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High doses of *Francisella noatunensis* induces an immune response in *Eleginops maclovinus*

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ABSTRACT

Francisella noatunensis subsp. *noatunensis*, the etiological agent of Francisellosis, affects a large number of farmed species such as *Salmo salar*. This species coexists with several native species in the same ecosystem, including *Eleginops maclovinus*. Our objective was to evaluate the susceptibility, presence of clinical symptoms, and the ability of *Eleginops maclovinus* to respond to Francisella infection. For this, healthy individuals were inoculated with 1.5×10^1, 1.5×10^5, and 1.5×10^{10} bact/μL of Francisella by intraperitoneal injection, subsequently the fish were sampled on days 1, 3, 7, 14, 21, and 28 post injection (dpi). At the end of the experiment, no mortality, nor internal and external clinical signs were observed, although in the high dose anaemia was detected. Additionally, bacteria were detected in all three doses, however there was replication at day 28 only in the liver in the high dose. Analysis of gene expression by qPCR showed that the spleen generated an immune response against infection from day 1 dpi, however at day 7 dpi most of the genes suffered repressed expression; observing over expression of the genes C3, NLRC3, NLRC5, MHCI, IgM. In contrast, expression in the anterior kidney did not vary significantly during the challenge. IgM quantification showed the production of antibodies in the medium and high doses. This study provides new knowledge about *Francisella* infection and the long-lasting and specific immune response generated by *Eleginops maclovinus*. It also demonstrates its susceptibility to Francisellosis where there is a difference in the immune response according to the tissue.

1. Introduction

Francisella spp. is the etiological agent of *Francisellosis*, which affects mammals and aquatic organisms [1]. In mammals, it produces clinical symptoms that acutely progress and trigger death [2]. In fish, it manifests as a granulomatous (chronic) disease with the target organs being spleen, kidney, and liver [3], but it can also be detected in heart, muscle, brain, testicles, ovary, and eye [4,5]; the clinical signs are anaemia, erratic swimming, anorexia, and exophthalmia [6,7]. The genera belonging to the Francisellaceae family are pleomorphic bacteria, predominantly coccoid, Gram-negative, strictly aerobic, facultative intracellular, with special nutritional requirements since they require cysteine and iron for their cultivation [7,10,15]. Their optimal growth temperature is between 22 and 25 °C [3], but they can be cultivated from 10 °C to 30 °C [9,16], depending on the species.

Within this genus there are 2 species of aquaculture importance, *Francisella noatunensis* subp. *noatunensis* and *Francisella noatunensis subp. orientalis*, which have been isolated from a wide variety of hosts, including fish and molluscs, such as Atlantic salmon (*Salmo salar*) [8–10], Atlantic cod (*Gadus morhua*) [6,11], grouper (*Epinephelus melanostigma*)

Abbreviations: C3, Complement protein 3; NLR, NOD-Like Receptor; MHC, Mayor Histocompatibility Complex; Ig, Inmunoglobulin; IL, Interleucin; LPS, Lipopolisacarid; NADP, Nicotinamide adenine dinucleotide phosphate; TLR, Toll Like Receptor; TNF, tumor necrosis factor; BSA, bovine serum albumin

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[12], sea bass (*Morone chrysops x Morone saxatillis*) [13], tilapia (*Oreochromis niloticus*) [4], and puye (*Galaxias maculatus*) [14]. Also, this bacteria (*Francisella* spp) has been isolated in different countries such as Chile, Norway, Costa Rica, and Japan [6,8–14], revealing the wide range of geographical distribution of this bacterium. This last can be attributed to: i) aquaculture massification [16,17] ii) environmental changes, which increases the likelihood of the establishment of new pathogens [18] iii) high levels of overcrowding in farming systems, which produce high levels of stress, making fish more susceptible to pathogen outbreaks [19].

The synergy between natural and anthropogenic effects could facilitate the spread of pathogens, which can eventually interact with the native fish fauna. An example of this is the finding of Contreras-Lynch [20], who detected the presence of *Piscirckettsia salmonis* in *Eleginops maclovinus*, a native species in Chile that is found within close proximity to salmon farming areas, where they search for unconsumed food [21]. The importance of this notothenoid species is given by to be native fish and its wide distribution in the Chilean territory, since it is distributed from the region of Magallanes and Chilean Antarctic to the region of Valparaiso [45], being able to act as an eventual vector of the infection, distributing it not only to crops, but also to other native species, due to *Eleginops maclovinus* is feeding of the rest of pellet and heces from Salmon cage [46].

