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Roles of Glyceraldehyde-3-Phosphate Dehydrogenase in *Edwardsiella tarda* Pathogenesis

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A research group demonstrated that the 37 kDA protein of *Edwardsiella tarda*, a causing causative agent of edwardsiellosis in fish, exhibited high antigenicity in Japanese flounder. The research group also showed that the N-terminus amino acid sequences of the 37 kDa protein were mapped to the N-terminus of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Using degenerated primer sets based on the known N-terminus sequence, the corresponding *E. tarda* DNA was amplified and cloned. The nucleotide sequences of the cloned gene revealed high homology with a bacterial gene for GAPDH, as we was expected. The amino acid sequence of *E. tarda* GAPDH (etGAPDH) revealed a <70% similarity with GAPDH proteins in other Enterobacteriaceae. With the application of artificial protein overexpression system in *Escherichia coli*, the recombinant etGAPDH (rGAPDH) was produced and purified. In this study, Using the purified rGAPDH, the etGAPDH specific polyclonal antibody has been was generated using the purified rGAPDHin this study. The immunoblotting analyses demonstrated that the location of the GAPDH protein is located with the association of is associated with the envelops of *E. tarda*. The rGAPDH was administrated into Japanese flounder via IP route for evaluation of the protective ability. Although the specific antibody titer against etGAPDH was increased about 3-fold after 4 weeks post-vaccination, the survival rates of vaccinated Japanese flounder and the control group with wild type *E. tarda* was were 12.5% and 0%, respectively. Our results indicated that rGAPDH is immunoreactive antigen but that it will not generate protective immunity in Japanese flounder.

Key words : *Edwardsiella tarda*, vaccine, pathogenesis

Introduction

Edwardsiella tarda is the causative agent of the systemic disease, edwardsiellosis, in many freshwater and marine fish in both farmed and wild populations around the world [16]. Edwardsiellosis causes high mortalities and severe economic losses in farmed fish, such as flounders [17], eels [27], tilapia [11], carp [19], channel catfish [14], and others. Several potential virulence factors of *E. tarda* have been reported. The abilities to invade epithelial cells [9,12], serum resistance [25], phagocyte-mediated killings [23], a type III secretion system (TTSS) [13], and production of toxin and enzymes, such as hemolysins [7], and catalyses [24] have been found to play important roles in *E. tarda* pathogenesis.

The outer membrane of pathogenic bacteria has an important role in the induction of immune response including humoral and cell-mediated immunity [10]. The outer membrane interacts with hosts in the bacterial pathogenesis during adherence, uptake of nutrients from the host, and eliminating host defense mechanisms, because the components of the outer membrane are easily recognized as foreign substances by the host immune defense system [21]. Up to date little is known about immune responses by induced *E. tarda* surface protein antigen. Recent studies emphasized the role of the outer membrane protein of pathogenic bacteria in protective immunity [2, 14, 19], which is often related to inducing neutralization of antibodies [2], inhibiting bacterial colonization in hosts [27], and inducing cell-mediated immunity [1].

In a recent study, the 37 kDa protein of *E. tarda* exhibited strong immune responses in fish [6]. The protein was existed in all of the *E. tarda* isolates, and highly conserved regardless of the serotype. The N-terminal amino acid sequence of this protein showed high homology to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [6]. GAPDH is one of a major enzyme, which is involved in energy production [28]. Some reports indicated that GAPDH binds to a cell membrane [1,15,26] and exists on the cell surface [8,18]. In this study, we were interested in the antigenic role of the GAPDH, which is conserved in different serotypes of *E. tarda*. We hypothesized that GAPDH is a surface protein as antigen to induce immune responses, and we wonder

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whether it can stimulate protective immunity. The aim of this report is identifying the role of GAPDH in *E. tarda* pathogenesis.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* used as hosts for cloning and protein expression were grown at 37℃ in Luria-Bertani (LB) broth or LB agar [3]. *E. tarda*, was isolated from flounder and provided by the National Fisheries Research and Development Institute (NFRDI) in the Republic of Korea, were grown at 27℃ in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco) containing 1% NaCl [22]. Antibiotics, when required, were added in the culture medium with the following concentrations; ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 30 μg/ml; and streptomycin, 50 μg/ml.

