A THESIS

FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

Identification, characterization, rapid detection technique development and vaccine production of major fish pathogen, *Pseudomonas anguilliseptica* isolated from farmed olive flounder, *Paralichthys olivaceus*

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CONTENT

국문초록

어류에 대한 소비량 증가로 양식어류의 공급확대에 대한 필요성이 커지고 있으며, 우리나라의 연간 양식어류 생산량 중 넙치 생산량은 전체 양식어류 생산량의 약 51%를 차지하고 있다. 이 중 제주지역의 양식넙치 생산량은 약 25,000 톤으로 국내 양식 넙치 생산량의 60% 이상을 차지하고 있어 국내 넙치양식 산업에서 중요한 역할을 하고 있다. 그러나 육상양식장 사육면적이 증가하고 있음에도 생산량은 오히려 감소 추세에 있으며 이는 고밀도 사육환경에 의한 스트레스와 질병발생으로 인한 폐사가 주요 원인으로 분석되고 있다. 도내 양식넙치에 주로 피해를 주고 있다고 알려져 있는 세균성 질병으로는 에드워드증 (Edwardsiellosis), 비브리오증 (Vibriosis), 연쇄구균증 (Streptococcosis) 등이 보고되어 있으며, 그 외에도 치어기에 주로 발병하여 피해를 준다고 알려져 있는 바이러스성 질병인 바이러스성 출혈성 패혈증 (Viral hemorrhagic septicemia) 및 기생충성 질병인 스쿠티카증 (Scuticocilliatosis) 등 연중 다양한 복합감염 형태를 보이고 있다. 이러한 각종 질병발생으로 인한 피해가 확산됨에 따라 도내 넙치양식은 어려움을 겪고 있으며 양식어가에서는 이를 극복하기 위해 많은 양의 항생물질을 사용하고 있어 내성균 증가, 근육 내 항생물질 잔류 및 해양생태계의 오염 등 다양한 문제를 야기하고 있다. 따라서 화학요법제 사용에 의한 사후치료보다는 천연물을 이용한 사료 첨가제 및 백신 개발은 안전한 양식수산물 생산과 질병 예방에 따른 생산량을 증가시킬 수 있는 가장 효과적인 대처방법으로 제시되고 있다.

2010년 이후 도내 넙치 치어의 70% 이상이 입식 되는 저수온기 (16~20℃)에 세균성 질병으로 인한 폐사량이 증가하고 있어 원인균 연구를 통한 감염특성 파악, 신속진단법 및 백신 개발에 대한 대책이 시급한 실정이다. 이를 위해 저수온기 도내 양식장을 대상으로 질병에 감염된 것으로 추정되는 양식어류 치어 (olive flounder, rock bream, seven-band grouper, red-spotted grouper, rockfish)를 채집하여 현미경

검경을 통한 기생충성 질병검사, TSA agar, BHI agar, Blood agar를 이용한 세균성 질병검사, PCR법을 이용한 바이러스성 질병검사를 실시하였다. 그 결과 현재까지 다양한 양식어류에 대해 주요 피해 질병으로 보고되어 있는 스쿠티카증, 바이러스성 출혈성 패혈증의 단독 및 혼합감염이 주요 질병으로 확인되었으며, 그 외 비브리오증, 활주세균증 (Flavobacteriosis)도 확인이 되었다. 그러나 도내 일부 양식장에서 채집된 샘플 중에서 슈도모나스증 (Pseudomonas septicemia) 원인균인 *Pseudomonas anguilliseptica*로 추정되는 병원균을 분리하고 확인한 결과 그람음성균으로 운동성이 있으며, catalase와 cytochrome oxidase 양성반응을 나타내었다. 또한 API 20E kit와 API ZYM kit를 이용하여 생화학적 특성을 확인한 결과 기존 *Pseudomonas anguilliseptica* 연구와 동일한 결과를 확인하였다.

분리 균주의 genotyping을 위해 *Pseudomonas anguilliseptica*의 16S rRNA gene specific primer로 보고되어 있는 Psan-F (5'-TTGGGAGGAAGGGCAGTAACC-3')과 Psan-R (5'-TGCGCCACTAAAATCTCAAG-3')을 이용하여 PCR을 실시한 결과 418 bp의 결과물을 얻었으며, 이를 NCBI GenBank에 등록되어 있는 *P. anguilliseptica*인 X99540, X99541, AB021376과 blast search를 이용하여 비교한 결과 99-100% 유사도를 보여 *P. anguilliseptica* (KC565867-KC565877)로 최종 확인되었다. 슈도모나스증의 경우 국내 및 일본에서는 돌돔과 뱀장어에 감염되어 체표 출혈, 체색흑화, 안구돌출, 복부팽만 등의 증상을 보이며, “Red spot disease” 또는 “Sekiten-byo”로 불리우고 있지만 넙치에서의 슈도모나스증은 현재까지 보고되어 있지 않다. 따라서 본 연구에서는 슈도모나스증 원인균의 분리, 감염특성 규명, 신속 진단법 개발, 백신 개발 등을 통해 저수온기 도내 양식넙치 치어에 발생하여 피해를 입히고 있는 슈도모나스증에 의한 폐사를 저감시킴으로써 양식경영의 안정성을 도모하고자 하였다.

본 연구에서는 31개의 *P. anguilliseptica* 균주를 양식넙치와 돌돔, 볼락, 능성어류에

서 분리하였으며, 그 중 현재까지 보고되어 있지 않은 양식넙치에서 분리한 11개의 균주를 실험에 사용하였다. 11개의 균주는 2010부터 2년 동안 도내 전 지역에서 분리하였으며, 분리 시 넙치 치어의 평균전장과 폐사율은 14 cm, 20-55%로 조사되었다. 병원성 확인을 위해 감염 농도, 사육 온도, 감염 방법에 따른 실험을 11개 균주를 대상으로 실시한 결과 동일한 감염패턴을 보였으며, 그 중 JEP4 균주 (KC565870)의 병원성이 가장 높은 것으로 확인되어 백신실험 균주로 최종 선발하였다. 병원성 실험 결과는 3×10^{8} $CFUml^{-1}$ 농도로 복강 내 접종한 그룹의 경우 두 (17℃과 20℃) 사육 수온 모두 90%의 누적폐사율을 보였으며, 3×10^{6} $CFUml^{-1}$ 농도로 감염시킨 그룹의 경우 75% (17℃)와 60% (20℃)의 누적폐사율을 각각 나타냄으로써 낮은 사육 수온과 고농도 감염에서 폐사가 급격히 증가하는 경향을 확인하였다.

현재까지의 슈도모나스증 제어 방법으로는 항생물질 투여, 수온 상승 등의 방법이 제시되고 있으나, 대부분의 기존 연구에서는 항생물질 경구 투여를 이용한 치료법에 대한 연구가 진행되어 왔다. *P. anguilliseptica*의 항생물질에 대한 감수성은 erythromycin, clindamycin, lincomycin, cephadroxil, cephalexin 등을 제외한 14종의 항생물질에 대해 높은 감수성을 보였으며, 특히 현재 국내 양식장에서 많이 사용되고 있는 oxytetracycline, oxolinic acid에서는 높은 항균활성을 보여 지금까지 보고된 결과와 일치하는 것을 알 수 있었다. 그러나 이러한 항생물질 사용은 저수온 사육환경에서 양식어류의 약물 대사와 투여 방법에 대한 문제를 야기하였으며, 이를 해결하기 위해 신속 진단법 개발을 통한 조기 항생물질 치료에 대한 필요성이 증가되었다. *P. anguilliseptica*의 진단은 *P. anguilliseptica*의 16S rRNA gene specific primer인 Psan-F과 Psan-R을 이용한 conventional PCR법이 유일하게 보고되어 있어, 본 연구에서는 *Pseudomonas putida, Pseudomonas chlororaphis, Pseudomonas stutzeri, Pseudomonas sp, Pseudomonas japonica, Pseudomonas xanthomarina, Pseudomonas tuomuerensis, Pseudomonas cremoricolorata, Pseudomonas*

koreensis, Pseudomonas umsongensis, Pseudomonas jinjuensis 등과 염기서열을 비교한 결과 특이성을 보이는 *P. anguilliseptica*의 71 bp를 target으로 specific primer (Ps16sr_F1: 5'-TGACGTTACCGACAGAATAAGCA-3'; Ps16sr_R1: 5'-CGCTTGCACCCTTCGTATTAC-3')와 probe (Ps16sr_TMP: 5'-6FAM-CGG CTA ACT TCG TGC CAG CAG CC-TAMRA3')를 제작하여 2004년 Romalde에 의해 보고된 conventional PCR법과의 검출 특이성과 민감도를 비교하였다. 그 결과 *P. anguilliseptica*에 대한 100% 검출 특이성과 100배의 검출 감도가 증가함을 확인하였으며, 인위감염을 통한 간, 신장, 비장, 뇌조직에서 *P. anguilliseptica* 감염 농도를 확인한 결과 비장에서 가장 낮은 C_T 값을 나타냄으로써 슈도모나스증 의심단계에서 비장조직을 이용한 조기 진단을 실시함으로써 진단 효과를 높일 수 있음을 확인하였다.

양식어류의 세균성질병 제어를 위한 항생물질 사용은 위에서 언급했던 항생제 내성 증가, 환경오염, 체내 잔류, 항생물질 구입에 따른 경제적 손실 등의 다양한 문제를 야기하고 있으며, 낮은 사육수온에서 어류 체내의 약물대사 및 사료섭이 감소에 따른 치료효과에 한계를 보임에 따라 어류질병의 예방적 측면 중 가장 이상적인 방안이라 할 수 있는 백신 개발 필요성이 절실히 요구되고 있다. 따라서 본 연구에서는 액체배지에 배양된 JEP4 균주 (KC565870)를 포르말린을 이용하여 불활화시킨 후 백신으로 사용하였으며, 백신 접종 후 방어효과를 분석함으로써 백신 개발의 필요성을 확인하였다.

제조된 백신의 방어효과 실험은 백신 농도를 3×10^7 $CFUml^{-1}$로 제작하여 1차 접종 2주 후 추가접종 (booster)을 실시하였으며, 4주 동안 넙치의 면화화를 유도하였다. 면역화된 넙치에 불화화되지 않은 JEP4 균주 (3×10^7 $CFUml^{-1}$)를 복강 내 접종하여 공격실험을 실시한 후 2주 동안 관찰을 통해 폐사어 수거 및 폐사원인 분석을 위한 배지배양 및 PCR 검사를 실시하였다. 사육수온 20℃에서의 폐사율은 백신을 접

종한 그룹의 경우 8%, 백신을 접종하지 않은 그룹의 경우 75%를 보여 89%의 상대생존율 (RPS)을 나타내었으며, 사육수온 17℃에서의 폐사율은 백신을 접종한 그룹의 경우 25%, 백신을 접종하지 않은 그룹의 경우 83%를 나타내어 69%의 상대생존율을 보였다. 또한 백신에 의한 항체생성은 응집항체가 측정을 통해 확인하였으며, 백신 접종그룹의 경우 대조구에 비해 높은 응집항체가가 확인됨에 따라 제조된 백신이 어체내에서 항체형성 및 방어효과를 유도하는 것으로 확인되었다. 백신 접종에 따른 선천면역반응 유도는 염증반응에 관여되어 있다고 보고된 사이토카인 발현량 측정을 통해 확인하였으며, 생성된 항체의 확인은 채혈된 넙치항혈청에 대한 western blotting을 이용하였다. 그 결과 100 kDa, 68 kDa, 60 kDa, 40 kDa의 단백질이 검출되었으며, 그 중 68 kDa, 60 kDa 단백질이 주요 항원결정기를 가지고 있는 것으로 확인됨으로써 추후 고효율 백신 개발을 위해서는 이들 단백질에 대한 연구가 반드시 필요하다고 사료된다.

또한, 불활화되지 않은 JEP4 균주 및 백신 투여 시 선천면역반응을 담당하는 TLR (Toll-like receptors), TNF (Tumor necrosis factor), IL (Interleukin), IFN (Interferon), CD40, Fas Ligand의 활성은 Real-time PCR (SYBR Green)법을 이용하여 발현정도를 측정한 결과 JEP4 균주 접종 시 접종 후 3시간 (3hpi)째 가장 높은 발현을 보여 기존 연구에서 보고되었던 그람음성균 감염에 따른 염증반응 발현패턴과 동일한 결과를 나타냄을 알 수 있었으며, 백신 투여 시 PBS를 접종한 대조구에 비해 TNFR-1, IRF7, Fas L, IL-1b, TLR2, CD40의 발현량이 증가함에 따라 선천면역반응을 유도하는 것을 알 수 있었다.

본 연구에서는 양식넙치의 슈도모나스증의 효과적인 제어를 위해 두 가지 측면으로 접근하였으며, 신속한 진단을 위해 검출 민감도를 향상시킨 primer와 probe를 제작함으로써 양식넙치의 슈도모나스증 조기 진단을 통한 항생물질 치료효과를 높이고, 양식돌돔, 능성어, 볼락 등의 다양한 슈도모나스증 감수성 어류에 대한 신속 진단 가능성

을 확인할 수 있었다. 또한, 백신 접종에 따른 방어효과 실험결과 80% 이상의 높은 상대생존율을 확인함에 따라 현장실증시험을 통한 양식현장 적용 시 양식수산물의 생산 안정성을 확보하는데 큰 힘이 될 수 있으리라 사료된다.

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INTRODUCTION

Among the global aquaculture production of olive flounder, Republic of Korea stands first with 70% of world's supply followed by Japan, in the year 2012 (Tveteras, 2012). Particularly 62% of domestic olive flounder is from Jeju Island, with the annual production of 24,575 mt (http//www.kostat.go.kr/, 2012). Such an increased production could be obtained from an intensive culture system of flounder production, which keeps the fishes at high densities and the possibility for exposure to disease pathogens. Pathogens can be of bacterial, parasitic, or viral. Under such conditions, the problems of the infectious fish diseases become serious and have considerable effects. The cumulative mortalities rate was 34.8% and caused mainly by scuticociliatosis, streptococcosis, viral hemorrahagic septicemia (VHS), gliding bacterial disease and also by some non-infectious loss. Particularly, scuticociliatosis and VHS of farmed juvenile olive flounder have lead to high moralities and reduced economical income for the fish farming industry in Jeju Island (Ko et al., 2004; Kim et al., 2010).

In addition, recently *P. anguilliseptica* had generated mass mortality in cultured striped beakperch, *Oplegnathus fasciatus* which were reared at low water temperature and fishes showed clinical signs of abnormal one-sided swimming on the water surface along with hemorrhagic lesions on the body surface (Ko et al., 2004; Kim et al., 2010). Recently in olive flounder farms located in Jeju Island showed increased mortality particularly in juvenile fishes reared at low water temperature (17-21°C). Pathogens identified for the mortality of cultured fishes reared at low temperatures includes gram negative bacteria and scuticociliates with clinical symptoms such as darkening skin, hemorrhaging and ulcer in fin bases, body surface (Personal communication).

Pseudomonas anguilliseptica, a Gram negative bacteria, the causative organism of ''red spot disease'' or "sekiten-byo" in Japanese eel *Anguilla japonica* (Temminck and Schlegel), was first isolated from pond-cultured in 1971 (Wakabayashi and Egusa, 1972) and its occurrence in tilapia *Oreochromis niloticus* cultured in Egyptian fish farm, caused Pseudomonas septicemia (Saleh et al., 2012). This opportunistic fish pathogen survives in low water temperature, the most significant

factor influencing disease in various fishes. Clinical symptoms of infected fishes showed abdominal distension of abundant ascetic fluid, hemorrhagic lesions on the body surface and internal organs and loss of skin mucus (Wiklund and Bylund, 1990; Wiklund and Lönnström, 1994; Berthe et al., 1995; Doménech et al., 1997).

P. anguilliseptica infection had been confirmed from various countries in several fish species of both fresh and marine water namely European eel (*Anguilla anguilla*), black seabream (*Acanthopagrus schlegeli*), ayu (*Plecoglossus altivelis*), sea trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), whitefish (*Coregonus* sp.), Baltic herring (*Clupea harengus*), rainbow trout (*Oncorhynchus mykiss*), orange-spotted grouper (*Epinephelus coioides*), gilthead seabream (*Sparus aurata*), turbot (*Psetta maxima*), and black spot seabream (*Pagellus bogaraveo*), farmed cod (*Gadus morhua*) (Kuo and Kou, 1978; Nakai and Muroga, 1982; Wiklund and Dalsgaard, 1987; Wiklund and Bylund, 1990; Wiklund and Lönnström, 1994; Berthe et al., 1995; Kusuda et al., 1995; Al-Marzouk, 1999; López-Romalde et al., 2003a; Ferguson et al., 2004). *P. anguilliseptica* known as a fastidious bacterium with slow growth and weak reaction on culture media used in the diagnostic purposes of fish diseases (Doménech et al., 1997; Daly, 1999; López-Romalde et al., 2003b). Additionally, different authors have reported the different phenotypical discriptions (Wakabayashi and Egusa, 1972; Wiklund and Bylund, 1990; Michel et al., 1992; Berth et al., 1995; Doménech et al., 1997; Daly, 1999). Also, in most cases affected fish do not show typical clinical symptoms due to *P. anguilliseptica* infection.

Conventional diagnosis method often rely on interpretation of clinical and histological signs in the infected fishes, pathogenic growth in or on a suitable medium, an analysis of morphological, phenotypic, or biochemical characteristics of the presumptive pathogen. Although these methods are fundamental to the development of any alternative diagnostic method, the accuracy and reliability of these techniques largely depend on competent expertise. Further, diagnosis of such fish pathogens required a culturing step, generally time-consuming and labor intensive. For example, assays for *Flavobacterium* or *Mycobacterium* species may require several days for growth with specialized media in suitable growth condition (Nematollahi et al., 2003; Van Trappen

et al., 2003). Detection of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonids, can take up to 12 weeks (Benediktsdottir et al., 1991). Moreover, these techniques rely on the ability of the organism to be cultured in vitro. This aspect considerably limits the applicability of these methods since possibly less than 1% of the microorganisms in an environmental sample may be cultured (Rappe and Giovannoni, 2003). Viruses are usually detected by designated virology laboratories using standard isolation protocol, electron microscopic studies, in vitro viral culture or, if available, serogical assays to detect viral antigens or test for the immune response against the virus (Leong, 1995; Lightner and Redman, 1998; Storch, 2000).

The disadvantages associated with traditional identification technique have triggered the research for alternative culture-independent detection and identification techniques, such as those based on the detection of serological techniques or nucleic acids-based techniques. Compared to traditional assays, these molecular techniques can avoid problems in investigating organisms for which no culture medium, cell lines, or detection method is available. In addition, these techniques are generally faster, more specific, more sensitive and more accurate. Nucleic acid-based techniques, especially if they make use of polymerase chain reaction (PCR), have the advantage of being exceedingly sensitive and specific and requiring reagents that are easily available. As a result, PCR-based techniques have increasingly been developed for fish pathogen diagnosis (Cunningham, 2002). The first stage in the development of nucleic acid-based diagnostic assays is the selection of specific sequences that can be used to identify pathogen, which is commonly applicable for bacteria and fungi, but viruses usually need different approaches.

Regarding bacteria, the most common strategy in the development of molecular diagnostic methods targeting the gene sequences involves the use of ubiquitously conserved genes that harbor specific sequences which includes ribosomal RNA (rRNA), operon, encompassing a 16S rRNA and 23S rRNA gene as well as an intergenic spacer (IGS) region (Ludwig and Schleifer, 1994; Call et al., 2003; Sachse, 2004; Toranzo et al., 2005, Drancourt et al., 2000; Hongoh et al., 2003;Warsen et al., 2004; Osborne et al., 2005).

However, in recent years, PCR based diagnosis has circumvent the conventional microbiological procedures in detecting the pathogens. Most of the PCR detection methods include the amplification of 16S rRNA genes for both Gram negative and Gram positive bacterial fish pathogens (Hiney and Smith, 1998; Osorio et al., 1999; Romalde and Toranzo, 2002) with low detection efficiency in carrier fish (Blanco et al., 2002). PCR assay is also used to quantify the amount of pathogenic DNA. Although it is relatively easy to quantify the amount of PCR products, it is more difficult to correlate and quantify the original amount of target DNA. For certain pathogens, however, this information may be necessary to make suitable disease management decisions and for monitoring the effects of these decisions. Though challenging, pathogen DNA may be quantified using competitive PCR (Siebert and Larrick, 1992). More recently, quantitative Real-time PCR (Heid et al., 1996) has been proven to be reliable with regard to both pathogen detection and quantification. This technology is more sensitive, more accurate, and less time-consuming than conventional end-point PCR, because it monitors the accumulation of PCR products during the reaction. This allows template quantification during the exponential phase of the reaction, before reaction components become limiting.

In addition, since there is no need to open the tubes in which the amplification takes place, the likelihood of post-PCR carry-over contamination is greatly reduced. Typically, DNA amplification is monitored each cycle based on the excitation of fluorescent dyes and detection of fluorescent emission (Heid et al., 1996; Mackay et al., 2002). In general, the initial amount of target DNA is related to a threshold cycle, which is defined as the cycle number at which fluorescence significantly increases above the background level. Target DNA is quantified using a calibration curve that relates threshold cycles to a specific amount of template DNA.

As extensively discussed in other reviews (Mackay et al., 2002; Hanna et al., 2005; Lievens et al., 2005; Espy et al., 2006), accumulating amplicon can be detected using either amplicon specific or non-specific detection method, i.e, sequence-specific probes or DNA-intercalating dyes, respectively. The use of a DNA-binding dye like SYBR Green is more straightforward and less expensive than using probes but is less specific since the fluorogenic molecule binds to all double

srranded DNA (dsDNA) present in the sample. Further, interpretation of the analysis may be disturbed by the formation of primer-dimer or aspecific PCR products. However, the risk of the latter can be reduced by using highly specific primer and stringent reaction condition and the accuracy of the reaction can be checked by a melting curve analysis at the end of the PCR run (Bustin, 2000; Mackay et al., 2002).

In contrast to amplicon non-specific detection chemistries, probe-based assays like those based on a TaqMan probe, offer the advantage of increased specificity, certainly in combination with specific primers (Livak et al., 1995). These probes consist of a single stranded short oligonucleotide labeled with a reporter fluorophore at the 5' end and a fluorogenic quencher at the 3' end. Because of the proximity of both groups, the fluorescent signal is quenched. During the annealing phase of each PCR cycle the probe hybridizes to a specific region within the amplified fragment. During the elongation phase, the probe is degraded by the 5'-exonuclease activity of the DNA polymerase causing the release of the reporter from the quencher, resulting in a fluorescent signal.

Real-time PCR assays have been developed for accurate detection and/or quantification of specific fish pathogen, including *Aeromonas* spp., *Flavobacterium* spp., *Vibro* spp., and DNA and RNA viruses (Overturf et al., 2001; Gilad et al., 2004; Balcazar et al., 2007; Kamimura et al., 2007). The assay, using a TaqMan probe, was 103 times more sensitive than the standard RT-PCR (Overturf et al., 2001).

Hence, the present study would be first reports, on the isolation and characterization of *P. anguilliseptica,* the most prevalent pathogenic microbes from the infected olive flounders, reared at low water temperature. Isolated microbes were characterized by phenotypic and genotypic methods and the phylogenetic relationship of the isolated pathogen was done using 16S rRNA gene sequences. The isolated microbes were tested for it pathogenicity in the farmed olive flounders at low rearing water temperature. Also, we developed and validated a rapid detection technique using Taqman probe Real-time quantitative PCR assay that targets a segment of the *P. anguilliseptica* 16S rRNA gene and hence to increase specificity and sensitivity of detection the target fish

pathogen.

In aquaculture, health management is very important and lack of effective diseases control and preventive measures would be the limiting factors for the stable production of flounder fish. Preventive measures by vaccination in combination with improved management techniques are an ideal method for controlling pseudomonas septicemia (Austin et al., 1993; Woo et al., 1999; Esteve-Gassent et al., 2004). A renewed interest in development of fish vaccine occurred in the early 1970s, and vaccination of cultured aquatic animal has become a viable fish health management tool for some diseases.