Added to the wide distribution of *Eleginops*, it is still unclear how *Francisella* could interact with native fish species and much less with the immunological mechanisms that employ these characteristics to cope with the infectious process. In fish the immune system is divided into innate and adaptive, both formed by humoral and cellular components [23]. The innate immune response is formed by macrophages, non-specific cytotoxic cells, and granulocytes [26], whose activation is given by the union and recognition of molecular patterns associated with pathogens by Toll and NOD receptors, which trigger the synthesis and release of pro-inflammatory cytokines such as: IL-1β, TNF, IL-18, IL-6 [27]. Subsequently, the adaptive immune system is activated by the production of cytokines that trigger the differentiation, maturation and proliferation of T and B lymphocytes [27]. It is important to note that fish have a low repertoire of antibodies (IgM, IgD and IgT), deficient immunological memory, and decreased affinity for immunoglobulins [22–24]. The latter is compensated by a greater repertoire of complement receptors and natural killer cells compared to mammals [22,25].

However, despite the robust immune response that can be generated to eradicate pathogens, microorganisms such as *Francisella* have articulated mechanisms to evade host immune responses such as LPS modification [28], phagosome escape [29], cleavage of C3 [30], inhibition of the assembly of the NADPH oxidase complex [31,32] and ubiquitination of MHC II [33]; which help the success and survival of the bacteria inside the host. This complex immune evacuation machinery makes it necessary to investigate not only the effects that this bacterium has on the species that are cultivated but also the physiological effects that it could generate in the native species that cohabit with the species of commercial crop. Oyarzun et al., 2018 evaluated the effect at the metabolic level of this bacterium on *E. maclovinus*, however, it is still unknown how the immune system in this species is regulated to fight this bacterial pathogen. Therefore, the aim of this study was to evaluate the innate and adaptive immune response of *Eleginops maclovinus* with three different doses of *Francisella noatunensis* in the main target organs: spleen and head kidney.

2. Materials and methods

2.1. Ethics statement

All experimental protocols complied with guidelines for the use of laboratory animals, as established by the Chilean National Commission for Scientific and Technological Research (CONICYT, Spanish acronym) and the Universidad Austral de Chile with protocol approved number 261/2016.

2.2. Fish

Immature juveniles of *Eleginops maclovinus* pathogen free were obtained from Fundación Chile. The fish were transferred to the Fish Vivarium at the High Biosecurity Wet Laboratory, Institute of Animal Pathology (Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia, Chile). These fish were kept in 500 L ponds with an average temperature of 13 ± 0.5 °C, plus a light regime of 12:12. Fish were fed once daily with a 1% body weight ratio of commercial dry pellet (Skretting Nutrece 100 ML; containing 48% protein, 22% fat, 13.5% carbohydrates, 8% moisture, and 8.5% ash). At the beginning of the experiment the fish had an average weight of 80grs. Prior to inoculation, fish were anesthetized with 2-Phenoxyethanol in 0.5 mL/L dose.

2.3. Bacteria

The *Francisella noatunensis* isolate was obtained from ADL Diagnostic Chile SpA. The bacterium was grown on trypticase soy agar enriched with haemoglobin [34] for five days at 20 °C. A colony was transferred to 5 mL of liquid medium [35] and incubated for approximately 3 days in constant motion, after incubation the bacteria were washed and resuspended in medium for subsequent quantification, which was performed in a Neubauer chamber, where the dilutions (1.5×10^1, 1.5×10^5 and 1.5×10^{10} bact/μL) were prepared with the same medium.

2.4. Infection

After acclimation, the fish were distributed in 8 tanks of 100 L, with two tanks per dose (1.5×10^1, 1.5×10^5 and 1.5×10^{10} bact/μL), with 19 fish per tank. The fish were injected i.p. with 0.1 mL of the inoculum. The fish control was injected with 0,1 mL of bacterial liquid medium [35]. And the fish inoculated with bacteria was done in exponential phase. They were kept in the same light and temperature conditions mentioned above.

2.5. Sampling

The animals were sacrificed on days 1, 3, 7, 14, 21, and 28, the day before each sampling feeding was stopped. First they were euthanized with an overdose of anaesthesia (1 mL/L) and cutting of the spinal cord. The blood was collected and centrifuged for 5 min at 2000 *g*, to obtain plasma. Spleen, liver, and anterior kidney were collected, which were stored at −80 °C until their use.