General DNA manipulations

DNA manipulations, including DNA preparation, ligation, restriction analysis, transformation and electrophoresis were carried out as described by Sambrook *et al* [20]. Genomic DNA of *E. tarda* was extracted by using the AccuPrep Genomic DNA Extraction Kit (Bioneer). PCR amplification was employed to obtain DNA fragments for cloning. The PCR conditions were as follows: denaturation at 95℃ for 30 sec, primer annealing at 50℃ for 30 sec, polymerization at 72℃ for 1 min, and a final extension at 72℃ for 10 min. Transformation of *E. coli* were done by either rubidium chloride-heat shock or electroporation (BioRad).

Primer design for amplifying of *E. tarda* GAPDH

The nucleotide sequences of the GAPDH gene of four Gram-negative bacteria, *Salmonella typhimurium, Yersinia pestis, Erwinia carotovora, E. coli*, were obtained by NCBI database, and the homologies of their GADPH nucleotide sequence were compared by using the NCBI homology alignment system. Both primers for the amplification of forward and reverse were designed from the conserved GAPDH nucleotide sequences at the 5′ and 3′ ends.

Heterologous overexpression of GAPDH

A DNA fragment encoding *E. tarda* GAPDH was amplified from chromosomal DNA using a pair of primers HY205F (GC *GGATCC* GCA TAC ATG CTG AAG) and HY206R (GC *AAGCTT* CAG TTT CAC GAA GTT), which were synthesized based on the *gapA* sequence of *E. tarda*. Italicized residues in the primer sequence show the cutting site of *Bam*HI and *Hin*dIII, respectively. The *Bam*HI-*Hin*dIII DNA fragment was cloned into pProEx^{TM}HTb vector (Invitrogen), resulting in the pBP356. *E. coli* BL21 (DE3) cells harboring pBP356 were grown in LB broth, and the recombinant protein was induced by the addition of 1 mM IPTG. Purification of the recombinant protein was performed by the instructions of the manufacturer (GE Healthcare). Most of the expressed recombinant protein existed in *E. coli* as an insoluble form. The proteins were solubilized using 8 M urea and purified by affinity chromatography with Ni^{2+}-sepharose (GE Healthcare).

SDS-PAGE and immunoblot analysis

Protein samples were solubilized in 2× digestion buffer [20% (v/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 200 mM β-mercaptoethanol, 100 mM Tris-HCl pH 6.8] by boiling for 5 min, and separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized by Coomassie brilliant blue G-250 (Sigma) staining.

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference
Strains		
E. tarda CK108	Wild type, isolate from flounder developed edwardsiellosis	NFRDI
E. coli DH5α	Transformation host for cloning vector	Promega
E. coli BL21 (DE3)	Host for overexpression, F$^-$ *ompT hsdS$_B$* ($r_B^- m_B^-$) *gal dcm* λ (DE3)	Promega
Plasmids		
pProEx^{TM}THb	Overexpression vector, LacI^q ColE1 Ap^R	Invitrogen
pGEM-T	Cloning vector for PCR product, ColE1 Ap^R	Promega
pBP237	1.0 kb DNA containing *gapA* in pGEX-T vector, Ap^R	This study
pBP356	1.0 kb *gapA* *Bam*HI-*Hin*dIII product from pBP237 in pProEX^{TM}THb	This study

For immunoblotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membrane in Towbin's buffer. The membrane was blocked with a blocking solution (5% skim milk in Towin's saline buffer) for 3 hr as described by Towbin *et al* [5]. The membrane was incubated with a suitable diluted rabbit or mouse antisera in the blocking solution for 2 hr and followed by 1:2,000 dilution of a horseradish peroxidase conjugated goat anti-rabbit IgG (Pierce) or anti-mouse IgG (Sigma) in the blocking solution. Immunoreactive bands were detected by the addition of 4-chloro-1-naphthol (Sigma) in the presence of H_2O_2 (Sigma). The reaction was stopped after 5 min by washing with several changes with a large volume of deionized water.

Preparation of the outer membrane protein fraction

E. tarda cells were collected by centrifugation at 8,000× *g* for 5 min, and suspended in 20 mM Tris-HCl (pH 8.0) and disrupted by French press (Thermo Electron Cooperation) at 18,000 psi. The lysate was centrifuged at 4,500× *g* for 5 min to remove unbroken cells. The total membrane pellet was obtained by centrifugation at 27,000× *g* at 4℃ for 1 hr, and treated with 1% lauryl sarcosine (Sigma). Then, the outer membrane protein was obtained as a pellet after centrifugation at 27,000× *g* at 4℃ for 1 hr. The pellet was resuspended in 10 mM Tris-HCl (pH 8.0).

