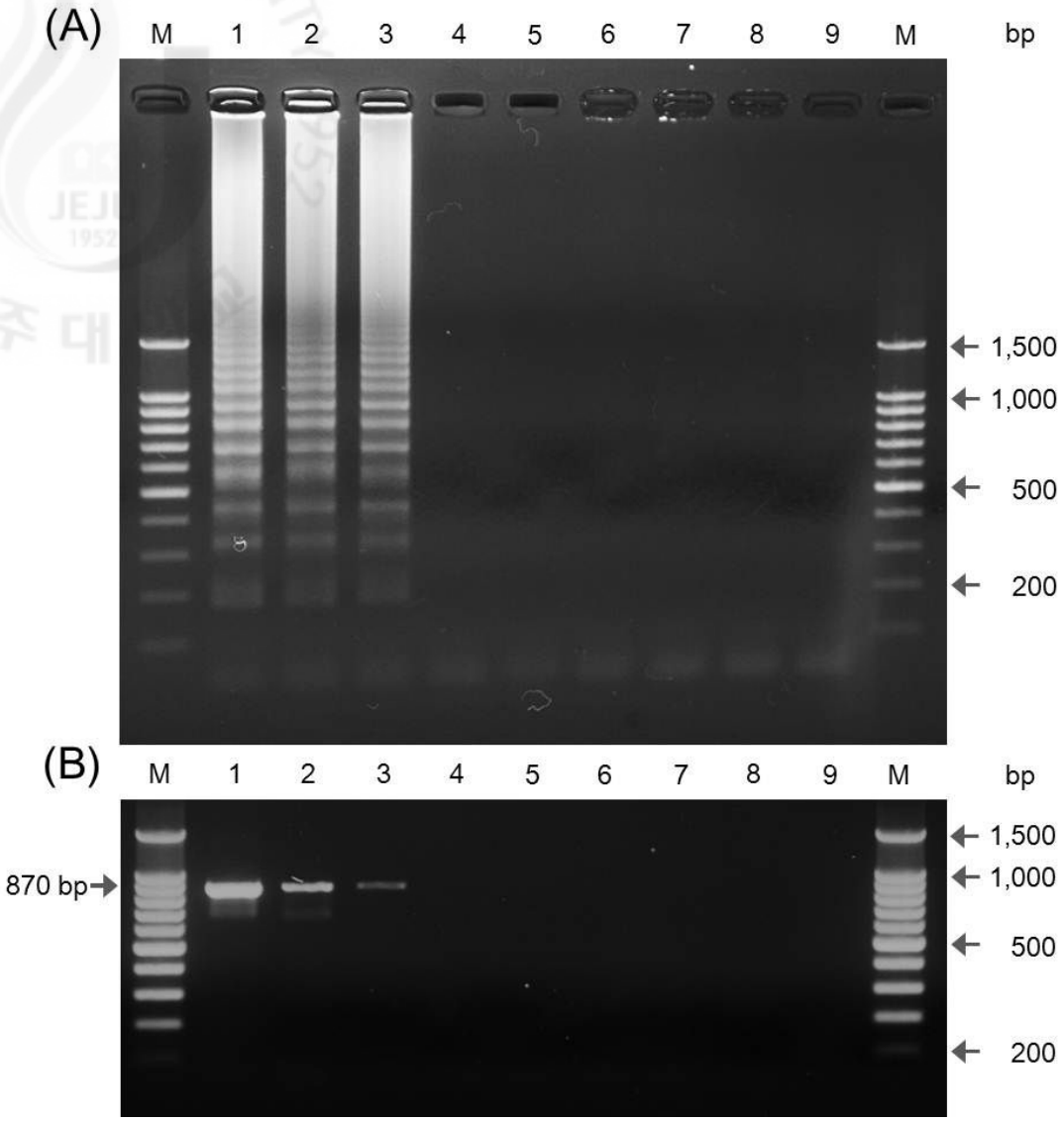

Figure 25. Sensitivity of *S. iniae* identification by LAMP and conventional PCR. Each sample was electrophoresed on a 1.5% agarose gel. (A) LAMP products. A product was seen typical ladder-like pattern on gel electrophoresis; (B) conventional PCR products using primers LOX-1 and LOX-2. A band of 870 bp was seen with positive samples. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); Lanes 1–8, amplification products using 10-fold serial dilutions of template DNA, which extracted variation of cell concentrations (1.0×10^8 CFU/ml, 1.0×10^7 CFU/ml, 1.0×10^6 CFU/ml, 1.0×10^5 CFU/ml, 1.0×10^4 CFU/ml, 1.0×10^3 CFU/ml, 1.0×10^2 CFU/ml, and 1.0×10^1 CFU/ml, respectively); Lane 9, distilled water.

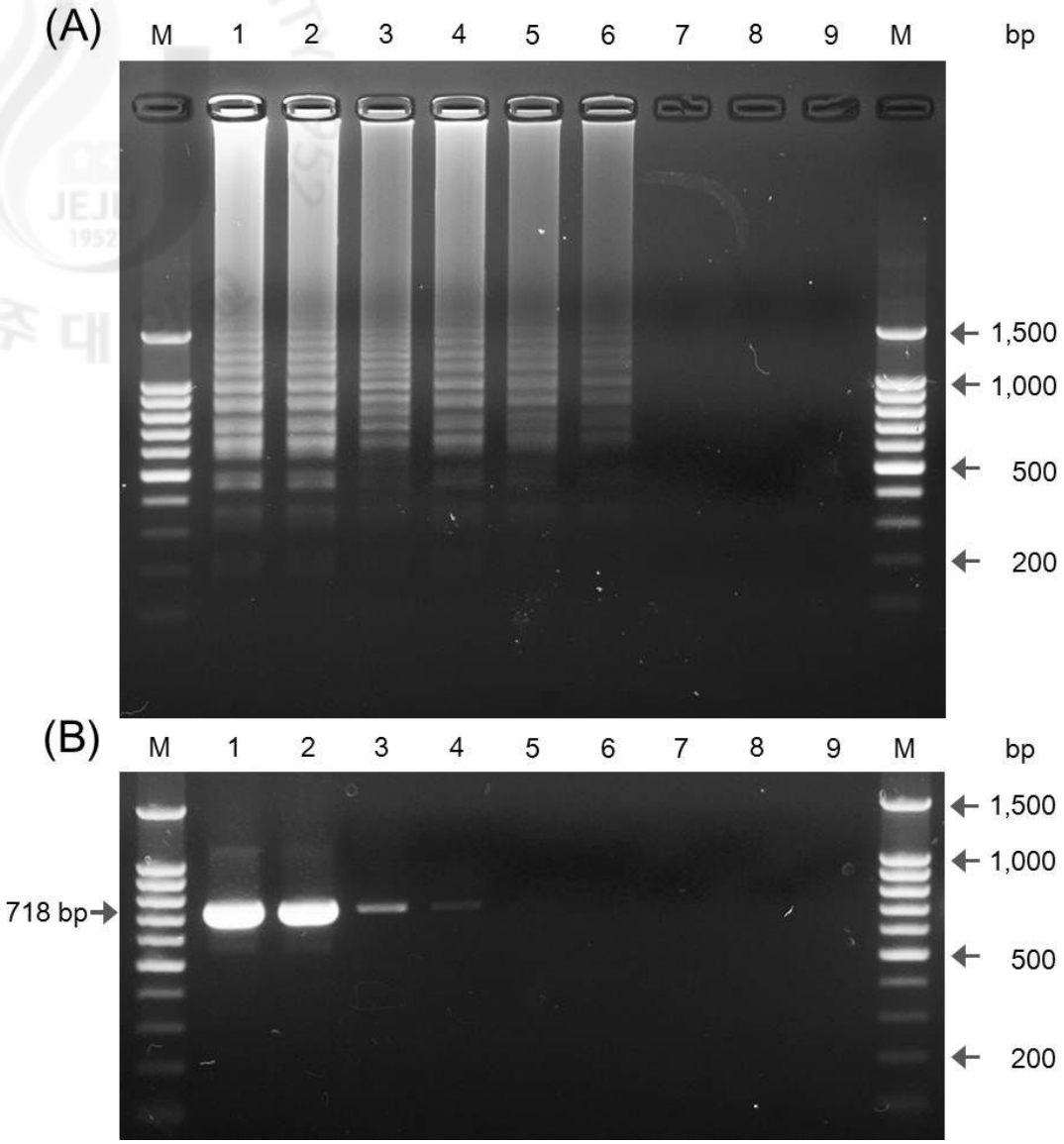

Figure 26. Sensitivity of *S. parauberis* identification by LAMP and conventional PCR. Each sample was electrophoresed on a 1.5% agarose gel. (A) LAMP products. A product was seen typical ladder-like pattern on gel electrophoresis; (B) conventional PCR products using primers Spa2152 and Spa2870. A band of 718 bp was seen with positive samples. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); Lanes 1–8, amplification products using 10-fold serial dilutions of template DNA, which extracted variation of cell concentrations (1.0×10^8 CFU/ml, 1.0×10^7 CFU/ml, 1.0×10^6 CFU/ml, 1.0×10^5 CFU/ml, 1.0×10^4 CFU/ml, 1.0×10^3 CFU/ml, 1.0×10^2 CFU/ml, and 1.0×10^1 CFU/ml, respectively); Lane 9, distilled water.

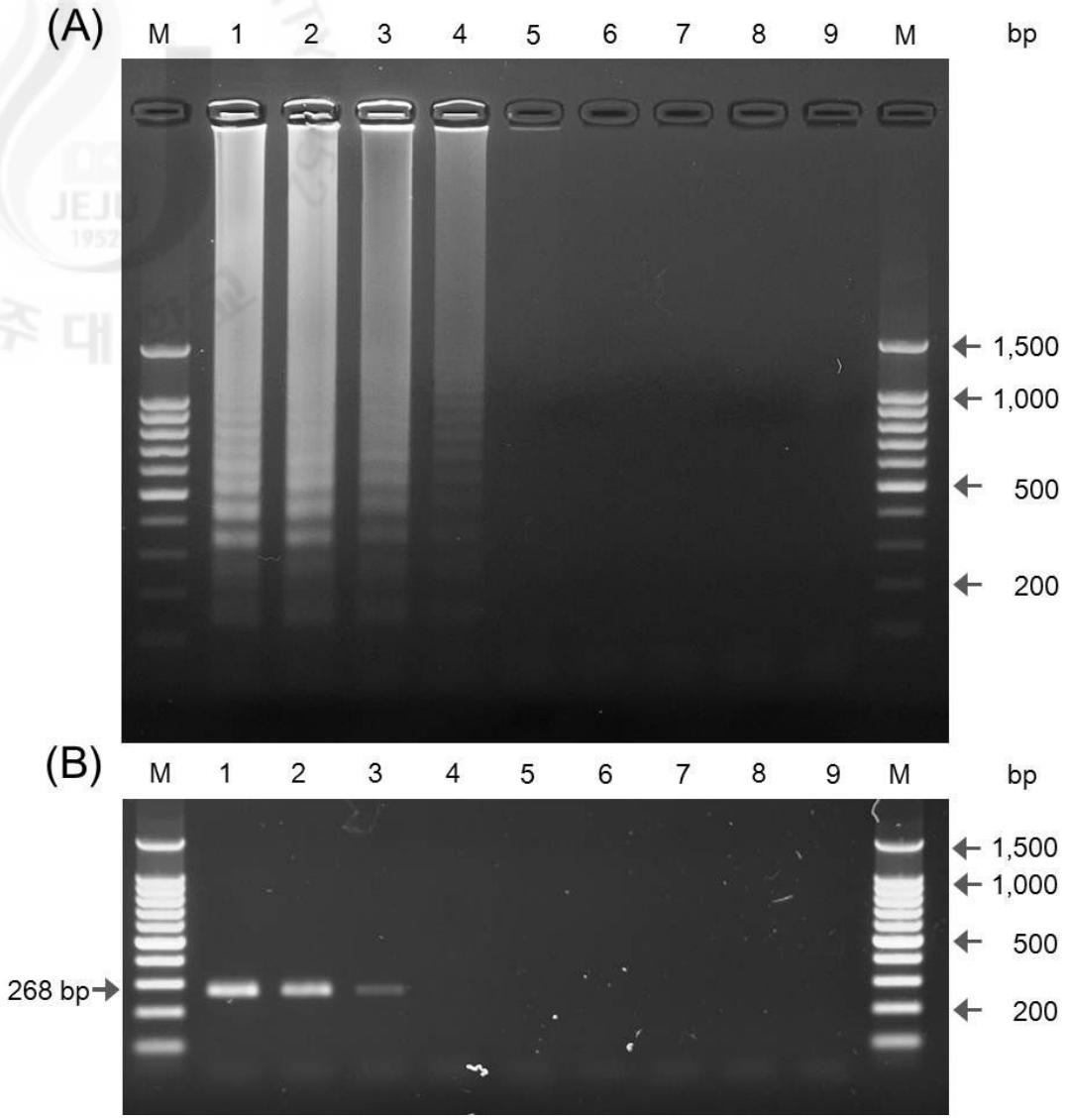

Figure 27. Sensitivity of *E. tarda* identification by LAMP and conventional PCR. Each sample was electrophoresed on a 1.5% agarose gel. (A) LAMP products. A product was seen typical ladder-like pattern on gel electrophoresis; (B) conventional PCR products using primers EDtT-F and EDtT-R. A band of 268 bp was seen with positive samples. Lane M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea); Lanes 1–8, amplification products using 10-fold serial dilutions of template DNA, which extracted variation of cell concentrations (1.0×10^8 CFU/ml, 1.0×10^7 CFU/ml, 1.0×10^6 CFU/ml, 1.0×10^5 CFU/ml, 1.0×10^4 CFU/ml, 1.0×10^3 CFU/ml, 1.0×10^2 CFU/ml, and 1.0×10^1 CFU/ml, respectively); Lane 9, distilled water.