Before the introduction of vaccines in aquaculture, bacterial diseases of fish were controlled in many countries by the use of a wide range of chemotherapeutics i.e. antibiotic therapy (Hastings, 1997). Furthermore, there were increasing concerns on the level of antibiotics in fish flesh intended for human consumption. According to Inglis et al., (1993) residues of antibacterial treatment in fish have had an indirect impact on human health. This also, has had environmental impact on aquatic species. The increasing problems associated with the usage of antibiotics to control fish diseases encourages researchers to investigate the possibility of developing vaccines against the major diseases of farmed fish and to emphasize the idea that prevention of disease is more important than therapy.

Today, vaccination is an integrated part of most salmon farms which has reduced the use of antibiotics, 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. At present only, five multinational animal health companies have acquired, joint venture with the small aquaculture companies. The major fish vaccine producing companies are 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), as the effective vaccines are the only way to resolve disease

problems with number of successful vaccines have been developed for aquaculture. In contrast, the approaches of controlling fish disease have been still dependent to a high proportion of chemotherapy in Korea. Therefore, Korean aquaculture industry is in need to develop an efficient fish vaccine in order to be competitive internationally.

Research and development of fish vaccine in Korea was started by National Fisheries Research and Development Institute (NFRDI). Kwon and Bang, (2004) reported as immersion vaccination against *E. tarda*, and Cho et al., (2006) reported β-hemolytic *S. iniae* vaccine to olive flounder. In 2005, the commercial products of immersion and injection vaccines developed against edwardsiellosis to prevent *E. tarda* infection in olive flounder aquaculture. In addition there were only a few reports on multivalent fish vaccine, which was reported and patent as multivalent vaccine against *E. tarda* and *S. iniae*. However development of multivalent vaccine was found to be inadequate for olive flounder aquaculture industry in Korea.

The purpose of vaccination is to induce protective immunity in an animal. The immunity is specific and should be long lasting. Vaccination is prophyllatic and more effective against infectious disease when compared to antibiotic treatment. In aquaculture, the vaccine should be effective and applicable against the most economically important disease. The commercially available vaccine used against fish bacterial disease contains inactivated bacterial cells. The current advance in technology offers a promising future for anti-bacterial vaccines in fish species. However, it is more difficult to develop effective and cheap anti-viral vaccines and anti-parasitic vaccines.

The injection administration gives the best and most long-lasting protection. The vaccine can be applied by injecting the fish either intra peritoneal (i.p) or intramuscularly (i.m). Also, injection has the advantage of delivering the right dose of vaccine antigen (Ellis, 1997; Smith, 1988). Injection is the commonly used method however, it is quite expensive due to the intensive labor forces needed to handle fish during the vaccination process.

Vaccines could be produced in various ways to develop protective immunity against the pathogen in the vaccinated fish. The vaccine components can be prepared as inactivated whole

cells, DNA or recombinant vaccines or subunit vaccines. The whole cell inactivated vaccines have a significant advantage over other types in their safety and easy development. Almost all the commercially available vaccines are inactivated whole bacterial cells. These vaccines are effective and protect fish against diseases (Ellis, 1997; Stevenson, 1997).

Fish immune mechanisms are thought to be similar to those in higher vertebrates, such that they have the ability to generate early innate immune response which is also required for the ignition of adaptive immunity. Conserved motifs of bacteria are recognized by the immune system as a sign of detection of pathogenic bacterial invasion and responsible for the activation of inflammatory response.

Hence, with these backgrounds of information, the present study aims

1. to isolate and characterize the pathogenic bacteria, *P. anguilliseptica* using phenotypic and genotypic assays.
2. to develop and validate a rapid detection technique using Taqman probe Real-time quantitative PCR assay that targets a segment of the *P. anguilliseptica* 16S rRNA gene and to increase specificity and sensitivity of detection the target fish pathogen.
3. to develop a formalin-killed vaccine against *P. anguilliseptica* infection and to evaluate its protective immunity by assessing the immune specific gene expression in olive flounder vaccinated and challenged consecutively.

Part Ⅰ. Identification, phenotyping and genotyping of *Pseudomonas anguilliseptica* isolated from olive flounder (*Paralichthys olivaceus*) cultured in low rearing temperature in Jeju Island

ABSTRACT

Pseudomonas anguilliseptica, a gram negative, slow growing, rod shaped bacteria and the causative organism of "winter disease" in various fish species. This pathogen were isolated from liver, spleen, kidney, brain, and hemorrhagic skin in diseased olive flounder since from various fish farms located in and around Jeju Island at rearing temperature 16-21°C. The bacteria were cultured on blood agar at 20°C for 1 week. Then the isolates were Gram stained and selected for phenotypic characteristics using API 20E and the enzymatic profile was analyzed using API ZYM kit. The results obtained by biochemical and API kit system, showed that the isolates are of homogeneous *P. anguilliseptica*. The phenotypic features of the isolates such as colony morphology, cell structure were determined by culturing on TS agar and electron microscopic study. DNA was extracted from each strain using RNeasy DNA kit. PCR primer obtained from previous report was used to amplify the fragment size 418 bp which was then as excised and purified using Gel PCR purification. The purified fragments were cloned into the pCR2.1-TOPO vector. Obtained 16S rRNA gene sequences of isolates were performed multiple sequence alignment using ClustalW program in MEGA. PCR amplification of 16S rRNA gene fragment analysis based on the 11 isolates, confirmed that isolates were *P. anguilliseptica*. Alignment of the 11 sequences showed 99-100% similarities with the other *P. anguilliseptica* strain X99540, X99541, and AB021376 in the phylogenetic tree. Antibiotic sensitivity assay of *P. anguilliseptica* showed that were sensitive to oxolinic acid and 13 other antibiotic disc. Pathogenicity assay of the isolated *P. anguilliseptica* was conducted with about 400 fishes, grouped (in duplicate) into control and experimental group, based on the criteria such as rearing water temperature: 17°C and 20°C, mode of infection: injection and

immersion and the dose of infecting pathogen: 3×10^6 and 3×10^8 CFUml^{-1}, each with 20 fishes in 100 L tanks. Control and two experimental group fishes were injected intraperitoneally with 100 µl of PBS and 100 µl of 3×10^6 and 3×10^8 CFUml^{-1} of *P. anguilliseptica,* respectively. The remaining two fish groups were exposed with 3×10^6 and 3×10^8 CFUml^{-1} by immersion for 1 hour. Result of the immersion infection assay shows 55 and 50% mortality of fishes reared at 17 and 20°C water temperature, respectively. However, fishes infected with IP shows increased cumulative mortality than immersion infected fish groups. All control groups showed no clinical sign and no pathogen isolated from skin as well as other internal organs.

MATERIALS AND METHODS

1. Fish

Olive flounder (*Paralichthys olivaceus*) fishes (15±5 g) were purchased from a local fish farm located in Jeju Island and acclimatized in the rearing facility for at least two weeks. Fishes were randomly screened for any pathogenic infection using microbiological and blood biochemical assays with blood, liver, kidney, spleen, skin and gill tissues in order to prevent interruption by other pathogens. Only pathogen free fishes were used for the challenge experiment with *P. anguilliseptica*. Fishes were maintained in concrete tanks with the volume of 5 tons of running seawater rearing system with the flow rate of approximately 7.2 ton/hr. Throughout the experiment fishes were maintained in the water temperature ranged from 18°C to 19°C, and fed two times daily with commercial dry pellets.

2. Isolation of pathogenic bacterial cells

The diseased fishes were collected since 2010 from various fish farms located in and around Jeju Island (Table 1). The bacterial isolations were made from liver, spleen, kidney, brain, hemorrhagic skin dissected from diseased fishes which includes olive flounder, rock bream, Red-spotted grouper and Seven-band grouper, rockfish reared at 17-20°C water temperature and incubated on Blood agar (bioMérieux, France) at 20°C for 1 week. Then subcultured on Tryptic Soy (TS) agar (BD, USA) and incubated at 20°C. Glycerol stock for all the isolated strains were prepared with 20% (v/v) of glycerol in TS broth and stored at -80°C. Isolates were performed for Gram staining and motility test from TS broth at 20°C. Pure cultures of the 31 strains were selected for phenotypic characteristics using API 20E kit (bioMérieux, France) at 25°C and the results were analyzed at 24 h and 48 h. Enzymatic profiles were obtained from API ZYM kit (bioMérieux, France) incubated for 4 h and 8 h at 30°C. Salt tolerance assay was conducted in TS agar, containing 0%, 1%, 2%, 3% NaCl supplements, at 20°C. Bacterial growth at different temperatures was performed in TS agar at 5°C, 10°C, 20°C, 30°C, 37°C, 45°C. The phenotypic features of the isolates and cell morphology, were determined by cultivating the isolates on Blood agar at 20°C.

General cell morphology was studied using phase contrast micrography and scanning electron micrography (SEM). Briefly, bacterial cells were treated in 2.5% Glutaraldehyde in Sorensens Phosphate Buffer, pH adjusted, over night. Then the cells were washed 3 changes of buffer for 5 min. Wash the cells in H_2O for 5 min after which the cells were immersed in 2% OsO_4 for 2 h. Wash the cells in 3 changes of H_2O for 5 min. Then the cells were dehydrate using a series of ethanol washes: starts with 50% ETOH and 50% H_2O 5 min, 75% ETOH and 25% H_2O 5 min, 95% ETOH and 5% H_2O 5 min and 100% ETOH-3 times for 5 min. Prepare to dry using a series of Hexamethyldisilizane (HMDS) washes: 50% (HMDS) and 50% ETOH 5 min, 75% (HMDS) and 25% ETOH 5 min, 100% (HMDS)-2 times 5 min. Let air dry at room temperature and mount the specimen on a stub with silver paint and coat with gold which was viewed under the SEM.

Table 1. *P. anguilliseptica* isolates from different fish species cultured in and around Jeju Island.

Fish species	Geographical origin	Source	Year of isolation	Isolation name	Isolated fish size(cm)	Accession number	Mortality (%)
olive flounder	Jeju, Seongsan	kidney, spleen, skin	2010	JEP1	11	KC565867	38
olive flounder	Jeju, Seongsan	kidney, spleen, liver	2010	JEP2	13	KC565868	40
olive flounder	Jeju, Seongsan	kidney, brain, skin	2011	JEP3	27	KC565869	22
olive flounder	Jeju, Daejeong	kidney, brain, liver	2011	JEP4	12	KC565870	50
olive flounder	Jeju, Aewol	kidney, liver	2011	JEP5	11	KC565871	30
olive flounder	Jeju, Pyoseon	kidney, brain	2011	JEP6	12	KC565872	30
olive flounder	Jeju, Pyoseon	kidney, brain	2011	JEP7	10	KC565873	36
olive flounder	Jeju, Pyoseon	kidney, brain	2012	JEP8	11	KC565874	20
olive flounder	Jeju, Pyoseon	kidney, brain	2012	JEP9	17	KC565875	50
olive flounder	Jeju, Pyoseon	kidney, spleen	2012	JEP10	9	KC565876	41
olive flounder	Jeju, Seongsan	kidney, spleen	2012	JEP11	19	KC565877	20
rock bream	Jeju, Namwon	kidney, spleen	2010	JERB1	11	-	11
rock bream	Jeju, Hangyeong	kidney, spleen, liver	2010	JERB2	13	-	15
rock bream	Jeju, Gujwa	kidney, brain, skin	2010	JERB3	27	-	9
rock bream	Jeju, Gujwa	kidney, liver	2010	JERB4	12	-	15
rock bream	Jeju, Hangyeong	kidney, spleen	2010	JERB5	11		20
rock bream	Jeju, Seongsan	kidney, spleen	2010	JERB6	12		11
rock bream	Jeju, Gujwa	kidney, spleen	2011	JERB7	10		9
rock bream	Jeju, Namwon	kidney, brain, liver	2011	JERB8	11		13
rock bream	Jeju, Hangyeong	kidney, brain, liver	2011	JERB9	17		17

Table 1. Continued

Fish species	Geographical origin	Source	Year of isolation	Isolation name	Isolated fish Size(cm)	Accession Number	Mortality (%)
rock bream	Jeju, Namwon	kidney, spleen, skin	2011	JERB10	12	-	16
rock bream	Jeju, Gujwa	kidney, spleen, liver	2011	JERB11	10	-	18
rock bream	Jeju, Seongsan	kidney, brain, skin	2011	JERB12	11	-	17
rock bream	Jeju, Daejeong	kidney, brain, liver	2012	JERB13	17	-	7
rock bream	Jeju, Gujwa	kidney, liver	2012	JERB14	9	-	19
rock bream	Jeju, Pyoseon	kidney, brain	2012	JERB15	19	-	10
rock bream	Jeju, Namwon	kidney, brain	2012	JERB16	9	-	11
rock bream	Jeju, Pyoseon	kidney, brain	2012	JERB17	11	-	15
seven-band grouper	Jeju, Pyoseon	kidney, brain	2012	JEGU1	8	-	7
red-spotted grouper	Jeju, Pyoseon	kidney, spleen	2012	JEGU2	7	-	6
rockfish	Jeju, Pyoseon	kidney, spleen	2012	JERCF1	7	-	7

3. DNA extraction of pathogenic bacterial cells

DNA was extracted from TS broth cultures of each strain using RNeasy DNA kit (Qiagen, Germany), eluted in 50 µl of Tris-acetate-EDTA buffer and stored at -20°C until required for PCR screening. Extracted DNA was measured for the maximum absorbance at 260 and 280 nm. Historically, the ratio of absorbance at these wavelengths has been used as a measure of purity in both nucleic acid and protein extractions. A ratio of ~1.8 is generally accepted as good quality DNA. Similarly, absorbance at 230 nm is measured to quantify the other contamination; therefore the ratio of A_{260}/A_{230} is also calculated. The 260/230 values for "pure" nucleic acid are higher than the respective 260/280 values. Expected 260/230 values are in the range of 2.0-2.2.

4. 16S rRNA gene sequence analysis of bacterial cells

The 16S rRNA gene specific primers namely Psan-F (5'-TTGGGAGGAAGGGCAGTAACC-3') and Psan-3 (5'-TGCGCCACTAAAATCTCAAG-3') obtained from the previous report were used to amplify the fragment size of 418 bp (Romalde et al., 2004). PCR amplification was performed in 25 µl reaction volume containing 2 µl genomic DNA, 15 pmol of each primer, in the presence of 10 Mm of each dNTP Mix, 5 U/µl of Diastar^{TM} Taq DNA polymerase (Solgent, Korea) and 10 × Taq reaction buffer (25 mM $MgCl_2$ mixed) and 5 × BandDoctor^{TM}. Amplification was conducted on Mycycler thermocycler (BIORAD, USA) and the temperature profile starts with initial denaturation at 95°C 3 min followed by 35 cycles of denaturing at 95°C for 20s followed by annealing at 63°C for 20s and extension at 72°C for 30s with a final extension step for 5 min at 72°C. Negative controls without template DNA were included to check for any contamination. The amplified products were electrophoresed on 1.5% agarose gel in order to check the specificity of the isolated pathogen. The single amplified PCR product with the target sized was excised and purified using Gel PCR purification system (Solgent, Korea). The purified fragment was then cloned into the pCR2.1-TOPO vector (Invitrogen, USA) following the manufacture's recommendations. The positive plasmid DNA were purified with QIAprep Spin Miniprep kit (Qiagen, Germany) and sequenced on ABI 3730XL DNA analyzer (Applied Biosystems, USA).

5. Phylogenetic analysis

Obtained 16S rRNA gene sequences were subjected to multiple sequence alignment using ClustalW program in MEGA ver. 4.1 (MEGA4.1: Molecular Evolutionary Genetics Analysis software). Nei's pair-wise genetic distance for each isolates were calculated and a phylogenetic tree was constructed using the neighbor-joining algorithm and the reliability of the branching was compared with other 16S rRNA gene sequences from the NCBI GenBank, using 1000 bootstrap samplings of the data.

6. Susceptibility assay of antimicrobial agents

Antibiotic susceptibility assay was done with discs diffusion method on Mueller-Hinton agar (BD, USA) using twenty different antibiotic discs (Oxoid, UK) prepared with antibiotics such as; oxolinic acid, oxytetracycline, doxycycline, flumequine, ciprofloxacin, norfloxacin, pefrofloxacin, ofloxacin, amoxicillin, ampicillin, cephadroxil, cephalexin, ceftiofur, gentamycin, neomycin, florfenicol, streptomycin, lincomycin, clindamycin, erythromycin at 20°C for 7 days.

7. Pathogenicity assay

Pathogenicity assay was conducted with the PCR verified disease free olive flounders (10±2 g) using *P. anguilliseptica* isolates. About 400 fishes were grouped (in duplicate) as control and experimental group, based on the criteria such as rearing water temperature: 17°C and 20°C, mode of infection: injection and immersion and the dose of infecting pathogen: 3×10^6 and 3×10^8 $CFUml^{-1}$, each with 20 fishes in 100 L tanks. Control and two experimental group fishes were injected intraperitoneally with 100 µl of PBS and 100 µl of 3×10^6 and 3×10^8 $CFUml^{-1}$ of *P. anguilliseptica,* respectively. The remaining two fish groups were exposed with 3×10^8 $CFUml^{-1}$ by immersion technique for 1 h in total volume of 5 L of saline (0.85% NaCl) at 17°C and 20°C water temperature (Table 2). Cumulative mortality was recorded for 25 days and the pathogens from dead fish were isolated from kidney, spleen, liver, brain and skin. The isolated strains grown on Blood agar were reconfirmed with PCR amplification of specific product. Assay period for all the groups were 25 days of post infection.

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

Mode of infection	Isolation name	Accession number	No. of fish / tank	Challenge dose ($CFUml^{-1}$)	Tank volume
Intra-peritoneal injection	JEP1	KC565867	20	3×10^6 / 3×10^8	100 L
	JEP2	KC565868			
	JEP3	KC565869			
	JEP4	KC565870			
	JEP5	KC565871			
	JEP6	KC565872			
	JEP7	KC565873			
	JEP8	KC565874			
	JEP9	KC565875			
	JEP10	KC565876			
	JEP11	KC565877			
Immersion	JEP4	KC565870	20	3×10^8	100 L

RESULTS

1. Phenotypic and biochemical characterization

The isolates were strict aerobic, Gram negative, and filamentous bacteria (Fig. 1), grew at 5-30°C, but not at 37°C. The causal organisms show growth at 0-3% NaCl concentration on TS agar and were positive for motility, Cytochrome oxidase and Catalase, but did not use organic carbon sources such as glucose, rhamnose, saccharose, mannitol, sorbitol, inositol, melibiose, amygladin, and arabinose. All isolates showed negative for biochemical tests except citrate utilization (Table 3). The enzymatic profiles using the API ZYM kit were showed in Table 4. The profiles of the 11 isolates were found to be very similar, except for L-leucyl-2-naphthylamide test.

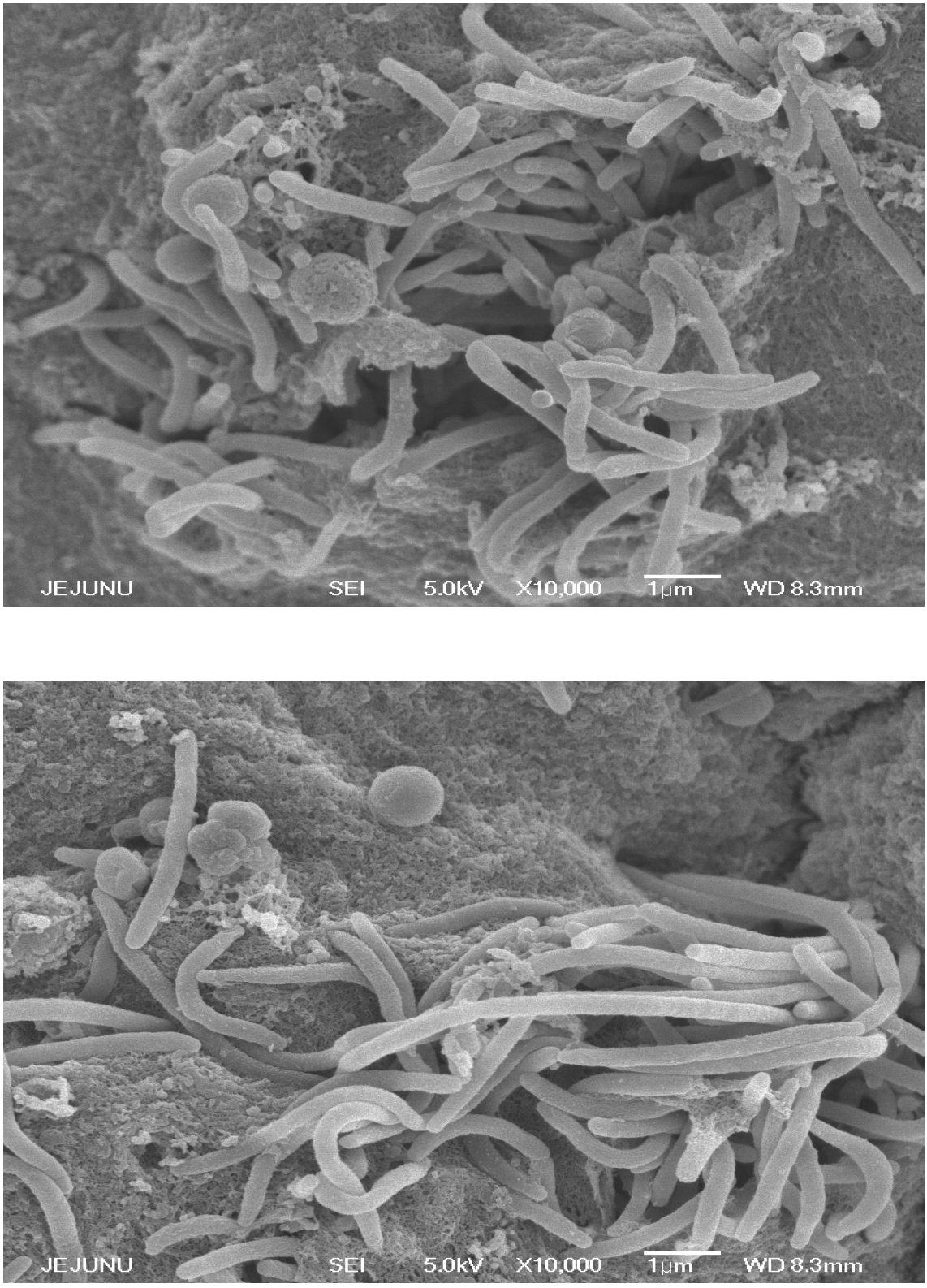

Fig. 1. Scanning electron micrograph (SEM) of is *P. anguilliseptica* isolated from infected olive flounder collected from Jeju Island.

Table 3. Phenotypic and biochemical characteristics of *P. anguilliseptica* isolates compare with previous studies.

Characteristics	Wakabayashi and Egusa (1972)	Doménech (1997) (n=11)	This study (n=11)
Gram stain	-	-	-
Haemolysis[a]	ND	-	-
Motility[a]	+	-	+
Cytochrome oxidase	+	+	+
Catalase	+	+	+
O/F test[a]	-	-	-
Growth at 5-30°C	+	+	+
Growth at 37°C	-	-	-
Growth at 0-3% NaCl	+	+	+
Growth on Macconkey agar	ND	+	+
Growth at TCBS agar	ND	-	-
ONPG (β-galactodidase)	-	-	-
ADH (Arginine dihydrolase)	ND	-	-
LDC (Lysine decaboxylase)	ND	-	-
ODC (Omithine decaboxylase)	ND	-	-
CIT (Citrate utilization)	+	+(9)	+(11)
NO_2, N_2 (Nitrate reduction)	-	-	-
H_2S (H_2S production)	-	-	-
URE (Urease)	-	-	-
IND (Indole production)	-	-	-
VP (Acetoin production)	ND	ND	-
GEL (Gelatinase)	-	-	-

ND: Not done; +: Positive reaction; -: Negative reaction; (n): n tested showing positive or negative reaction.