ELISA (Enzyme-linked immunosorbent assay)

ELISA 96-well plates (Corning) were coated with 1 ng/μl of GAPDH in 50 mM sodium carbonate (pH 9.6). The plates were blocked with 200 μl of blocking buffer (1% BSA in PBS, pH 7.4) per each well to avoid non-specific binding. After remove blocking buffer, 100 μl of serially diluted samples were added into each well, and washed 5 times with 250 μl of PBS. Then, the plates were incubated with biotin conjugated goat anti-rabbit IgG or anti-mouse IgG (Southern Biotech) (1:10,000 in 0.1% BSA in PBS), and washed with PBS. Finally, the plates were incubated with alkaline phosphatase labeled streptavidin (Southern Biotech) (1:3,000 in 0.1% BSA in PBS). The ρ-nitrophenylphosphate (1 mg/ml) (Sigma) in 0.1 M diethanolamine buffer (pH 9.8) was used as the substrate. The optical density of the color reaction was read at 405 nm with an automated ELISA reader (GE Healthcare).

Results and Discussion

Determination of nucleotide sequence of *E. tarda gapA*

To identify nucleotide sequence of *E. tarda* GAPDH gene, the DNA fragment encoding the *gapA* was amplified by PCR. An approximately 1.0 kb length of amplified DNA fragment was cloned into pGEM-T vector (Promega), resulting in pBP237, and its nucleotide sequence was analyzed

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Atg act atc aaa gta ggt atc aac ggt ttt ggc cgt atc ggc cgt att gtt ttc cgt gct
 M   T   I   K   V   G   I   N   G   F   G   R   I   G   R   I   V   F   R   A
gct cag gaa cgt tct gac atc gaa atc gtt ggc atc aac gat ctg ctg gat gcc aac tac
 A   Q   E   R   S   D   I   E   I   V   G   I   N   D   L   L   D   A   N   Y
atg gca tac atg ctg aag tac gac tct act cac ggt cgt ttc aac ggc act gtt gaa gtg
 M   A   Y   M   L   K   Y   D   S   T   H   G   R   F   N   G   T   V   E   V
aaa gat ggc cac ctg atc gtt aac ggt aaa aaa atc cgt gtt acc gct gaa aga gat ccg
 K   D   G   H   L   I   V   N   G   K   K   I   R   V   T   A   E   R   D   P
gct aac ctg aag tgg aac gaa atc ggc gtt gac gtt gtt gcc gaa gcg acc ggt ctg ttc
 A   N   L   K   W   N   E   I   G   V   D   V   V   A   E   A   T   G   L   F
ctg acc gac gaa acc gcg cgt aag cac atc gcc gcc ggt gcc aag aaa gtc gtc atg act
 L   T   D   E   T   A   R   K   H   I   A   A   G   A   K   K   V   V   M   T
ggc ccg tct aaa gat gct acc ccg atg ttc gtt atg ggc gta aac cac aag aac tac gct
 G   P   S   K   D   A   T   P   M   F   V   M   G   V   N   H   K   N   Y   A
ggc cag gag atc gtt tcc aac gca tcc tgc acc acc aac tgc ctg gct ccg ctg gct aaa
 G   Q   E   I   V   S   N   A   S   C   T   T   N   C   L   A   P   L   A   K
gtc ctg aac gac aac ttc ggc atc gtt gaa gcg ctg atg acc acc gtt cac gct acc acc
 V   L   N   D   N   F   G   I   V   E   A   L   M   T   T   V   H   A   T   T
gcg acc cag aaa act gtc gac ggc ccg tcc atg aaa gat tgg cgc ggc ggc cgc ggc gct
 A   T   Q   K   T   V   D   G   P   S   M   K   D   W   R   G   G   R   G   A
agc cag aac atc atc ccg tcc tct acc ggt gca gcc aag gcc gtt ggc aag gtc atc ccg
 S   Q   N   I   I   P   S   S   T   G   A   A   K   A   V   G   K   V   I   P
gag ctg aac ggc aaa ctg acc ggt atg gct ttc cgc gtt ccg acc ccg aac gtc tcc gtt
 E   L   N   G   K   L   T   G   M   A   F   R   V   P   T   P   N   V   S   V
gtt gac ctg act gct cgc ctg gcc aag ccg gcg acc tat cag cag atc tgt gac gtg atg
 V   D   L   T   A   R   L   A   K   P   A   T   Y   Q   Q   I   C   D   V   M
aag gcc gct tct gaa ggc gaa atg aaa ggc gtt ctg ggc tac acc gac gaa gcc gtc gtt
 K   A   A   S   E   G   E   M   K   G   V   L   G   Y   T   D   E   A   V   V
tct acc gac ttc aac ggc gaa gta tgc acc tcc gtg ttt gat gcc gac gcc ggt atc tct
 S   T   D   F   N   G   E   V   C   T   S   V   F   D   A   D   A   G   I   S
ctg aac gac aac ttc gtg aaa ctg gtt tct tgg tat gac aac gaa act ggc tac tcc aac
 L   N   D   N   F   V   K   L   V   S   W   Y   D   N   E   T   G   Y   S   N
aaa gtt ctg gat ctg atc gct cac atc tcc aag taa ggg gga ttg gga gag gtt ttc ttt
 K   V   L   D   L   I   A   H   I   S   K   -
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Fig. 1. Full length nucleotide sequence of the *E. tarda gapA* and its deduced amino acid sequence. The 990 bp of nucleotide and encoded amino acid sequence are shown.