5. Optimum temperature and time for detection

Even if the Bst DNA polymerase has the optimal activity at 65 ℃, several reports showed this enzyme can amplify DNA templates at several lower temperature conditions in the LAMP reaction (Endo et al., 2004; Iwamoto et al., 2003; Parida et al., 2004; Poon et al., 2004; Yoshikawa et al., 2004). The effect of temperature on the LAMP reaction in three primer sets such as SiLct, SpAroK, and EtFimB for species-specific detection of each species bacteria were determined that as shown in Figure 28, The specificity of amplification of *S. iniae* was optimum at 60 °C and 65 °C with SiLctO primer set as observed by agarose gel electrophoresis and visually by the formation a typical ladder-like pattern (Figure 28A), but no such typical pattern product was detected at 40, 55, and 70 °C, respectively. The case of *S. parauberis,* the amplification temperatures were optimum at from 55, 60, and 65 °C with SpAroK primer set as observed the formation a typical ladder-like pattern in wide temperature range (Figure 28C), but no such typical pattern product was detected at 40 and 70 °C, respectively. Finally, the amplification temperatures of LAMP reaction of detection for *E. tarda* was optimum at 60 °C and 65 °C with EtAroB primer set as observed the formation a typical ladder-like pattern (Figure 28E), Amplification of other temperature conditions was faintly detected at 40, 55, and 70 °C however these faint bands were not observed the LAMP typical ladder-like pattern. Previous reports (Iwamoto et al., 2003; Savan et al., 2004; Yoshikawa et al., 2004) have determined, which amplified products can be detected less than 60 min in the LAMP assay. Therefore we investigated the several LAMP reaction lengths from 0 to 100 min with three primer sets such as SiLct, SpAroK, and EtFimB for species-specific detection of each species bacteria. We observed that increasing the length of the LAMP reaction with SiLctO primer set increased, Amplification was faintly detected at 40 min, and initially detected typical pattern product clearly at 60 min to 100 min (Figure 28B). The case of *S. parauberis* and *E. tarda* increasing the length of the LAMP reactions increased the target yield, and amplification of was initially detected at 40 min, and reached maximal at 60 min to 100min (Figure 28D and 28F).

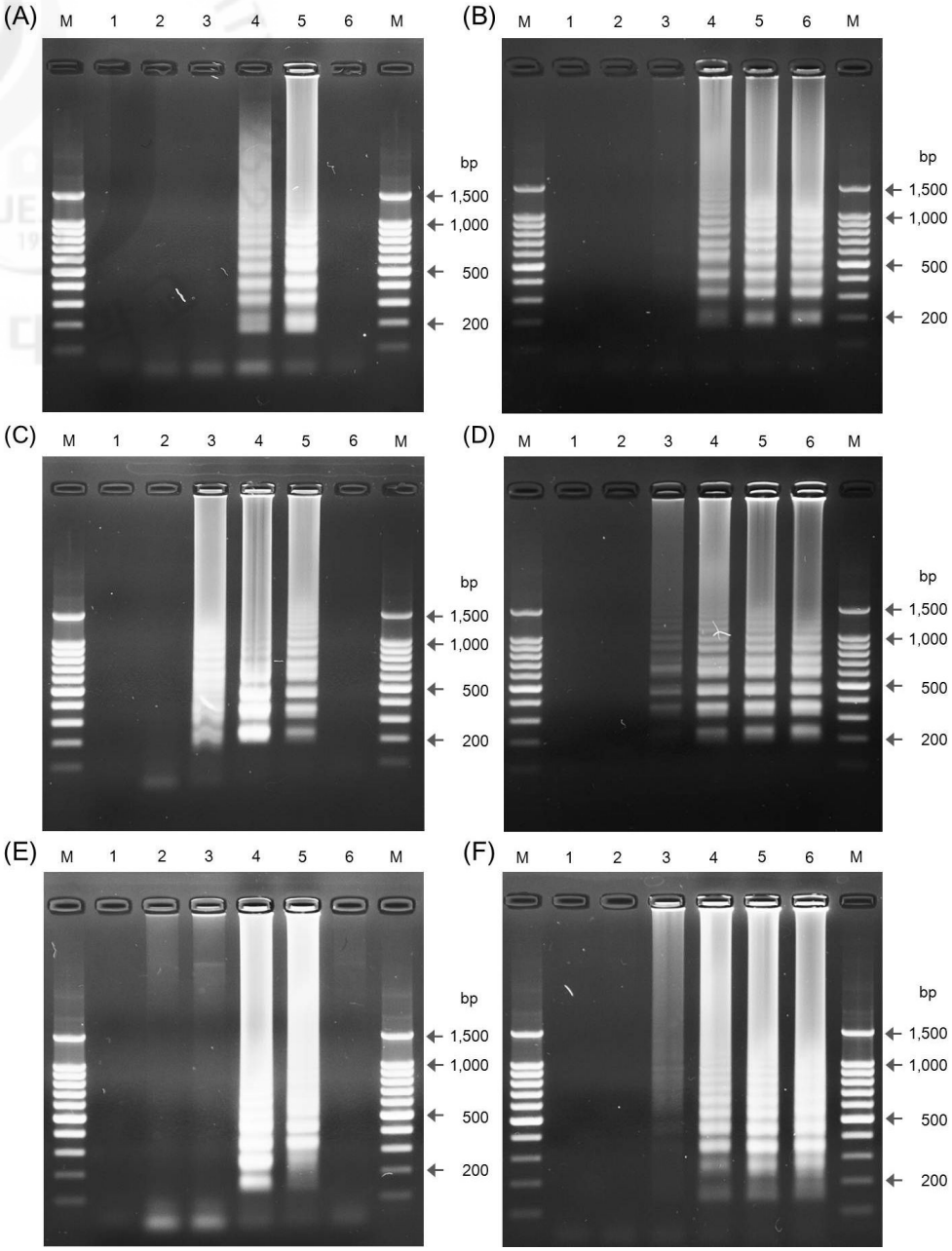

Figure 28. Optimum temperature and time of the LAMP reaction of three primer sets for detection of *S. iniae*, *S. parauberis*, and *E. tarda*. (A) Effect of temperature with SiLctO primer set, (C) Effect of temperature with SpAroK primer set, (E) Effect of temperature with EtFimB primer set, Lane 1, without DNA template in the reaction, amplification at 65 **℃**; Lane 2, amplification at 45 **℃**; Lane 3, amplification at 55 **℃**; Lane 4, amplification at 60 **℃** ; Lane 5, amplification at 65 **℃**; Lane 6, amplification at 70 **℃**. (B) Effect of reaction length with SiLctO primer set, (D) Effect of reaction length with SpAroK primer set, (F) Effect of reaction length with EtFimB primer set, Lane 1, amplification for 0 min; Lane 2, amplification for 20 min; Lane 3, amplification for 40 min; Lane 4, amplification for 60 min; Lane 5, amplification for 80 min;; Lane 6, amplification for 100 min; Lanes M, 100 bp DNA Marker (Intron biotechnology, Inc., Korea);

6. Effect of Mg^{++} concentration

Because free Mg^{++} availability affects primer annealing and DNA polymerase activity (Saiki et al., 1998), the effect of Mg^{++} concentrations ranging from 2 to 12 mM on the LAMP reaction was determined in LAMP reaction with three primer set such as SiLctO, SpAroK, and EtAroB respectively. LAMP reaction with SiLctO was observed typical amplified bands at 4mM and 8mM, but other Mg^{++} concentrations could not amplified LAMP products (Figure 29A). Whereas LAMP reaction with other primer sets such as SpAroK and EtAroB were indicated typical amplicons in the wide range of Mg^{++} concentrations, compared to amplified with SiLctO primers. The LAMP reactions detection range with SpAroK was between 4 mM and 12 mM and EtAroB was between 4 mM and 8mM, respectively (Figure 29B and 29C). In addition, all amplification in Mg^{++} high concentrations were indicated increasing sharpness of a typical ladder-like bands pattern of LAMP reaction compered to low Mg^{++} concentration trials, except at higher concentration outside the range of amplification trials. Consequently, Optimum $MgSO_4$ concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 4mM, 6mM, and 4mM, respectively.

7. Effect of betaine concentration

Betaine has been used in DNA amplification (AbuAl-Soud and Radstrom, 2000; Frackman et al., 1998). The LAMP reaction in the presence of different concentrations of betaine was tested with three primer sets such as SiLctO, SpAroK, and EtAroB. As shown in Figure 30, we observed that increasing betaine concentrations increased the LAMP reaction products with all of the three primer sets. Results of LAMP reactions with SiLctO, and EtAroB were clearly amplified wide range of betaine concentration between 0.2 M and 1.0 M (Figure 30A and 30C). The case of SpAroK was also amplified same range of betaine concentration, but in 0.2 M trial was faintly amplified a typical ladder-like band of LAMP reaction (Figure 30B). As a these results, Optimum betaine concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 0.4 M, 0.8 M, and 0.2 M, respectively.

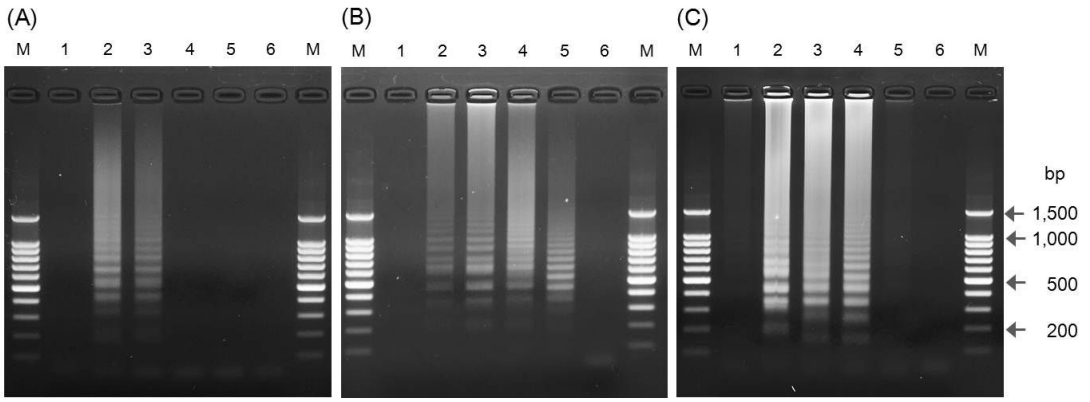

Figure 29. Optimum $MgSO_4$ concentrations of the LAMP reaction of three primer sets for detection of *S. iniae*, *S. parauberis*, and *E. tarda***.** (A) Effect of $MgSO_4$ concentrations with SiLctO primer set, (B) Effect of $MgSO_4$ concentrations with SpAroK primer set, (C) Effect of $MgSO_4$ concentrations with EtFimB primer set, Lane 1, 2 mM $MgSO_4$; Lane 2, 4 mM $MgSO_4$; Lane 3, 6 mM $MgSO_4$; Lane 4, 8 mM $MgSO_4$; Lane 5, 10 mM $MgSO_4$; Lane 6, without DNA template in the reaction, amplification with 4mM $MgSO_4$.

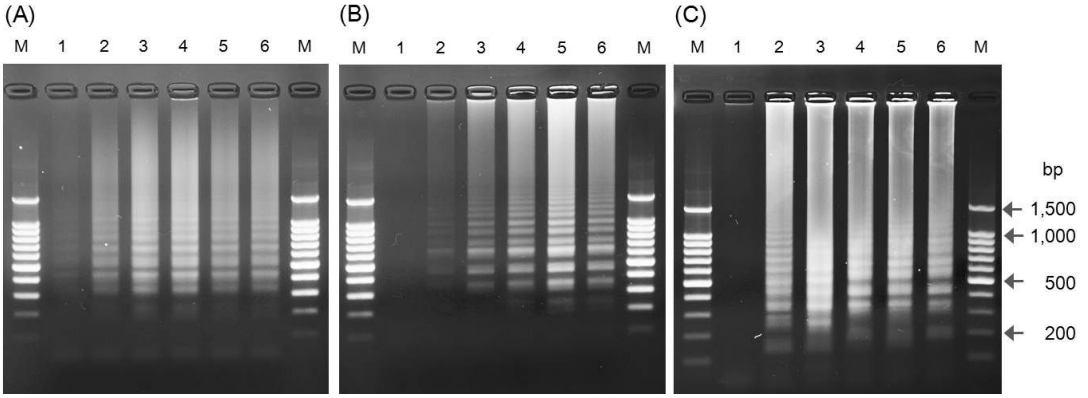

Figure 30. Optimum betaine concentrations of the LAMP reaction of three primer sets for detection of *S. iniae*, *S. parauberis*, and *E. tarda***.** (A) Effect of betaine concentrations with SiLctO primer set, (B) Effect of betaine concentrations with SpAroK primer set, (C) Effect of betaine concentrations with EtFimB primer set, Lane 1, 0 M betaine; Lane 2, 0.2 M betaine; Lane 3, 0.4 M betaine; Lane 4, 0.6 M betaine ; Lane 5, 0.8 M betaine; Lane 6, 1.0 M betaine.

8. Effect of deoxynucleotide triphosphate concentration

It is known that the deoxynucleotide triphosphate concentration affects the specificity of DNA polymerase amplification (Innis et al., 1988). The LAMP reactions in the presence of different concentrations of deoxynucleotide triphosphate were amplified with three primer sets such as SiLctO, SpAroK, and, EtAroB. In these results, LAMP reaction with SiLctO and SpAroK primers, the deoxynucleotide triphosphate concentrations ranging from 0.2 to 0.8 mM amplified the target DNA, but EtAroB primer trial was ranging from 0.2 to 1.0mM (Figure 31). In addition, SiLctO trial was indicated specific difference of sharpness of amplified bands related to deoxynucleotide triphosphate concentration (Figure 31A), however the other trials were demonstrated no specific difference related to deoxynucleotide triphosphate concentration (Figure 31B and 31C). Consequently, Optimum deoxynucleotide triphosphate concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 0.8 mM, 0.2 mM, and 0.2 mM, respectively.

9. Effect of Bst polymerase concentration

It is that the polymerase concentration in polymerase chain reaction affects cost of experiment. Therefore, minimizing of polymerase concentration has to improve of economic value in the molecular diagnosis technique. Thus in the present study investigated that the LAMP reactions in the presence of different concentrations of bst polymerase were amplified with three primer sets such as SiLctO, SpAroK, and EtAroB. In these results, LAMP reaction with three primers sets, the bst polymerase ranging from 4 U to 12 U in 25μl reaction volume enough amplified the target DNA (Figure 32). However, the results of 4 U bst concentration trial with SpAroK was faintly amplified a typical ladder-like band of LAMP reaction compared to other concentrations such as 8 U and 12 U (Figure 32B). Consequently, Optimum bst polymerase concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 4 U, 8 U, and 4 U in 25 μl reaction volume, respectively.