[a]Motility, Oxidation of glucose, Fermentation of glucose, Haemolysis were performed in commercial medium.

Table 4. API ZYM profile of *P. anguilliseptica* isolates compared with the previous study strains.

Hydrolysis of the following substrates	Wakabayashi and Egusa (1972)	Doménech (1997) (n=11)	This study (n=11)
2-naphthyl-phosphate	+	-	-
2-naphthyl-butyrate	-	+(9)	+
2-naphthyl-caprylate	+	+(9)	+
2-naphthyl-myristate	+	-	-
L-leucyl-2-naphthylamide	+	+(9)	+(2)
L-valyl-2-naphthylamide	-	-	-
L-cystyl-2-naphthylamide	-	-	-
N-benzoyl-DL-arginine-2-naphthylamide	-	-	-
N-glutaryl-phenylanine-2-naphthylamide	-	-	-
2-naphtyl phosphate	+	+(7)	+
Naphthol-AS-Bl-phosphate	+	-	+
6-Br-2-naphthyl- αD-galactopyranoside	-	-	-
2-naphthyl-βD-galactopyranoside	-	-	-
Naphthol-AS-Bl-βD-glucuronide	-	-	-
2-naphthyl-αD-glucopyranoside	-	-	-
6-Br-2-naphthyl-βD-glucopyranoside	-	-	-
1-naphthyl-N-acetyl-βD-glucosaminide	-	-	-
6-Br-2-Naphthyl-αD-mannopyranoside	-	-	-
2-naphthyl-αL-fucopyranoside	-	-	-

API reaction scores 0 and 1 were considered negative, scores 2 to 5 were considered positive; (n): n tested showing positive or negative reaction.

2. Phylogenetic analysis

PCR amplification of 16S rRNA gene fragments were seen in all 31 isolates from 11 olive flounder, 17 rock bream, 2 grouper, and one rockfish showed the amplification of 418 bp (Fig. 2A, 2B, 2C). Of which the 11 isolates from olive flounder were sequenced and deposited in the NCBI GenBank (accession number KC565867-KC565877). The BLAST search and phylogenetic analysis based on the sequences of 11 isolates, confirmed that the isolates are *P. anguilliseptica*. The genetic distance (Table 5A, 5B) and the phylogenetic tree was constructed for the 11 isolates (Fig. 3A) along with the other 16S rRNA gene sequences of *P. anguilliseptica* from NCBI GenBank (X99540, X99541, AB021376, AM114532, DQ178233 and DQ178234) (Fig. 3B). Alignment of the sequenced showed 99-100% similarities with the other *P. anguilliseptica* strain (X99540, X99541, AB021376).

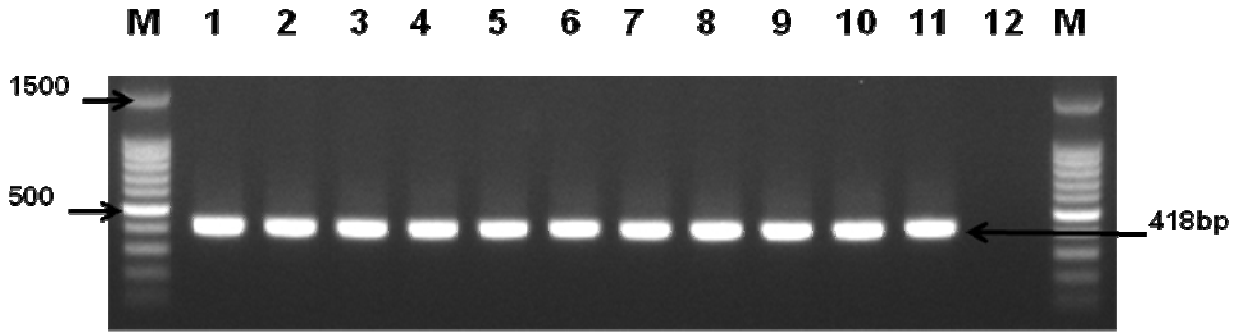

Fig. 2A. PCR Amplification of the specific gene fragments (418 bp) from 11 isolates of *P. anguilliseptica*. Lanes: M, 100-1500 bp ladder molecular size marker (iNtRON, Korea); 1, JEP1; 2, JEP2; 3, JEP3; 4, JEP4; 5, JEP5; 6, JEP6; 7, JEP7; 8, JEP8; 9; JEP9; 10, JEP10; 11, JEP11; 12, negative control (no DNA); 13, M: 100-1500 bp ladder molecular size marker; 14.

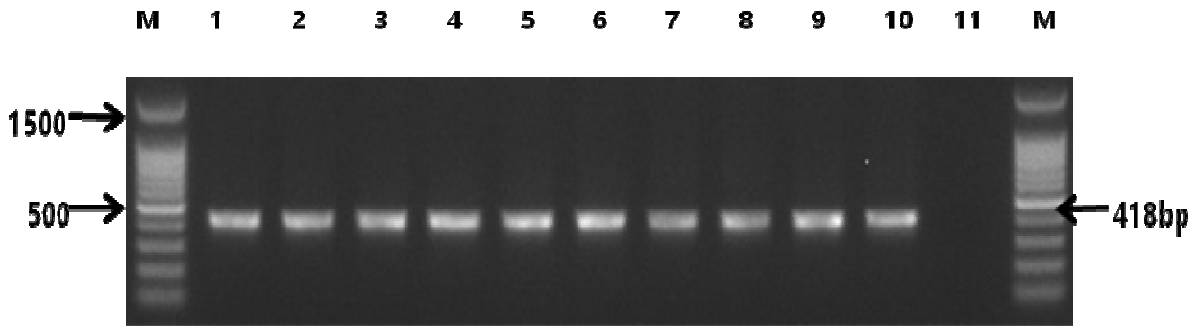

Fig. 2B. PCR Amplification of the specific gene fragments (418 bp) from 10 isolates of *P. anguilliseptica*. Lanes: M, 100-1500 bp ladder molecular size marker (iNtRON, Korea); 1, JERB1; 2, JERB 2; 3, JERB 3; 4, JERB 4; 5, JERB 5; 6, JERB 6; 7, JERB 7; 8, JERB 8; 9; J JERB 9; 10, JERB 10; 11, negative control (no DNA); 12, M: 100-1500 bp ladder molecular size marker; 13.

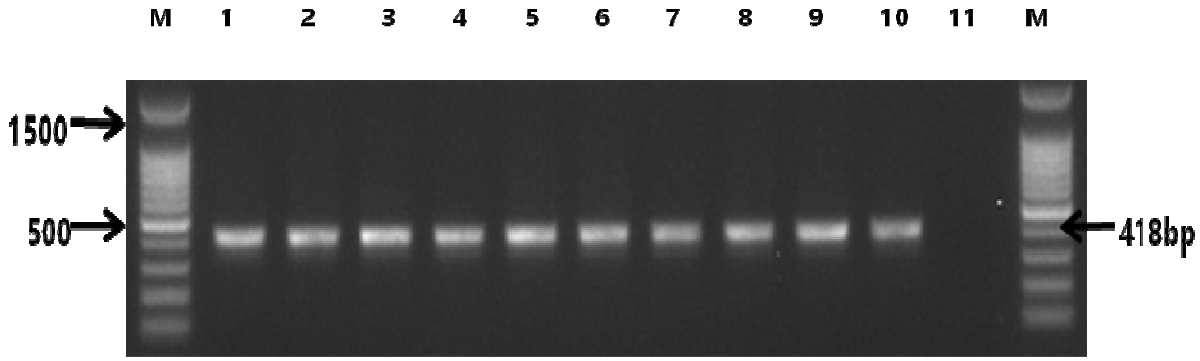

Fig. 2C. PCR Amplification of the specific gene fragments (418 bp) from 10 isolates of *P. anguilliseptica*. Lanes: M, 100-1500 bp ladder molecular size marker (iNtRON, Korea); 1, JERB 11; 2, JERB 12; 3, JERB 13; 4, JERB 14; 5, JERB 15; 6, JERB 16; 7, JERB 17; 8, JEGU1; 9; JEGU2; 10, JERCF1; 11, negative control (no DNA); 12, M: 100-1500 bp ladder molecular size marker; 13.

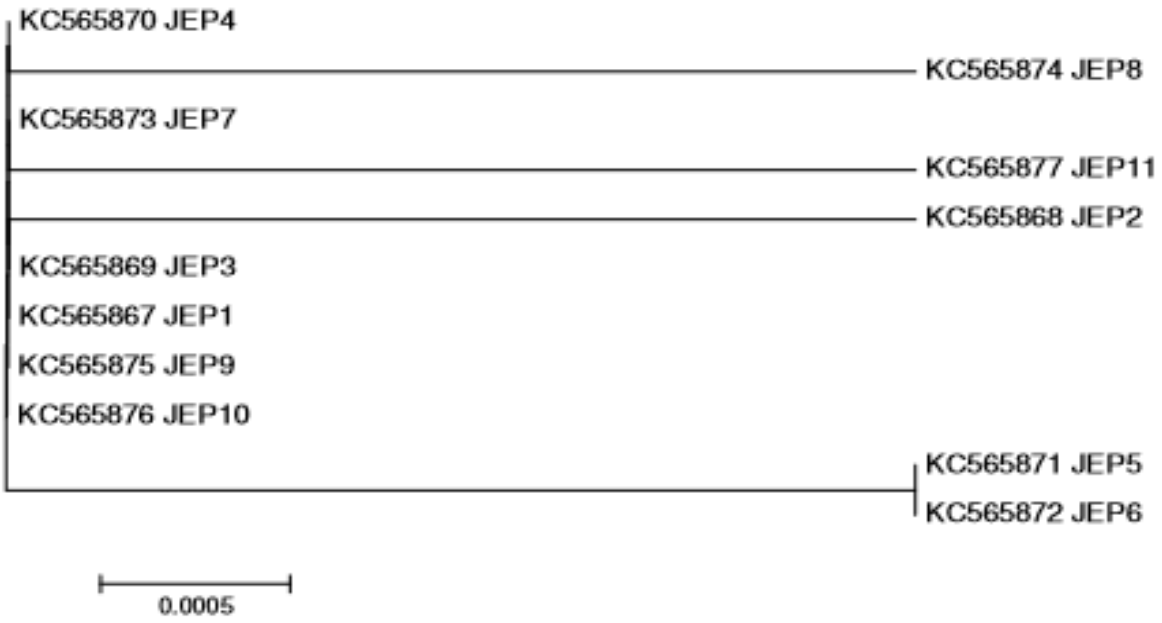

Fig. 3A. Molecular phylogenetic tree constructed based on the 16S rRNA nucleotide sequence of 11 *P. anguilliseptica* isolates. Bootstrap values at 1000 times construction are shown at the major nodes in the tree. The scale bar is a genetic distance marker (number of replacement nucleotides per site). The number indicates the GenBank accession number.

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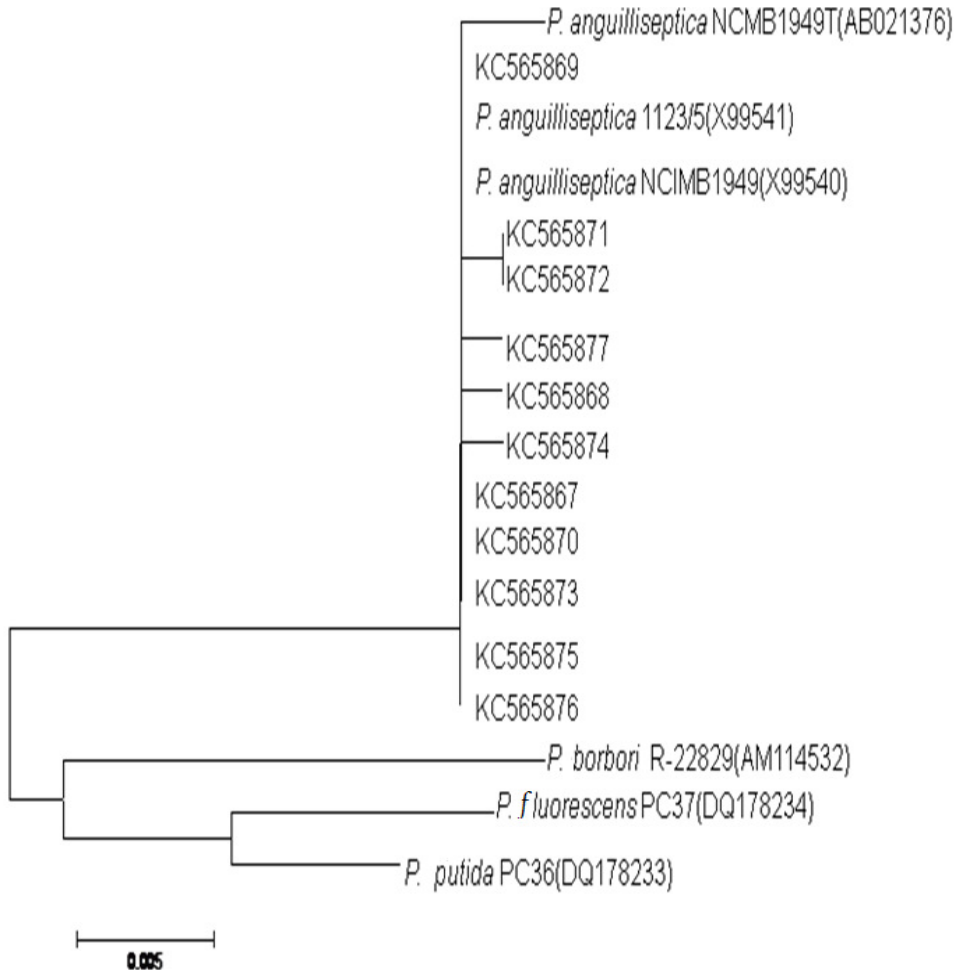

Fig. 3B. Molecular phylogenetic tree constructed based on the nucleotide sequence of 16S rRNA of 11 isolates from Jeju Island and 6 from previous studies of *Pseudomonas* sps. Bootstrap values at 1000 times construction are shown at the major nodes in the tree. The scale bar is a genetic distance marker (number of replacement nucleotides per site). The number in parentheses indicates the GenBank accession number.

Table 5A. Pairwise distance matrix (Nei, 1972) of Estimates of Evolutionary Divergence between eleven *P. anguilliseptica* 16S rRNA gene sequence.

	KC565867	KC565868	KC565869	KC565870	KC565871	KC565872	KC565873	KC565874	KC565875	KC565876	KC565877
KC565867	****										
KC565868	0.002	****									
KC565869	0.000	0.002	****								
KC565870	0.000	0.002	0.000	****							
KC565871	0.002	0.005	0.002	0.002	****						
KC565872	0.002	0.005	0.002	0.002	0.000	****					
KC565873	0.000	0.002	0.000	0.000	0.002	0.002	****				
KC565874	0.002	0.005	0.002	0.002	0.005	0.005	0.002	****			
KC565875	0.000	0.002	0.000	0.000	0.002	0.002	0.000	0.002	****		
KC565876	0.000	0.002	0.000	0.000	0.002	0.002	0.000	0.002	0.000	****	
KC565877	0.002	0.005	0.002	0.002	0.005	0.005	0.002	0.005	0.002	0.002	****

The p-distances were calculated from 16S rRNA gene sequences of 11 *P. anguilliseptica*.

Table 5B. Pairwise distance matrix (Nei, 1972) of Estimates of Evolutionary Divergence between eleven *P. anguilliseptica* and other *Pseudomonas* species 16S rRNA gene sequence.

	KC565867	KC565868	KC565869	KC565870	KC565871	KC565872	KC565873	KC565874	KC565875	KC565876	KC565877	AB021376	X99541	X99540	AM114532	DQ178234	DQ178233
KC565867	****																
KC565868	0.002	****															
KC565869	0.000	0.002	****														
KC565870	0.000	0.002	0.000	****													
KC565871	0.002	0.003	0.002	0.002	****												
KC565872	0.002	0.003	0.002	0.002	0.000	****											
KC565873	0.000	0.002	0.000	0.000	0.002	0.002	****										
KC565874	0.002	0.003	0.002	0.002	0.003	0.003	0.002	****									
KC565875	0.000	0.002	0.000	0.000	0.002	0.002	0.000	0.002	****								
KC565876	0.000	0.002	0.000	0.000	0.002	0.002	0.000	0.002	0.000	****							
KC565877	0.002	0.003	0.002	0.002	0.003	0.003	0.002	0.003	0.002	0.002	****						
AB021376	0.003	0.005	0.003	0.003	0.005	0.005	0.003	0.005	0.003	0.003	0.005	****					
X99541	0.000	0.002	0.000	0.000	0.002	0.002	0.000	0.002	0.000	0.000	0.002	0.003	****				
X99540	0.000	0.002	0.000	0.000	0.002	0.002	0.000	0.002	0.000	0.000	0.002	0.003	0.000	****			
AM114532	0.036	0.037	0.036	0.036	0.038	0.038	0.036	0.037	0.036	0.036	0.037	0.039	0.036	0.036	****		
DQ178234	0.034	0.036	0.034	0.034	0.036	0.036	0.034	0.036	0.034	0.034	0.036	0.038	0.034	0.034	0.032	****	
DQ178233	0.031	0.032	0.031	0.031	0.032	0.032	0.031	0.032	0.031	0.031	0.032	0.034	0.031	0.031	0.031	0.016	****

The p-distances were calculated from 16S rRNA gene sequences of 14 *P. anguilliseptica* and 3 other *Pseudomonas* species.

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X99541          TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
X99540          TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
KC565876        TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
KC565875        TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
KC565873        TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
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KC565869        TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
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KC565871        TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGACAGAATAAGCA 60
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AB021376        TTGGGAGGAAGGGCAGTAACCTAATACGTTATTGTTTTGACGTTACCGGCAGAATAAGCA 60
DQ178234        TTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCA 60
DQ178233        TTGGGAGGAAGGGCATTAACCTAATACGTTGGTGTCTTGACGTTACCGACAGAATAAGCA 60
AM114532        TTGGGAGGAAGGGCAGTAAGCGAATACCTTGCTGTTTTGACGTTACCGACAGAATAAGCA 60
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X99540          CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATT 120
KC565876        CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATT 120
KC565875        CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATT 120
KC565873        CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATT 120
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AM114532        CCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATT 120
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Fig. 4. Continued

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X99540          ACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGTAAGTTGGAAGTGAAATCCCCGGGCTCAA 180
KC565876        ACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGTAAGTTGGAAGTGAAATCCCCGGGCTCAA 180
KC565875        ACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGTAAGTTGGAAGTGAAATCCCCGGGCTCAA 180
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DQ178234        ACTGGGCGTAAAGCGCGCGTAGGTGGTTAGTTAAGTTGGATGTGAAATCCCCGGGCTCAA 180
DQ178233        ACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAA 180
AM114532        ACTGGGCGTAAAGCGCGCGTAGGTGGTTGGTTAAGTTGGATGTGAAAGCCCCGGGCTCAA 180
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Fig. 4. Continued

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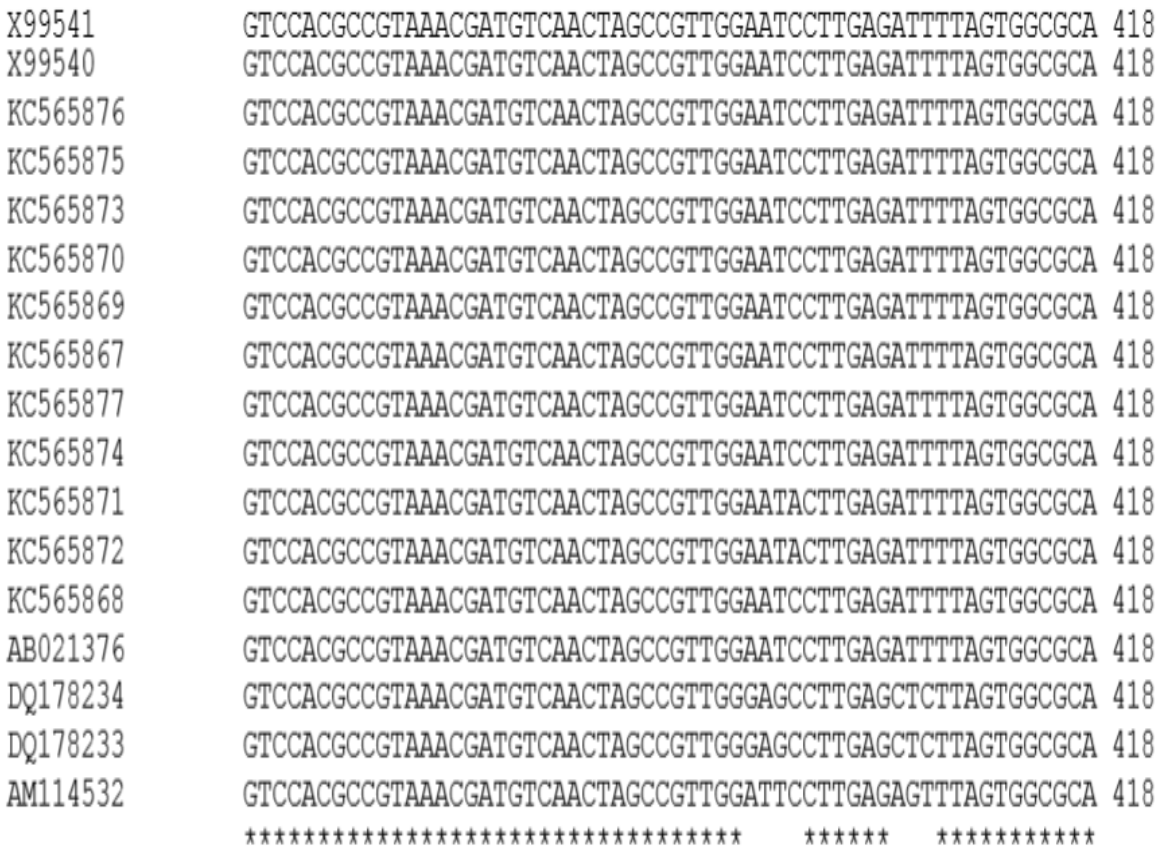


Fig. 4. Multiple sequence alignment of 418 bp of *P. anguilliseptica* 16S rRNA gene fragments. KC565867-77 are *P. anguilliseptica* isolates from olive flounder from Jeju Island; X99540-41 are *P. anguilliseptica* from sea bream (from previous report); AB021376 is *P. anguilliseptica* from eel; DQ178234 is *P. fluorescens;* DQ178233 is *P. putida*; and AM114532 is *P. borbori.*

3. Susceptibility to antimicrobial agents

Antibiotic sensitivity assay of the isolates showed that all strains were sensitive to oxolinic acid, oxytetracycline, doxycycline, flumequine, ofloxacin, ciprofloxacin, norfloxacin, pefrofloxacin, amoxicillin, ampicillin, gentamycin, neomycin, florfenicol, streptomycin; and resistance to clindamycine, cephadroxil, ceftiofur, cephalexin, lincomycin and erythromycin (Table 6).

Table 6. Antibiotic sensitive assay of *P. anguilliseptica* based on paper disc method.

Antibiotic agent	Symbol	Concentration (μg)	Inhibition zone (mm)	Sensitivity reaction
Oxolinic acid	OA	2	28	S
Oxytetracycline	OT	30	36	S
Doxycycline	DO	30	34	S
Flumequine	UB	30	28	S
Ofloxacin	OFX	5	28	S
Ciprofloxacin	CIP	5	26	S
Norfloxacin	NOR	10	26	S
Pefrofloxacin	PEF	5	28	S
Amoxicillin	AMC	30	34	S
Ampicillin	AMP	10	36	S
Cephadroxil	CFR	30	16	R
Cephalexin	CL	30	12	R
Gentamycin	CN	10	25	S
Neomycin	N	10	26	S
Florfenicol	FFC	30	30	S
Clindamycine	DA	2	12	R
Erythromycin	E	15	14	R
Ceftiofur	EFT	30	15	R
Streptomycin	S	10	28	S
Lincomycin	MY	2	10	R

S: sensitivity; R: resistance.