by DNA auto sequencer (ABI PRISM 377, Perkin Elmer) (Fig. 1). The DNA fragment contained the full sequence of the *gapA*, and the GAPDH was composed of 331 amino acid residues. The homologies of the GAPDH amino acid sequence between *E. tarda* and the other Enterobacteriaceae family were compared by using the BLAST search engine. The GAPDH of *E. tarda* showed <70% similarity with other Enterobacteriaceae GAPDH proteins (Table 2).

Subcellular location of GAPDH protein in *E. tarda*

To verify the location of the GAPDH in the outer membrane, we generated an antiserum against the *E. tarda* outer membrane proteins (OMPs). The OMPs of *E. tarda* were prepared as described above and 20 μg of *E. tarda* OMPs were administrated to BALB/c by the intraperitoneal route. After 4 weeks of post administration, BALB/c mice were boosted with the same amount of *E. tarda* OMPs. After 2 weeks of post boosting, the blood of BALB/c was collected from the vein on the marginal part of the eyes. The recombinant GAPDH, prepared as described in Materials and Methods (Fig. 3A), was detected by immunoblot analysis using *E. tarda* OMPs specific polyclonal antibodies, and the same position of reactive band was also detected in *E. tarda* total lysate (Fig. 2). These results suggest that the GAPDH exists in the outer membrane as well as cytosol.

Preparation of GAPDH specific polyclonal antibodies

To generate GAPDH specific antibodies, 120 μg of the recombinant GAPDH emulsified with the same volume of Freund's complete adjuvant (Sigma) as antigen. The antigen was administrated to the New Zealand White rabbit by intramusclar, after 3 weeks post administration, the rabbit was boosted with the same amount of antigen used in the primary administration mixed with incomplete Freund's adjuvant (Sigma). The bloods were collected from the vein on the marginal part of the ears at every step. Two weeks after boosting, the rabbit sacrificed to collect sera. The production of anti-GAPDH polyclonal antibodies was evaluated by immunoblot assay and ELISA. The recombinant GAPDH showed a strong reaction (Fig. 3B) and specific antibodies titer measured $Log_2 9$ to $Log_2 13$, which were sera after the first administration and boosted, respectively (Fig. 4). These results indicated that anti-GAPDH antibodies were successfully produced.

Table 2. Amino acid similarity of *E. tarda* GAPDH compared with other bacteria

Bacteria	Identities (%)
Yersinia pestis biovar Medievalis str. 91001	839/994 (84%)
Shigella flexneri 2a str. 301	632/731 (86%)
Escherichia coli O157:H7	632/731 (86%)
Escherichia coli K-12	631/731 (86%)
Erwinia carotovora subsp. atroseptica	637/742 (85%)
Salmonella typhimurium LT2	620/725 (85%)
Serratia sp.	590/689 (85%)

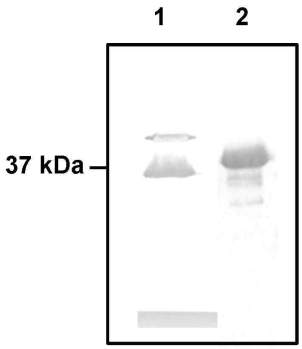

Fig. 2. Confirmation of the GAPDH protein in *E. tarda*. Protein samples were subjected to immunoblot analysis. The interested proteins were visualized by reactivity with mouse anti-*E. tarda* outer membrane proteins (OMPs) antibodies. Lane 1, *E. tarda* total lysate; 2, recombinant GAPDH.