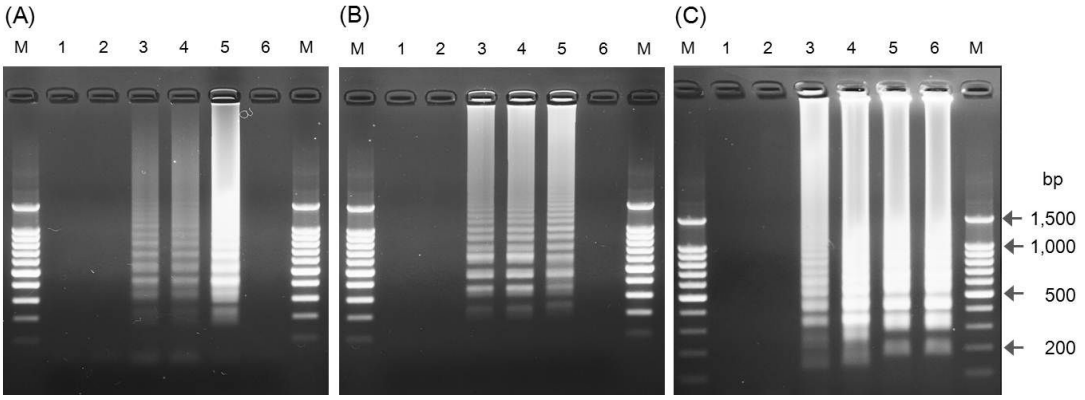

Figure 31. Optimum deoxynucleotide triphosphate (dNTP) concentrations of the LAMP reaction of three primer sets for detection of *S. iniae*, *S. parauberis*, and *E. tarda*. (A) Effect of dNTP concentrations with SiLctO primer set, (B) Effect of dNTP concentrations with SpAroK primer set, (C) Effect of dNTP concentrations with EtFimB primer set, Lane 1, without DNA template in the reaction, amplification with 400 μM dNTP; Lane 2, 0 mM dNTP; Lane 3, 0.2 mM dNTP; Lane 4, 0.4 mM dNTP ; Lane 5, 0.8 mM dNTP; Lane 6, 1.0 mM dNTP.

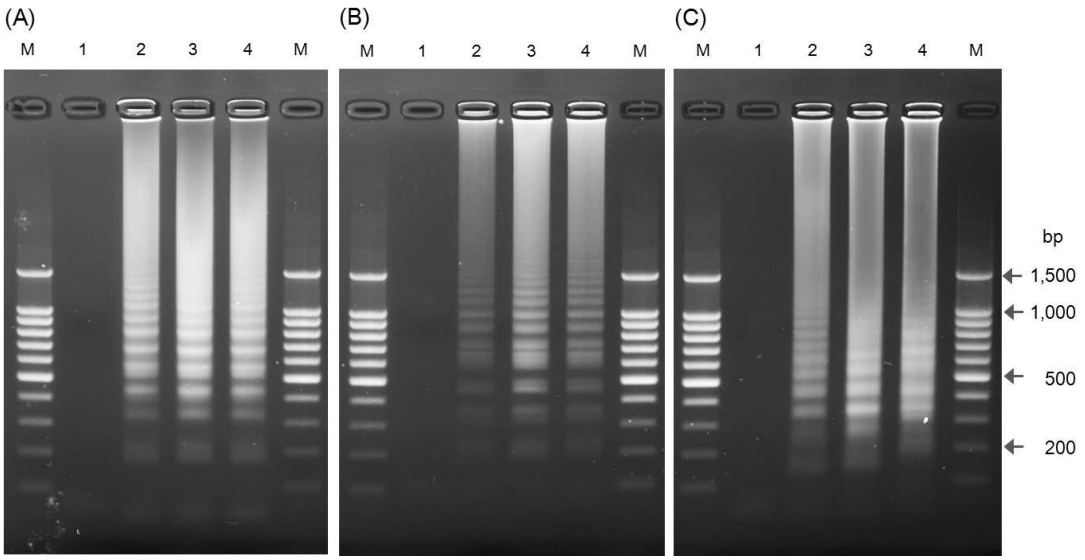

Figure 32. Optimum bst polymerase concentrations of the LAMP reaction of three primer sets for detection of *S. iniae*, *S. parauberis*, and *E. tarda*. (A) Effect of bst polymerase concentrations with SiLctO primer set, (B) Effect of bst polymerase concentrations with SpAroK primer set, (C) Effect of bst polymerase concentrations with EtFimB primer set, Lane 1, without DNA template in the reaction, amplification with 8 U bst polymerase; Lane 2, 4 U bst polymerase; Lane 3, 8 U bst polymerase; Lane 4, 12 U bst polymerase.

DISCUSSION

Disease diagnosis has been mainly based on clinical signs supported by isolation and identification of the aetiological agent. The streptococcusis and edwardsielosis of olive flounder has very typical symptoms, but it is difficult to diagnose the infection in its initial stages or during mild infection. Outbreaks of streptococcusis and edwardsiellosis are first seen in the form of infections affecting only a few fish. When early detection and proper chemotherapy are not under taken, the infection spreads to the surrounding areas, creating streptococcusis and edwardsiellosis epidemic. Controls of these diseases are attempted through monitoring and stocking specific-pathogen-free stocks. However, diagnosis of *S. iniae*, *S. parauberis*, and *E. tarda* is made only on the basis of isolation and biochemical identification and of detection of antigens in infected organs like spleen, kidney, and liver. However, a rapid and sensitive detection technique is required to detect streptococcusis and edwardsiellosis in fish at early stages to control the disease at the source. Therefore Molecular diagnostic techniques, such as PCR assays and multiplex PCR array, are increasingly used to detect and identify these bacterial pathogens of olive flounder (Mata, 2004; Sakai, 2009). The LAMP is one of the various PCR techniques that is a novel and effective aspect of diagnosis. Notomi (2000) suggested that loop-mediated isothermal amplification is a sensitive strand displacement technique. This method amplifies target DNA from a few copies to 10^9 copies in less than an hour under isothermal conditions. It is an offshoot of the basic strand displacement techniques which have been described thoroughly (Notomi et al., 2000). Briefly, four highly specific primers are constructed from the target DNA, one set of primers anneal to the target region one after the other on the same strand and the primer which anneals at the later stage displaces the strand formed by the first primer with the help of Bst DNA polymerase. The Bst polymerase has a strand displacement activity. This takes place on both strands and the primers are designed such that loops are formed. The reaction is carried out under isothermal conditions as denaturation of the strand

takes place by strand displacement. The reactions produce a series of stem-loop DNAs with various lengths. The four primers hybridize against six distinct sequences in the target DNA making it highly specific (Notomi et al., 2000). In addition, The LAMP reaction is carried out at 60–65 ℃ for 45–60 min and the reaction terminated at 80 ℃ for 2 min (Fig. 1). The main advantage of the technique is that it does not need thermocyclers. As the amplification in made under isothermal conditions, only a water bath or heating block is needed to maintain the required temperature (Notomi et al., 2000). For this reason,in this study developed a novel approach method of rapid molecular diagnosis known as LAMP that is gaining popularity among researchers due to its simple operation, rapid reaction, and easy detection in order to detect major fish pathogens efficiently. All the *S. iniae*, *S. parauberis*, and *E. tarda* isolates were selected five strains, respectively that were according to genotyped results on above by RAPD profiles. In order to design the species-specific LAMP DNA oligonucleotide primers, first searched the nucleic acid sequences of each three bacterial species such as *S. iniae*, *S. parauberis*, and *E. tarda* deposited in the GenBank database, and used the BLAST program to choose the species-specific gene sequences. Based on these results, we choose specific gene in each bacterial species which were LctO, AroK, and AroB gene used for species-specific LAMP reation in three bacterial species such as *S. iniae, S. parauberis*, and *E. tarda*, respectively. These primers set of four primers composed of two outer primers which initiate strand displacement and two inner primers which structure “the loop” through the reaction. To determine the specificity of the primers of the LctO, AroK, and AroB gene, species-related strains and other fish bacterial pathogens were tested. To observed that the each primer only amplified the DNA of target species bacterial strains. These LAMP products were shown a typical ladder-like pattern on gel electrophoresis which indicated that stem-loop DNA with inverted repeats was formed. This result was indicated that the LAMP reaction was highly specific to *S. iniae*, *S. parauberis*, and *E. tarda* strains, respectively. The LAMP reaction does not progress without the hybridization of six distinct sequences in the target DNA by four different highly specific

primers, thus it is highly specific. Furthermore, the ability of the method to amplify from fewer copies of initial target DNA than PCR has been conclusively demonstrated (Gunimaladevi et al., 2004, 2005; Kono et al., 2004; Savan et al., 2004). The efficiency of LAMP does not seem to be affected by the presence of non-target genomic DNA in the reaction mixture (Notomi et al., 2000), which is highly desirable in development of a diagnostic system. Detection of target DNA by LAMP compared with detection by two-step nested-PCR was at least equal or more sensitive (Gunimaladevi et al., 2004, 2005; Kono et al., 2004; Savan et al., 2004). As mentioned above, the present study was investigated similar results that LAMP is equal sensitive or more sensitive than conventional DNA based detection systems for detection of *S. iniae*, *S. parauberis* and *E. tarda.* This high sensitivity of the LAMP system makes it susceptible to false positives because of carry-over or cross-contamination. Briefly described these results, the detection limit of LAMP for *S. iniae* could be reached at 1.0×10^{-6} CFU/ml dilution level. With previous primers for detecting *S. iniae*, the detection limit of PCR could be also reached at 1.0×10^{-6} CFU/ml dilution level. Whereas two LAMP arrays for detecting *S. parauberis* and *E. tarda* were indicated detection limit that was higher than conventional PCR. First of these results, the detection limit of LAMP for *S. parauberis* could be reached at 1.0×10^{-3} CFU/ml dilution level, and conventional PCR primers for detecting *S. parauberis*, which could be reached at 1.0×10^{-5} CFU/ml dilution level. In case of the detection limit of LAMP for *E. tarda* that could be reached at 1.0×10^{-5} CFU/ml dilution level, and conventional PCR primers for detecting *E. tarda*, which could be also reached at 1.0×10^{-6} CFU/ml dilution level. These results revealed that the LAMP assay was 10-100 fold sensitive than the PCR assay in *E. tarda* and *S. parauberis*, respectively. Especially the results of LAMP for detecting *S. parauberis* was extremely higher than conventional PCR consequently, the SpAroK primer set could be effectively use than other PCR-based diagnosis protocols in the fields of olive flounder aquaculture. The optimizing of LAMP reaction is very important in order to develop of commercial detection kit. Therefore this study investigated optimization of LAMP reaction

for each experiment. Even if the Bst DNA polymerase has the optimal activity at 65 °C, several reports showed this enzyme can amplify DNA templates at several lower temperature conditions in the LAMP reaction (Endo et al., 2004; Iwamoto et al., 2003; Parida et al., 2004; Poon et al., 2004; Yoshikawa et al., 2004). In present study, the effect of temperature on the LAMP reaction in three primer sets such as SiLct, SpAroK, and EtFimB for species-specific detection of each species bacteria. We were determined that amplification of *S. iniae* was optimum at 60 °C and 65 °C with SiLctO primer set. The case of *S. parauberis*, the amplification temperatures were optimum at from 55, 60, and 65 °C with SpAroK primer set that was indicated wide amplification temperature range. Finally, the amplification temperatures of LAMP reaction for *E. tarda* were optimum at 60 °C and 65 °C with EtAroB primer. Previous reports (Iwamoto et al., 2003; Savan et al., 2004; Yoshikawa et al., 2004) have determined, which amplified products can be detected less than 60 min in the LAMP assay. Therefore we investigated the several LAMP reaction lengths from 0 to 100 min with three primer sets such as SiLct, SpAroK, and EtFimB for species-specific detection of each species bacteria. We observed that increasing the length of the LAMP reaction with SiLctO primer set increased, Amplification was faintly detected at 40 min, and initially detected typical pattern product clearly at 60 min to 100 min. The case of *S. parauberis* and *E. tarda* increasing the length of the LAMP reactions increased the target yield, and amplification of was initially detected at 40 min, and reached maximal at 60 min to 100min. Because free Mg^{++} availability affects primer annealing and DNA polymerase activity (Saiki et al., 1998), the effect of Mg^{++} concentrations ranging from 2 to 12 mM on the LAMP reaction was determined in LAMP reaction with three primer set such as SiLctO, SpAroK, and EtAroB respectively. LAMP reaction with SiLctO was observed at 4mM and 8mM. Whereas LAMP reaction with other primer sets such as SpAroK and EtAroB were indicated typical amplicons in the wide range of Mg^{++} concentrations, compared to amplified with SiLctO primers. The LAMP reactions detection range with SpAroK was between 4 mM and 12 mM and EtAroB was between 4 mM and 8mM, respectively. Consequently, Optimum MgSO4

concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 4mM, 6mM, and 4mM, respectively. Betaine has been used in DNA amplification (AbuAl-Soud and Radstrom, 2000; Frackman et al., 1998). The LAMP reaction in the presence of different concentrations of betaine was tested with three primer sets such as SiLctO, SpAroK, and EtAroB. As a these results, Optimum betaine concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 0.4 M, 0.8 M, and 0.2 M, respectively. It is known that the deoxynucleotide triphosphate concentration affects the specificity of DNA polymerase amplification (Innis et al., 1988). The LAMP reactions in the presence of different concentrations of deoxynucleotide triphosphate were amplified with three primer sets such as SiLctO, SpAroK, and EtAroB. In these results, LAMP reaction with SiLctO, and SpAroK primers, Optimum deoxynucleotide triphosphate concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 0.8 mM, 0.2 mM, and 0.2 mM, respectively. It is that the polymerase concentration in polymerase chain reaction affects cost of experiment. Therefore, minimizing of polymerase concentration has to improve of economic value in the molecular diagnosis technique. Therefore, this study was investigated that the LAMP reactions in the presence of different concentrations of bst polymerase were amplified with three primer sets such as SiLctO, SpAroK, and EtAroB. In these results, Optimum bst polymerase concentrations of the LAMP reaction of SiLctO, SpAroK, and EtAroB were confirmed 4 U, 8 U, and 4 U in 25 μl reaction volume, respectively. Several reports on LAMP-mediated diagnostic methods have been developed for bacterial pathogens of fish. The first use of LAMP for detection of an aquaculture pathogen was reported for edwardsiellosis (Savan et al., 2004). *E. tarda* was detected from infected Japanese flounder, *Paralichthys olivaceus*. LAMP primers were designed by targeting the haemolysin gene. A LAMP-based diagnostic system has been recently developed for detection of *E. ictaluri* from diseased catfish, *Ictalurus punctatus* (Yeh et al., 2005). The LAMP primers were designed targeting the eip18 gene. The LAMP method has also been applied to the detection of nocardiosis (Itano et al., 2006). Nocardiosis is a serious pathogen affecting yellowtail,

Seriola quinqueradiata, and amberjack, *S. aureovittata*. Nocardial diagnosis has been mainly achieved by isolation and biochemical identification. Recently, PCR detection has been possible for nocardiosis (Kono et al., 2001; Miyoshi and Suzuki, 2002). Although several report had been published, the LAMP research of most of the fish bacterial pathogens. Especially, streptococcusis of LAMP techniques is not yet reported such as *S. parauberis*. In conclusion, LAMP, a rapid and highly sensitive system for detecting streptococcusis and edwardsiellosis, has been designed. This is the meaningful report for detection *S. iniae* and *E. tarda* in terms of diagnostic aspects; in addition, this is first report of the use of a LAMP technique, which has applications for the detection of *S. parauberis*. This method can be effectively used for diagnosis of streptococcusis and edwardsiellosis in olive flounder and in a aqua farming environment.