4. Pathogenicity by intra-peritoneal challenge

The infected fishes showed darkening of skin, hemorrhaging in fin bases and ulcer over the body surface (Fig. 5) and results in the death of the fishes. The cumulative mortality of six fish groups (two control and four experimental group; two doses: 3×10^6 and 3×10^8 at 2 rearing temperatures: 17°C and 20°C) were shown in Fig. 6. The 17°C fish group received 3×10^8 CFUml^{-1} showed 90% cumulative mortality. Among the eleven *P. anguilliseptica* isolates, fishes infected with JEP4 isolate with 3×10^8 CFUml^{-1} of cells, reared at 17°C showed rapid infection and high cumulative mortality while other isolates showed significantly similar mortality pattern. For instance, no mortalities were observed in any experimental groups challenged with *P. anguilliseptica* until third or fourth day of post infection (dpi). However, 3×10^8 CFUml^{-1} dose at 20°C group showed 90% mortality on the 9th dpi while the 3×10^6 CFUml^{-1} dose group fishes showed 60% mortality after 11th day of post infection and 3×10^8 CFUml^{-1} dose group showed 90% mortality on the 6th dpi while the 3×10^6 CFUml^{-1} dose group fishes showed 75% mortality after 8th day of post infection at 17°C. Hence, JEP4 isolate was selected for further studies. Control groups showed no clinical signs and no pathogens were isolated from skin as well as other internal organs. PCR amplification of the infected fish groups reconfirmed the specificity of the infected microbe and no other microbial contamination was found in any of the challenged fishes groups.

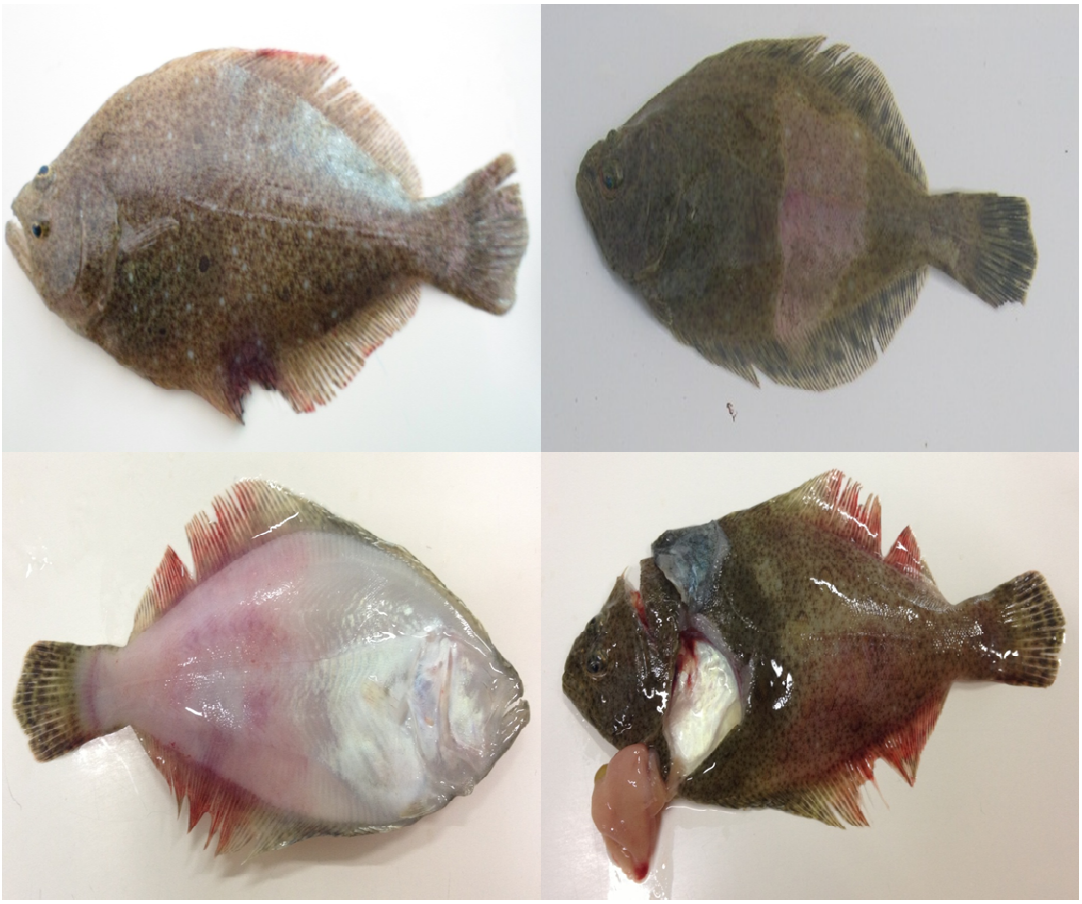
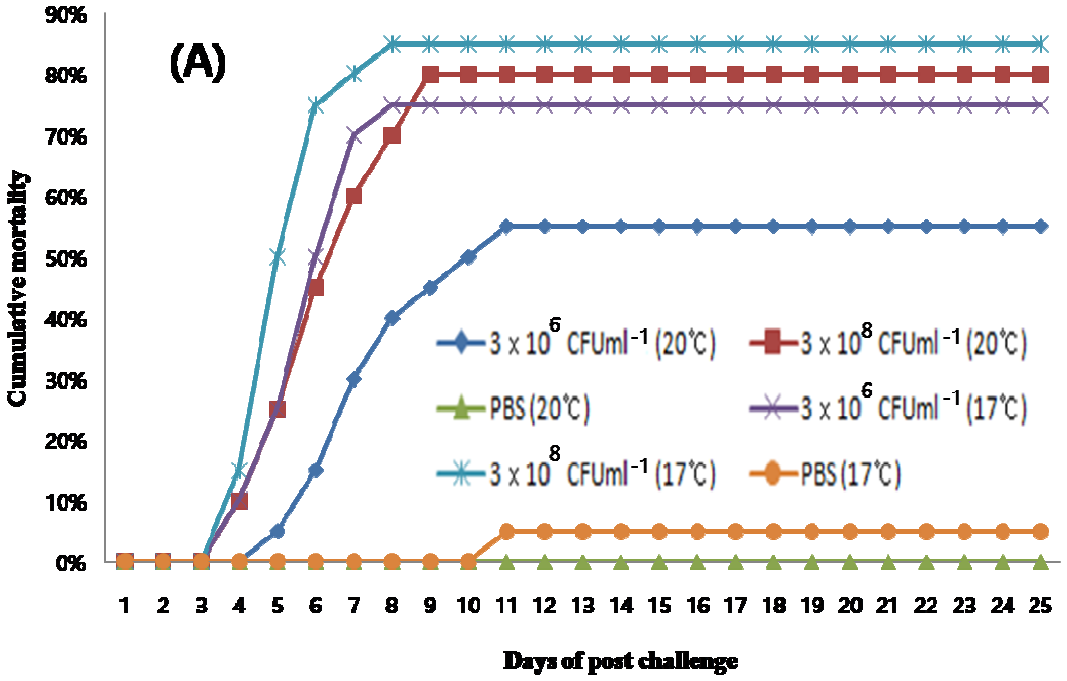
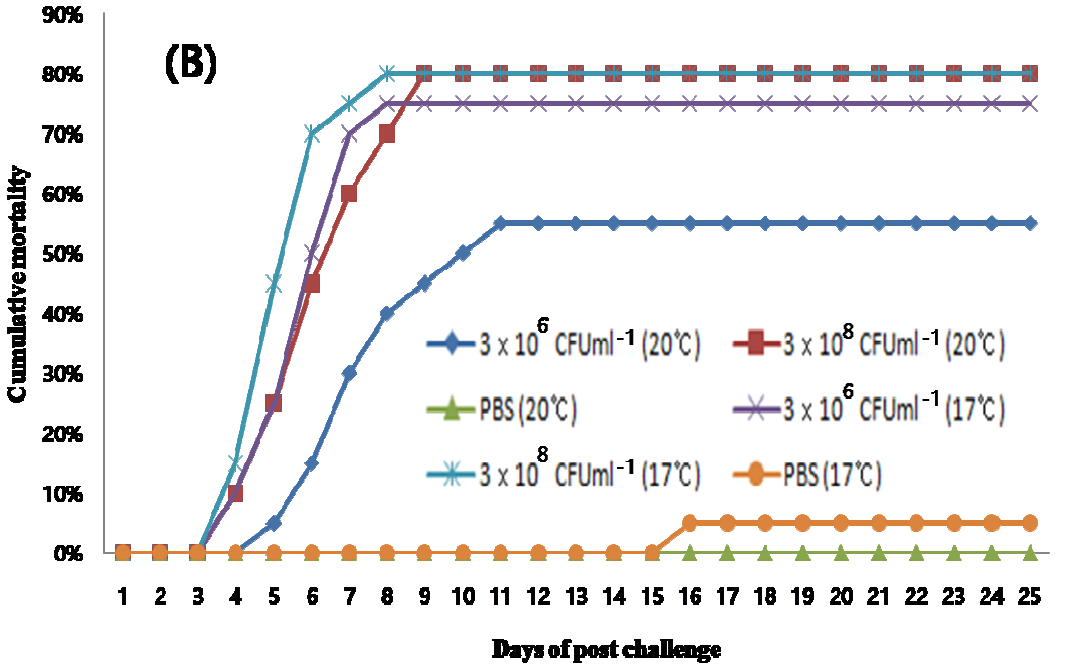


Fig. 5. Infected flounder fish showing unique clinical signs due to the infection of *P. anguilliseptica*.





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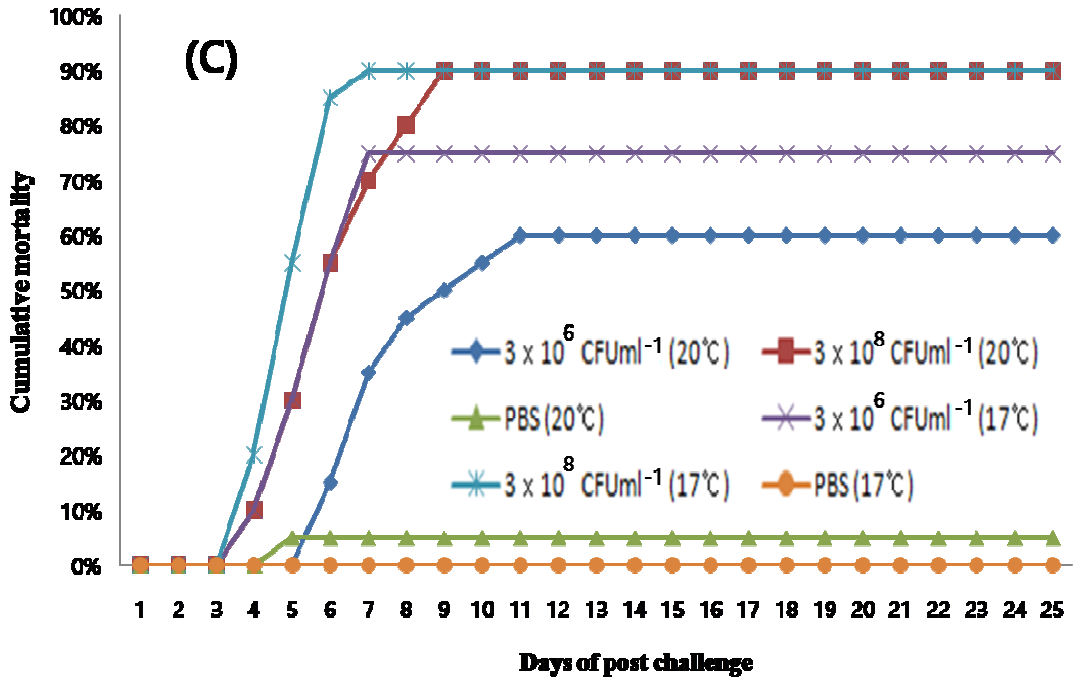

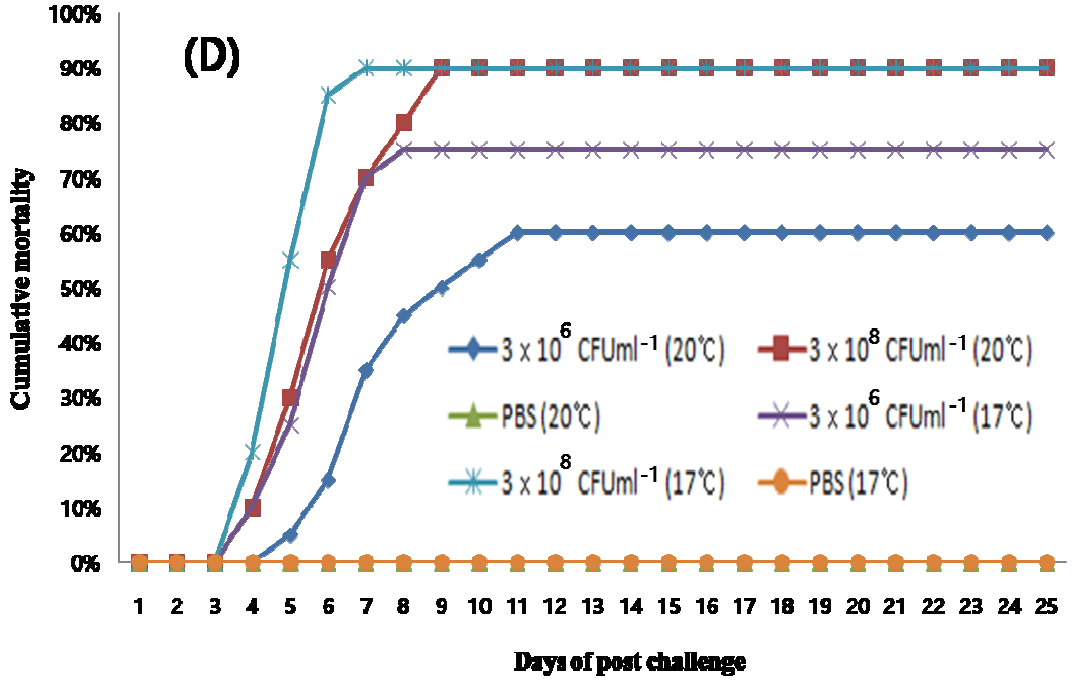

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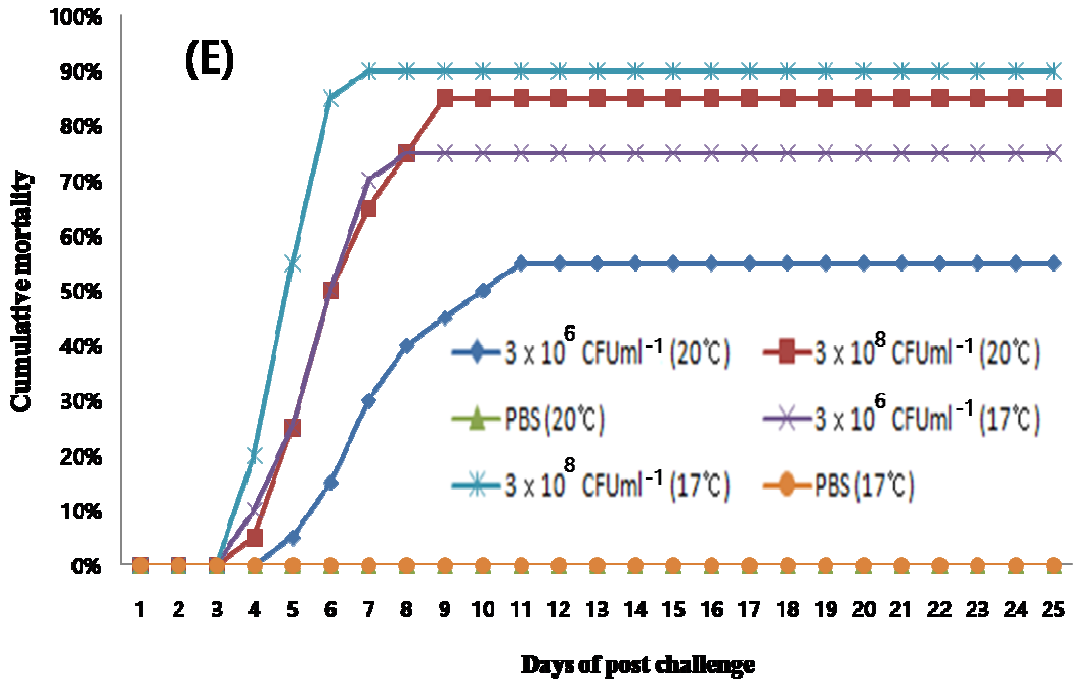

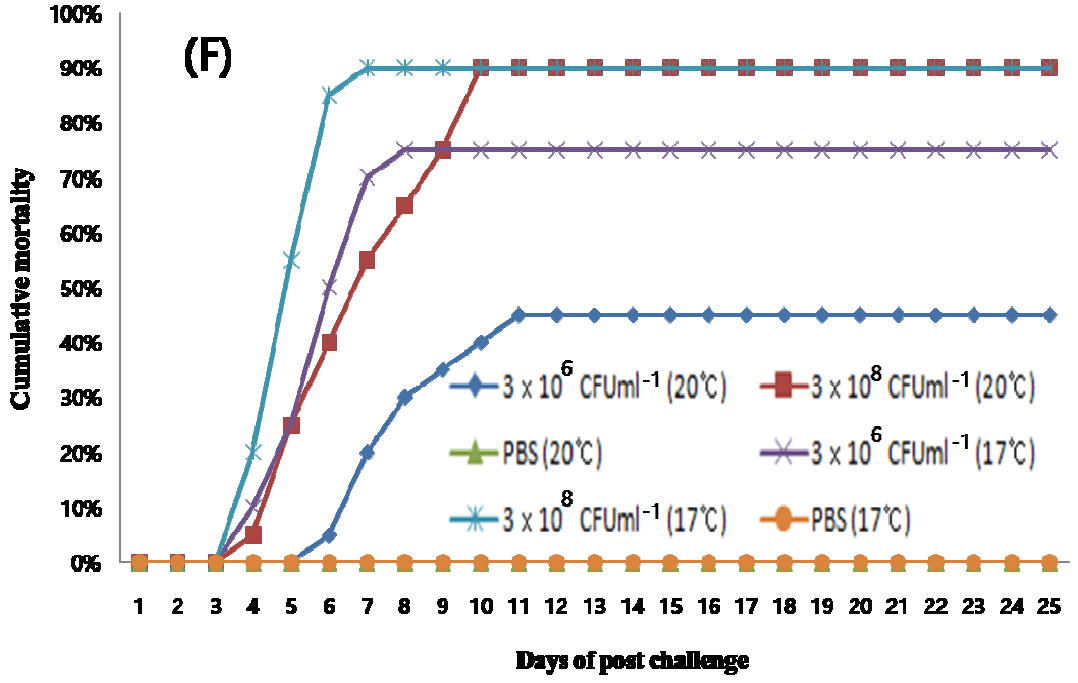

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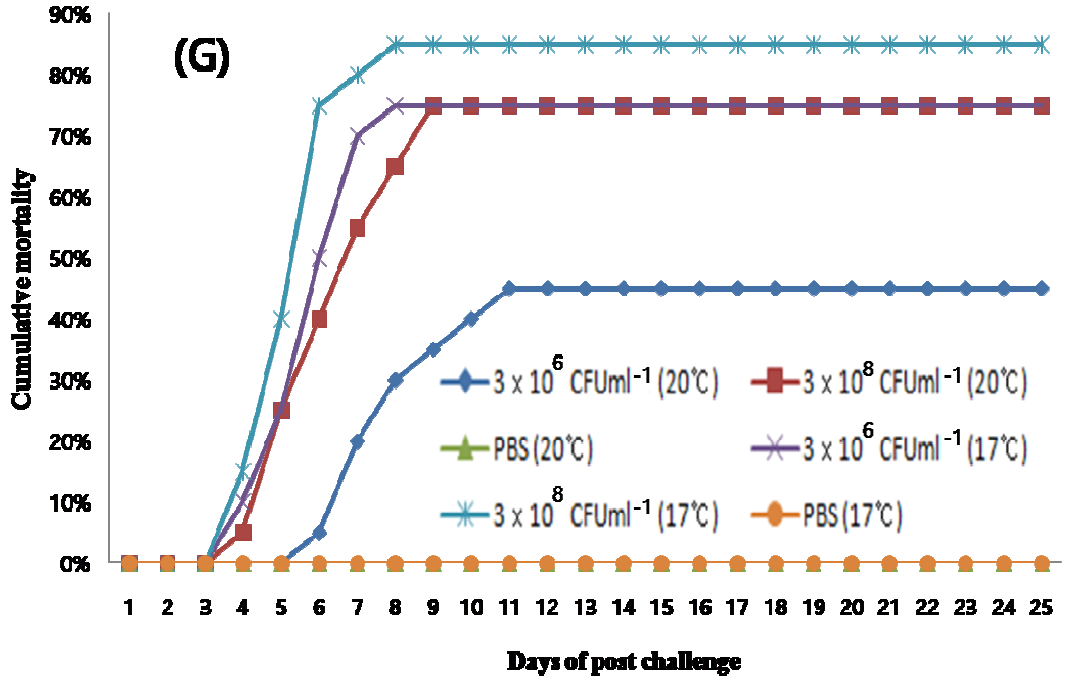