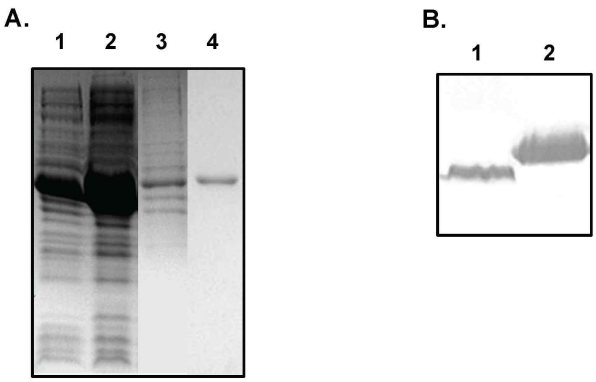

Fig. 3. Overexpression and immunoblot analysis of the GAPDH. (A) SDS-PAGE analysis of overexpressed GAPDH. The $(His)_6$-tagged GAPDH expression in *E. coli* BL21 (DE3) harboring pBP356 was induced by addition of 1 mM IPTG into LB broth. Lane 1, cell lysates; 2, soluble fraction; 3, insoluble fraction. of *E. coli* BL21 (DE3) harboring pBP356 (B) Immunoblot analysis for confirmation of generated antibodies against GAPDH. Lane 1, *E. tarda* outer membrane protein (OMPs); 2, the recombinant GAPDH.

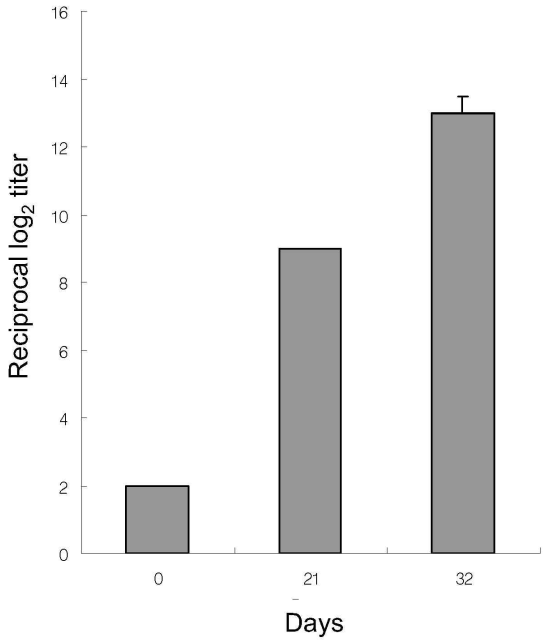

Fig. 4. Titration of the anti-GAPDH IgG from rabbit. Specific antibody titer by ELISA in the sera of rabbit after immunization of GAPDH. The sera were diluted in a series of two-fold.

Evaluation of the protection ability of GAPDH challenged with *E. tarda* in flounder

The Japanese flounder (body weight 50 to 100 g) was maintained in the National Fisheries Research and Development Institute under laboratory conditions. The Japanese flounders were vaccinated with 10 μg and 100 μg of the recombinant GAPDH by intraperitoneal route. After 4 weeks of post-vaccination, the fish were challenged with 5.0×10^8 CFU of *E. tarda*. At 4-7 days after challenge, all the fish in the unvaccinated group had died and most of the fish in the vaccinated group with GAPDH had also died. Survival rate of the vaccinated groups was 12.5% (Fig. 5). A specific antibody titer against GAPDH was detected in the vaccinated flounder sera by using ELISA. The antibody titer was increased three-fold after 4 weeks (data not shown).