Part Ⅲ. Efficacy test of three monovalent vaccine and one trivalent oil adjuvant vaccine administered to olive flounder, *Paralichthys olivaceus* against streptococcusis and edwardsiellosis

ABSTRACT

The second aspect is the development of multivalent vaccine of major bacterial fish pathogens in olive flounder that mentioned above. Ultimately effective vaccines are the only way to resolve disease problems and a number of highly successful vaccines have been developed for aquaculture. In contrast, the approaches of controlled fish disease have been still dependent to high proportion of chemotherapy in Korea recently. Therefore aquaculture industry of Korea is important to efficient development of fish vaccine in order to be competitive internationally. However development of multivalent vaccine is very insufficient in aquaculture industry of olive flounder in Korea. Therefore, we used oil base adjuvant base approach to evaluate the multivalent vaccine formulation in order to give effective immunoprotection of major bacterial pathogens in olive flounder. Three monovalent and one trivalent vaccines for *S. iniae*, *S. parauberis*, and *E. tarda* were injected into olive flounder to investigate the efficacy levels of the vaccines. The vaccinated groups were required to completely recover for just one days post-vaccination, and vaccinated flounder showed a complete recovery rate from the beginning of immunization; therefore these vaccines were gave low level of stress. In order to choice of challenge periods, the present study was selected 28 day of post-vaccination according to accumulated temperature. The average growth of the FO-5 group was increased by approximately 10% at the termination of immunization periods, as compared to that of the control fish group. In addition, the other vaccinated groups were also increased or similar levels as compared to the control group at same periods. These results might be induced from an decreased chances that was infectious

target pathogens in the fish tanks, therefore vaccinated fish could be avoid stress by pathogens. During the immunization periods, vaccinated groups were investigated the cumulative mortalities. The FO-1 injection group was occurred tiny mortality that only two fish were died in four weeks, which were calculated 0.008 % of cumulative mortality rate. However the control and other vaccinated groups were showed any mortality. The blood biochemical and physiological parameters are important for general health, toxicology, and bio-monitoring. According to these results, the vaccines in the present study might be gave no stress to the olive flounder. The superoxide anion production of respiratory burst activity of immunization by vaccination was measured via NBT reduction and MPO release activities. All the vaccinated groups did not significantly increase the NBT reduction activity after second, and fourth weeks post-vaccination of all the vaccinated groups significantly increased when compared to the control. The release of myeloperoxidase by the azurophilic granules of neutrophils during oxidative respiratory burst activity was significantly higher in all of the vaccinated groups than in the controls. The serum lysozyme activity was not indicated to differ significantly between the control and immunized group with FO-4 during immunized period. However the lysozyme activities were significantly higher in the immunized groups with the other vaccines groups than in the control group. In addition, the FO-5 vaccination group was extremely shown high lysozyme activity at one week post-vaccination, and it was decreased lysozyme activity to the level of other vaccination groups. The FO-1 vaccination group that injected trivalent vaccine was increased to 2^{nd} weeks post-vaccination, and it was maintained the lysozyme activity until 4^{th} weeks post-vaccination. Especially, trivalent vaccine was shown higher lysozyme activity compared to other monovalent vaccines, on 2^{nd} weeks and 4^{th} weeks post-vaccination. In these results, lysozyme activity might more strongly protect against the invasion of certain pathogenic bacteria. The phagocytosis activity of head-kidney leucocytes were significantly increased in all the vaccinated groups on 1^{st}, 2^{nd}, and 4^{th} weeks post-vaccination. In addition, FO-3 vaccination group was shown highest phagocytosis activity during all the immunization

period, and the FO-1 vaccination group that injected trivalent vaccine was increased to 2nd weeks post-vaccination, and it was maintained the phagocytosis level until 4th weeks post-vaccination. In these result, the immunization by the vaccines may induce cell-mediated immunity in the vaccinated olive flounder. The serum collected from all immunized fish by monovalent vaccine and trivalent vaccine showed agglutination activity against formalin killed *S. iniae*, *S. parauberis*, and *E. tarda*, respectively. A weak agglutination activity of serum were observed in the fish immunized with FO-1, FO-3, and FO-4 vaccines against *S. iniae* and *S. parauberis*, and the activity was significantly increased at 1st week post-vaccination. The trivalent vaccinated group on $2^{2.6}$ and $2^{4.2}$ were highest agglutination titers during immunization period against *S. iniae* and *S. parauberis*, respectively. However, FO-1 and FO-5 vaccinated groups were appeared high agglutination activities, it was increased to 2nd weeks post vaccination, and it were maintained the agglutination activities until 4th weeks post vaccination. The trivalent vaccinated group was $2^{8.4}$ of highest agglutinating antibodies level against *E. tarda.* In addition, agglutination titers of trivalent vaccinated groups were lower than each monovalent vaccine during immunization period. In order to examine the immunoprotective effect of monovalent and trivalent vaccines, the fish were challenged with homologous *S. iniae*, *S. parauberis*, or *E. tarda* strain monitored for mortality. The cumulative mortalities of FO-1, FO-3 and saline vaccinated fish were 7.5 %, 2.5 %, and 82.5 %, respectively, which yield a protection efficacy, in terms of RPS, of 90.31 % , 96.97 % for FO-1, FO-3 when compared to control group at termination of challenge test. By the termination of the monitored period, the cumulative mortalities of FO-1, FO, 4, and control groups were 20 %, 10 %, and 65%, respectively, and the mean survival rates, in terms of RPS, were 69.23 % and 84.62 %, respectively. The cumulative mortalities of FO-1, FO, 5, and control groups were 32.5 %, 47.5 %, and 85 %, respectively. In addition, the mean survival rates, in terms of RPS, were 44.12 % and 61.76 %, respectively. These results indicate that FO-1 as trivalent vaccines exhibited low protections, when compared to monovalent vaccines such as FO-3, FO-4, and FO-5. However, the trivalent vaccine FO-1

was indicated sufficient efficacy against the major bacterial pathogens such as *S. iniae*, *S. parauberis*, and *E. tarda*, therefore we consider that the trivalent vaccine in this study could be used successfully in commercial olive flounder farms.

MATERIALS AND METHODS

1. Fish

Olive flounder (*Paralichthys olivaceus*) (10.5 ± 1.2 cm, 10.5 ± 4.1 g at vaccination) were purchased from a local fish farm in Jeju Island and acclimatized in the rearing facility at least two weeks before vaccination. Before vaccination, fish (5%) were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen in order to prevent of interruption by other pathogens. Any bacteria could be detected from any of the examined tissues of the sampled fish. Fish were maintained in rearing tanks that was running seawater rearing system, and volume of rearing tanks were five tons and flow rate of seawater was approximately 7.2 ton/hr. During the immunization water temperature range was between 19.8 and 27.5 °C, and vaccinated fish were fed two times daily with commercial dry pellets.

2. Vaccine preparation

S. iniae JJU-019, *S. parauberis* JJU-045, and *E. tarda* JJU-054 are fish pathogens that were mentioned above. These strains were used in manufacturing of three monovalent and one trivalent oil adjuvant vaccines by pharmaceutical company specializing in aquaculture (Pharmaq AS, Norway) that was provided to evaluate efficacy of vaccination in olive flounder. In this study, three kinds of monovalent vaccines and a trivalent vaccine target to three bacterial species such as *S. iniae*, *S. parauberis*, and *E. tarda*, these vaccines were used immunization of olive flounder. The trivalent vaccine used three species, *S. iniae*, *S. parauberis*, and *E. tarda.* The trivalent vaccine was labeled FO-1, and the monovalent vaccines against *S. iniae*, *S. parauberis*, and *E. tarda* were labeled FO-3, FO-4, and FO-5, respectively. The test vaccine was stored between 2 and 8 °C until used. For vaccination, only sterile vaccination equipment was used. In the case of accidental self-injection, seek prompt medical advice. In rare circumstances, self-injection may result in anaphylaxis in individuals, and it is recommended that operators have ready access to adrenaline for

emergency use. During vaccination the use of protective equipment (e.g. safety bow) is recommended. The control substance used in this study was sterile saline (at 0.9% NaCl).

3. Identification (marking) and allocation of study groups

The experimental fish were identified by visual implant elastomer of individual fish at the time of vaccination. The trial groups were kept in separate tanks during the period of immunization. The study groups were marked at vaccination by injection of yellow and red visual implant elastomer (VIE) underneath the skin of the fish. The markings were conducted according to the description issued by the manufacturer. The VIE marks in FO-1 and FO-3 were placed on the right and left sides by the yellow VIE, FO-4 and FO-5 were placed on the right and left sides by the red VIE, respectively (Figure 33). Prior to vaccination, the fish were slightly anaesthetized (sedated) for limit body movement and stress during the marking.

4. Vaccination

A volume of 50 µl vaccines were injected at a spot located on the lower posterior dorsal side of the abdominal cavity of the fish. All vaccines along with the negative control substance were injected at a dose of 50 µl/fish. Sterile Kaycee vaccination guns were used for vaccination, and the length of the needle was selected to ensure that the vaccine was deposited within the abdominal cavity without penetrating the internal organs. It is recommended to use a needle of 3 mm x 0.6 mm when vaccinating olive flounder weight range 10-15 g. Prior to vaccination, the fish were slightly anaesthetized (sedated) for limit body movement and stress during the vaccination. After vaccination and marking, the fish were transferred to another freshly prepared tank (one tank per vaccine group) which were immunized until at least 450 day degrees (dd : temperature x days = degree days) post-vaccination. After at least 450 dd elapsed post-vaccination, the fish of the study groups (four vaccinated and one control) were collected and transferred to the challenge facilities.

5. Blood sampling

Blood samples of five fish / group were collected randomly from caudal vein using a vacationer fitted 1-ml 27-G needle on weeks 1, 2, and 4 after vaccination. Individual fish were anaesthetized with MS-222 ($NaHCO_3$ and tricaine methane sulphonate; Sigma Chemicals). Individual fish were sampled only once to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. To evaluate the blood physiological parameters and immunological assay feeding was ceased for 24 h prior to sampling. One half of each blood sample was immediately used for hematological examination, while the other half was mixed with heparin anticoagulant and kept frozen at 4 °C. The serum tubes were placed at room temperature and allowed to clot for 2 h. Sera were separated by centrifugation at 1500 g for 20 min and sera from the same groups were pooled before being stored at −70 °C for direct agglutination, biochemical and immunological analyses.

6. Blood biochemistry

Serum biochemical parameters, such as serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities, low density cholesterol (LDL), triglycerides (TG), phosphorus, hemoglobin (Hb), phosphotus (PHO), concentration of total protein (TP), and glucose (GLU) were determined in ch100 plus blood chemistry autoanalyzer (SEAC, Italy) by using analysis kits (STANBIO, Taxas, USA).

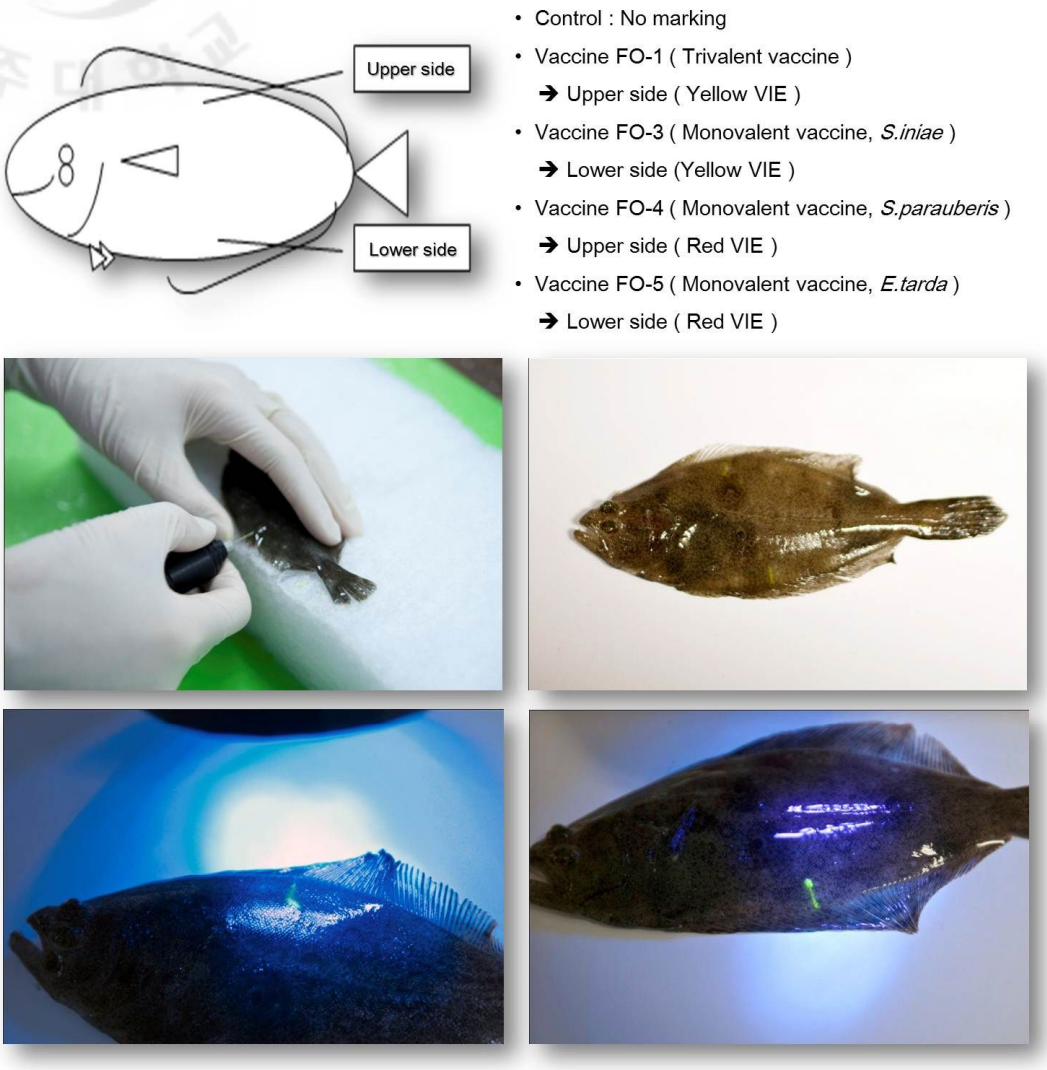

Figure 33. Marking of vaccination groups by visual implant elastomer (VIE).