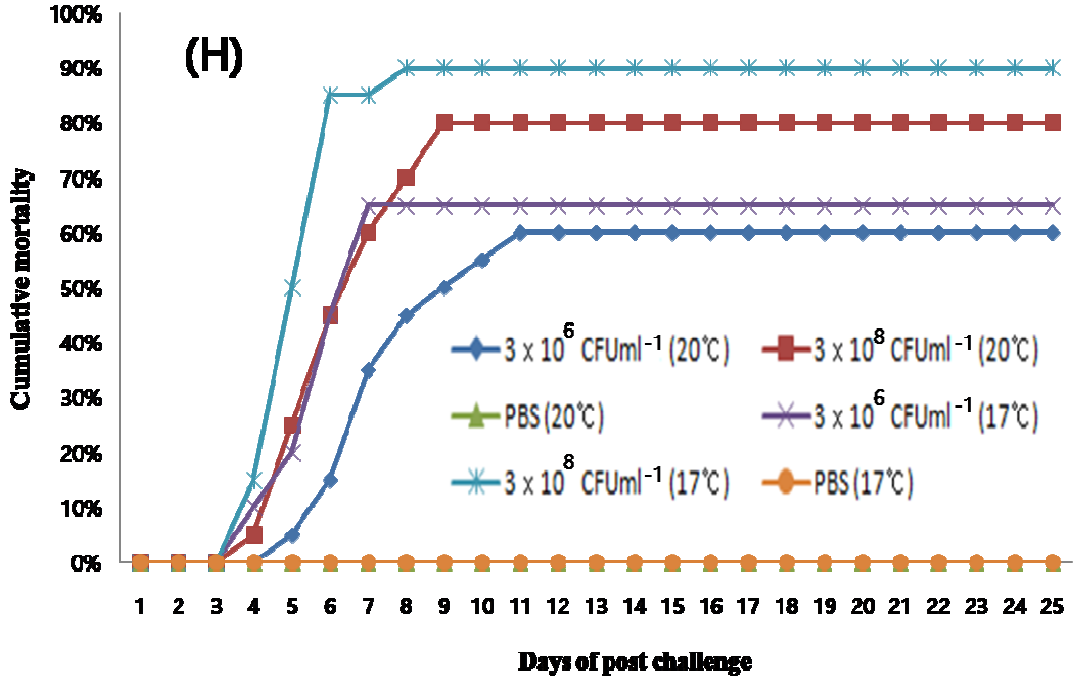

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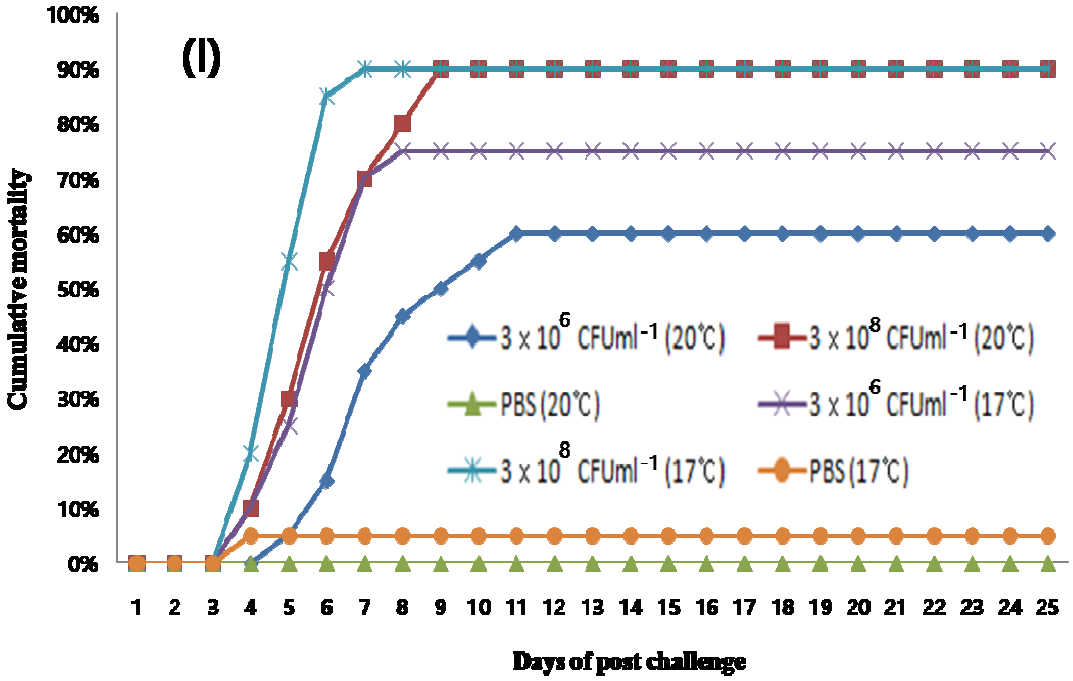
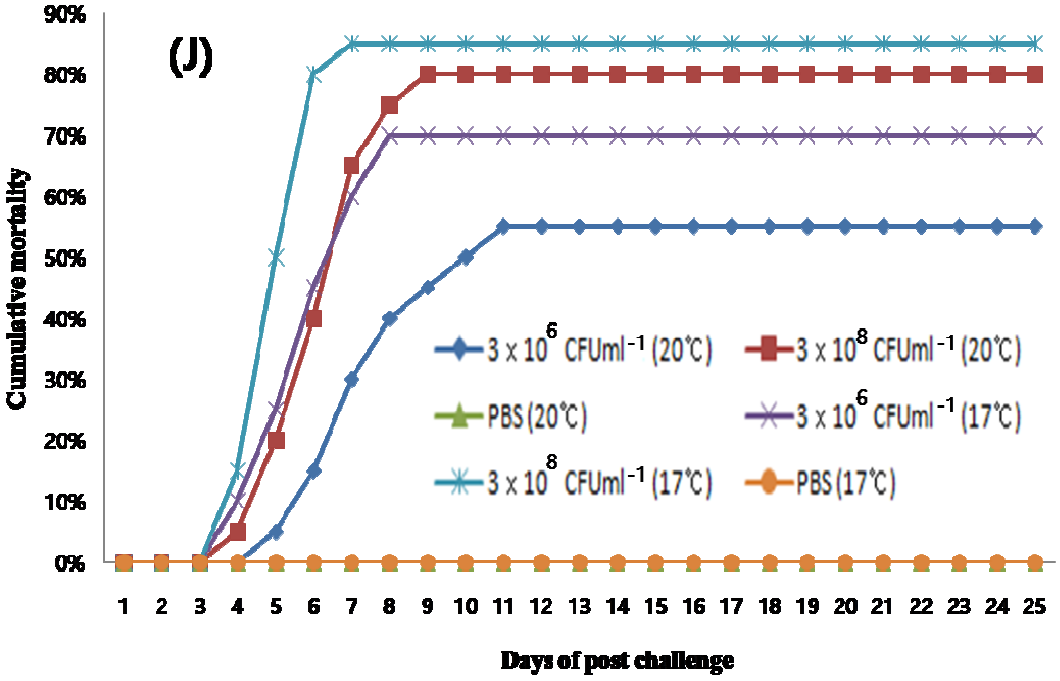




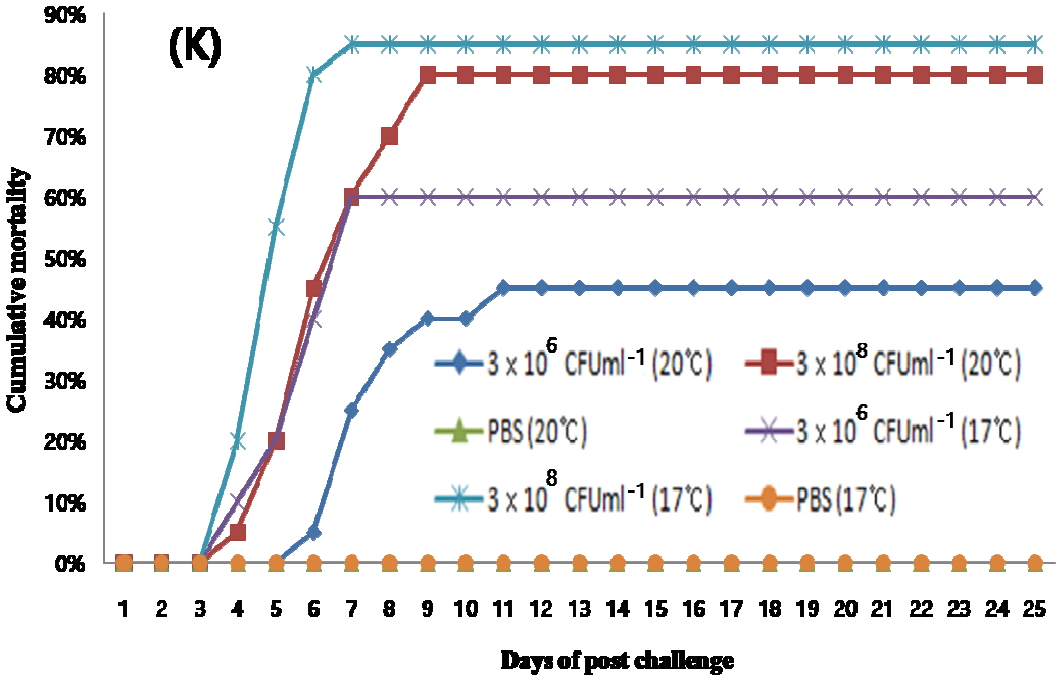


Fig. 6. Cumulative mortality of olive flounder infected with *P. anguilliseptica* intraperitoneally and reared at 17°C and 20°C. A, JEP1; B, JEP2; C, JEP3; D, JEP4; E, JEP5; F, JEP6; G, JEP7; H, JEP8; I, JEP9; J, JEP10; K, JEP11.

5. Pathogenicity by immersion challenge

The infected fishes showed symptoms of the same as that of intra-peritoneal injection group (Fig. 5) and finally results in the death of the fishes. The cumulative mortality and the relative pathogenicity of isolated pathogens were shown in Fig. 7. No mortalities were observed in either group of flounder challenged with *P. anguilliseptica* until 5th day of post infection (dpi). However, fishes reared at 17°C and 20°C temperature received 3×10^8 CFUml^{-1} dose showed 55 and 50% mortality respectively. Control groups showed no clinical signs and no pathogens were isolated from skin as well as other internal organs. Based on these results, we concluded that the intra-peritoneal injection mode is more effective than that of immersion challenge method.

PCR amplification of the infected fish groups reconfirmed the specificity of the infected microbe and no other microbial contamination was found in any of the challenged fishes groups.

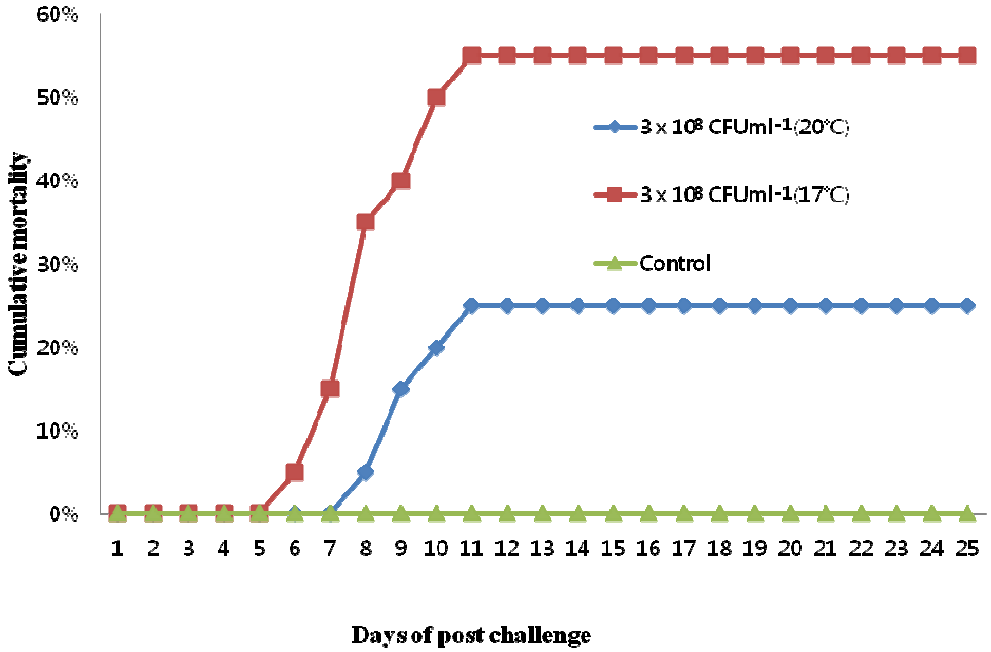


Fig. 7. Cumulative mortality of immersion challenged olive flounder with *P. anguilliseptica* JEP4.

DISCUSSION

Occurrence of high mortality in many flounder fish farms particularly in low rearing water temperature in and around Jeju Island had made the huge production loss to the olive flounder farmers. The identified causative agent of such a huge mortality in flounder fishes due to Pseudomonas septicemia is *P. anguilliseptica.* It was considered as a limiting factor for the culture of a number of fish species of great economic importance (Barnabe, 1990; Doménech at al., 1997; Romalde et al., 2003; López-Romalde et al., 2003a). The condition was more severe in fishes reared at low water temperature. This condition is called as "winter disease" and could be caused by several microbial fish pathogens including other Gram-negative bacteria such as *Aeromonaus hydrophila* and viruses (Bovo et al., 1995; Doimi, 1996). "Winter disease" in sea bream has been associated mainly with low water temperature, although other stressful condition, such as variation in salinity, nutritional deficiencies, immune status, or high fish densities, can also play a role as predisposing disease factors (Bovo et al., 1995). We observed an increase in the mortality in those farms where a decrease in the water temperature and salinity were simultaneously observed. These results reinforce the view that "winter disease" is probably a multi-factorial process favored by different undesirable environmental conditions acting in concert (Doménech et al., 1997).

In the present study, *P. anguilliseptica* was isolated as pure culture from the kidney, liver, brain, skin and body fluids of diseased flounder fish and not from the healthy fishes of the same fish farm. The morphology of *P. anguilliseptica* were non-sporing rods, measuring about 2μm by 4μm and the tendency to form long, filamentous cells were observed in blood agar medium (Wakabayashi and Egusa, 1972). The results obtained by biochemical and phenotypic test as well as API kit system, showed that the isolates were homogeneous, matching previous reports of *P. anguilliseptica* (Wakabayashi and Egusa, 1972; Doménech et al., 1997, Wiklund and Bylund, 1990; Berthe et al., 1995). This was confirmed with the other diseased fishes collected from eleven different flounder fish farms located in Jeju Island. Previous reports on sea-bream (Berthe et al., 1995), suggesting that *P. anguilliseptica* could be the causative agents of winter diseases also in olive flounder, *Paralichthys olivaceus.* This was confirmed with the specific amplification and

sequencing of 418 bp fragment of 16S rRNA gene, a crucial method in recognition of *P. anguilliseptica,* which was further confirmed by more detail phenotypic tests. Use of 16S rRNA gene sequence in the diagnosis of unusual bacterial pathogens had been reported in earlier study (Romalde et al., 2004).

The eleven isolated strains were sensitive to the chemotherapeutic agents such as oxolinic acid, oxytetracycline, doxycycline, flumequine, ofloxacin, ciprofloxacin, norfloxacin, pefrofloxacin, amoxicillin, ampicillin, gentamycin, neomycin, florfenicol, streptomycin and resistance to clindamycin, cephadroxil, ceftiofur, cephalexin, lincomycin and erythromycin. Earlier reports found that oxolinic acid was effective against fish disease and help in regaining the normal feeding health status (Jo, 1978; Al-Marzouk, 1999) and also reported that some strains have been found resistant against sulfisoxazole, erythromycin, penicillin, amoxicillin (Wiklund and Bylund, 1990; Al-Marzouk, 1999; López-Romalde et al., 2003b). This sensitivity can possibly be explained on the basis of the relative lack of use of chemotherapeutic agents for controlling diseased in fishes.

Increasing the rearing water temperature in order to control the pathogenic infection could be practically difficult and it would probably increase the physiological stress in fish. Disease in sea bream, *Sparus aurata* L., caused by *P. anguilliseptica* infection tends to occur at 12°C (Doménech et al., 1999) and the disease was found to declines with rising water temperatures in eels (Stewart et al., 1983). Such an approach to disease control was not very effective. Instead, the fishes treated with in-feed antibiotics responded well, with declining mortality and reducing incidence of clinical disease (Nakai and Muroga, 1979).

The optimum growing temperature of *P. anguilliseptica* is ranged around 15 to 18°C and in the present study, fishes infected with 3×10^8 $CFUml^{-1}$ of cells, reared at 17°C showed rapid infection and high cumulative mortality. During stressful conditions such as change in water temperature, the immune system of fish become partially depressed and are more susceptible to infection by ubiquitous or opportunistic bacterial fish pathogens like *P. anguilliseptica* (Tort et al., 1996). Temperature is a well-know environmental cue in fish. To control a disease like “winter disease” rising the rearing water temperature above 26°C had shown significant effect on mortality (Muroga

et al., 1973).

Mode of infection of pathogens is very crucial in any challenge experiments. Present study showed the two modes of infection namely intra-peritoneal injection (IP) and immersion. Among the two methods, fishes reared at 17 and 20°C, received *P. anguilliseptica* through IP injection showed significantly increased mortality when compared with that of immersion method. Previous studies had shown the rapid multiplication of *P. anguilliseptica* and subsequently developed disease in intramuscularly inoculated eels reared at 12°C, while *P. anguilliseptica* injected fishes reared at 28°C showed rapid clearance of bacterial cell (Nakai et al., 1985). This clearly indicates the temperature dependence infection of *P. anguilliseptica.* Several reports on temperature effect on *P. anguilliseptica* infection in various fish species namely, Atlantic salmon *Salmo salar* L., rainbow trout, *gairdneri*, sea trout S*, trutta* L., and whitefish, *Coregonus* sp. which were reared at 15-18°C in Finland (Wiklund and Bylund, 1990) and farmed gilthead sea bream *Sparus aurata* along in French.

Part Ⅱ. Rapid detection of *P. anguilliseptica* from olive flounder (*Paralichthys olivaceus*) using Real-Time PCR Taqman Fluorescent probes technique

ABSTRACT

Marine fish pathogens including bacteria, fungi, viruses, parasites and protozoa are the major threat to the aquaculture and public health. Lack of accurate and rapid detection technique in the identification of fish pathogens is the main limitation in the fish disease diagnosis and management sector. Due to advancement in the molecular biology and bio-instrumentation, diseases diagnoses become more accurate and reliable. Particularly, after the invention of most important molecular biological technique namely polymerase chain reaction (PCR), the disease diagnosis has become more accurate. In aquaculture, identification of pathogen free seed fishes is very crucial and it could be done with PCR screening of the fish seeds for several fish pathogens. Many PCR methods have been developed, each requiring its own protocol, equipment and expertise. In this regards, Real-time quantitative Taqman probe based detection assay targeting the 16S rRNA gene of fish pathogen *P. anguilliseptica* was developed. Using the 16S rRNA gene sequences of *Pseudomonas* species, including *P. anguilliseptica,* a 71 bp Taqman probe was designed as it rich in single nucleotide polymorphisms (SNPs), conserved within the *P. anguilliseptica* strains but varied from other non-*P. anguilliseptica* strains. The designed probe was very sensitive in detecting 300 pg of infected fish genomic DNA and in 1.22×10^{0} plasmid copies/µl. The probe was very specific in detecting only *P. anguilliseptica* when compared with the conventional PCR, biochemical and microbiological assays. Disease free flounder fishes were experimentally infected with 100 µl of 3×10^{6} and 3×10^{8} $CFUml^{-1}$ of *P. anguilliseptica* intra-peritoneally. Cumulative mortality of the

infected and control fishes were recorded. Result shows that the cumulative mortality of fishes received 3×10^8 CFUml^{-1} doses have 90% on 9th dpi while 3×10^6 CFUml^{-1} have 60% mortality on 11th dpi. The C_T value obtained for different organs of the infected flounder fishes reveals that spleen and kidney of infected *Paralichthys olivaceus,* would be suitable organ for the rapid detection. This assay represents a novel approach in diagnosing *P. anguilliseptica* infection in olive flounder.

MATERIALS AND METHODS

1. *P. anguilliseptica* culture and genomic DNA extraction

The diseased fishes were collected since 2010 from various fish farms located in and around Jeju Island (Table 7). The bacterial isolations were made from liver, spleen, kidney, brain, hemorrhagic skin dissected from diseased fishes which includes olive flounder, rock bream, grouper and rockfish reared at 17-20°C water temperature and incubated on Blood agar (bioMérieux, France) at 20°C for 1 week. Then sub-cultured on Tryptic Soy (TS) agar (BD, USA) and incubated at 20°C. Glycerol stock for all the isolated strains were prepared with 20% (v/v) of glycerol in TS broth and stored at -80°C.

DNA was extracted from TS broth cultures of each strain using RNeasy DNA kit (Qiagen, Germany). Briefly, 25 mg (10 mg for spleen) of tissue samples from infected fishes were homogenized in 100 µl of ATL buffer. Then 20 µl of proteinase K was added and incubated at 56°C for 1h or till complete lysis of the tissue with occasional vortexing. After complete lysis of the tissue, add 4 µl of RNase A (100 mg/ml) and incubate at 25°C for 2 min. Then the samples were centrifuged at maximum speed for 3min and collect the supernatant in 2 ml tube and then loaded to Qiacube (Qiagen, Germany) instrument. From then the genomic DNA was eluted automatically in 50 µl of Tris-acetate-EDTA buffer and that was stored at -20°C until required for PCR screening.

Table 7. Species, strain and threshold cycle for all bacterial strains tested (n=5).

Species	Strain	Origin	Host fish	C_T±SEM	16S rRNA gene sequence accession no.
Pseudomonas anguilliseptica	JEP1	Jeju, Seongsan	olive flounder	20.07±0.31	KC565867
	JEP2	Jeju, Seongsan	olive flounder	19.56±0.49	KC565868
	JEP3	Jeju, Seongsan	olive flounder	21.53±0.19	KC565869
	JEP4	Jeju, Daejeong	olive flounder	22.13±0.23	KC565870
	JEP5	Jeju, Aewol	olive flounder	20.14±0.34	KC565871
	JEP6	Jeju, Pyoseon	olive flounder	21.34±0.33	KC565872
	JEP7	Jeju, Pyoseon	olive flounder	19.56±0.12	KC565873
	JEP8	Jeju, Pyoseon	olive flounder	19.17±0.13	KC565874
	JEP9	Jeju, Pyoseon	olive flounder	23.34±0.23	KC565875
	JEP10	Jeju, Pyoseon	olive flounder	24.22±0.15	KC565876
	JEP11	Jeju, Seongsan	olive flounder	23.23±0.43	KC565877
	JERB1	Jeju, Namwon	rock bream	26.23±0.41	-
	JERB2	Jeju, Hangyeong	rock bream	25.24±0.26	-
	JERB3	Jeju, Gujwa	rock bream	17.28±0.68	-
	JERB4	Jeju, Gujwa	rock bream	18.13±0.12	-
	JERB5	Jeju, Hangyeong	rock bream	21.27±0.14	-
	JERB6	Jeju, Seongsan	rock bream	22.12±0.28	-
	JERB7	Jeju, Gujwa	rock bream	27.33±0.82	-
	JERB8	Jeju, Namwon	rock bream	25.28±0.68	-
	JERB9	Jeju, Hangyeong	rock bream	21.23±0.60	-

NA = No amplification.

Table 7. Continued.

Species	Strain	Origin	Host fish	C_T ± SEM	16S rRNA gene sequence accession no.
Pseudomonas anguilliseptica	JERB10	Jeju, Namwon	rock bream	28.07±0.68	-
	JERB11	Jeju, Gujwa	rock bream	22.46±0.84	-
	JERB12	Jeju, Seongsan	rock bream	29.13±0.22	-
	JERB13	Jeju, Daejeong	rock bream	18.23±0.21	-
	JERB14	Jeju, Gujwa	rock bream	19.24±0.42	-
	JERB15	Jeju, Pyoseon	rock bream	18.54±0.37	-
	JERB16	Jeju, Namwon	rock bream	22.76±0.20	-
	JERB17	Jeju, Pyoseon	rock bream	28.27±0.19	-
	JEGU1	Jeju, Pyoseon	seven-band grouper	25.53±0.26	-
	JEGU2	Jeju, Pyoseon	red-spotted grouper	27.23±0.74	-
	JERCF1	Jeju, Pyoseon	rockfish	29.33±0.61	-
Other *Pseudomonas species*					
P. fluorescens	ATCC			NA	-
P. putida	ATCC			NA	-
P. stutzeri	ATCC			NA	-
Other fish pathogens					
Vibrio alginolyticus	ATCC			NA	-
V. harveyi	ATCC			NA	-
Edwardsiella tarda	ATCC			NA	-
Streptococcus parauberis	ATCC			NA	-
S. iniae	ATCC			NA	-

NA = No amplification.

2. Taqman probe Real-time PCR assay

Nucleotide sequences of the 16S rRNA gene were retrieved from relevant *Pseudomonas* species, including *P. anguilliseptica* using NCBI Enterz Nucleotide Database search tool (http://www.ncbi.nlm.nih.gov/). A 71 bp region containing high concentrations of single nucleotide polymorphisms (SNPs), conserved within *P. anguilliseptica* strains but varied from other non-*P. anguilliseptica* strains, was identified. Oligonucleotide primers Ps16sr_F1 (5'-TGACGTTACCGACAGAATAAGCA-3') and Ps16sr_R1 (5'-CGCTTGCACCCTTCGTATTAC-3') and a Taqman probe, Ps16sr_TMP (5'-6FAM-CGG CTA ACT TCG TGC CAG CAG CC-TAMRA-3'); 6FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethyl-rhodamine quencher. The qPCR assay was optimized and validated using DNA extracted from *P. anguilliseptica,* isolated from infected flounder fish, non target *Pseudomonas* species and other bacterial species grown on TS broth (Table 7). The genomic DNA was diluted in Nuclease-Free water and 10-fold serial dilutions were prepared before quantification by the Taqman qPCR assay. The reaction mixture for qPCR assay consisted of 5 nM of specific primer set, 2.5 nM of probe and 2 µl of genomic 10-fold diluted genomic DNA in a final volume of 20 µl in Taqman universal master mix (Applied Biosystems, USA). Following an initial 10 min denaturation step at 95°C, and 40 cycles of amplification profile: 95°C for 30s (denaturation) and 60°C for 60s (annealing/extension). The result was analyzed using MX Pro in the Mx 3000P (Stratagene, USA).

