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ORIGINAL ARTICLE

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Persistence and Immunogenicity of *Edwardsiella Piscicida* phoP/Q Mutants in Channel Catfish (*Ictalurus punctatus*)

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Abstract

Edwardsiella piscicida is a Gram negative, invasive and intracellular pathogen lead to develop hemorrhagic septicemia in wide varieties of freshwater fish species worldwide. Previously, two novel *E. piscicida* mutant strains namely Δ*phoP* and Δ*phoQ* were constructed and the data were published. In the present study, the resistance of in-frame deleted mutant strains Δ*phoP/Q* were investigated for their immunogenicity at the levels of blood and serum killing activities in comparison with *E. piscicida* wild-type (WT) pathogenic strain C07-087. Moreover, tissue persistence and expression profile of some immune relevant genes were monitored in head kidneys, spleens and livers of the intra-peritoneal immunized channel catfish fingerlings model. Results revealed that Δ*phoP* and Δ*phoQ* showed a significant decreased resistance against host killing activities compared to *E. piscicida* WT strain. At all tested levels, the tissue persistence showed that the mutants held the capability to attack and spread in the immunized channel catfish investigated tissues. Meanwhile, live bacteria could be noticed in livers, spleens and anterior kidneys up to 7 days after immunization. Furthermore, significant up-regulation levels of IL-1β, INFγ, CD4-1, MHC class I and MHC class II were detected in anterior kidneys and spleens of WT, Δ*phoP* and Δ*phoQ* vaccinated fish compared to non-immunized control group at 14 and 21 days post immunization. In conclusion, our findings proved that Δ*phoP* and Δ*phoQ* mutant strains have the aptitude to motivate both innate and adaptive immune responses and also they have the prospective requirements as successful live vaccine candidates.

Keywords

Channel Catfish, *Edwardsiella piscicida,* Mutant, Strains and Vaccine

1. Introduction

Huge economic loses at the level of wide varieties of freshwater fish aquaculture systems, were triggered by *Edwardsiella piscicida* which considered one of the main reasons for development of hemorrhagic septicemia in channel catfish **(Mohanty and Sahoo, 2007; Abayneh et al., 2013).** Lately, it has arisen as an imperative fish pathogen of channel and hybrid catfish in the US **(Griffin et al., 2020).** Channel catfish agonized from *E. piscicida* infection, presented the classical clinical picture of hemorrhagic septicemia which characterized by diffusible hemorrhages allover fish body, epidermal and dermal erosive lesions that developed mainly to become ulcers, abdominal distention produced from accumulation of edematous fluid and internal hemorrhages **(Griffin et al., 2019).** Two component systems (TCSs) are one of the most important virulence factors of Edwardsiella species that take part in occurrence of the infection in the fish host **(Wang et al., 2010).** The interconnected signal transduction systems as TCSs are enabling bacterium to governor their expression profile in reply to environmental influences as temperature and pH **(Chamnongpol and Groisman, 2002; Chakraborty et al., 2010).** Environmental stimuli as temperature could be detected by PhoP/Q system in *Edwardsiella spp.* Furthermore, it controls the type III and type VI secretion systems throughout direct triggering of genes which direct activate these systems as esrB gene **(Chakraborty et al., 2010).** Inside host, replication of *E. piscicida* occurs intracellular, mainly within fish phagocytes **(Xiao et al.,**

2013), so development of live attenuated mutant strains from this pathogen are expected to produce proper live vaccine candidates holding ability to motivate the adaptive immunity (Munang'andu, 2018). Previously, two novel *E. piscicida* live attenuated strains (*ΔphoP* and *ΔphoQ*) had constructed by in-frame deletion of the phoP and phoQ genes, that were able to significantly prevent *E. piscicida* infection in channel catfish host (Sayed et al., 2021). Intracellular microbes characterized by their aptitude to retain infection in the host even in the attendance of innate and/or adaptive immunity (Byndloss and Tsolis, 2016). Mostly, those microorganisms are retained in a state of latency as asymptomatic disease. Although the live attenuated bacteria are checked by the adaptive immunity, they are not completely abolished from the host, such as live attenuated *mycobacterium tuberculosis, salmonella enterica* and *E. tarda* (Cooper et al., 1993; Tascon et al., 1998; Vergne et al., 2005; Monack, 2012; Zhao-Lan et al., 2013). By the time, identification and categorization of various immune-regulatory genes of teleost have endorsed the expression analyses of the immune-genes followed infection with WT strains or vaccination by live attenuated vaccines (Campos-perez et al., 2000; Xiao et al., 2011; kordon et al., 2019).