2.6. RNA isolation

Total RNA was extracted with the column (Total mini Kit RNA, Geneaid®) following the manufacturer's instructions. The obtained samples were treated with DNase I (1 U/g RNA, Invitrogen). RNA concentration and purity were assessed via A260/280 and A260/230 ratios on a spectrometer (Nano, Maestrogen®). Samples were then stored at −80 °C until use.

Complementary DNA (cDNA) was synthetized by the reverse transcription of RNA using a cDNA synthesis kit (Promega) following the manufacturer's recommendations. The bacterial cDNA was synthetized using Random primer hexamers (Thermo Fisher). cDNA concentration and the A260/280 and A260/230 ratios were measured, and samples were diluted to 100 ng/μL.

Table 1
Primers used for conventional PCR and real-time qPCR analysis of mRNA and DNA expression.

Gene	Forward	Reverse	Accession numbers	Reference
FtsZ	TACCATACTCAGCGGCTTTC	GCGCCTGTAGTTGCTGAAGT	–	[39]
FopA	TACTGGTGCATGGGATGTTG	TCTTGGAGCCATTGTCTGAA	DQ825766.1	[39]
C3	ACTGCAGGGAGTCTTTAGATTTG	TGTGGGCCAGTACTCGATCCAGG	–	[38]
TLR1	CAACGCTATCTGATCCCCAAGCAA	AAAGCCGACGCTCAGTGTTTGT	–	[38]
TLR8	TCCTGCAGAACTCTCACTTCCT	TCTGACCACATTCCTCAGGTTT	MF945983	[38]
NLRC3	TACGATTGCTCCCGTGAAGCTGTT	GTACCGAGCTCTACGCTCACTTTT	MF945985	[38]
NLRC5	AGTGATCAATCCGTGGGCGTTT	TCTGGAGTCGAGGCTCATATCAGT	MF945986	[38]
IL-1b	TGCAACATGAGCCAGATGTGGA	CGTTGATTCTGAGCCCTTCATCCT	–	[38]
MHC I	CCAGAGTTTGTGACTGTTGGGT	GTGATTCTGCTCATCCAGTCCTGT	–	[72]
MHC II	CTACGAGTTCTACCCCAAACCCAT	CAGTCGCTGTCAGCCAGTTCTT	MF945987	[38]
IgMs	TGAAAGACTTCTACCCGCATGAGG	AACTGGCCATAAGCGGAAAAGG	MF945988	[38]
18S	CGTTGATTCTGAGCCCTTCATCCT	TTGAGTCAAATTAAGCCGCA	–	[73]

Abbreviations: FtsZ, cell division protein; FopA, *Fancisella* outer membrane protein A; C3, protein Complement 3; TLR1, Toll –like receptor 1; TLR8, Toll-like receptor 8; NLRC3, Nod-like receptor 3; NLRC5, Nod-like receptor 5; IL-1b, Interleukin 1 beta; MHC I, major histocompatibility complex I; MHC II, major histocompatibility complex II; IgMs, soluble inmunoglubulin M; 18S, 18 subunit ribosomal.

2.7. DNA isolation

Bacterial DNA was extracted from 60 mg of liver. DNA was isolated by using a bacterial DNA isolation kit (Matchery-Nagel). DNA concentration and purity were assessed via A260/280 and A260/230 ratios on a spectrometer and diluted to 100 ng/μL.

2.8. qPCR analysis

Primers were obtained from previous publications (Table 1). Melting curve analysis and sequencing were used to test for non-specific amplifications and primer-dimer formations. Real-time quantitative PCR (qPCR) analysis was carried out using the AriaMx Real-Time PCR System (Agilent, Stratagene). A total reaction volume of 12 μL was obtained using SYBR Green (Thermo Fisher®). Reaction runs were as follows: incubation for 10 min at 95°C, followed by 40 cycles of 10 s at 90 °C and 30 s at 60 °C, and a final melting curve of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 95 °C. mRNA gene expressions were normalized to the ribosomal gene 18S using the comparative ΔΔCt method [36]. Each real-time qPCR was performed in triplicate.