Fluorescence intensity, as an indicator of amplicon concentration, was calculated from the equation $\Delta R_n=(R_n^+)-(R_n^-)$ (reporter signal fluorescence minus normalized background). The signal amplification (ΔR_n) was then plotted against PCR cycles to generate cycle threshold number (C_T) values. The cycle threshold number (C_T) value is the initial cycle in which amplification is detected as exceeding an arbitrary threshold. Standard curves for quantification were plotted from triplicate samples by using C_T values of 10-fold dilutions of plasmid DNA template. The concentrations were from 10^0 to 10^6 copies. The C_T values for experimental samples were also calculated from the mean of triplicate PCRs.

3. Specificity, sensitivity and amplification efficiency to Taqman PCR technique

The specificity of the qPCR probe and PCR primers were evaluated by amplification of DNAs from all *P. anguilliseptica* isolates and other pathogenic bacterial species listed in Table 7, under the optimized PCR conditions. Sensitivity of the developed qPCR probe was tested with different dilutions of *P. anguilliseptica* infected flounder fish DNA ranged from 30 µg to 30 pg in different organs like kidney, spleen, liver, brain, gill, muscle and skin. PCR efficiency was calculated by the slope value for the standard curve as described previously.

4. Challenge experiment

Healthy flounder fishes were obtained and divided into control and experimental groups (each group 30 fishes) in triplicate. After a week of habituation, experimental group fishes were intra-peritoneally injected with 100 µl of *P. anguilliseptica* (3×10^8 $CFUml^{-1}$) while, the control fish group received 0.1 ml of phosphate buffered saline (PBS). Fishes were maintained in 100 L aquaria at 20°C with continues aeration and cumulative mortality were daily recorded for a period of 25 days. Infected dead fishes were collected and kidney, spleen, liver and brain were dissected and genomic DNA was extracted. The extracted DNA was used in calculating the bacterial titration in different organs of the challenged fishes.

5. Statistical analysis

The relationship between the C_T values of Real-time PCR and the concentration of purified DNA or the number of *P. anguilliseptica* strains in pure culture was determined by standard curve, followed by regression analyses using Microsoft™ Excel.

RESULTS

1. Challenge experiment

The cumulative mortality and the relative pathogenicity of isolated pathogens were shown in Fig. 8. No mortalities were observed in either group of flounder challenged with *P. anguilliseptica* until third day of post infection (dpi). However, 3×10^8 CFUml^{-1} dose group showed 90% mortality on the 9th dpi while the 3×10^6 CFUml^{-1} dose group fishes showed 60% mortality after 11th day of post infection. Control groups showed no clinical signs and no pathogens were isolated from skin as well as other internal organs. PCR amplification of the infected fish groups reconfirmed the specificity of the infected microbe and no other microbial contamination was found in any of the challenged fishes groups.

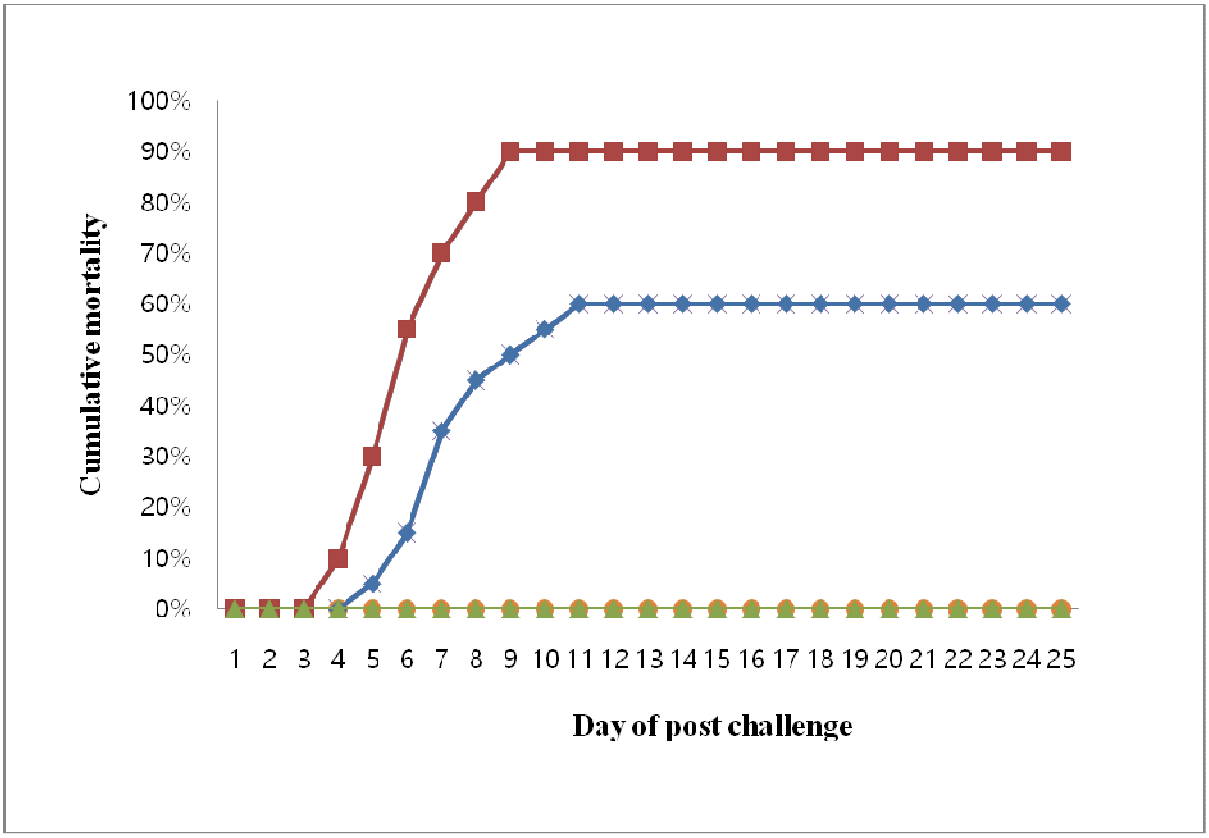


Fig. 8. Cumulative mortality of olive flounder infected with *P. anguilliseptica* intra-peritoneally reared at 20°C.

2. Taqman probe detection assay

Real-time PCR conducted on isolated *P. anguilliseptica* confirmed that the primers and probe were specific only for *P. anguilliseptica* as there was no or very less fluoresence intensity was detected with other *Pseudomonas* and other fish bacterial pathogens. The probe used in this study is designed from the variable region of 16S rRNA gene and is specific to *P. anguilliseptica.* The qPCR assay specically detected all the eleven *P. anguilliseptica* isolates from Jeju Island and no amplification was found with other *Pseudomonas* species and non-*Pseudomonas* species (Table 7). The detection limit for purified *P. anguilliseptica* infected flounder fish genomic DNA was 300 pg of DNA, determined by performing 10-fold serial dilution (30 µg to 30 pg per reaction), and followed by qPCR amplification. The results were further compared against the conventional PCR results (Fig. 9) to determine the sensitivity and specificity (Fig. 10A). To calculate the number of plasmid copy initially present in the reaction, a standard curve was constructed to correlate the threshold cycle C_T value with varying concentration of plasmid DNA. Standard curve using the C_T values was constructed with the R^2 value of 0.99 (Fig. 10B). Lesser the amount of template DNA (bacterial genomic DNA concentration) in the sample would have higher the C_T value. Figure 11 shows the differential accumulation of *P. anguilliseptica* in kidney, spleen, liver and brain of the infected flounder fishes. The spleen followed by the kidney shows less C_T value indicates the higher concentration of *P. anguilliseptica* and hence, spleen and kidney of the infected fishes are suitable organs for the rapid detection.

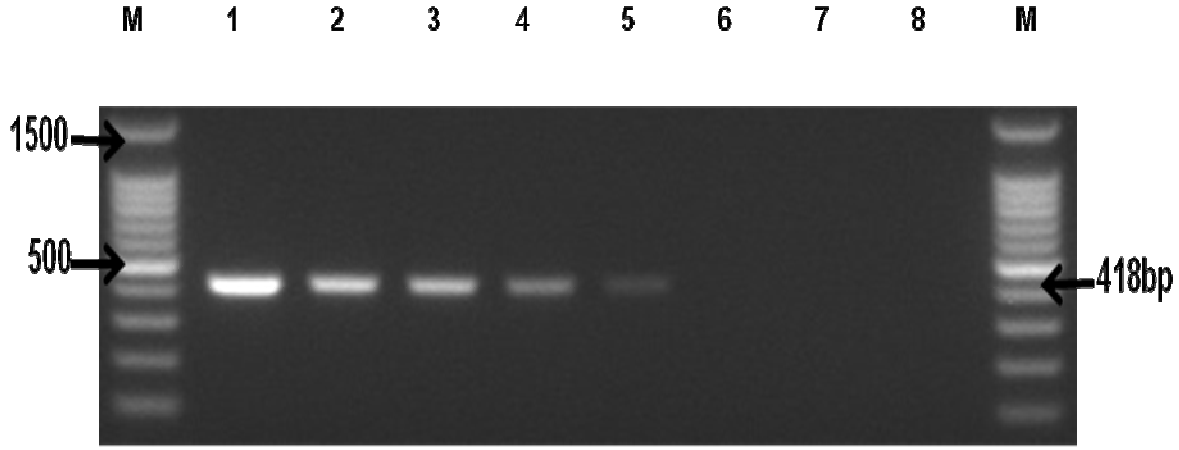


Fig. 9. Specificity of conventional PCR to different concentration of genomic DNA (titer=30 μg/ml) 10-fold dilutions.

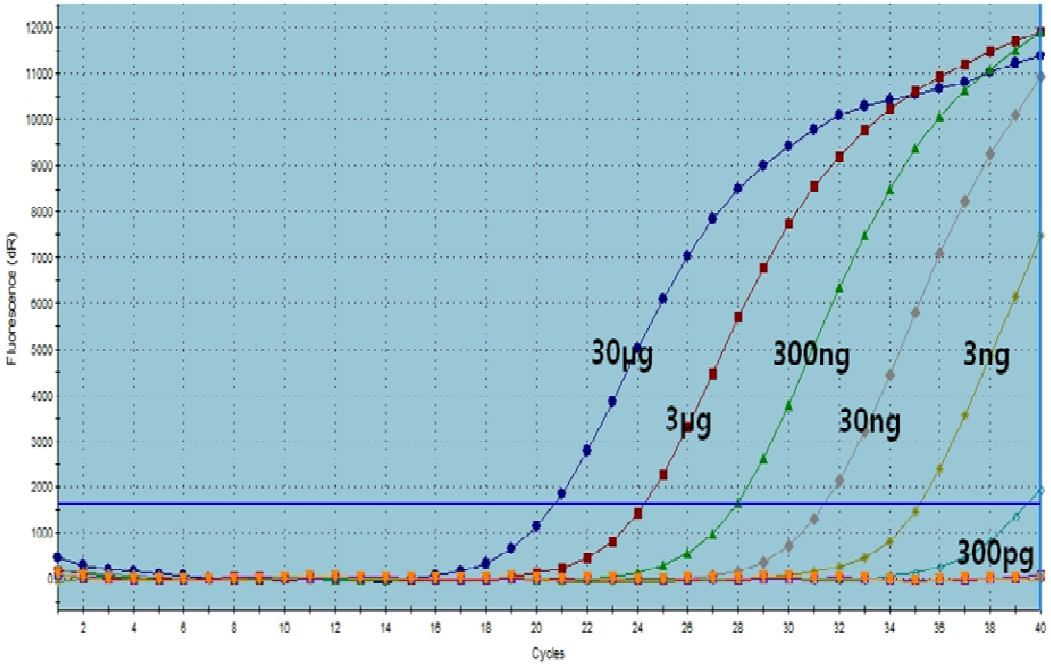


Fig. 10A. Plot of amplification from different concentrations of genomic DNA (titer=30 μg/ml) 10-fold dilutions.

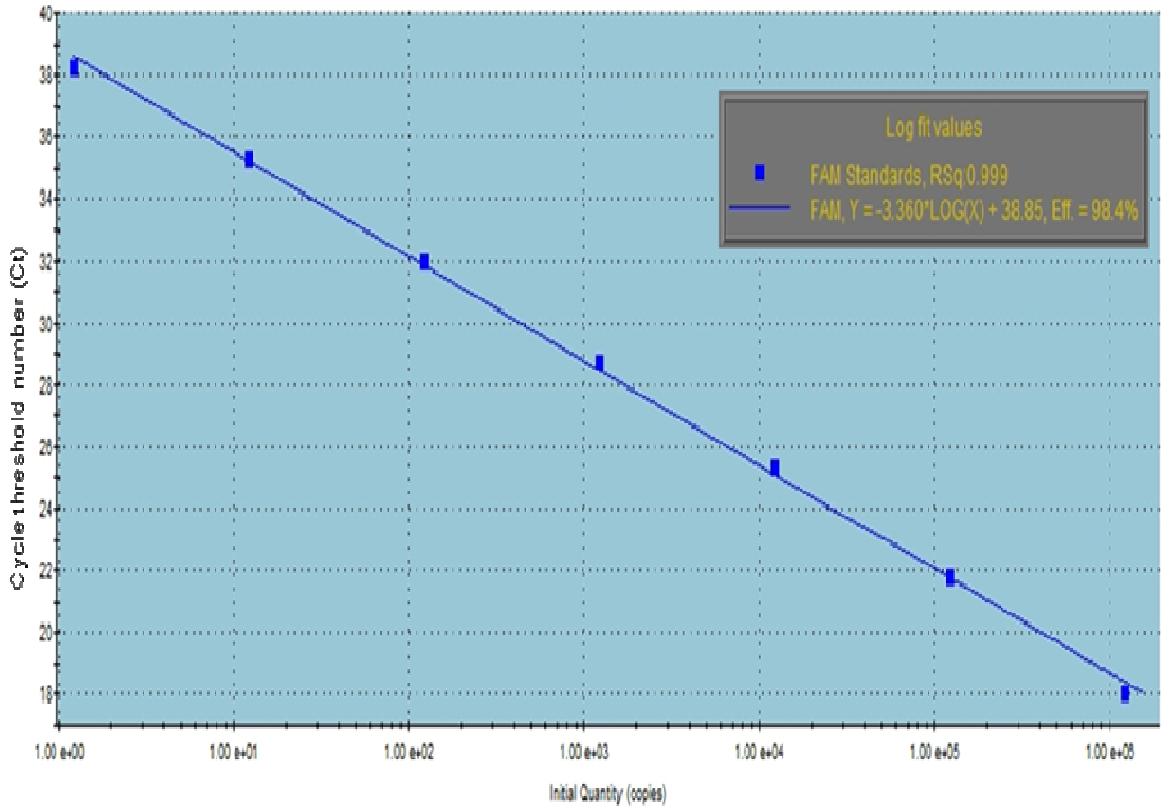


Fig. 10B. Standard curve generated using a 10-fold dilution series of the number of copies of plasmid (titer=1.22 $\times 10^6$ copies/μl) and C_T values.

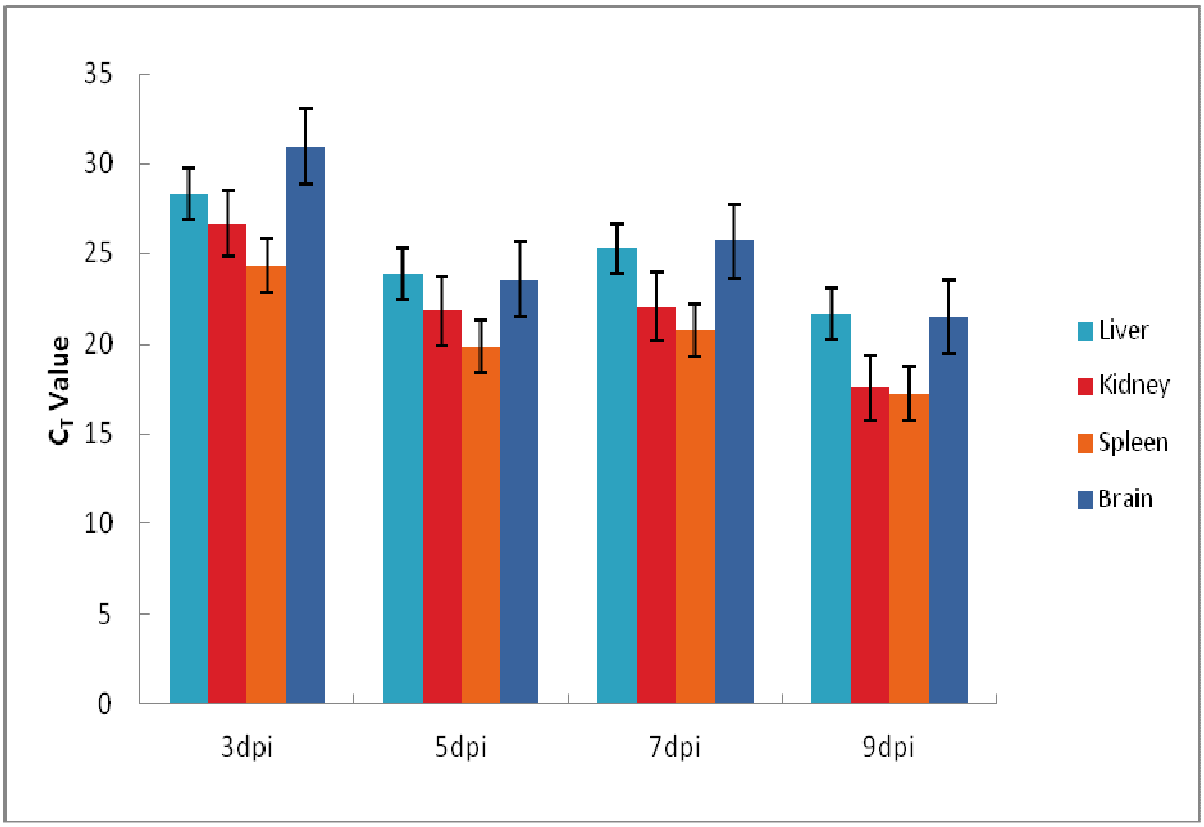


Fig. 11. Taqman probe Real-time detection of *P. anguilliseptica* in vital organs of infected flounder fishes (n=5).

DISCUSSION

Fish diseases pose a universal threat to the global aquaculture industry and in turn to the public health. Major fish pathogens include bacteria, fungi, viruses and protozoa cause considerable economic loss to the fish cultivators. Lack of rapid, accurate and reliable methods of detecting and identifying fish pathogens are the main limitations in fish diseases diagnosis and management. This limitation triggered the search for an alternative efficient diagnostic technique with the advent of molecular biological method particularly polymerase chain reaction (PCR). PCR had have been used successfully in identifying the pathogen and quantify its concentration using Real-time quantitative PCR method. However, in this chapter, a rapid detection method using a probe based Real-time PCR analysis in detecting the fish pathogens specifically *P. anguilliseptica.*

P. anguilliseptica, causative organism of "winter diseases" in many fish species, recently its infection was reported in olive flounder fish farm present in and around Jeju Island. Previous reports on sea-bream (Berthe et al., 1995), suggesting that *P. anguilliseptica* would be the causative agents of winter diseases also in olive flounder, *Paralichthys olivaceus.* This was confirmed with the specific amplification and sequencing of 418 bp fragment of 16S rRNA gene, a crucial method in recognition of *P. anguilliseptica,* which is further confirmed by more detail phenotypic tests. Use of 16S rRNA gene sequence in the diagnosis of unusual bacterial pathogens had been reported earlier (Romalde et al., 2004).

Present study highlights the rapid detection technique using Real-time Taqman probe method which was more sensitivity and specificity than the conventional diagnostic methods. The minimum detection limits of our Taqman probe is found to be 300 pg of infected flounder fish DNA, which is most sensitive when compared with previous reported on maximum detection limit of 0.7 pg/μl of *P. anguilliseptica* DNA (Romalde et al., 2004). Other studies using Real-time PCR have shown the increased specificity in detecting fish pathogens including *Aeromonaus* spp, *Flavobacterium* spp., *Vibrio* spp. and virus using probe based assays (Overturf et al., 2004; Gilad et al., 2004; Balcazar et al.,

2007; Kamimura et al., 2007). The C_T value obtained by the Taqman probe Real-time PCR analysis of genomic DNA extracted from spleen, kidney, liver and brain was inversely proportional to the bacterial cell accumulation in the vital organs of the infected fishes. The bacterial concentration was found to be high in the spleen followed by kidney of the infected fish group sampled at different time point (Fig. 11). In teleost fish, spleen and kidney are the major lymphoid organs (Zapata et al., 2006) and spleen in particular involved in immune reactivity and blood cell formation (Galindo-Villegas and Hosokowa, 2006). Moreover, presence of competing, non-*P. anguilliseptica* cells or DNA had minimal impact on the detection of *P. anguilliseptica.* This is a significant factor for accurate detection of target organism, *P. anguilliseptica* within a matrix of other microbial and host cells.

Moreover, differences between infected tissues from diseased fish and healthy tissues seeded with a laboratory-grown bacterium have been reported (Hiney and Smith, 1998), which can be originated by variations in the accessibility of the pathogen and/or the presence of PCR inhibitors. Therefore, the developed PCR assay was applied to DNA extracted from different organs obtained from experimentally infected fish. *P. anguilliseptica* was detected in practically all the infected fish, even at concentrations much lower than LD_{50} values, while no amplification was observed in non infected fish used as negative controls. The results obtained in the analysis of natural samples clearly validate the method to be used in the field, since it was possible not only to detect the pathogen in tissues from diseased fish but also in apparently healthy fish (data not included). Further, studies are needed to enhance the knowledge of the genetic, physiological and virulence factors of this pathogen, along with the information of its distribution, incidence of infection and rate of transmission throughout the population. The *P. anguilliseptica* specific qPCR assay developed in this study would provide significant insights into the dynamics of pathogen invasion and spread within populations. In addition, present study suggests that spleen and kidney could be the suitable organ for the rapid detection of pathogenic microbes such as *P. anguilliseptica* in olive flounder fish, *Paralichthys olivaceus.* This method could be applicable for the diagnosis of several other fish pathogens with species specific probe.

Part Ⅲ . Efficacy of formalin-killed *Pseudomonas anguilliseptica* vaccine on immune gene expression pattern and protection of farmed olive flounder (*Paralichthys olivaceus*)

ABSTRACT

Due to economic benefits, particularly in aquaculture industries, formalin-inactivated whole cell vaccines (bacterin) are used commonly as a preventive measure of aquatic diseases. Bactrial infection is one of the major disease problems in farming flounder fishes, which includes streptococcosis by *streptococcus iniae,* edwardsiellosis by *Edwardsiella tarda,* Pseudomonas septicemia by *P. anguilliseptica.* Formalin killed bacterial vaccine was prepared and used against *P. anguilliseptica* infections in farmed olive flounder, *Paralichthys olivaceus.* Anti sera was prepared against formalin-killed *P. anguilliseptica* vaccine in flounder fish and rabbit. The efficacy of the prepared vaccine was validated using serological assays like agglutination titers, immunoblotting and immune gene expression profiles. Fishes reared at 17°C and 20°C water temperature were vaccinated with 3×10^7 CFU ml^{-1} along with positive (live bacteria cells) and negative control (PBS) intra-peritoneally. Fishes were also sampled from zero hour of post injection (hpi) to 28 days of post injection (dpi) for the immune gene expression assay using Real-time PCR. Mean percent mortality and relative percent survival (RPS) were calculated for the vaccinated and challenged fishes. Results reveal that the vaccinated fishes reared at 17°C and 20°C water temperature showed significantly increased RPS (69 and 89 respectively) and decreased mortality from 83±0.6% and 74±0.7% in challenged control fishes to 25±0.8% and 8±0.8% in vaccinate and

challenged fish groups respectively. Immunoblotting result reveals that rabbit anti-Ps serum is more effective in capturing antigenic protein when compared to the fish anti-Ps. Vaccinated fishes showed significant increase in the expression of TNFR-1, FasL, IRF7, TLR2, IL-1b and CD40 gene transcripts when compared with that of the control fishes. The up-regulation of these genes along with the increased RPS values suggest that the formalin-killed cells of *P. anguilliseptica* could play an important role in immunizing olive flounder fishes against *P. anguilliseptica* infection. Thus increasing the immunity of the flounder fish with the formalin-killed *P. anguilliseptica* would enhance the disease resistance and in turn helps to increase flounder production in South Korea.