Release of pro-inflammatory cytokines as IL-1β and IFN-γ is the motivator of the anti-microbial response as they rouse the onset of the innate immune responses represented by activation of granulocytes and production of reactive oxygen species (ROS) from macrophages (Kobayashi and DeLeo, 2009; Iyer and Cheng, 2012; Ivashkiv and Donlin, 2014). On the other hand, lymphocytic cells (B and T-lymphocytes) play the main role in the adaptive immune responses (Dixon and Stet, 2001). In teleost, T- cytotoxic and T-helper cells govern the cell- mediated immunity especially in case of intra-cellular infection (Kato et al., 2013). These cells carry different cell markers that considered the main way for cellular differentiation (Forlenza et al., 2008). CD4-1 and MHC I and II are the most important cell markers, they are responsible for the recognition and presentation of the foreign antigens, so they deliberated an essential constituent of the vertebrate adaptive immune system (Anthony, 2017). The current study's objective was to assess the changes induced by *E. piscicida* live attenuated vaccines (Δ*phoP* and Δ*phoQ*) on the innate and adaptive immune responses of the immunized channel catfish fingerlings.

2. Materials and Methods

2.1. Ethics Statement

Under protocol (IACUC19_388) approved by Mississippi State University Institutional Animal Care and Use Committee, all fish experiments were accomplished. Channel catfish fingerlings, specific pathogen free (SPF) were gotten from the fish hatchery at the College of Veterinary Medicine, Mississippi State University.

2.2. Evaluation of Resistance against Non-Immunized Channel Catfish Serum

Δ*phoP* and Δ*phoQ* mutant strains that were previously constructed (Sayed et al., 2021) were tested against serum killing activity according to the procedures described by (Li and Sun, 2018) with modifications. Briefly, brain heart infusion (BHI) broth tubes were streaked with *E. piscicida* WT, Δ*phoP* and Δ*phoQ* strains. Growing bacteria were left to reach the exponential phase (3-4 hrs at 28ºC). Phosphate buffer saline (PBS) was used to wash the harvested bacteria two times before re-suspension. 50µL fish non-immune serum was mixed with approximately 10^6 of *E. piscicida* WT, *Ep*Δ*phoP* and *Ep*Δ*phoQ* bacterial cells per milliliter, at the same time another 10^6 of *E. piscicida* WT bacterial cells per milliliter, were mixed with PBS that used as control. At 28°C, two sets of mixtures were exposed to incubation with agitation 2 hrs for one set of mixture and 4 hrs for the anther set. Subsequently, the mixtures were serially diluted and plated in triplicate on BHI agar plates. At 30°C the plates were kept for 24 hrs and the appeared colonies on the plates, were counted. Survival rates of the tested bacterial strains were calculated by using the following formula;

(Number of serum-treated bacterial cells) / (number of control bacterial cells) × 100.

2.3. Evaluation of Resistance against Blood Killing Activity

This assay was performed according to (Quan et al., 2018) with modifications. Concisely, sterile syringes loaded with tri-potassium salts of ethylene diamine tetra acetic acid (EDTA) (Thermo Scientific®, Pittsburg, US) were used to withdraw blood from non-immunized SPF channel catfish in concentration 1.5 mg EDTA per ml blood. *E. piscicida* WT, Δ*phoP* and Δ*phoQ* strain cultures were adjusted at a concentration of 10^6 colony forming units (cfu) per ml. 100µL from the growing cultures were mixed with 900µL from the un-coagulated blood and the mixtures were incubated at 28°C. After 2 and 12 hrs. 100µL from each mixture were serially diluted in PBS and were plated on BHI agar plates in triplicates. The plates were incubated at 30°C for 24 hrs. After that the appeared colonies were counted and the concentration of each strain per ml blood was calculated.