2.9. Haematocrit

The sample was collected in heparinized haematocrit capillaries (Micro Haematocrit Tubes, Vitrex), immediately the capillaries were sealed with plasticine at one end, subsequently centrifuged (Haematokrit 200, Hettich) for 5 min at 12000 rpm. The reading was made by calculating the proportion of the globular mass with respect to the total length of the sample in the capillary, this was done with a microhematocrit rule (Haematokrit 200, Hettich) [43].

2.10. ELISA assay

Serum samples were subjected to indirect enzyme-linked immunosorbent assays (ELISA). The ELISA plates were seeded with total protein extracts (1 μg/dL of *Francisella noatunensis* overnight at 4 °C). The extracts were obtained by following the protocol described by Oliver [37]. After incubation, plates were washed with 0.05% PBS Tween and blocked with BSA for 1 h; after which, serum samples from each fish (1:100 dilution) were added and incubated for 2.5 h at 17 °C. After washing, 100 μL of *anti*-IgM (commercial mouse monoclonal antibody IgG, Ango, 1:1000 dilution) was added to each well and incubated for 1 h at room temperature. The plates were then washed again, and 100 μL of *anti*-IgG conjugated to peroxidase (commercial goat monoclonal antibody, Jackson Immuno Research; 1:3000 dilution) was added and left to incubate for 1 h at room temperature. After incubation, the plates were washed again, and the enzyme substrate (TMB Reagent) was added. The reaction was stopped after 30 min with 2 M H_2SO_4. Absorbance was measured at 450 nm on a Musltiskan Go plate reader (Thermo Fisher Scientific) with the MultiskanGo software. The produced colour intensity was proportional to the concentration of *anti-Francisella* IgM in the sample. As described by the protocol established by Martinez et al. [38].

2.11. Statistical analyses

Assumptions of variance normality and homogeneity were tested. Data were logarithmically transformed when needed to fulfil conditions for parametric analysis of variance (ANOVA). Two-way ANOVA was performed using the different doses and experimental times as factors of variance, followed by a *post-hoc* Tukey test to identify differences between groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Mortality rates during the Francisella noatunensis infection challenge

No mortality, or external pathological changes related to *Francisellosis* were observed in any of the treatments during the course of the challenge.

3.2. Detection and replication of F. noatunensis in the liver

Detection of *Francisella* was evident in the three doses at day 28 pi (Fig. 1). Although no replication was observed in the low and medium doses on day 28 (Fig. 2). No DNA or bacterial RNA was detected in the control condition injected with medium.

3.3. Haematocrit

A decrease in haematocrit was observed from day 3 post injection in all infected groups, although no significant difference was observed compared to the control group. The low dose maintained a haematocrit that was very similar to the control group after day 7 post injection. The medium dose had a lower haematocrit than the control group throughout the experiment, with a value close to 24% at each time point, although this was not significantly different. Meanwhile, the haematocrit of the fish that received a high infection dose was lower than the control group and from day 14 post injection this difference was statistically significant ($P < 0.05$) (Fig. 3).

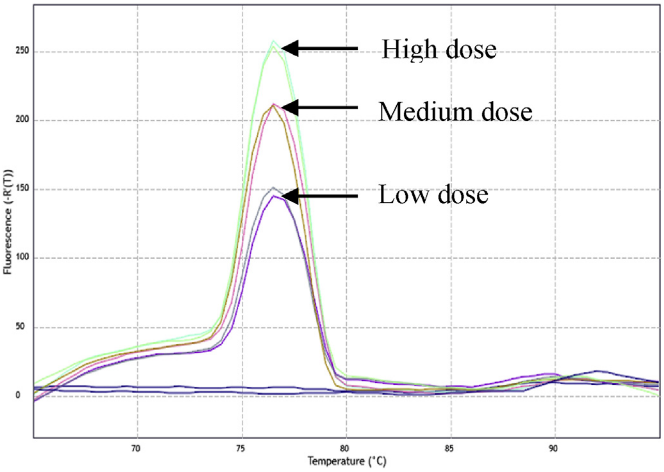

Fig. 1. The Fluorescence melting curve from the amplification of the FopA gene from *Francisella* in SYBR Green qPCR using the DNA of four different treatments. Basal lanes are the controls injected with medium. Genic expression was measured in the liver at day 28 pi. N = 2 per condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