MATERIALS AND METHODS

1. Fish

Juvenile olive flounder fishes (mean weight of 15±3 g) were obtained from commercial fish farm in Jeju Island. The fish were habituated for two weeks in aerated water at 17°C and 20°C in 500 L tanks prior to experiments. During experiment, the fish were maintained with standard culture conditions and fed twice a day with commercial pelleted diet. Also, Fishes were confirmed to be negative for *P. anguilliseptica* infection by culturing fish tissue in the brain heart infusion (BHI) agar (BD, USA).

2. Vaccine preparation

Formalin killed *P. anguilliseptica* vaccine was prepared as described previously (Klesius et al., 1999). Briefly, *P. anguilliseptica* (KC565870) isolated from infected flounder fish collected from Jeju Island, was cultured in BHI broth for 1 week at 20°C. The bacterial pellet was collected by centrifugation (5000 × g for 10 min at 4°C), washed twice in sterile PBS (pH 7.4) and re-suspended in 30 ml of PBS. Inactivation was done by adding 10% neutral buffered formalin to give a final concentration of 3% formalin at room temperature for 24 h. The bacterium was confirmed to be killed by the lack of growth on the BHIA plates following 7 days at 20°C. The formalin-killed cultured was then centrifuged at 7000 × g for 30 min to separate to the cell pellet and supernatant. The cell free culture fluid was concentrated 20-fold, filter sterilized (0.2 μm) and used to re-suspend the cell pellet at V/V of 10:1. The final concentration of the vaccine was approximately 3×10^7 $CFUml^{-1}$ which was based on the lethal dose (LD_{70}) determined by the pathogenicity assay (data not included).

3. Vaccination-preparation of fish anti-Ps sera

The fishes were divided into four groups (n=150; in duplicate), two control (17°C and 20°C rearing water temperature) and two vaccinated group (17°C and 20°C rearing water temperature). The vaccinated fish groups received about 100 µl of 3×10^7 $CFUml^{-1}$ of *P. anguilliseptica* bacterin, while the control fishes received 100 µl PBS intra-peritoneally. Then booster vaccination was done after two weeks of interval. The mortality was recorded for 6 weeks period and all dead fish were examined to confirm the re-isolation of the cultured pathogen from kidney, spleen, liver, brain, gill, skin on the BHI agar. The blood sample was collected from the live fishes by every two weeks interval, from both the vaccinated and control fishes of two temperature groups. The collected blood was allowed to clot for 1 h at 25°C and then centrifuged at 1000 × g for 10 min. Collected serum was stored at -20°C until assayed for antibody titer and western blotting.

4. Preparation of rabbit anti-Ps sera

Rabbit was immunized with intravenous injection of PBS-diluted suspension (3×10^7 $CFUml^{-1}$) of formalin-killed *P. anguilliseptica* cells twice weekly in consecutive doses of 0.2, 0.4, 0.8, and 1.0 ml. One week after the last injection, the rabbit was bled from the ear vein. Two weeks later, this immunization procedure was repeated, now with 1.0 ml doses throughout. The antiserum was stored at -20°C.

5. Agglutination assay

Antibody titer against vaccinated olive flounder serum by *P. anguilliseptica* was determined by micro-titration agglutination test with rabbit anti-Ps as positive control. Briefly, each well of a 96-round well micro-titration plate was coated with 50 µl of phosphate-buffered saline (PBS) solution and then 50 µl of vaccinated olive flounder sera collected from 2^{nd}, 4^{th} and 6^{th} week of post vaccination, were added to the first well of each row, mixed and then 50 µl of diluted serum was serially diluted into the remaining wells. Doubling dilutions of positive and negative sera were included on every plate as controls. To each well, 50 µl of 3×10^7 $CFUml^{-1}$ *P. anguilliseptica* (KC565870) cells suspension was added and mixed. The plate was covered and incubated in humidified air at 25°C for 18 h. The highest serum dilution that showed a circular diffuse button with fuzzy edges at the bottom of the well was considered a positive reaction and a circular compact cell button was considered to be a negative reaction. Antibody titer was expressed as log base for each isolate studied.

6. Blood sampling

Blood samples of five fishes/group were collected randomly from caudal vein using a vacationer fitted with 1 ml 27-G needle on 2^{nd} and 4^{th} week after vaccination. Fishes were anaesthetized with MS-222 ($NaHCO_3$ and tricaine methane sulphonate; Sigma Chemicals) and sampled only once to avoid the influence due to multiple bleeding and handling stress on the fishes. 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.

7. Blood biochemistry

Serum biochemical parameters, such as serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), total protein (TP), cholesterol (CHOL), triglycerides (TRIG) were determined in VetTest 8008 chemistry analyzer (IDEXX, USA) by using analysis VetTest kits (IDEXX, USA).

8. Denaturing polyacrylamide gel electrophoresis SDS-PAGE and immunoblotting analysis

For the protein extraction, 20 ml of 1 week BHI broth culture of *P. anguilliseptica* was centrifuged for 10 min for 3000 × g. Then the pellet was washed twice with distilled water and sonicated for three times at 30W for 30s with frequent incubation on ice for 20s. Subsequently, samples were re-suspended in gel loading buffer (0.9 ml phosphate buffer 0.1 M (pH 6.8), 5% β-mercaptoethanol, 5% sucrose and 20% SDS) was added to the suspension and the mixture was heated at 100°C for 2 min. The extracted protein (10 µg/µl) was separated in 4-12% precast NuPAGE Bis-Tris Mini gels in duplicate by using the method of Laemmli (1970). After separation one gel was staining with SimplyBlue SafeStain (Invitrogen, USA), while the other one was subjected to Western blotting using the *P. anguilliseptica* polyclonal antibody raised in rabbit and flounder fish as primary antibody. The gel was cut into two half and the bacterial protein was electrophoretically transferred to two PVDV membranes separately using a iBlot apparatus (Invitrogen, USA) following the manufacturer's instruction. The blotted membranes were blocked with blocking buffer and then incubated separately with primary antibody (diluted 1:1000 in iBlot antibody diluting mixture) and then with goat anti-rabbit IgG (diluted 1:250 in iBlot antibody diluting mixture) secondary antibody. Then the blots were immunostained to produce a color reaction by using iBlot Western Detection Chromogenic kit (Invitrogen, USA).

9. Immune gene expression of vaccinated fishes

For the immune gene expression analysis, formalin killed *P. anguilliseptica* vaccinated fishes (n=5) reared at 17°C water temperature (effective invation of pathogen was observed at low rearing temperature) were collected after zero, one, three, six, nine, twelve hours of post injection (hpi) and 1, 2, 3, 5, 7, 9, 17, 24, 28 days of post injection (dpi). Total RNA was extracted from head-kidney of infected flounder fishes using the RNeasy Plus Mini Kit (Qiagen, Germany), following the manufacturer's instructions. RNA samples were eluted in 50 µl DEPC-water and RNA concentration was determined by spectrophotometer at 260 nm and subsequently adjusted to 0.1 µg/µl for each sample. Aliquots were stored at -80°C until used.

Synthesis of cDNA from total RNA was performed by mixing 2.5 ng/µl of random primers (Invitrogen, USA) and 9 µl of extracted total RNA in nuclease free water. The mixture was incubated at 95°C for 5 min, and held on ice for at least 1 min. A reverse transcription mixture containing 200 U/µl Superscript III RT (Invitrogen, USA), 0.5 mM dNTPs and 0.05 M DTT in 1 × first strand buffer was then added. Then the mixture was incubated at 25°C for 10 min, followed by 50 min at 50°C and the reaction was stopped by heating at 85°C for 5 min. the synthesized cDNA was stored at 4°C until use.

10. Primer design and quantitative PCR

Seven sets of primers were designed from NCBI Genbank database using the Primer Express 3 software (Applied Biosystems, USA) and were used in SYBR Green-based PCR analysis (Table 8). Reaction were carried out in a final volume 25 µl reaction volume containing 2 µl 10-fold diluted genomic DNA, 200 nM of each primer sets using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent technologies, USA) (in triplicate). β-actin gene was used as endogenous control for the Real-time gene expression analysis. The PCR reaction was carried out following an initial 10 min *Taq* activation step at 95°C, and 35 cycles of amplification profile: 95°C for 30s, with appropriate annealing temperature (Table 8). The result was analyzed using MX Pro-Mx3000P Multiplex Quantitative PCR system Software (Stratagene, USA) and the relative expression ratio (R) of mRNA was calculated according to the formula $2^{-\Delta\Delta C_T} = 2^{-(\Delta C_T\,(test) - \Delta C_T\,(\beta\text{-actin}))}$ (Livak et al, 2001). Real-time PCR efficiencies were acquired by the amplification of dilution series of cDNA according to the equation $10^{(-1/slope)}$ and were consistent between target genes and β-actin. The results are presented as means with standard deviations.

Table 8. Quantitative Real-time PCR primer sequences of olive flounder immune genes.

Primer name	Nucleotide sequence (5' → 3')	Tm	Accession number	Amplicon size(bp)
FasL F	GTTGGCCGGAAAACAGCA	59	AB206381	166
FasL R	TGGGGTGGCTCTTGGAGA			
TNFR-1 F	AGATCCATGACCTGCTGA	50	AB080946	145
TNFR-1 R	GGACCTCTCATAGGCACA			
CD40 F	TGGTGGTGATGCTGTGCA	57	AB081752	166
CD40 R	CCACACTCTGCGCACTGA			
TLR2 F	TGCTGCAGTACCCCAGGA	57	AB109393	111
TLR2 R	GGTTGTCGGACAGGTCCA			
IL-1b F	TGCACCCTTCACCCACCA	60	AB070835	117
IL-1b R	CGACACGCTCCAGATGCA			
IRF7 F	GCAGGGATGAGGACAGCA	57	GU017419	112
IRF7 R	TTGAGTGCGCAGCGGAA			
β-actin F	AGGCGCAGAGCCTTGATG	57	HQ386788	191
β-actin R	GTCAAGCGCCAAAAATAACTG			

11. Vaccinated fish challenge assay

For the challenge experiment, after four weeks of vaccination, forty fishes from each vaccinated and control fish groups were injected intra-peritoneally with 100 µl of homologous isolate of *P. anguilliseptica* (KC565870), containing 3×10^7 CFUml^{-1}. Mortalities were recorded for 14 days and any clinical signs in survivors were noted. The cause of death and pathological signs were verified by re-isolation of bacteria from kidney sample of freshly dead fish or survivors. Cumulative mortality was registered and relative percent survival (RPS) was calculated according to the formula: RPS = [1 - % mortality in vaccinated group/% mortality in control group] ×100.

12. Statistical analysis

The means (±SD) of assayed parameters were calculated for each group of fishes. Two-sample Student's t-tests were used to compare values from individual experimental fish groups with those from controls. Measurements were considered significant if $P<0.05$.

RESULTS

1. Blood biochemical constituents

The blood biochemical and physiological parameters are important for general health, toxicology, and bio-monitoring. The aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), total protein (TP), cholesterol (CHOL) and triglyceride (TRIG) of both control and vaccinated fish groups showed no significant variation ($P>0.05$) till 4^{th} week of post-vaccination (Table 9). These results suggests that the vaccination of flounder fishes with formalin killed *P. anguilliseptica* vaccines, used in the present study could not found increase any internal body stress.

Table 9. Serum biochemical parameters of control and vaccinated olive flounder from the 1st day of pre-vaccination and 2nd & 4th week of post-vaccination.

Parameters	Weeks	Groups					
		17°C			20°C		
		Control	3×10^{6} CFUml^{-1}	3×10^{8} CFUml^{-1}	Control	3×10^{6} CFUml^{-1}	3×10^{8} CFUml^{-1}
AST (U/L)	0	38.2 ± 2.8	42.2 ± 2.1	38.2 ± 6.4	44.2 ± 2.4	48.1 ± 1.6	41.6 ± 4.2
	2	44.8 ± 4.6	44.6 ± 4.2	47.1 ± 4.8	40.2 ± 1.4	42.2 ± 3.2	40.7 ± 3.4
	4	40.8 ± 4.5	39.4 ± 4.4	40.0 ± 4.2	47.2 ± 4.4	38.5 ± 6.4	44.5 ± 2.6
ALT (U/L)	0	8.4 ± 0.6	8.8 ± 0.9	7.8 ± 0.6	8.8 ± 0.6	8.4 ± 0.6	8.2 ± 0.8
	2	8.9 ± 0.6	7.2 ± 0.8	8.6 ± 0.4	8.2 ± 0.8	8.0 ± 0.2	8.0 ± 0.2
	4	7.8 ± 0.8	8.5 ± 0.4	9.0 ± 0.4	9.0 ± 0.6	7.8 ± 0.6	8.8 ± 0.2
GLU (mg/dL)	0	18.8 ± 4.2	20.0 ± 5.8	20.2 ± 5.4	19.6 ± 5.2	22.6 ± 6.2	19.4 ± 5.4
	2	21.4 ± 3.6	19.6 ± 5.9	21.8 ± 4.8	20.2 ± 3.8	19.6 ± 6.4	26.4 ± 3.8
	4	19.2 ± 4.5	18.7 ± 3.0	22.2 ± 4.6	22.2 ± 6.8	20.8 ± 4.2	21.7 ± 7.2
TP (g/dL)	0	3.8 ± 0.8	4.8 ± 0.8	4.3 ± 0.8	4.2 ± 0.2	4.0 ± 0.5	4.8 ± 0.4
	2	4.1 ± 0.4	4.0 ± 0.6	4.4 ± 0.6	4.8 ± 1.2	4.2 ± 0.8	3.9 ± 0.6
	4	3.9 ± 0.4	3.9 ± 0.2	4.6 ± 0.9	3.9 ± 0.8	3.8 ± 0.6	4.6 ± 0.8
CHOL (mg/dL)	0	102.0 ± 13.5	121 ± 12.0	113.7 ± 11.6	122.0 ± 9.4	122.6 ± 9.2	121.6 ± 12.3
	2	121.8 ± 8.6	128 ± 10.2	102.1 ± 10.4	134.4 ± 10.4	114.2 ± 8.8	119.8 ± 6.7
	4	113.2 ± 10.6	118 ± 9.8	124.4 ± 8.3	112.6 ± 13.6	113.5 ± 11.4	124.6 ± 9.4
TRIG (mg/dL)	0	130.2 ± 8.8	114.4 ± 11.2	126.5 ±10.8	131.6 ±12.6	130.4 ± 11.8	136.4 ± 10.9
	2	124.7 ± 12.4	138.2 ± 14.6	138.4 ± 12.4	130.4 ±14.8	118.6 ± 12.4	128.6 ± 13.4
	4	118.6 ± 10.2	135.3 ± 11.8	114.0 ± 6.2	128.8 ±11.6	130.2 ± 18.4	118.3 ± 11.4

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GLU: Glucose, TP: Total protein, CHOL: cholesterol, TRIG: Triglycerides.

2. Protection of fish after vaccination

Vaccinated and challenged flounder fishes with homologous *P. anguilliseptica* KC565870 isolates (3×10^7 CFU ml^{-1}) had shown significant ($P<0.05$) difference in mean percent mortality between two rearing water temperature and also with that of challenged control fish groups (Table 10). Highest mean percent mortality, 25±0.8 with 69% RPS was shown in the vaccinated and challenged fishes reared at 17°C water temperature while fishes reared at 20°C showed significantly ($P<0.05$) less mortality (8%) with increased RPS (89%). However, the vaccinated fishes showed increased RPS and decreased mortality when compared with that of non-vaccinated control fishes. Cumulative mortality of the vaccinated and non-vaccinated control fishes reared at 17°C and 20°C was shown in Fig. 12. Vaccinated fishes reared at the low temperate shows significantly high cumulative mortality than fishes reared at 20°C. Agglutination titer of vaccinated and challenged fishes reared at 17°C and 20°C water temperature did not differ significantly ($P>0.05$) and there is no correlation was found with RPS values among different fish groups.

Table 10. Mortality and antibody titer of formallin-killed *P. anguilliseptica* and no vaccinated olive flounder after challenge with *P. anguilliseptica* (KC565870) of 3×10^7 CFU ml^{-1}.

Group number	Rearing temperature	Vaccination	Mean % mortality (S.E.M)	RPS	Serum antibody response (average of agglutination titer, log_2)	
					2 weeks	4 weeks
Group 1	17°C	Vaccine	25±0.8	69	1.0±0.66	3.8±0.46
Group 2	17°C	PBS	83±0.6	0	0	0.2
Group 3	20°C	Vaccine	8±0.8	89	1.4±0.44	4.0±0.88
Group 4	20°C	PBS	74±0.7	0	0	0

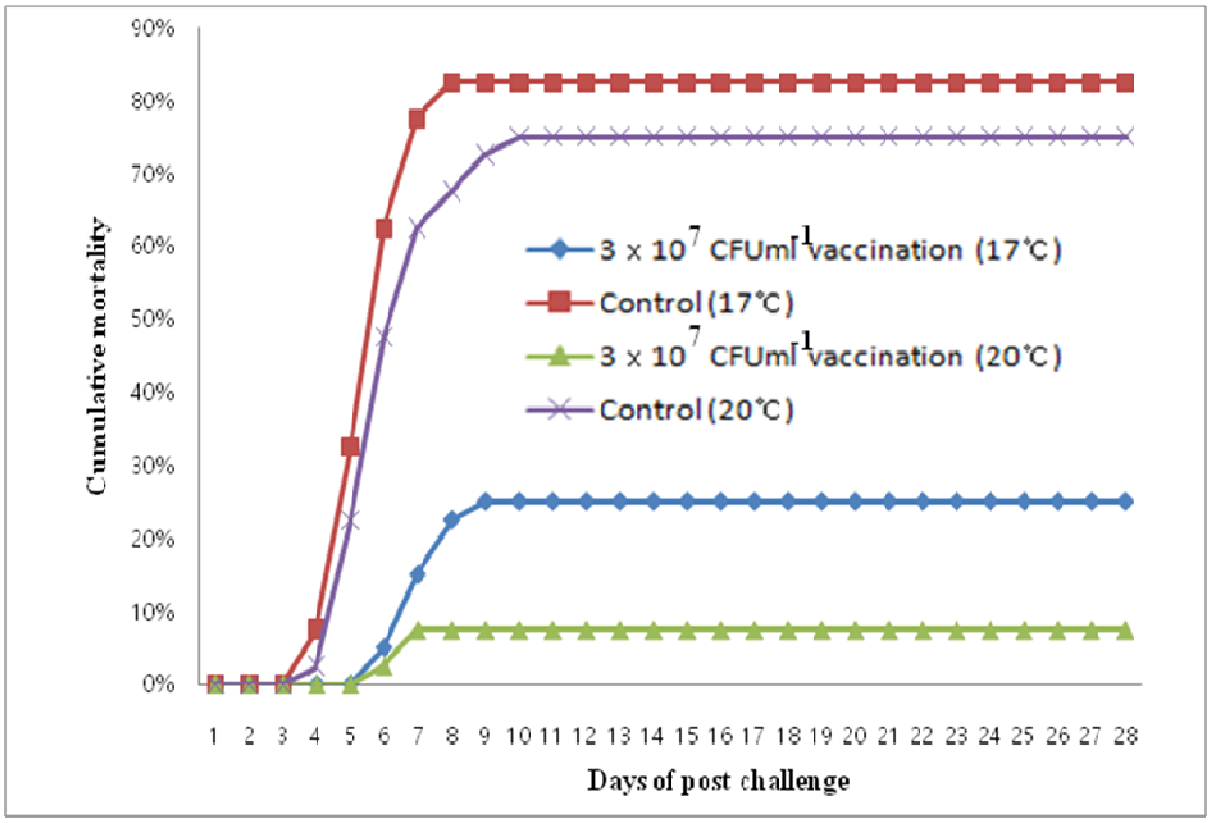


Fig. 12. Cumulative mortality of non vaccinated control and vaccinated flounder fish groups reared at 17°C and 20°C challenged with *P. anguilliseptica.*

3. Antigenic protein recognition of vaccinated fishes

Fig. 13A show the SDS-PAGE profile of *P. anguilliseptica* protein and Fig. 13B shows the immunoblotting of the rabbit anti-Ps and flounder fish anti-Ps sera against *P. anguilliseptica.* Immunoblotting result reveals that rabbit anti-Ps serum is more effective in capturing antigenic protein when compared to the fish anti-Ps. About 4 fragments (molecular weight of approximately 100 kDa, 68 kDa, and 40 kDa) are clearly detected of which ~ 60 kDa fragment is more prominent. However, fish anti-Ps sera captured the 68 kDa protein fragment.

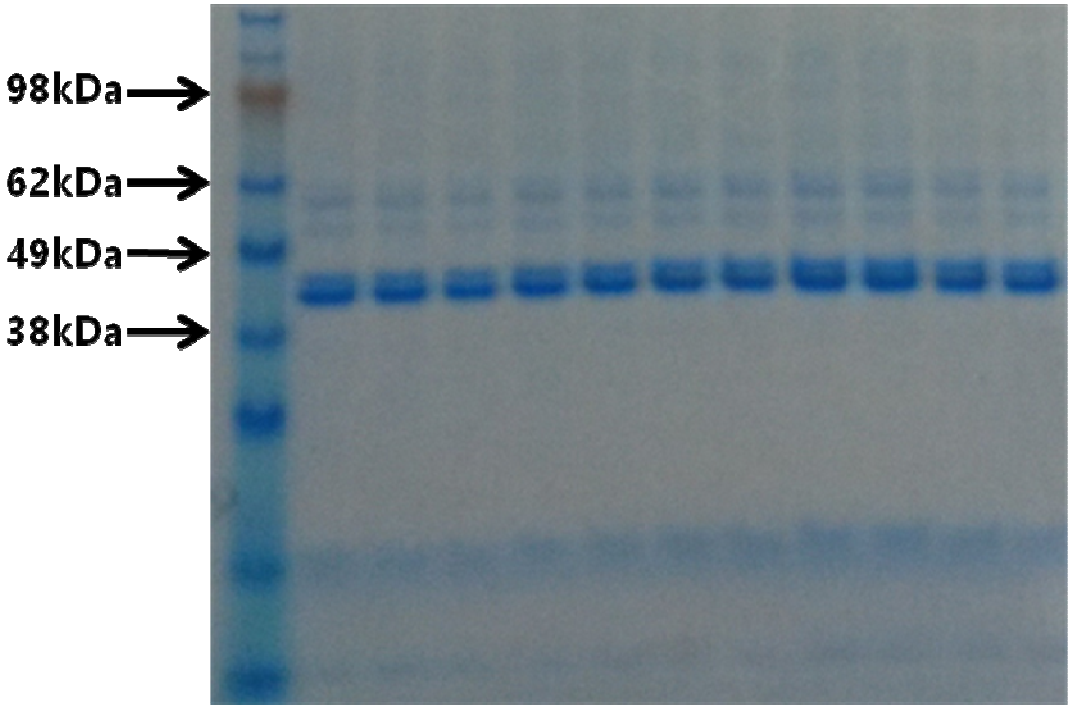


Fig. 13A. SDS-PAGE protein profile of *P. anguilliseptica* isolated from diseased flounder fishes. Lane 1 (M) is the pre-stained molecular weight protein marker. Lane 2-12 are of 11 isolates.