2.4. Persistence of ΔphoP and ΔphoQ in Immunized Channel Catfish

One hundred and sixty SPF channel catfish fingerlings were maintained at a rate of 40 fish per group. One of the groups was intra-peritoneal (i.p.) injected with *E. piscicida* WT strain at dose 3.5×10^7 cfu/fish, two groups were immunized with Δ*phoP* and Δ*phoQ* at doses 3.1×10^7 and 3×10^7 cfu/fish in addition to another group act as negative control that was injected with PBS. At sampling time points (3, 5, 7 and 10 days post immunizations), three fish was removed from each group and euthanized by transfer to water containing 1.5 gm of MS-222 per liter. By aseptic technique, liver, spleen and anterior kidney were taken from each fish. Each tissue sample was weighted, suspended in 0.1ml of sterile PBS solution and

subjected to fine homogenization. After that suspensions were serially diluted in PBS and 150µL aliquots from each sample were streaked on BHI plates in triplicates for quantification. After 24 hrs. colonies were counted and the concentration of each strain per gram tissue was calculated.

2.5. Expression analyses of some Immune Related Genes Following Vaccination

2.5.1. RNA Extraction from Different Tissues

About 120 six-month old SPF channel catfish fingerlings were kept at a rate of 30 fish per group. Classified groups were injected i.p. at doses 3.5×10^7, 3.1×10^7 and 3×10^7 cfu/fish with *E. piscicida* WT, Δ*phoP* and Δ*phoQ* respectively. At 14 and 21 days post vaccination (dpv), anterior kidney and spleen tissues were assembled from the euthanized channel catfish. The collected tissue samples were sited immediately into sterile RNase-free tubes and the tissues were immersed in RNA later (Ambion®, TX, USA) for 24 hrs. before starting RNA extraction. Trizol (Invitrogen®, USA) was used to extract total RNA from tissue samples. NanoDrop ND-1000 spectrophotometer (Thermo Scientific®, TX, USA) was used to check the quantity and quality of the extracted RNA.

2.5.2. cDNA Synthesis Procedure

Total RNA was converted to cDNA that was used for RTqPCR by using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific®, TX, USA). According to manufactures' structure cDNA reactions were held in 20µl reaction tubes. Each tube was holding RNA (2.5µl), Reaction Mix (4µl), Enzyme Mix (2µl) and highly purified water (11.5µl). The reactions were run at 28°C for 7 minutes then at 55°C for 25 minutes finally; reactions were terminated by incubation at 87°C for 4 minutes.

2.5.3. Quantitative Real-Time PCR

Oligonucleotide sequences for detection and evaluation the expression levels of IL-1β, INFγ, CD4-1, MHC class I and MHC class II are listed in **(Table, 1)** according to **(Kordon et al., 2019),** they were commercially obtained from Eurofins® Genomics, US. FastStart Sybr Green Universal Master Kit (ROX®; Switzerland) was used for running real-time PCR. Each qPCR reaction was held in 20µl reaction tube that contained primers (0.5µl per each), Syber Green Master Mix (10µl), cDNA (2µl) and highly purified water (7µl). RTqPCR Applied Biosystems® US was used to accomplish quantitative PCR reactions. Thermal cycler was adjusted with 40 cycles, of initial denaturation (94°C for 10 sec), denaturation (94°C for 30 sec), annealing (58°C for 45 sec) and extension (74°C for 30 sec). Each sample was handled in triplicates. For normalization, cycle threshold (Ct) difference, between the reference gene (18S rRNA) and genes of interest was calculated in addition to use the formula $2^{-\Delta\Delta Ct}$ to get fold changes for each gene according to **(Karsi et al., 2004; Kordon et al., 2019).** Values of fold changes were used for statistical analysis to determine significant differences among fish groups that represent negative control, constructed mutants and *E. piscicda* WT.

2.6. Statistical analysis

SAS program for Windows v9.4 (SAS Institute, Inc., Cary, NC) was used to assess significance among the tested groups depending upon one-way ANOVA test. For evaluation of growth of the mutant strains under stress conditions and persistence in tissues, comparisons were made to WT. For gene expression analyses of of IL-1β, INFγ, CD4-1, MHC class I and MHC class II genes, comparisons were made to the negative control. The significance level was defined as ($p<0.05$).

Table 1. Gene names, oligonucleotide sequences and GenBank accession numbers used in RT-qPCR.