7. Separation of leukocytes from head kidney

Head kidney cells were acquired in accordance with the methodology described by Secombes (1990), with minor modifications. In brief, the head kidney was aseptically removed and placed in a petri dish containing cooling HBSS (Hank's Balanced Salt Solution, Sigma). Small pieces of head kidney were pushed through 100 μm nylon mesh, and the cell suspension was layered onto a 1.077 density histopaque (Sigma, USA) gradient. After 30 min of centrifugation at 400 ×g at 4 °C for 15 min, the interface cells were gently collected and dispensed into tubes, containing HBSS solution, and then it washed twice in HBSS. For investigating phagocytosis, viable cells were adjusted to 2×10^6 cell/ml in the same medium, after estimating the cell viability via trypan blue exclusion.

8. Nitroblue tetrazolium reduction analysis (NBT)

The production of oxidative radicals by neutrophils in blood during the respiratory burst was measured via NBT assays, in accordance with the description of Anderson and Siwicki (1994). In brief, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, and then 50 μL was extracted and dispensed into Ependorff tubes. For the solubilization of the reduced formazan product, 1 mL of dimethyl formamide (Sigma, Lo, USA) was added and centrifuged for 5 min at 2000 rpm. Finally, the supernatant was acquired and the extent of reduced NBT was determined at an optical density of 540 nm with a microreader (Packard Spectrocount™, Austria). Dimethyl formamide was used as the blank.

9. Myeloperoxidase (MPO) activity

MPO generates hypochlorous acid (HClO) from hydrogen peroxide (H_2O_2) and the chloride anion (Cl^-) during the neutrophil respiratory burst. HClO kills pathogens entering the body. Total MPO content in the kidney cell was measured in accordance with the methods of Quade and Roth (1997). 15 μL of cells were distributed into each well and added to 135 μL

of HBSS (background), 0.02% CTAB (cetyl trimethyl ammonium bromide; lysed) and 50 nM PMA (phorbol myristate acetate in DMSO; stimulated). After that, 45 µL of 20 mM TMB (3,3′,5,5′-tetramethylbenzidine hydrochloride, Sigma) and 5 mM H_2O_2 were added. The color change reaction was halted after 2 min via the addition of 53 µL of 4 M sulfuric acid (H_2SO_4). The optical density (OD) was read at 450 nm with a microplate reader (Packard Spectrocount™, Austria). The percentage release of MPO was calculated via this formula:

% release =[(OD stimulated □ OD background) / (OD lysed □ OD background)] × 100.

10. Phagocytosis assay

This assay depends on the principle of, spectrophotometric measurement of congo red-stained yeast cells which have been phagocytized by cell. To perform the assay, leukocyte suspension (250 µl) was mixed with autoclaved and congo red-stained yeast cell suspension (providing a yeast cell: leukocyte ratio of 40:1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml HBSS was added and 1ml of histopaque 1.077 was injected into the bottom of each sample tube. The samples were centrifuged for 5 min at 850 × g to separate macrophages from free yeast cells. Obtained macrophages were washed twice in HBSS. The cells then were resuspended in 1 ml trypsin-EDTA solution (5.0 g/l trypsin and 2.0 g/l EDTA, Sigma) and incubated at 37 ˚C for 12 h. The absorbances of the samples were read in spectrophotometer (510 nm) using trypsin- EDTA as blank C (Jeney et al., 1997).

11. Lysozyme activity

The lysozyme functions by attacking peptidoglycans (found in the cell walls of bacteria, especially gram-positive bacteria) and hydrolyzing the bond that connects N-acetylmuramic acid to the fourth carbon atom of N-acetylglucosamine. Lysozymes can function as an innate opsonin to some extent, or function as a lytic enzyme. A turbidometric assay utilizing

lyophilized *Micrococcus lysodeikticus* cells (Sigma, St. Louis, USA) was employed in order to determine lysozyme activity. A minor modified the method previously developed by Kumari and Sajoo (2006) was used to determine the lysozyme activity. *M. lysodeikticus* suspended in 0.02 M sodium citrate buffer (pH 5.5) at a concentration of 0.2 mg/mL was added to 15 μL of serum samples in 96-well microtiter plates. Immediately after the addition of 150 μL of *M. lysodeikticus*, the optical density was determined. The absorbance was measured at 5 min intervals for 60 min at 450 nm. A unit of lysozyme activity was defined as the quantity of sample required to induce a reduction in absorbance of 0.001/min.

12. Measurement of serum agglutination titer

We measured the serum agglutination titer following the method of Vivas et al. (2004), with minor modifications. The serum (20 μL) was diluted with 60 μL PBS then serially double diluted into a 96-well microtiter plate. We then added 20 μL of a washed suspension of formalin-killed bacteria, adjusted to an O.D. 600 = 0.7 in PBS, to the serum dilutions. The plates were incubated at 25 °C for 2 h and incubated overnight at 4 °C. The final dilution which caused agglutination was recorded 16 h after incubation.

13. Challenge test

The challenge bacterial strains were used as mentioned above such as *S. iniae* JJU-019, *S. parauberis* JJU-045, and *E. tarda* JJU-054, respectively. The challenge method was carried out by IP injection. The challenge dose was 0.1 ml/fish, and the point of injection was at the same point as that for vaccination. Challenge of vaccinated fish was performed at min 450 dd. The challenge pressure of *S, iniae*, *S. parauberis*, and *E. tarda* used for 1.0×10^4 CFU/fish, 1.0×10^6 CFU/fish, and 1.0×10^4 CFU/fish, respectively. We conducted the challenge test twice for efficiency of vaccination. Registration of mortality post-challenge was performed daily until mortality was no longer observed in the experimental groups (minimum of 3 consecutive days post-challenge). Fish died post-challenge were subjected to

pathological signs were recorded. Further, all fish that survived the challenge period were checked for clinical symptoms of disease. Post-challenge, re-isolation, and identification of the challenge pathogen were conducted from the dead fish. The relative percent survival (RPS) was calculated at the time point corresponding to 60% mortality in the control group (RPS60), and RPS at the endpoint (RPSEND) was calculated at the time point when each challenge experiments were terminated. RPS was calculated using the following formulas:

$RPS60 = (1\text{-}M_{60}{}^{a} / 60) \times 100, \quad RPSEND = (1\text{-}M_{vacc}{}^{b} / M_{con}{}^{c}) \times 100$

[a] Mortality of vaccination group at the time point that mortality of control group was reached 60%

[b] Mortality of vaccination group

[c] Mortality of Control

RESULTS

1. Immunization and accumulated temperature.

Three monovalent and one trivalent vaccines for *S. iniae*, *S. parauberis*, and *E. tarda* were injected into olive flounder to investigate the efficacy levels of the vaccines. Previous study, vaccinated flounder over a long period showed no response to feed, that supposed to decrease of recovering rate because water temperature was low during that period (Data not shown). However, in this study all of the vaccine flounder were immediately recovered fed, this result was considered to that high water temperature was affected to fed recovery in vaccinated flounder. To briefly explain, several vaccinated groups were required to completely recover for just one days post-vaccination, and vaccinated flounder showed a complete recovery rate from the beginning of immunization therefore these vaccines were gave low level of stress (Figure 34). In order to choice of challenge periods, we selected 28 day of post-vaccination according to accumulated temperature that is knew affecting to immunization level. In knowhow of Pharmaq AS, the accumulated temperatures need to be reached at least 450 days degree (dd), that is required for enough immunization in fish. Therefore, the present study conducted challenge test to investigation of the vaccine efficacy at 28 day of post-vaccination that was approximately 660 dd of accumulated temperatures (Figure 34).

2. Growth performances in immunization periods

The growth of olive flounders vaccinated or control diets were evaluated for 4 weeks and sized and weighed every 1week in the immunization periods. The average growth of the FO-5 group was increased by approximately 10% at the termination of immunization periods, as compared to that of the control fish group (Figure 35). In addition, the other vaccinated groups were also increased or similar levels as compared to the control group at same periods. These results might be induced from an decreased chances that was infectious target pathogens in the fish tanks, therefore vaccinated fish could be avoid stress by pathogens.

3. Safety of three monovalent vaccines and one trivalent vaccine

During the immunization periods, intraperitoneally injection groups of three monovalent vaccines, one trivalent vaccine, and control were investigated the cumulative mortalities. The FO-1 injection group was occurred tiny mortality that only two fish were died in four weeks, which were calculated 0.008 % of cumulative mortality rate (Data not shown). However the control and other vaccinated groups were showed any mortality The Dead flounder of vaccinated FO-1 were not investigated any pathological symptoms. In addition, flounders of the control and vaccinated groups randomly picked out at least five fish in order to investigate of histopathological inspection, which were observed any hisotopathological changes.

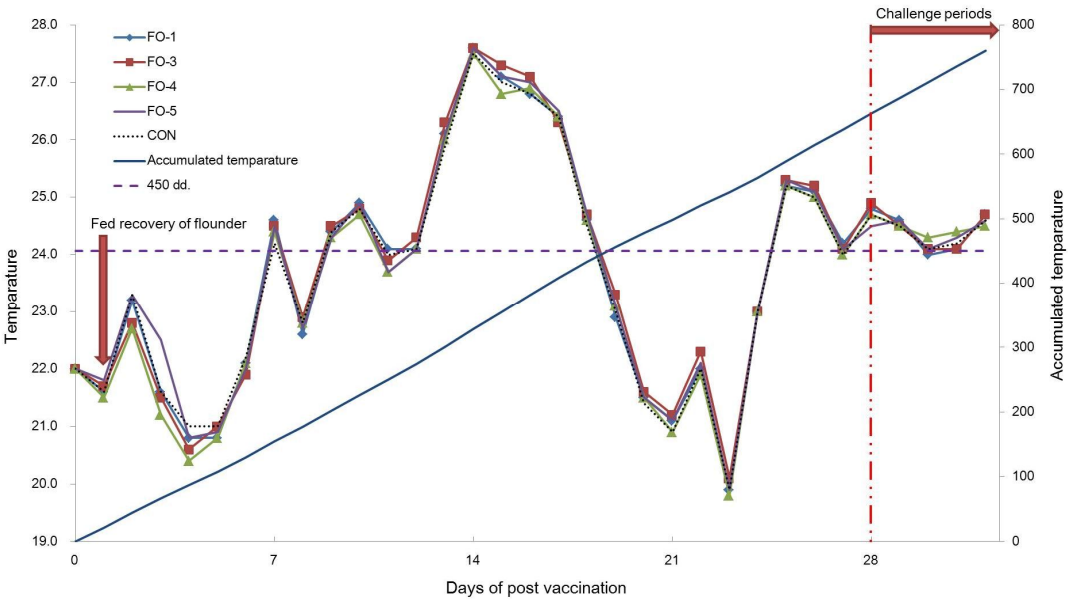

Figure 34. Major issues during rearing for immunization and accumulated temperature.

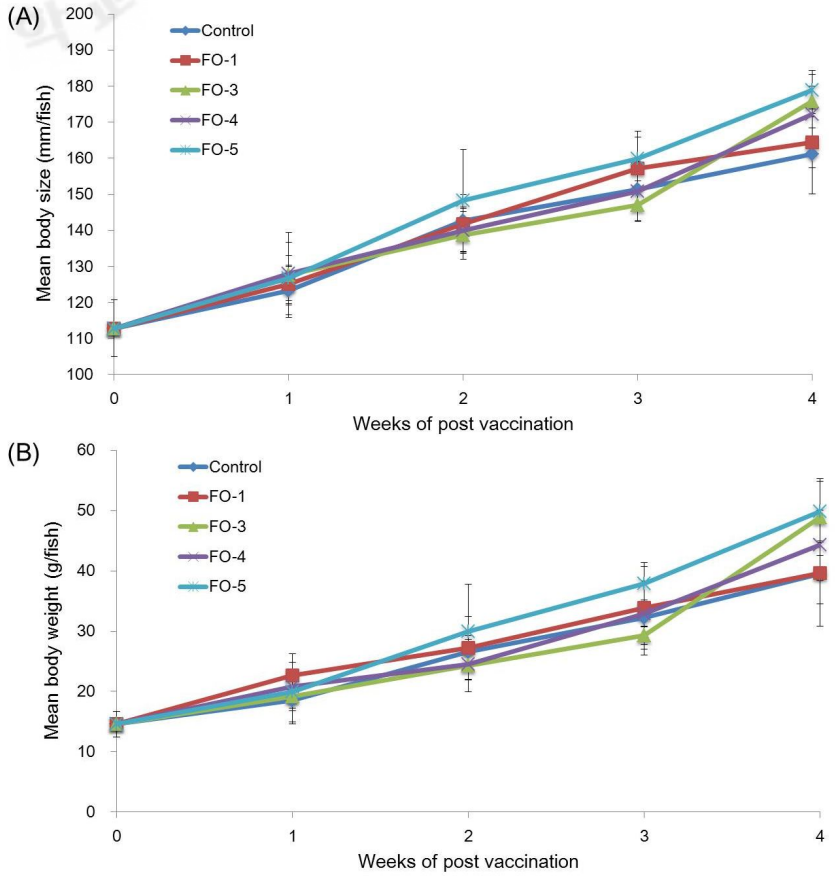

Figure 35. Growth performances of olive flounder that were vaccinated with FO-1, FO-3, FO-4, and FO-5 and control for 4 weeks in the immunization periods. (A) Body size gain of vaccinated groups and control, (B) Weight gain of vaccinated groups and control.