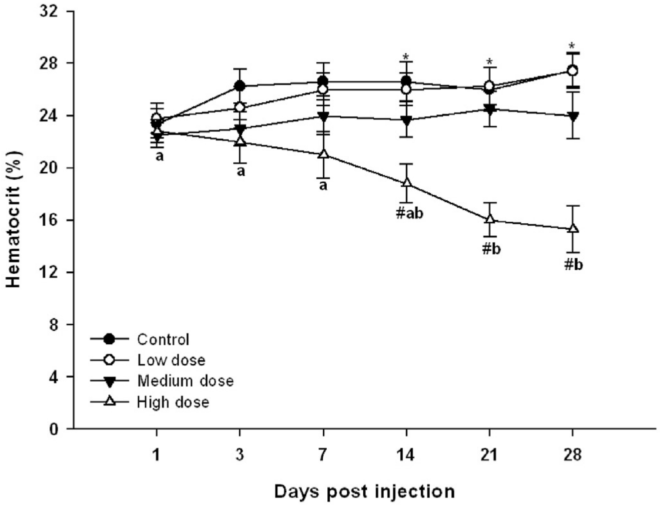

Fig. 3. Average haematocrit percentage in the blood of each treatment condition over time. Symbols indicate statistical differences between different treatment groups at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

3.4. Expression of immune genes

3.4.1. Genes involved in the innate immune response

In the spleen, C3 gene expression increased in the 3 doses from day 1 to day 3 pi compared to the control group. Subsequently, the high dose increased until day 28, with a 16 fold increase compared to the control. However, expression in the low and medium doses fell to basal levels (Fig. 4a). In head kidney, C3 gene expression decreased significantly on the first day post injection in all 3 doses. On day 3 expression levels began to increase until day 7, then fell and showed a similar expression pattern over the following two weeks of the experimental period (Fig. 4b).

TLR1 gene expression in the spleen on the first day post injection was overexpressed at low and high doses until day 7, where it then abruptly dropped. Subsequently, it increased in all three doses on day 14, falling to basal levels on day 28 (Fig. 5a). When evaluating TLR1 gene expression in the anterior kidney, no significant differences were observed in the three doses throughout the challenge (Fig. 5b).

In the spleen, TLR8 gene expression in the 3 doses was down regulated on the first day post infection and then increased on day 3. However, this difference was significant only in the low dose (compared to the control). Gene expression levels then decreased at day 7 dpi and rose again at day 14 in all three doses; gradually decreasing to basal levels after two weeks (Fig. 6a). In the head kidney, TLR8 expression decreased significantly in the three doses on day 1 pi, then on day 3 it increased to basal levels; which was maintained for the remainder of the challenge (Fig. 6b).

In spleen, NLRC3 gene expression decreased the day after injection in all 3 doses, on day 3 pi gene expression increased by over two fold in all three doses, then decreased again at day 7 pi. The highest expression levels were observed at day 14 in the high dose, which was significantly different compared to the control. It subsequently decreased gradually in all three doses, until the end of the challenge (Fig. 7a). In the anterior kidney, gene expression decreased significantly in the three doses at day 1 pi, however, this returned to baseline levels at day 3 and did not show variations in its expression during the course of the challenge (Fig. 7b).

When evaluating NLRC5 gene expression in the spleen, an increase was observed in all 3 doses on the first day pi (not significant), where subsequently the expression increased in the lowest dose by 15 fold at day 3. Additionally, gene expression in the high dose remained high during the course of the challenge, presenting its highest increase on day 21 pi, with a 25 fold increase. Finally the 3 doses returned to basal expression values at the end of the challenge (Fig. 8a). NLRC5 gene expression in the anterior kidney showed a gradual decrease in all three doses until day 7, returning to baseline values at day 14 in the low and high doses, where in medium dose it increased 4 fold compared to the control, and then decreased until the end of the challenge. In the low dose expression levels were increased 8 fold on day 28; this difference was significantly different compared to the other treatments (Fig. 8b).

IL-1b gene expression in spleen decreased significantly the day following injection in all 3 doses and subsequently increased significantly at day 3. However, expression levels returned to baseline at day 7. During the remainder of the challenge, there were variations in expression levels which were only significant in the high dose on days 21 and 28 pi (Fig. 9a). In the anterior kidney, IL-1b expression varied significantly but showed no expression pattern (9b).

3.4.2. Genes involved in the adaptive immune response

MHC I gene expression in the spleen was increased from the first day after injection in the low and high doses and was maintained during the first 3 days, however it returned to normal values at day 7.