Genes	Sequences (5`-3`)	Acc. Numbers	Reference
18S rRNA	F-GAGAAACGGCTACCACATCC R-GATACGCTCATTCCGATTACAG	(AF021880)	(Kordon et al., 2019)
IL-1β	F-TGATCCTTTGGCCATGAGCGGC R-AGACATTGAAAAGCTCCTGGTC	(DQ157743)	
INFγ	F-TTGGGCAAAGTAGAGGACACC R-TGTTTCCACACTGCCTGTTCG	(NC_030434)	
CD4-1	F-GATGTCATCATTGTAGATCTCG R-GAGGTAGCTGGCATTTCACTCC	(DQ435305)	
MHC I	F-GCACAACAAACCAGACGAGA R-TCGTTGTCCTCCAGTTTCAA	(AF103001)	
MHC II	F-GACACCAGGACATGGGAGGTG R-CGAGGAAGAAAGTTCCGGTAG	(AF103002)	

3. Results

3.1. Evaluation of Resistance against Non-Immunized Channel Catfish Serum

E. piscicida WT exhibited noticeable serum resistance, as 86.3 relative percent survivals of the WT bacteria was detected after 2 hrs. incubation with non-immunized channel catfish serum. On the other side, Δ*phoP* and Δ*phoQ* mutant strains showed 70.8% and 70.1% percent survivals after the same period of incubation, which is significantly ($P < 0.05$) lower than that of *E. piscicida* WT **(Fig. 1a).** After 4 hrs. incubation period the survival rates showed further significant ($P < 0.05$) reduction to reach 79.1%, 62.4% and 60.9% for *E. piscicida* WT, *Ep*Δ*phoP* and *Ep*Δ*phoQ* strains respectively **(Fig. 1b).**

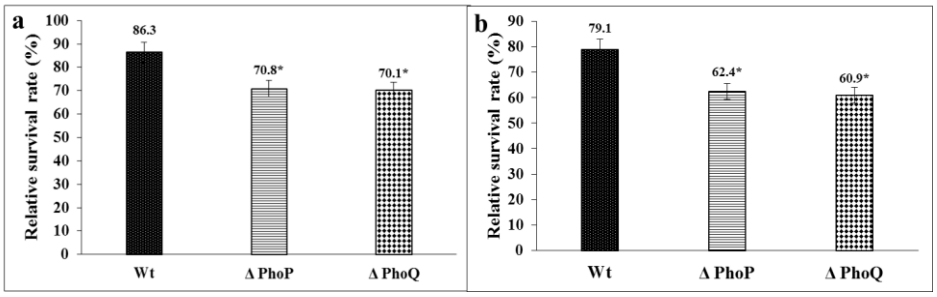

Fig. 1. Relative survival rates of *E. piscicida* WT, Δ*phoP,* and *p*Δ*phoQ* mutant strains after 2 hours (a) and 4 hours (b) incubation periods with serum from non-immunized channel catfish fingerlings. The data represent means (±SE) of four replicates. Significant differences are indicated with asterisks ($p < 0.05$).

3.2. Evaluation of Resistance against Blood Killing Activity

After 2 hrs. incubation period, concentrations of *E. piscicida* WT, Δ*phoP* and Δ*phoQ* strains in un-coagulated blood samples were 2×10^7, 1.6×10^7 and 1.5×10^7 cfu/ml respectively **(Fig. 2a).** Furthermore, after 12 hrs. of incubation, Δ*phoP* and Δ*phoQ* mutant strains showed significantly ($P< 0.05$) decreased concentrations (0.7×10^7 and 0.8×10^7 cfu/ml) compared to concentration of *E. piscicida* WT strain (1.5×10^7) **(Fig. 2b).**

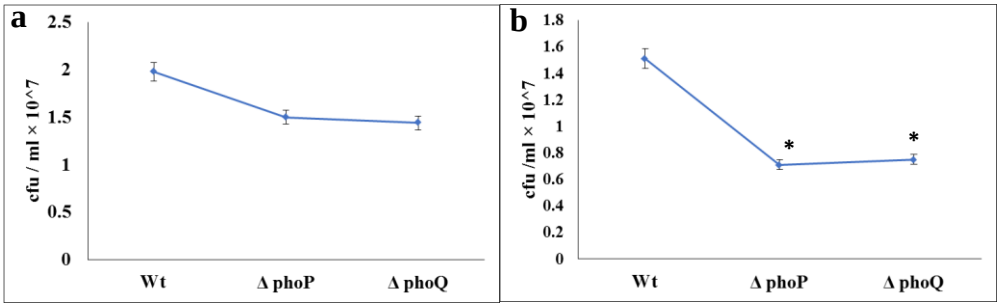

Fig. 2. Concentrations of *E. piscicida* WT, Δ*phoP,* and Δ*phoQ* mutant strains after 2 hours (a) and 12 hours (b) incubation periods with anti-coagulated blood from non-immunized channel catfish fingerlings. The data represent means (±SE) of four replicates. Significant differences are indicated with asterisks ($p < 0.05$).