4. Blood biochemical constituents

The blood biochemical and physiological parameters are important for general health, toxicology, and bio-monitoring. The aspartate aminotransferase, low density cholesterol, triglyceride, and hemoglobin concentration significantly increased in all of the vaccinated groups when immunization periods with all the vaccine on 1st and 4th weeks post-vaccination. However, alanine aminotransferase, glucose, and phosphorus concentration did not significant change (Table 13). According to these results, the vaccines in the present study might be gave no stress to the olive flounder.

5. Superoxide anion production

The superoxide anion production of respiratory burst activity of immunization by vaccination was measured via NBT reduction. The superoxide anion production of respiratory burst activity of the blood leukocytes are shown in Figure 36A. All the vaccinated groups did not significantly increase the superoxide anion production on first week when compared to control group. However, after second, fourth weeks post-vaccination of all the vaccinated groups significantly increased when compared to the control.

Table 13. Serum biochemical parameters of olive flounder control, vaccinated groups on the time point that vaccinated day, 1st , and 4th weeks of post-vaccination.

Parameters	Weeks	Groups				
		Control	FO-1	FO-3	FO-4	FO-5
AST (U/L)	0	33.3 ± 4.6	24.2 ± 3.8	36.2 ± 5.4	30.1 ± 3.6	21.8 ± 4.6
	1	20.7 ± 3.4	24.6 ± 5.2	39.1 ± 8.1	47.2 ± 6.9	33.7 ± 6.4
	4	23.8 ± 3.8	38.4 ± 6.0	28.0 ± 11.5	33.5 ± 8.3	29.5 ± 5.4
ALT (U/L)	0	6.0 ± 0.8	6.8 ± 0.9	5.1 ± 0.6	5.3 ± 0.6	4.3 ± 0.6
	1	4.9 ± 0.6	6.2 ±0.8	4.0 ± 0.4	5.1 ± 0.6	4.5 ± 0.4
	4	3.8 ± 0.6	4.5 ± 0.4	5.7 ± 0.4	6.4 ± 0.8	5.9 ± 0.5
LDL-C (mg/dL)	0	18.6 ± 6.2	19.0 ± 5.8	20.2 ± 5.0	21.3 ± 7.2	19.4 ± 5.4
	1	22.4 ± 4.6	26.6 ± 5.9	17.8 ± 7.8	28.7 ± 9.0	26.4 ± 3.8
	4	19.2 ± 6.5	15.7 ± 3.0	24.2 ± 4.6	23.9 ± 4.8	21.7 ± 7.2
TG (g/dL)	0	6.0 ± 0.6	5.5 ± 0.8	4.3 ± 0.8	5.9 ± 0.5	4.2 ± 0.4
	1	5.3 ± 0.4	5.7 ± 0.2	4.4 ± 0.6	6.2 ± 0.8	5.5 ± 1.2
	4	4.5 ± 0.8	6.4 ± 0.8	4.6 ± 0.9	4.2 ± 0.4	4.6 ± 1.1
Hb (g/dL)	0	17.8 ± 5.2	16.8 ± 5.4	21.4 ± 5.6	19.4 ± 6.4	22.4 ± 5.5
	1	22.4 ± 7.4	29.0 ± 9.5	24.8 ± 8.5	28.1 ± 8.1	35.8 ± 11.7
	4	15.3 ± 5.6	27.1 ± 6.8	28.0 ± 10.1	26.8 ± 9.4	27.1 ± 4.7
PHO (mg/dL)	0	8.6 ± 1.8	8.8 ± 2.4	10.5 ± 0.8	9.5 ± 1.2	6.1 ± 2.2
	1	9.5 ± 1.6	5.7 ± 2.4	9.8 ± 2.4	7.2 ± 3.0	6.5 ± 1.4
	4	7.7 ± 2.4	9.9 ± 1.4	8.2 ± 2.8	7.2 ± 2.8	7.4 ± 2.8
TP (g/dL)	0	4.0 ± 0.5	4.3 ± 0.4	3.7 ± 0.6	2.6 ± 0.2	3.4 ± 0.3
	1	2.8 ± 0.4	3.8 ± 0.2	2.1 ± 0.4	4.2 ± 0.2	3.9 ± 0.7
	4	3.7 ± 0.3	4.6 ± 0.5	4.4 ± 0.3	3.5 ± 0.5	2.9 ± 0.4
GLU (mg/dL)	0	30.3 ± 0.6	16.4 ± 0.9	26.5 ± 0.8	31.1 ± 0.8	26.4 ± 0.9
	1	24.7 ± 0.8	31.1 ± 0.8	27.4 ± 0.8	28.6 ± 2.4	30.6 ± 3.6
	4	29.6 ± 1.4	25.3 ± 1.5	14.0 ± 1.0	30.2 ± 1.4	28.3 ± 2.4

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDL-C: Low density cholesterol, TP: Total protein, GLU: Glucose, PHO: Phosphorus, TG: Triglycerides, Hg: Hemoglobin, *: $P < 0.05$.

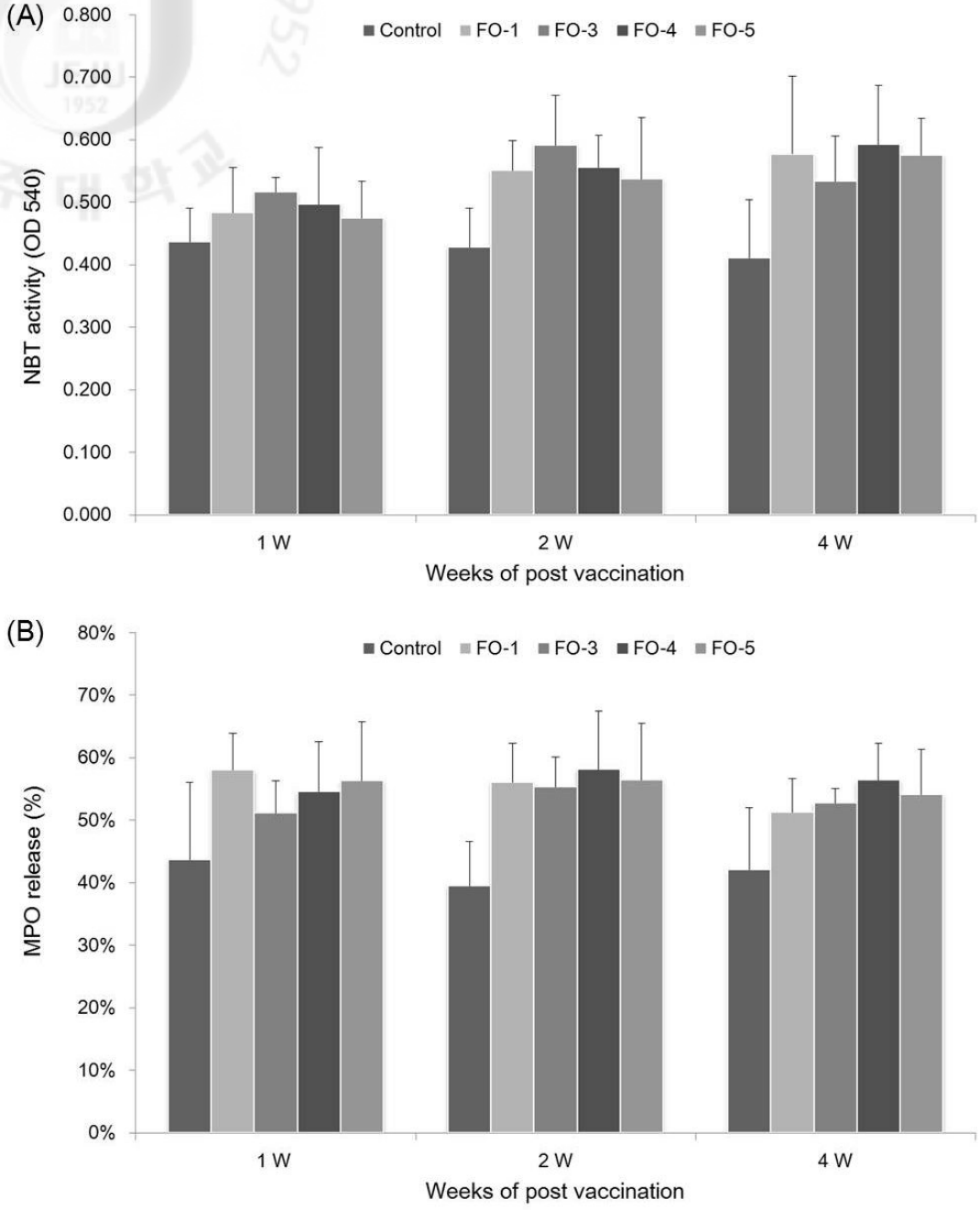

Figure 36. Nitroblue tetrazolium (NBT) and myeloperoxidase (MPO) activities of three monovalent vaccines, one trivalent vaccine and control groups. (A) Nitroblue tetrazolium (NBT) activity measured at OD 540nm, (B) Myeloperoxidase (MPO) release percentage.

6. Myeloperoxidase (MPO) release activity

This assay for the measurement of MPO release from neutrophil granules was recently adapted for use with fish kidney neutrophils, and is employed to determine the effects of immunization by vaccination, stress, anesthetics, and dietary immunomodulators on neutrophil function (Palić et al., 2006 a,b). The release of myeloperoxidase by the azurophilic granules of neutrophils during oxidative respiratory burst activity was significantly higher in all of the vaccinated groups than in the controls (Figure 36B). MPO is an antimicrobial compound. This result considered that the fish immunized by these vaccines evidenced improved their health.

7. Lysozyme activity

Lysozyme is an important hydrolytic enzyme with a protein character in the non-specific defense system. The serum lysozyme activity was not indicated to differ significantly between the control and immunized group with FO-4 during immunized period (Figure 37). However the lysozyme activities were significantly higher in the immunized groups with the other vaccines groups than in the control group (Figure 37). In addition, the FO-5 vaccination group was extremely shown high lysozyme activity at one week post-vaccination, and it was decreased the level of lysozyme activity at other immunized periods. The FO-1 vaccination group that injected trivalent vaccine was increased to 2^{nd} weeks post-vaccination, and it was maintained the lysozyme activity until 4^{th} weeks post vaccination. Especially, trivalent vaccine was shown higher lysozyme activity compared to other monovalent vaccines, when 2^{nd} weeks and 4^{th} weeks post-vaccination. In these results, lysozyme activity might more strongly protect against the invasion of certain pathogenic bacteria.

8. Phagocytosis activity

The phagocytic cells are the most important cellular components of the innate immune system of fish (Zang et al., 2009). It is a primitive defense mechanism (Macarthur and Fletcher, 1985), and an important characteristic of the innate immune system (Seeley et al., 1990). The phagocytosis activity of head-kidney leucocytes were significantly increased in all the vaccinated groups on 1^{st}, 2^{nd}, and 4^{th} weeks post-vaccination (Figure 38). In addition, FO-3 vaccination group was shown highest phagocytosis activity during all the immunization period, and the FO-1 vaccination group that injected trivalent vaccine was increased to 2^{nd} weeks post-vaccination, and it was maintained the phagocytosis level until 4^{th} weeks post-vaccination. On the other hand, FO-4 and FO-5 groups were also increase to 1^{st} weeks post-vaccination; thereafter it did not show change of phagocytosis levels, significantly. In these result, the immunization by the vaccines may induce cell-mediated immunity in the vaccinated olive flounder.

9. Direct serum agglutination

Induction of specific agglutinating antibodies alone appears to be insufficient to protect from bacterial infection in fish. However, measuring of agglutinating antibodies is need to determine the immunization level by vaccination.

The serum collected from all immunized fish by monovalent vaccine and trivalent vaccine showed agglutination activity against formalin killed *S. iniae*, *S. parauberis*, and *E. tarda*, respectively. A weak agglutination activity of serum were observed in the fish immunized with FO-1, FO-3, and FO-4 vaccines against *S. iniae* and *S. parauberis*, and the activity was significantly increased at 1^{st} week post-vaccination (Fig. 39A and 39B). The trivalent vaccinated group was $2^{2.6}$ and $2^{4.2}$ of highest agglutination titers during immunization period against *S. iniae* and *S. parauberis*, respectively. In addition, agglutination titers of trivalent vaccinated groups were lower than each monovalent vaccines during immunization period.

However, FO-1 and FO-5 vaccinated groups were appeared high agglutination activities, it was increased to 2^{nd} weeks post-vaccination, and it were maintained the agglutination activities until 4^{th} weeks post-vaccination (Figure 39C). The trivalent vaccinated group was $2^{8.4}$ of highest agglutinating antibodies level against *E. tarda,* and it was also lower than monovalent vaccinated group, during immunization period.

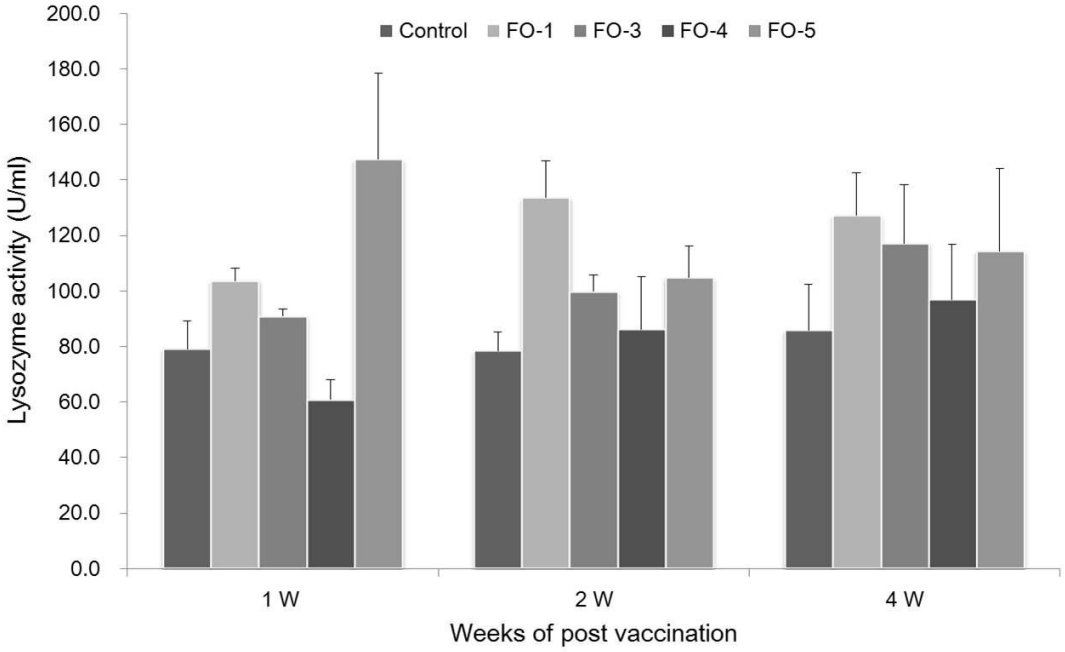

Figure 37. Lysozyme activity of three monovalent vaccines, one trivalent vaccine and control groups at 1, 2, and 4 weeks of post-vaccination.