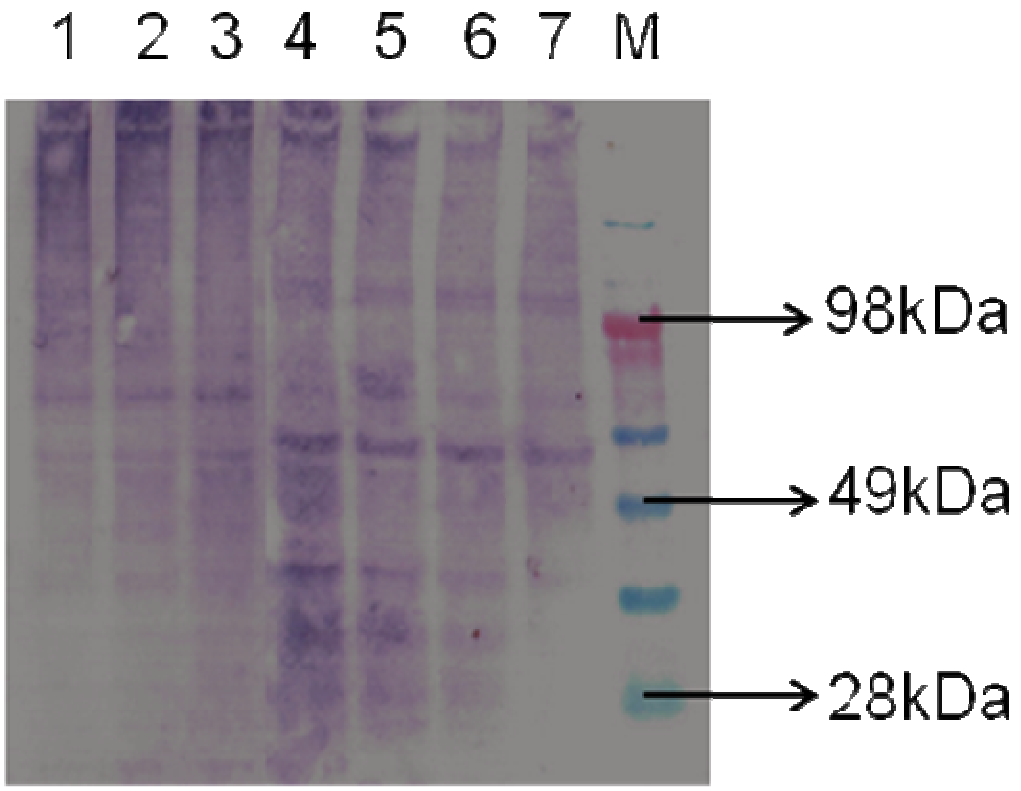
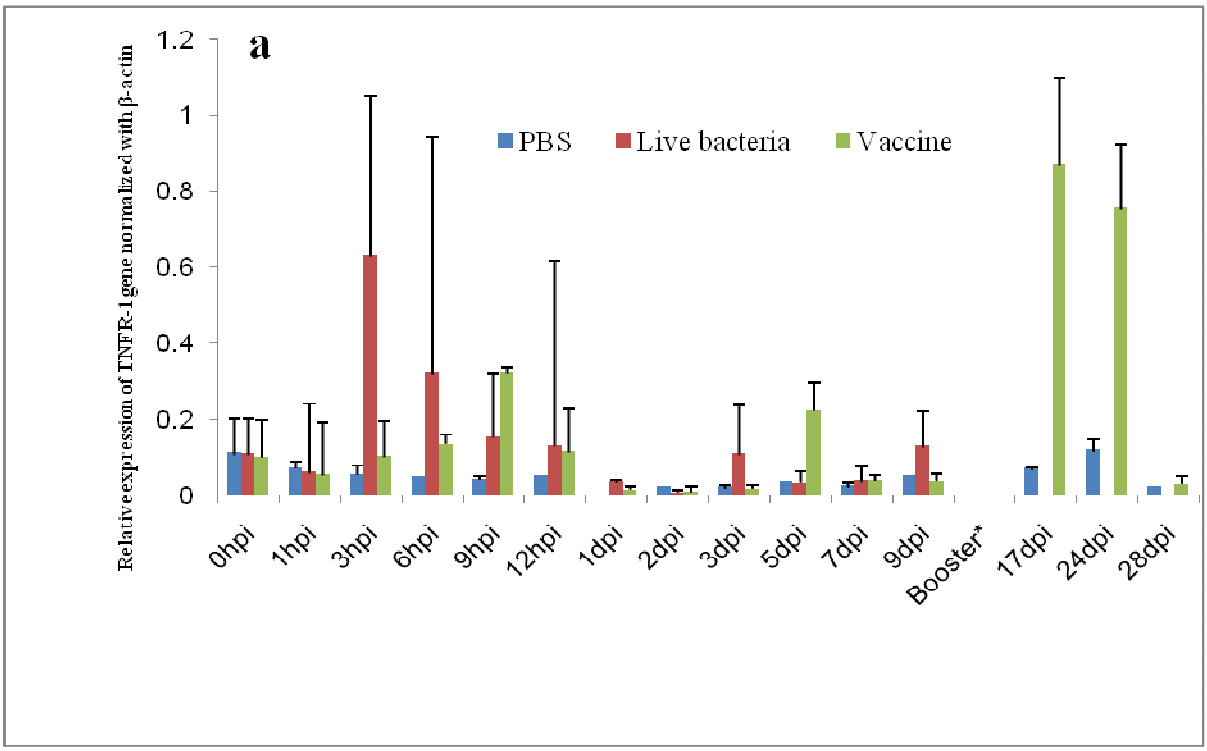


Fig. 13B. Immunodominant proteins captured by the vaccinated fish and rabbit sera. Western blots of *P. anguilliseptica* proteins recognized by flounder fish anti-Ps sera (Lane 1-3) and rabbit anti-Ps sera (Lane 4-7). Lane 8 (M) is the pre-stained molecular weight protein marker.

4. Real-time PCR expression of immune gene in vaccinated fishes

The cDNA obtained from the control, pathogen and vaccine (formalin-killed pathogens) fish groups at different sampling period were subjected to Real-time PCR expression of six immune related genes namely TNFR-1, IRF7, Fas-L, IL-1b, TLR2 and CD40 and all normalized with the internal control gene β-actin (Fig. 14). The expression profile of all six genes was significantly high at 3hpi in pathogen injected fishes when compared with control and vaccinated fish groups. However, vaccinated fishes showed increased expression of TLR2 (at 6hpi), FasL (at 12hpi) IL-1b and TNFR-1 (at 9hpi) after late hours of post injection. Pathogen injected fish group shows 95% mortality at 9dpi and hence, gene expression profile for this fish group is available till 9dpi only. The chronic vaccination study for 28 days reveals that the expression of genes was found up-regulated after the vaccinated fishes were given booster dose of the vaccine on 16^{th} day of vaccination. Immune gene expression was found to 3-fold increased at 17dpi and 24dpi. IRF7 mRNA level of vaccinated fishes were found to be significantly low when compared that of pathogen injected fishes and the booster injection of the vaccine up-regulates its transcripts level. Expression of CD40 mRNA transcript level was found significantly ($P<0.05$) lower than pathogen infected fishes, but on booster vaccination, its transcript level significantly increase at the 17dpi and 24dpi.



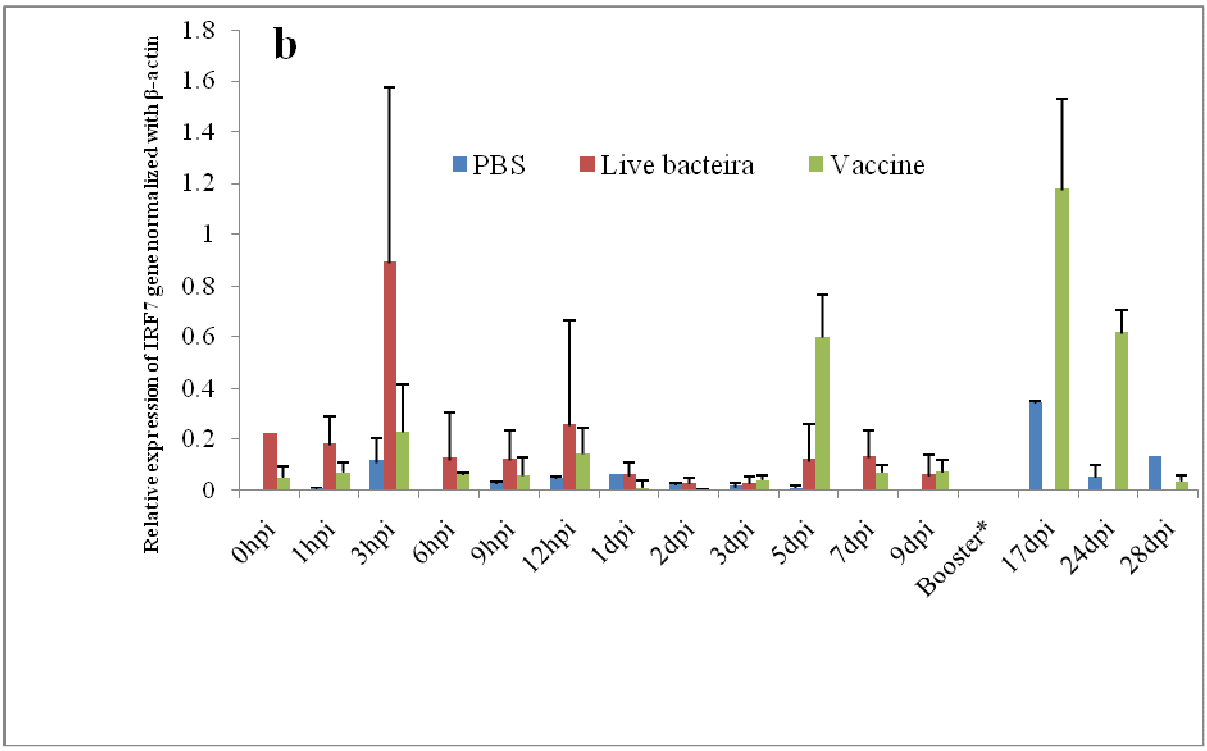
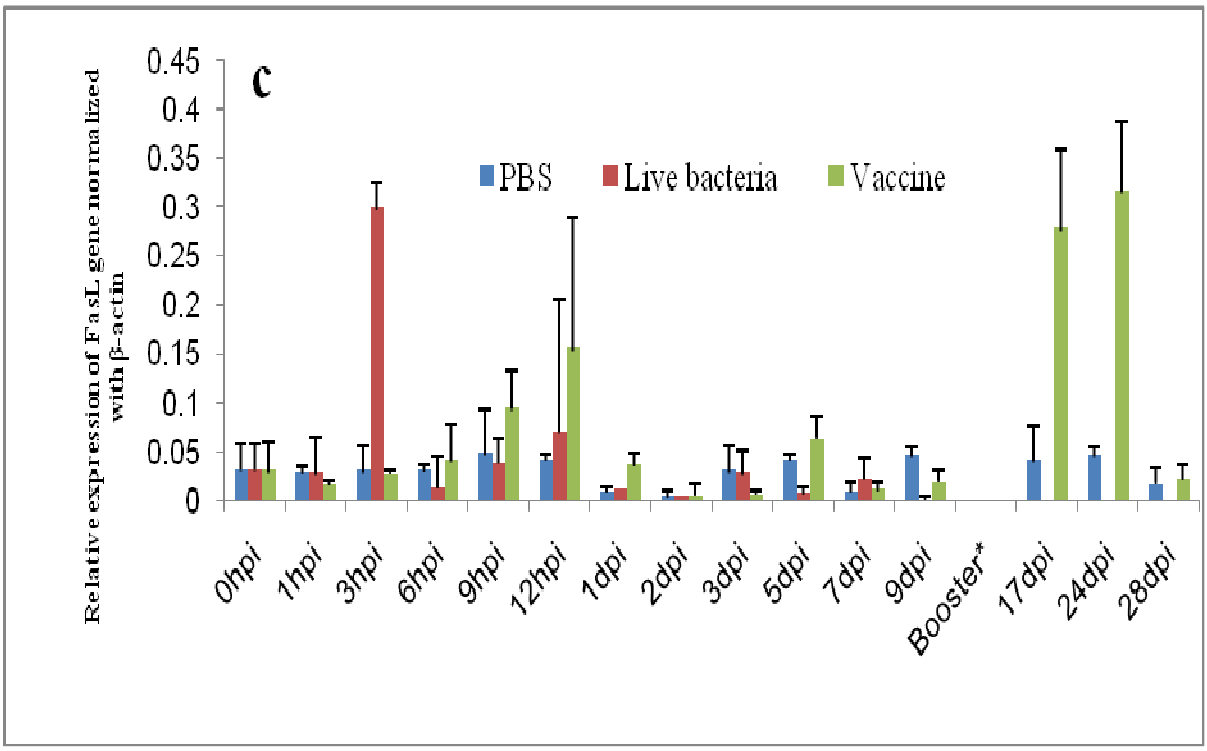
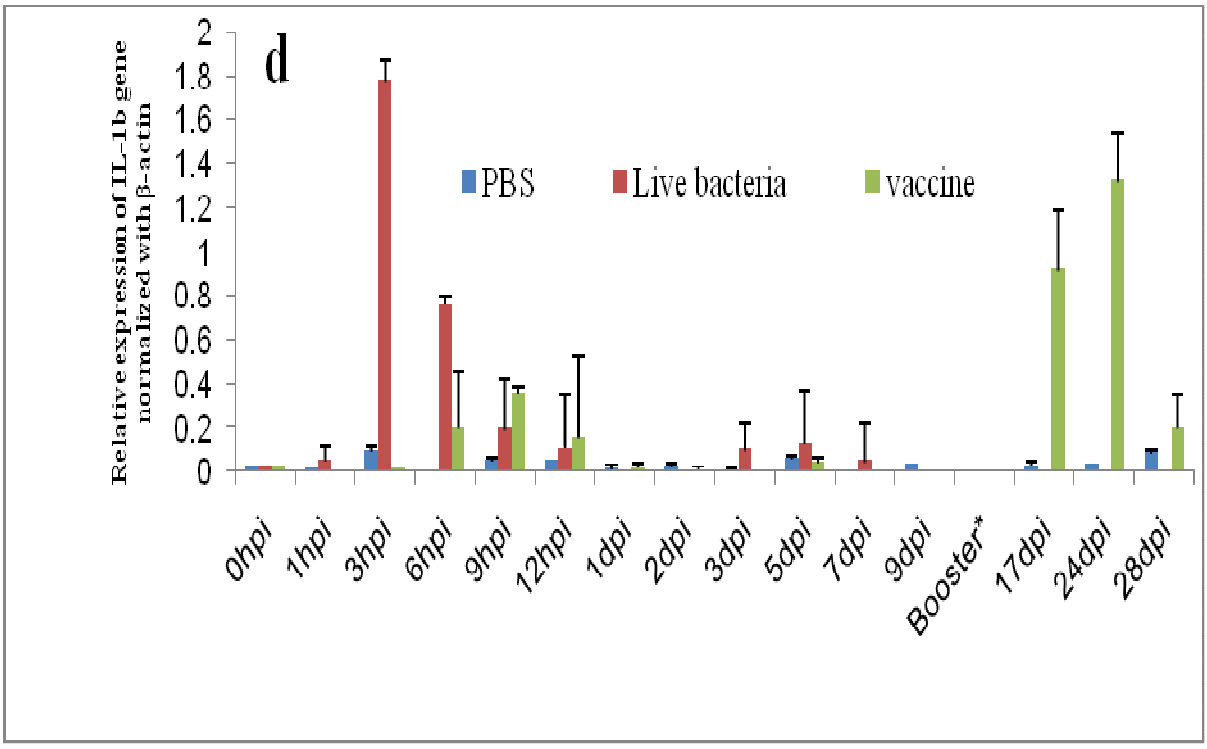


Fig. 14. continued





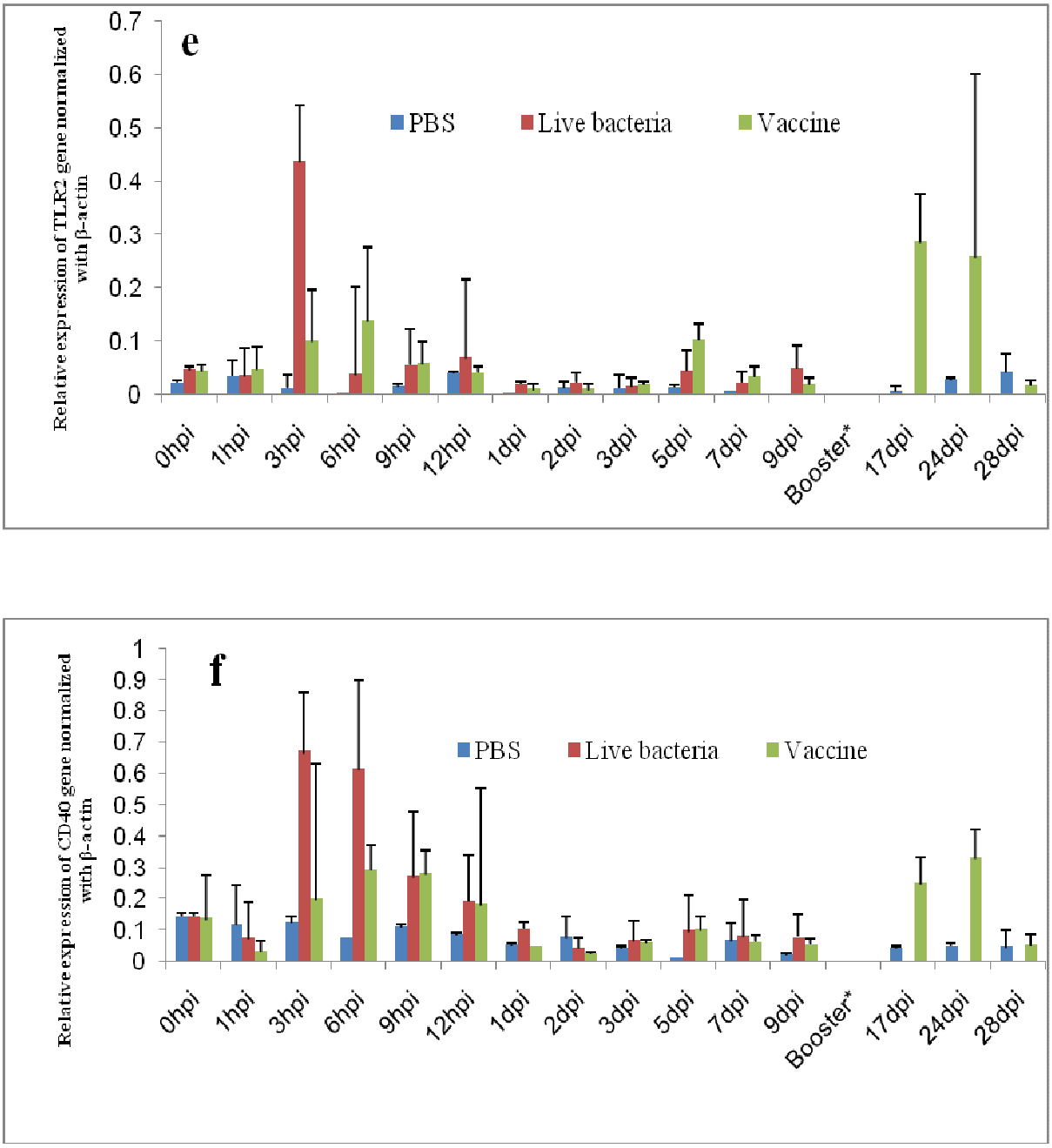


Fig. 14. Relative mRNA expression of six immune gene of olive flounder vaccinated with formalin killed *P. anguilliseptica*. Fig. 3a) Relative mRNA expression of TNFR-1, b) IRF7, c) FasL, d) IL-1b, e) TLR2 and f) CD40 in olive flounder, *Paralichthys olivaceus*, injected intraperitoneally with PBS, live pathogen and vaccine, by SYBR green qPCR. All samples were normalized using β-actin expression as an internal control. Relative levels of immune gene mRNA were analyzed by the $2^{-\Delta C_T}$ (the C_T value of the target immune gene minus the C_T value of the β-actin gene) method. Data are presented as mean±S.D. (n=5).

DISCUSSION

P. anguillisetica is a dreadful fish pathogen causing severe economic losses to the olive flounder farmers particularly in Jeju Island. *P. anguillisetica* infections have caused high mortalities and huge losses to Japanese eel farms since from the first disease outbreak in 1971 (Muroga et al., 1981). Prevalence of this dreadful pathogenic bacterium at low water temperature makes the satiation worst.

In aquaculture, due to its economic benefit, formalin-inactivated whole cell vaccines (bacterin) are commonly used as a fish vaccine against many bacterial diseases. Protective efficacy correlates well with the ability of the vaccine to stimulate a humoral response of producing the specific antibodies to protect the fish from bacterial infection (Bercovier et al., 1997). Bacterial infection is one of the major problems in farming Japanese flounder, including streptococcosis caused by *Streptococcus iniae* (a Gram-positive bacteria) (Nguyen et al., 1999), edwardsiellosis caused by *Edwardsiella tarda* (a Gram-negative bacteria) (Nakatsugawa, 1983). Conserved molecular motifs of bacteria are recognized by the immune system as a sign for detection of pathogenic bacterial invasion, which in turn activates inflammatory response (Medzhitov et al., 2000a; Medzhitov et al., 2000b)

The efficacy of the prepared vaccine in protecting the fish to the experimental disease are validated along with the serological data including agglutination titers, western-blot analysis and immune gene expression profiles that the vaccine elicited a protective immune response. Previous studies with agglutination titer 1:30 had been reported with the fishes vaccinated against *Streptococcus iniae* (Eldar et al., 1997). Immunoblotting of *P. anguilliseptica* protein against fish anti-Ps sera clearly reveals that vaccinated fish recognize *P. anguilliseptica* antigenic epitopes, though less when compared with that of antibodies of mammalian (rabbit in the present study) origin. Production of specific antibodies against antigenic protein moieties is the most important humoral response influencing the state of protection against disease (Schultz et al., 2003).

Some teleosts are considered to be low responders with regard to antibody production (Solem et al., 2006). Hence, knowledge of gene transcription involved in the innate responses in fish may contribute to our understanding of innate resistance and susceptibility towards various pathogens. In the present

study, expression level of six immune related genes namely TNF, IRF, Fas-L, IL, TLR and CD40 was assessed in the *P. anguilliseptica* vaccinated olive flounder fish using Real-time PCR analysis. The mRNA transcripts of the assessed genes show elevated level after 3hpi and return to the normal level after 12hpi. Again elevated level was found when the fishes were challenged at the 16th day of vaccination, maintained few days and return back to the normal level. Fas ligand (FasL) and TNFα belong to the TNF superfamily. The two death inducers bind to their respective receptors, Fas receptor and TNF receptor, to activate death domains in the infected host cell (Barber, 2001; Chávez-Galán et al., 2009; Schultz et al., 2003). The sequence and functional properties in olive flounder and other fishes were analyzed because of the importance of these two death initiators (Ordas et al., 2007; Xiao et al., 2007; Kurata et al., 2011; Kurobe et al., 2007). The vaccinated olive flounder expressed TNFα at an early stage but down regulated it to control levels in most of the later sampled fish, while the expression of TNFα after challenge showed elevated levels. Turbot infected with VHSV showed elevated TNFα expression within 1dpi that was later reduced to normal levels (Ordas et al., 2007). Although there has been some work on fishes response to recombinant TNFα, their response during apoptosis induction is not well characterized. Kurobe et al. (2007) sequenced and verified the cell destructive properties of FasL in olive flounder. TLRs are major components of the pattern recognition receptor repertoire that detects and differentiates between invading microorganisms and direct the vertebrate immune system to eliminate infection (Muhammad et al., 2005).

Hence, in the present study the formalin killed *P. anguilliseptica* vaccine could be used to immunize the culture olive flounder as it elucidate antibody production and immune gene expression. Immunization has played an important role as good farming practices in the control of infectious disease (Vervarcke et al., 2005). Previous studies on formalin killed vaccine of *P. angulliseptica* appeared to be more effective in the prevention of Pseudomonas septicemia in *O. niloticus* (Nakai et al., 1982) and in trout (Romaled et al., 2005). Thus the formalin killed bacterin vaccine of *P. anguilliseptica* could be effectively used to enhance the disease resistance in olive flounder fish and in turn helps to increase the aquaculture production of *Paralichthys olivaceus*.

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