3.3. Persistence of ΔphoP and ΔphoQ in Immunized Channel Catfish

At all tested sampling time points, it was found that concentrations of *E. piscicida* WT in livers, spleens and anterior kidneys **(Fig. 3a)** of the WT injected fish group, were significantly (*P<0.05*) higher than those concentrations of both Δ*phoP* **(Fig. 3b)** and Δ*phoQ* **(Fig. 3c)** immunized fish groups **(Fig. 3a)** in all examined tissues. The highest numbers of bacteria per gram of tissue were detected in head kidneys, with 7.6×10^7, 6.7×10^7 and 6×10^7 cfu/gm of tissue for *E. piscicida* WT, Δ*phoP* and Δ*phoQ*, respectively at day 3 post injections **(Fig. 3 a-c).** Bacterial load in spleen were decreased by 2×10^7, $1.7\times10^7$and 1×10^7 cfu/gm for *E. piscicida* WT, Δ*phoP* and Δ*phoQ*, respectively. Liver had the lowest numbers of bacteria per gram of tissue, with about 3.2×10^7, 2.3×10^7 and 2.4×10^7 cfu/gm of tissue for *E. piscicida* WT, Δ*phoP* and Δ*phoQ*, respectively at the same time point **(Fig. 3).** Numbers of bacteria in anterior kidney, spleen and liver endured to decrease reaching to the lowest concentration at day 7 post injections. At all tested level, viable bacterial cells could not be noticed for *E. piscicida* WT and the constructed mutant strains at day 10 post injections.

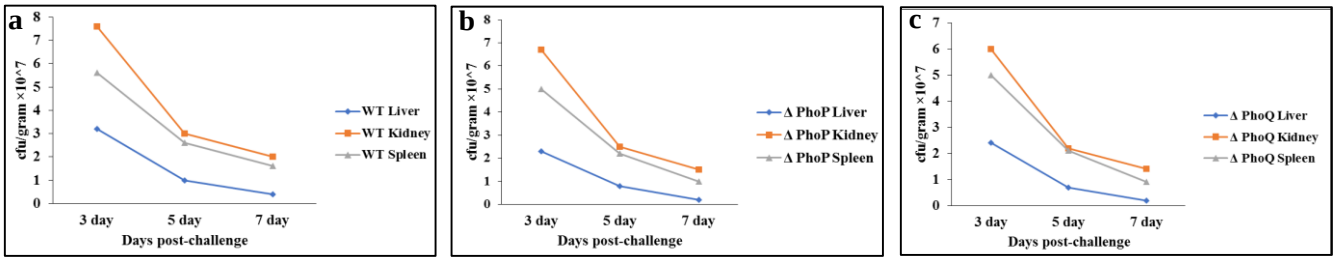

Fig. 3. Concentrations of *E. piscicida* WT (a), Δ*phoP* (b) and Δ*phoQ* (c) in anterior kidney, spleen and liver of channel catfish at day 3, 5 and 7 after intra-peritoneal injections. Numbers shown represent the averages of the $\log_{10}$ transformations of the number of bacteria per gram of tissue.

3.4. Expression Analyses of Some Immune Related Genes Following Vaccination

Transcription profiles of pro-inflammatory genes (IL-1β and INF-γ) revealed significant up-regulation levels in both spleens and anterior kidneys of *E. piscicida* WT exposed fish group at day 14 and day 21 post injection compared to negative control group **(Fig. 4 a, b).** Concerning to mutants' immunized groups, IL-1β showed significant up-regulation level ($P<0.05$) in anterior kidney and spleen of the vaccinated fish at the same time points post immunizations. The highest up-regulation level was observed in Δ*phoQ* vaccinated group by 58- fold at day 14 post vaccinations in anterior kidney. Furthermore, marked INF-γ significant ($P<0.05$) up-regulation level was detected in spleen of fish immunized with Δ*phoP* mutant strain at day 14 and day 21 post immunizations **(Fig. 4b).** In contrast, Δ*phoQ* immunized fish group, showed non-significant up-regulation level of INF-γ in both kidney and spleen at the same time points **(Fig. 4 a, b).** Moreover, CD4-1, MHC I and MHC II genes, exhibited significant ($P<0.05$) up-regulation expression levels in the all examined groups at day 14 post immunization followed by little decline at day 21 post vaccination in both anterior kidneys and spleens.