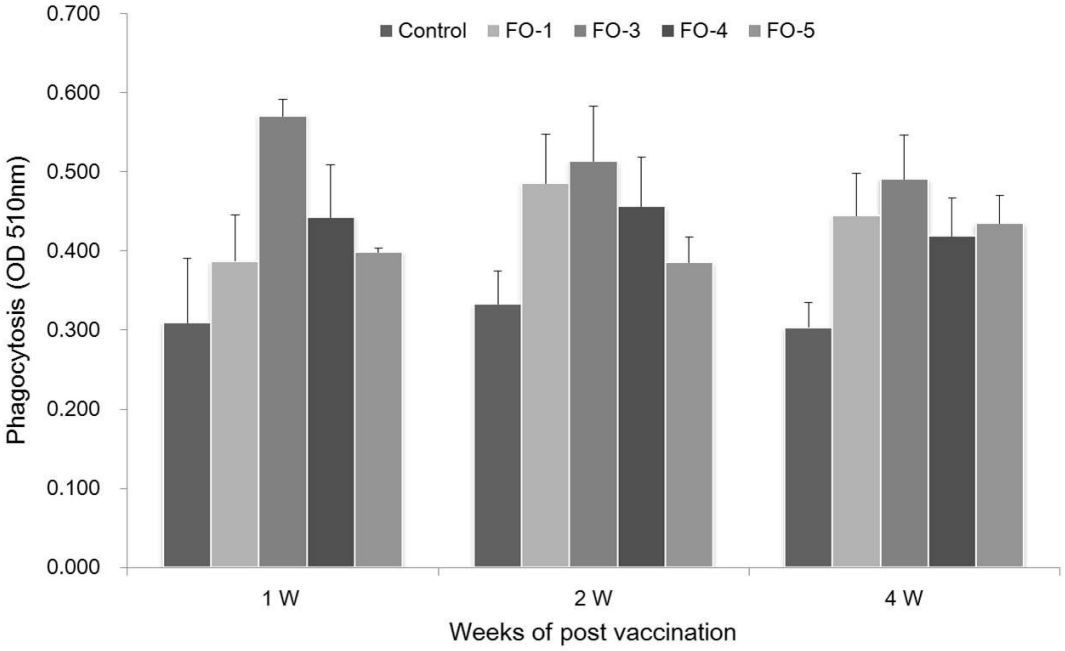

Figure 38. Phagocytosis activity of three monovalent vaccines, one trivalent vaccine and control groups at 1, 2, and 4 weeks of post-vaccination.

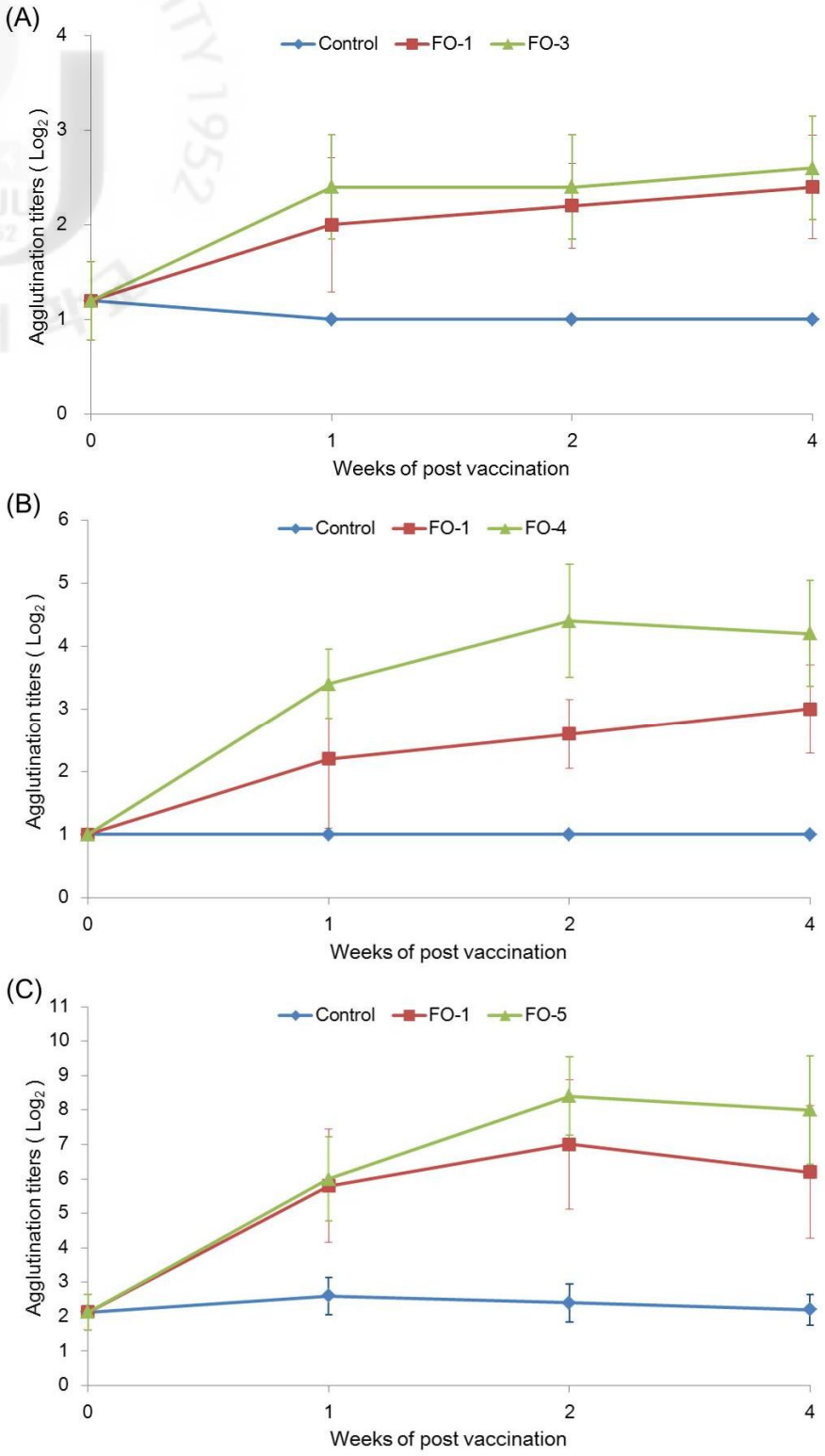

Figure 39. Change in agglutination against *S. iniae*, *S. parauberis*, and *E. tarda* FKC of serum collected from vaccinated olive flounder with monovalent and trivalent vaccines. (A) The agglutination titers against *S. iniae* FKC, (B) The agglutination titers against *S. parauberis* FKC, (C) The agglutination titers against *E. tarda* FKC.

10. Efficacy of oil adjuvant vaccines administered to olive flounder

In order to examine the immunoprotective effect of three monovalent and one trivalent vaccines, which were injected into olive flounder, the immunized olive flounder by monovalent (FO-3, FO-4, and FO5) and trivalent (FO-1) vaccination that were reared until 28 days post-vaccination, therefore the vaccinated fish have to immunized fully for efficacy investigation of these vaccines. The fish were challenged with homologous *S. iniae*, *S. parauberis*, or *E. tarda* strain monitored for mortality. The results of challenge with *S. iniae* strain, the cumulative mortalities of FO-1, FO-3 and saline vaccinated fish were 7.5 %, 2.5 %, and 82.5 %, respectively (Table 14), which yield a protection efficacy, in terms of RPS, of 90.31 % , 96.97 % for FO-1, FO-3 when compared to control group at termination of challenge test. In addition, challenged experiment with the homologous *S. iniae* strain was appeared acute mortalities in control group, which was appeared initial mortality at 5th days post-challenge and then it was extremely increased cumulative mortality until the 8th day post-challenge. However immunized fish by trivalent vaccine was successfully protected the pathogen, that showed a very lower mortality rate than non-immunized fish and similar to mortality of immunized fish by monovalent vaccine (Figure 40A). The immunized fish by FO-1 and FO-4 vaccines were challenged with the homologous *S. parauberis* strain. In the control fish, mortality began to occur at 10th days post-challenge, while in the vaccinated groups, mortality lately occurred at 22-26 days post-challenge. By the termination of the monitored period, the cumulative mortalities of FO-1, FO, 4, and control groups were 20 %, 10 %, and 65%, respectively (Figure 40B). in addition, the mean survival rates, in terms of RPS, were 69.23 % and 84.62 %, respectively (Table 14). The immunized fish by FO-1 and FO-5 vaccines were challenged with the homologous *E. tarda* strain. In the control fish, mortality began to occur at 7th days post-challenge, while in the vaccinated groups, mortality lately occurred at 7-11 days post challenge. By the termination of the monitored period, the cumulative mortalities of FO-1, FO, 5, and control groups were 32.5 %, 47.5 %, and 85 %, respectively (Figure 40C). in addition, the mean survival rates, in terms of RPS, were

44.12 % and 61.76 %, respectively (Table 14). In addition, challenge test with the homologous *S. parauberis* and *E. tarda* strains were monitored chronic mortalities pattern in contrast challenge with *S. iniae* strain (Figure 40). The mortality pattern might be affect immunoprotection against these bacterial pathogens because rearing environment of fish can occur actively horizontal infection. These results indicate that FO-1 as trivalent vaccines exhibited low protections, when compared to monovalent vaccines such as FO-3, FO-4, and FO-5. However, the trivalent vaccine FO-1 was indicated sufficient efficacy against the major bacterial pathogens such as *S. iniae*, *S. parauberis*, and *E. tarda*, therefore we consider that the trivalent vaccine in this study could be used successfully in commercial olive flounder farms.

Table 14. Cumulative mortality and relative percent survival of monovalent and trivalent vaccinated olive flounder challenged with virulent isolates of *S. iniae*, *S. parauberis*, and *E. tarda*.

Vaccination	Isolated used for challenge	Challenge dose (CFU/fish)	dpc_{60} [a]	RPS_{60} [b]	dpc[c]	Cumulative mortality (%)	RPS[d]
Monovalent vaccine							
FO-3	*S. iniae* JJU-019	1.0×10^4	-	97.15%	29	2.5	96.97%
FO-4	*S. parauberis* JJU-045	1.0×10^6	-	85.80%	29	10.0	84.62%
FO-5	*E. tarda* JJU-054	1.0×10^4	-	80.80%	29	32.5	61.76%
Trivalent vaccine							
FO-1	*S. iniae* JJU-019	1.0×10^4	7	94.29%	29	7.5	90.91%
	S. parauberis JJU-045	1.0×10^6	28	71.60%	29	20.0	69.23%
	E. tarda JJU-054	1.0×10^4	18	42.40%	29	47.5	44.12%
Control	*S. iniae* JJU-019	1.0×10^4	7	-	29	82.5	-
	S. parauberis JJU-045	1.0×10^6	28	-	29	65.0	-
	E. tarda JJU-054	1.0×10^4	18	-	29	85.0	-

[a] dpv_{60}, days post challenge at the time point corresponding to 60% mortality in the control group.

[b] RPS_{60}, relative percent of survival at the time point corresponding to 60% mortality in the control group.

[c] dpv, , days post challenge.

[d] RPS, relative percent of survival.

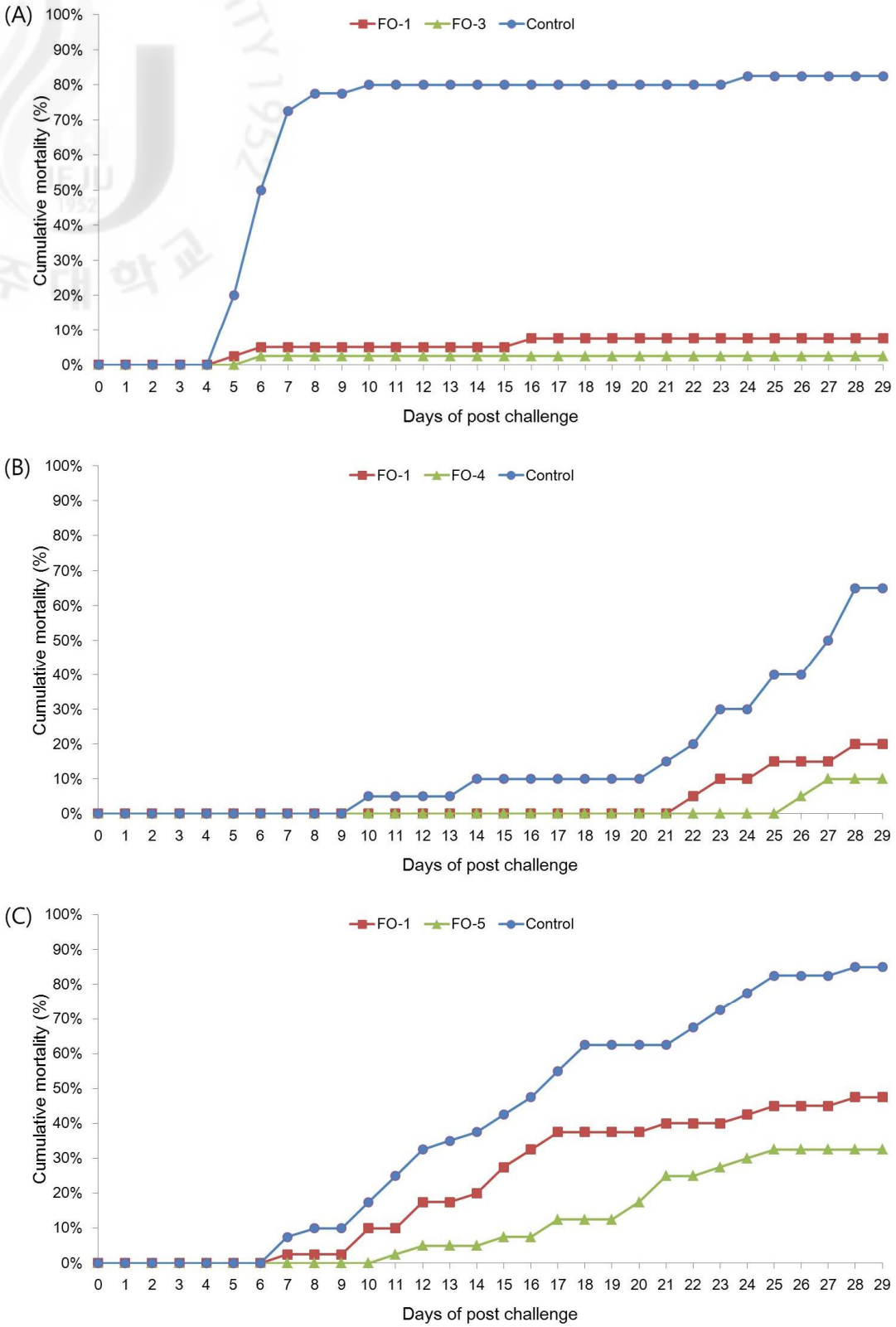

Figure 40. Daily mean percent cumulative mortality of olive flounder vaccinated with three of the monovalent and one of the trivalent vaccines and challenged with *S. iniae*, *S. parauberis*, and *E. tarda* through intraperitoneal injection at 28 days post-vaccination.