E. tarda is causes a systemic fish disease called edwardsiellosis [4]. In a recent study, the 37 kDa protein of *E. tarda* was suggested to have and antigenicity [13]. The 37 kDa protein was identified as a GAPDH, which is involved in energy production [28]. We focused on the roles of GAPDH in *E. tarda* pathogenesis, To investigate the roles of GAPDH, the *E. tarda gapA* gene was amplified and its nucleotide sequence was analyzed. The nucleotide and amino acid sequence of GAPDH revealed a strong similarity with other

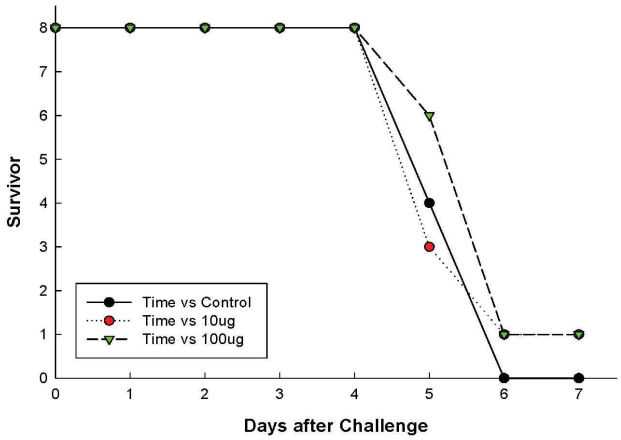

Fig. 5. Protection ability of GAPDH challenged with *E. tarda* in Japanese flounder. The recombinant GAPDH and PBS was immunized as a vaccinated and control, respectively. The Japanese flounders were vaccinated with 10 and 100 μg of GAPDH. After 4 weeks of post vaccination, the fish were injected with 5.0×10^8 CFU of *E. tarda*, and mortality observation were recorded for 8 days.

Enterobacteriaceae GAPDH proteins. Its high homologue indicates that the *gapA* gene is well conserved in the evolution process. To confirm the location of GAPDH, we generated anti-*E. tarda* OMPs antibodies, and detected the recombinant GAPDH by immunoblot analysis using specific antibodies. To examine the role of GAPDH as a protective antigen, the protective ability of GAPDH against *E. tarda* infection in the Japanese flounders was performed. Fish were intraperitoneally immunized with the recombinant GAPDH, and the fish were challenged with *E. tarda*. No significant difference between the vaccinated and the unvaccinated groups was observed. The reason for this result could be explained that challenge dose of *E. tarda* was excessed. Indeed, the LD_{50} of *E. tarda* was measured to be 3.0×10^6 CFU, but the challenge dose of *E. tarda* used for this study was 5.0×10^8 CFU, almost an 200-fold higher dose. This report is study to identify antigen and examine whether it is functioning as a vaccine candidate. The results of this study will be used as a basis to develop an effective vaccine.

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초록 : *Edwardsiella tarda*의 glyceraldehyde-3-phosphate dehydrogenase가 병원성에 미치는 영향

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*Edwardsiella tarda*는 그람 음성의 장내세균과의 주요 어병세균으로 어류에 edwardsiellosis를 유발하는 전신감염성 병원체이다. 최근 병원성 세균의 외막 단백질들은 세균성 감염에 있어서 숙주와 반응하여 면역반응을 유도하는 것으로 여겨져 연구가 되고 있다. 일본의 연구팀은 어류에서 에드워드병의 원인체인 *E. tarda*의 37 kDa 단백질이 넙치에서 높은 항원성을 제시하는 것을 보고하였다. 또한 그 연구자들은 37 kDa 단백질의 N-말단 아미노산 서열이 GAPDH와 대응하는 것을 밝혔다. 본 연구에서는 다른 세균에서 알려진 N-말단 서열을 기반으로 primer를 제작하여 이에 상응하는 *E. tarda* DNA를 증폭하고 클로닝하였다. 이 DNA단편의 염기서열은 예상한 바와 같이 세균의 GAPDH유전자인 *gapA*와 높은 상동성이 있고, *E. tarda* GAPDH (etGAPDH)의 아미노산 서열은 다른 장내세균의 GAPDH와 70% 이상의 상동성을 보이는 것을 확인하였다. *E. tarda*의 외막단백질에 특이적으로 반응하는 항체를 이용하여 *E. tarda*의 GAPDH가 외막에 존재한다는 것을 증명하였고, *gapA*의 염기서열을 바탕으로하여 재조합 GAPDH를 과발현 시켰다. 과발현된 재조합단백질 GAPDH는 GAPDH 특이적인 항체를 제조하는데 사용되었고, 또한 넙치에 면역시켜 단일 단백질 백신으로서의 활용도를 모색하였다. 비록 재조합 GAPDH가 면역된 넙치에서 GAPDH에 특이적인 항체가 증가하였음에도 불구하고, *E. tarda*로 공격실험을 하였을 때 면역된 넙치의 생존율이 12.5%로 측정되어 면역된 그룹과 면역되지 않은 그룹간에 큰 차이가 없는 것이 확인되었다.