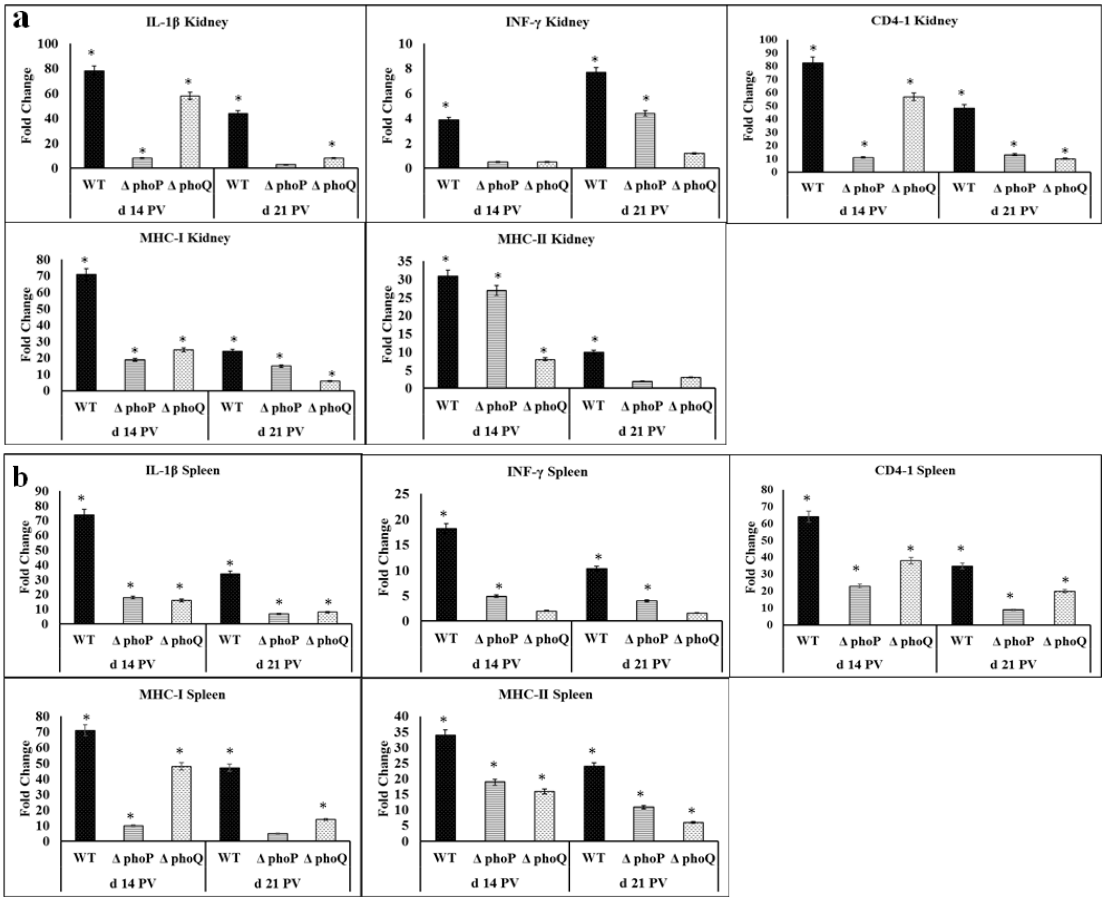

Fig. 4. Fold changes of immune related genes, IL-1β, INF-γ, CD4-1, MHC I and MHC II in anterior kidney (a) and spleen (b) of intra-peritoneal injected channel catfish fingerlings with *E. piscicida* WT, Δ*phoP* and Δ*phoQ* strains at days 14 and 21 post vaccination (PV).