DISCUSSION

In Korea, the aquaculture had been developed with the evolving scientific technology, production losses were also increased by infection of various pathogens, recently. However, cultured fish mortality is not only criterion to evaluate the damage effects of its disease. The morbidity that leads to weight loss and decreasing growth rate in surviving fish also contributes substantial economic losses to the fish farmers. Recently, control of pathogens is extremely important factor to maintain healthy fish to increase production. Various fish bacterial diseases are known to be caused by bacterial pathogens such as *S. iniae*, *S. parauberis*, *E. tarda*, *L. garvieae*, *V. anguillarum*, and *V. haveyi*. They have been identified as most infectious agents in fish disease mostly. In addition, streptococci and edwardsiellosis including *S. iniae*, *S. parauberis*, and *E. tarda* are most occurred disease in olive flounder (Park, 2009). The main strategies for disease control are chemotherapeutics, medicinal herbs, vaccines, and immunostimulants. In Korea, 27 antibacterial agents have been used including 23 antibiotics and 4 sulfa medicines. The mass use of these medicines often caused in occurrence of drug-resistant bacteria, remnants of antibiotics in the cultured fish and environmental diffusion. Therefore, the strategy of vaccination would be alternative and more promising aspect to prevent fish from various bacterial diseases (Park, 2009). As the host control aspect, we have been used vaccines that with good health management appears to be the most effective way to prevent diseases in aquaculture farms. Therefore, vaccination has become one of the efficient method to prevent fish diseases among countries such as Europe and North America. In addition, several vaccines against the bacterial pathogens were also developed in Korea. Vaccines against the bacteria including *E. tarda* and *S. iniae* were already developed and commercially available recently. The effectiveness of immersion vaccine of *E. tarda* against edwardsiellosis of olive flounder was evaluated in a field trial test (Park 2009; Bang et al., 2000; Cho et al., 2006). However development of multivalent vaccine is very insufficient in aquaculture industry of olive flounder in Korea.

Therefore, we used oil base adjuvant base approach to evaluate the multivalent vaccine formulation in order to give effective immune protection of major bacterial pathogens in olive flounder, in this study. Three monovalent and one trivalent vaccines for *S. iniae*, *S. parauberis*, and *E. tarda* were injected into olive flounder to investigate the efficacy levels of the vaccines. The vaccinated groups were required to completely recovered for just one days post-vaccination, and vaccinated flounder showed a complete recovery rate from the beginning of immunization therefore these vaccines were gave low level of stress. In order to choice of challenge periods, we were selected 28 day of post-vaccination according to accumulated temperature. The average growth of the FO-5 group was increased by approximately 10% at the termination of immunization periods, as compared to that of the control fish group. In addition, the other vaccinated groups were also increased or similar levels as compared to the control group at the same periods. These results might be induced from an decreased chances that was infectious target pathogens in the fish tanks, therefore vaccinated fish could be avoid stress by pathogens. During the immunization periods, vaccinated groups were investigated the cumulative mortalities. The FO-1 injection group was occurred tiny mortality that only two fish were died in four weeks, which were calculated 0.008 % of cumulative mortality rate. However the control and other vaccinated groups were showed any mortality. In addition, The blood biochemical and physiological parameters are important for general health, toxicology, and bio-monitoring. The stress caused by vaccination of fish is major factor, which reluctant reason of aquaculture farmers. Overall, these results did not induce stress by these mono valent vaccines and multi valent vaccine in olive flounder of these trials. It is desirable that investigations on vaccine efficacy require to determine what defense mechanisms are of primary importance to achieve successful protection against the bacterial pathogen. Fish have many non-specific and specific humoral and cell-mediated mechanisms to resist bacterial diseases (Ellis, 1999). Therefore, we had been investigated innate immune response of immunized olive flounder by these vaccines. Leucocytes play an important role in non-specific or innate immunity and

their count can be considered as an indicator of the health status of fish. Both macrophages and neutrophilic granulocytes are characterised by having high phagocytic activity against the invading microorganisms, acting as the first line of defence to eliminate infectious agents. During phagocytosis, stimulation of the cell membrane triggers the production of oxygen free radicals by generating superoxide anion (O^{2-}) and its derivatives such as hydrogen peroxide (H_2O_2) and hydroxyl free radicals (OH^-). This process is known as respiratory burst. These reactive oxygen intermediates have been reported to have potent bactericidal activities against fish bacterial pathogens (Hardie et al., 1996; Itou et al., 1997). The superoxide anion production of respiratory burst activity of immunization by vaccination was measured via NBT reduction and MPO release activities. All the vaccinated groups did not significantly increase the NBT reduction activity after second, whereas fourth weeks post-vaccination of all the vaccinated groups significantly increased when compared to the control. The release of myeloperoxidase by the azurophilic granules of neutrophils during oxidative respiratory burst activity was significantly higher in all of the vaccinated groups than in the controls. The phagocytosis activity of head-kidney leucocytes were significantly increased in all the vaccinated groups on 1st, 2nd, and 4th weeks post-vaccination. In addition, FO-3 vaccination group was shown highest phagocytosis activity during all the immunization period, and the FO-1 vaccination group that injected trivalent vaccine was increased to 2nd weeks post-vaccination, and it was maintained the phagocytosis level until 4th weeks post-vaccination. In these result, the immunization by the vaccines may induce cell-mediated immunity in the vaccinated olive flounder. Lysozyme is widely distributed in bacteriophages, microbes, plants, invertebrates, and vertebrates (Jollès and Jollès, 1984). It is an enzyme that catalyzes the hydrolysis of sugar chains that constitute the cell walls of most bacteria. Lysozyme possesses lytic activity that enables it to function as a non-specific biodefense molecule and provides defense against pathogens. In this study, the serum lysozyme activity was not indicated to differ significantly between the control and immunized group with FO-4 during immunized period. However the lysozyme activities were significantly higher in the

immunized groups with the other vaccines groups than in the control group. In addition, the FO-5 vaccination group was extremely shown high lysozyme activity at one week post vaccination, and it was decreased lysozyme activity to the level of other vaccination groups. The FO-1 vaccination group that injected trivalent vaccine was increased to 2nd weeks post vaccination, and it was maintained the lysozyme activity until 4th weeks post-vaccination. Especially, trivalent vaccine was shown higher lysozyme activity compared to other monovalent vaccines, when 2nd weeks and 4th weeks post vaccination. In these results, lysozyme activity might more strongly protect against the invasion of certain pathogenic bacteria. The serum collected from all immunized fish by monovalent vaccine and trivalent vaccine showed agglutination activity against formalin killed *S. iniae*, *S. parauberis*, and *E. tarda*, respectively. A weak agglutination activity of serum were observed in the fish immunized with FO-1, FO-3, and FO-4 vaccines against *S. iniae* and *S. parauberis*, and the activity was significantly increased at 1st week post-vaccination. The trivalent vaccinated group was 22.6 and 24.2 of highest agglutination titers during immunization period against *S. iniae* and *S. parauberis*, respectively. However, FO-1 and FO-5 vaccinated groups were appeared high agglutination activities, it was increased to 2nd weeks post vaccination, and it were maintained the agglutination activities until 4th weeks post-vaccination. The trivalent vaccinated group was 28.4 of highest agglutinating antibodies level against *E. tarda*. In addition, agglutination titers of trivalent vaccinated groups were lower than each monovalent vaccine during immunization period. According to previous studies, some researches that were to developed vaccine for prevent these bacterial diseases of olive flounder had been reported in Korea. The two-fold diluted *E. tarda* formalin-killed cells (FKC) were administrated by immersion for two minutes to 3,000 fingerlings and 5,000 juveniles of olive flounder. Relative percent survivals (RPS) for two vaccinated groups of the fingerlings and juveniles were 94.9%, and 92.4%, respectively (Bang et al., 2000). Streptococcal diseases were also considered as a serious problem because of significant economic losses in aquaculture of olive flounder. Fortunately, effective *S. iniae* FKC vaccine for olive flounder

was developed and the fish immunized intraperitoneally with the 0.3% as prime and booster exhibited the RPS of 66.7% and 87.5%, respectively (Cho et al., 2006). However, the researches on multivalent vaccine of olive flounder are very rare. Especially, this study that research of develop vaccine against these bacterial pathogens including *S. iniae*, *S. parauberis*, and *E. tarda* is first report in Korea. Therefore, we were investigated the efficacy of these vaccination in olive flounder directly. In order to examine the immunoprotective effect of monovalent and trivalent vaccines, the fish were challenged with homologous *S. iniae*, *S. parauberis*, or *E. tarda* strain monitored for mortality. The cumulative mortalities of FO-1, FO-3 and saline vaccinated fish were 7.5 %, 2.5 %, and 82.5 %, respectively, which yield a protection efficacy, in terms of RPS, of 90.31 % , 96.97 % for FO-1, FO-3 when compared to control group at termination of challenge test. By the termination of the monitored period, the cumulative mortalities of FO-1, FO, 4, and control groups were 20 %, 10 %, and 65%, respectively, and the mean survival rates, in terms of RPS, were 69.23 % and 84.62 %, respectively. The cumulative mortalities of FO-1, FO, 5, and control groups were 32.5 %, 47.5 %, and 85 %, respectively. In addition, the mean survival rates, in terms of RPS, were 44.12 % and 61.76 %, respectively. These results indicate that FO-1 as trivalent vaccines exhibited low protections, when compared to monovalent vaccines such as FO-3, FO-4, and FO-5. However, the trivalent vaccine FO-1 was indicated sufficient efficacy against the major bacterial pathogens such as *S. iniae*, *S. parauberis*, and *E. tarda*, therefore we consider that the trivalent vaccine in this study could be used successfully in commercial olive flounder farms.

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ACKNOWLEDGEMENT

구체적인 목표가 있는 사람은, 조금 돌아가더라도 확고한 신념과 목표가 있다면 그 꿈을 이룰 수 있습니다. 더불어 그 꿈을 이루기 위해서는 그 길을 올바르게 갈 수 있도록 인도해 주는 안내자와 나침반이 필요하다고 생각합니다. 제가 해양미생물 실험실의 일원이 되어 지금까지 힘들어하고 방황할 때 마다 항상 주변에서 충고와 격려해 주신 모든 분들 덕에 미약하나마 박사학위논문을 마칠 수 있었던 것 같습니다. 박사과정 동안, 너무나도 부족한 저를 언제나 믿어주시고 기회를 주신 허문수 교수님께 먼저 머리 숙여 마음깊이 감사의 인사를 드리고 싶습니다. 가는 길이 힘들어 포기하고 싶을 때도, 저를 감싸주시고 제게 올바른 길로 갈 수 있도록 지도해 주신 교수님께 더 좋은 결과를 보여 드리지 못한 것 같아 너무나 송구스럽습니다.

그리고 대학원 기간동안 학술적인 조언을 아끼지 않으시고 이끌어 주시고 바쁘신 가운데도 학위논문을 검토해 주신 이승헌 교수님, 전유진 교수님, 이경준 교수님, 진창남 소장님 특히 연구과제를 통해 많은 조언과 관심을 가져주신 이제희 교수님, 대학원 기간동안 많은 가르침과 관심을 주신 송춘복 교수님, 여인규 교수님, 최광식 교수님, 이영돈 교수님, 김기영 교수님, 정준범 교수님께 깊은 감사드립니다. 대학원생 생활을 하면서 많은 관심과 격려를 아끼지 않으신 고대희 계장님, 실험에 있어 어려워하는 저를 바쁘신 와중에도 관심을 가져 주시며 많은 가르침을 주셨던 강봉조 연구사님께 진심으로 감사의 마음을 전합니다. 졸업 후에도 항상 마음 써주시고 격려해 주신 양병규 연구사님, 장영환 연구사님, 철영이형, 영건이형, 만철이형, 민주, 선경, 윤범, 봉근, 용재, 동민 그리고 항상 힘들 때 챙겨주었던 태원, 학위논문 준비에 많은 도움을

주었던 하리 그리고 항상 고생하는 실험실 후배들 익수, 동휘, 민선, 경미, 지운, 원용이 특히 학위논문을 준비하는데 실질적으로 많은 도움을 준 창영, 승현이 모든 해양미생물 실험실원들에게 고맙다는 말을 전합니다.

그리고 항상 의지할 수 있어 제일 큰 힘이 되어주고 실험에 관해서도 항상 자기 일처럼 도와주고 고민해 주었던 동기 승홍, 송헌, 경주 그리고 대학 생활동안 함께한 해양자원공학 실험실, 어류분자생리학 실험실, 분자유전학 실험실, 어류유전육종 실험실 선배님, 후배님들과 외국인 유학생들에게도 감사의 마음을 전합니다. 항상 친구 중에 박사하나 생긴다고 농담하며 걱정, 격려, 응원해준 내 친구들에게도 감사드립니다. 항상 철없는 둘째아들을 걱정하시며 저희 형제 뒷바라지 하시느라 고생하시는 아버지, 어머니 그리고 우리 형, 동관이에게 평소에는 말하지 못했지만 항상 감사하다고 말씀드리고 싶습니다. 더불어 이번 졸업논문은 노르웨이의 어류백신 제조업체인 Pharmaq 의 연구지원을 받아 연구를 진행하였습니다. 마지막으로 박사학위를 진행하는 동안 늘 응원해준 예신 이은주에게 앞으로 존경받을 수 있는 사람이 되도록 더욱 노력하고 감사한 마음 전하고 싶습니다.