4. Discussion

Edwardsiellosis is considered one of the most important bacterial emerging diseases that affecting wide varieties of fish hosts including channel and hybrid catfish **(Abayneh et al., 2013).** *E. piscicida* a facultative anaerobic, Gram-negative, rod-shaped bacterium, represents the most common *Edwardsiella species* isolated from channel catfish **(Griffin et al., 2013).**

Cutaneous petechial hemorrhages, distended abdomen, scattered ulcers and erosions all over the body especially around bases of fins with high mortalities are the main clinical picture that was observed in channel catfish agonized to *E. piscicida* infection **(Reichley et al., 2018).** Oxidation and acidic circumstances in addition to deprivation from certain nutrients are examples of series of stresses that challenge the establishment of *E. piscicida* infection inside the fish host **(Yamasaki et al., 2015).** The adaptation and growth of *E. piscicida* bacterium in harsh conditions is controlled by many virulence factors **(Leung et al., 2011).**

PhoP/Q is one of the TCSs that widely present in most Edwardsiella species **(Perez et al., 2009).** The main functions of PhoP/Q system are mainly associated with regulation the response of bacteria to several parameters inside and outside the host **(Perez et al., 2009; Chakraborty et al., 2010; Vadyvaloo et al., 2015).** Pathogenesis of *E. piscicida* is a very complicated process. The mechanisms by which *E. piscicida* resist serum and blood killing activities are still unclear. In the present study, role of phoP and phoQ genes of *E. piscicida* in resistance to host stresses generated by serum and blood of the fish host were investigated. Survivability of Δ*phoP* and Δ*phoQ* mutant strains had assessed and compared to the survival rate of *E. piscicida* WT strain after incubation with serum from non-immunized channel catfish. It was found that Δ*phoP* and Δ*phoQ* mutant strains exhibited significantly decreased survival rates (62.4% and 60.9%) in channel catfish serum compared to 79.1 % for *E. piscicida* WT after 4 hrs. incubation period, proposing that *phoP* and *phoQ* genes have a role in protection of *E. piscicida* WT against the killing activity of host serum. Many mechanisms that clarified the bacterial serum resistance have been illustrated recently. Inhibition of classical and alternative complement systems through lipopolysaccharide (LPS) modifications in bacterial cell membranes, is considered the most common mechanism that gave an clarification for the bacterial serum resistance **(Rooijakkers and van Strijp, 2007; Hovingh et al., 2016; Abreu and Barbosa, 2017).** Interestingly, PhoP/Q system has regulatory role associated with direct and indirect activation of BasS/R system which consider the main regulator for LPS modifications of the bacterial cell membrane of Gram negative bacteria **(Wosten et al., 2000; Gibbons et al., 2005; Perez and Groisman, 2007; Chen and Groisman, 2013).** This may gave an explanation to the lower survival rates of Δ*phoP* and Δ*phoQ* mutant strains compared to *E. piscicida* WT strain under serum stress condition. In agreement with our results **(Sayed et al., 2021)** declared that phoP and phoQ genes are required for proper growth and adaptation of *E. piscicida* to oxidative and acidic killing conditions. Moreover, phagocytosis that was achieved by neutrophils and other non-specific phagocytes in blood of the host, are essential to be resisted by bacterial cells **(Der Maten et al., 2017).**

In the current study, anti-coagulated blood from non-immunized channel catfish host was used to accomplish blood killing activity on the constructed mutants in comparison with the *E. piscicida* WT strain. Under blood stress condition, significant reductions in growth of Δ*phoP* and Δ*phoQ* mutant strains were observed when compared with the growth of *E. piscicida* WT strain especially after 12 hrs. incubation period **(Fig. 2 a, b),** indicating that the absence of *phoP* and *phoQ* genes weaken the resistance of *E. piscicida* against the bactericidal effect of fish blood. In agreement to our results, **Thompson et al., (2011)** discovered the role of PhoP/Q system of *Salmonella typhimurium* in avoidance of phagocytosis that was achieved by murine macrophages. **Oyston et al., (2000)** mentioned that *Yersinia pestis ΔphoP* mutant strain revealed a lower survival rate in macrophage cell cultures. Also, **Miller et al., (1989)** declared that *Salmonella typhimurium* Δ*phoP/Q* mutant strains were more sensitive than WT strain. At the level of tissue persistence, it is known that live attenuated microbes are able to persist and replicate in different host tissues in addition to its ability to escape from the host immunity **(Brown et al., 1993; Byndloss and Tsolis, 2016).** In the present study, the relative tissue distributions of *E. piscicida* WT strain was alike to the tissues distribution of Δ*phoP* and Δ*phoQ* mutant strains however; *E. piscicida* WT accomplished higher tissue concentrations than Δ*phoP* and Δ*phoQ* mutant strains in the parenchymatous organs of the immunized channel catfish fingerlings **(Fig. 3 a-c).** At all levels tested, anterior kidneys had the highest tissue concentrations for WT and mutant strains **(Fig. 3 a-c).** In head kidneys, the concentration of *E. piscicida* WT strain was almost 1 $\log_{10}$ higher than concentrations of Δ*phoP* and Δ*phoQ* mutant strains at day 3 post injections, by the time WT and mutants' concentration began to decrease gradually. Regarding to tissues microbial concentration, spleen occupied the second place after head kidneys followed by livers in all examined groups **(Fig. 3 a-c).** Similar results were obtained by **Baldwin and Newton, (1993)** and **Lawrence et al., (1997)** after exposure of channel catfish to *Edwardsiella Ictaluri* by immersion and oral routs, where the highest concentration was noticed in the head kidneys followed by spleens and livers. Concerning to immune system of teleost, anterior kidney and spleen are considered the most important hemopoietic compartments in fish that have the function of generating all blood elements **(Zapata et al., 2006; Koppang et al., 2010; Abdelhamed et al., 2017).**

Regarding to the innate immune response, our data showed that, highest up-regulation expression level of IL-1β was obtained in the anterior kidney of WT-treated group, followed by fish groups immunized with Δ*phoP* and Δ*phoQ* mutant strains at day 14 post immunization, compared to the sham immunized group. After that, gradual reduction in IL-1β expression occurred in all treated groups 3 weeks post

vaccination. Similarly spleens of fish groups immunized with Δ*phoP* and Δ*phoQ* showed significant up-regulation level of IL-1β at day 14 followed by gradual decline at day 21 post immunization. Taking together, IFN-γ showed increased level of expression in anterior kidney of fish groups exposed to WT and Δ*phoP* strains at day 21 post immunizations while, their spleens showed significant up-regulation levels at day 14 and day 21 post vaccinations. Similar findings were obtained by **Kordon et al., 2019.** The up-regulation expression levels of IL-1β and IFN-γ genes could be explained as the early stage of anti-microbial response is refereed by pro-inflammatory cytokines as IL-1β and IFN-γ **(Taechavasonyoo et al., 2013).** The pro-inflammatory response is mainly started by release of IL-1β followed by activation of lymphocytes and macrophages that resulted in enhancing their microbicidal and cytotoxic activities in addition to promotion synthesis of prostaglandins **(Alvarez-Pellitero, 2008; Dinarello, 2009; Iyer and Cheng 2012; Ivashkiv and Donlin, 2014).** Besides, IFN-γ plays an important role in control the production and release of bactericidal molecules such as reactive oxygen species (ROS) by macrophages and other cells of the innate immune system **(Becskei and Grusby, 2007; Kobayashi and DeLeo, 2009).**

Taking together, cell-mediated immunity in fish, is the most important part of the adaptive immune responses which expressed by T-lymphocytes **(Dixon and stet, 2001).** T-lymphocytes included T-helper and T-cytotoxic cells that carry glycoprotein receptors as CD4-1, MHC I and II which responsible for capturing and destruction of the foreign antigens and other intra-cellular infected cells **(Weiss and littman, 1994; Croisetiere et al., 2008; Forlenza et al., 2008; Xu et al., 2010; Kato et al., 2013).** Our data revealed that, CD4-1, MHCI and MHCII genes were significantly up-regulated in anterior kidneys and spleens of fish groups immunized by Δ*phoP* and Δ*phoQ* mutant strains with higher expression levels in the anterior kidneys and spleens of fish group exposed to *E. piscicida* WT. The nature of *E. piscicida* as an intracellular pathogen, which required CD4-1, MHCI and MHCII glycoprotein receptors for recognition, capturing and destruction of the infected cells, gave the explanation of the high up-regulation expression levels of CD4-1, MHCI and MHCII genes **(Xiao et al., 2013; Yamasaki et al., 2015).**

5. Conclusion:

In conclusion, our results reveal the role of phoP and phoQ genes of *E. piscicida* in resistance against hostile circumstances, represented by the host immune killing mechanisms which facilitate tissues pervasion of this pathogen. More importantly, Δ*phoP* and Δ*phoQ* mutant strains can trigger innate immunity and motivate adaptive immune responses proposing that Δ*phoP* and Δ*phoQ* strains are prospective live attenuated vaccines against *E. piscicida* infection in channel catfish.

6. Conflict of Interest

The authors declare no conflict of interest.

7. Acknowledgment

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