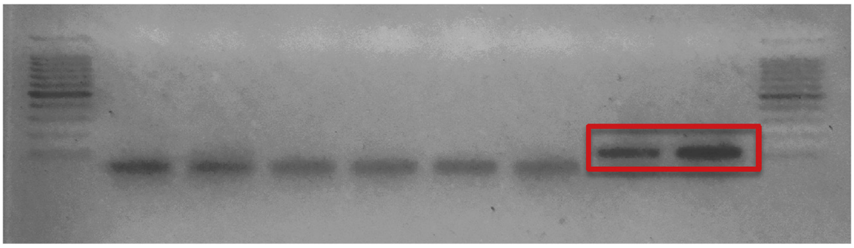

Fig. 2. Agarose gel analysis of the FtsZ replication gene by conventional PCR, using RNA from the four different treatments. Lane 1: 100 pb DNA molecular marker, lane 2–3: negative control, lane 4–5: low dose, lane 6–7: medium dose, lane 8–9: high dose, lane 10: 100 pb DNA molecular marker. Genic expression was measured in the liver at day 28 pi. N = 2 per condition.

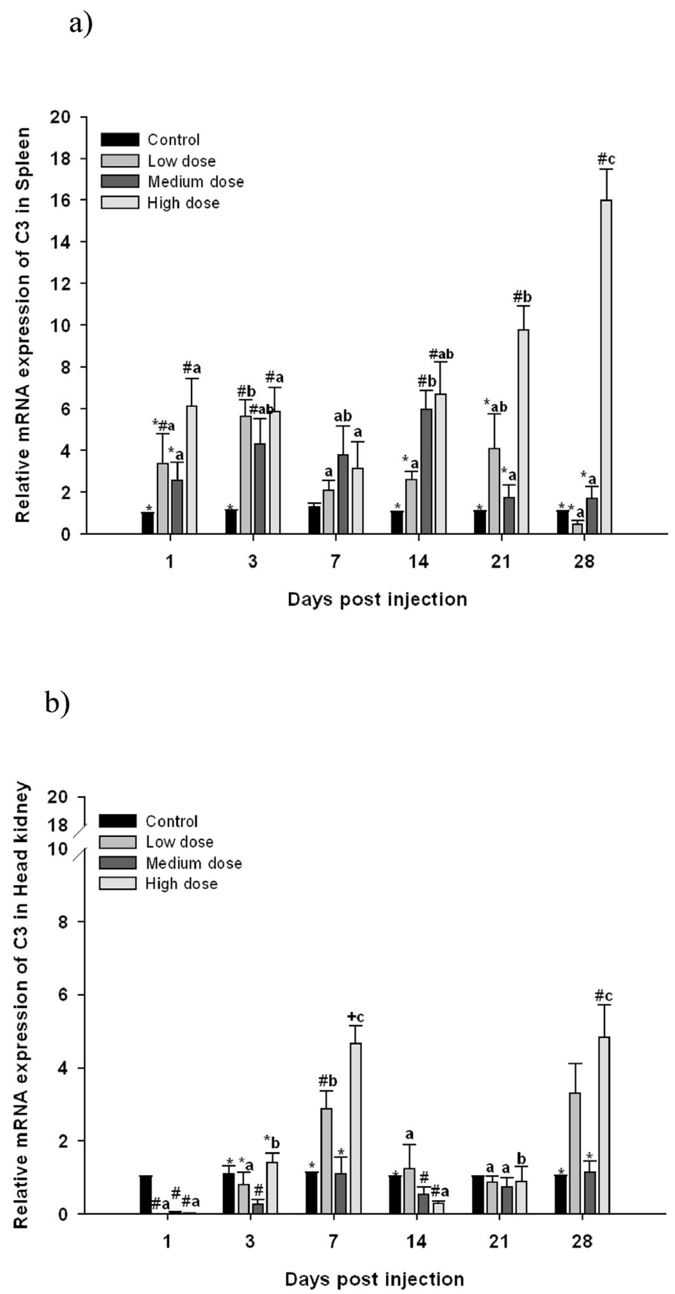

Fig. 4. C3 gene expression analysis in spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different doses of *Francisella noatunensis* 1,5 × 10^1 (▢) 1,5 × 10^5 (▣), 1,5 × 10^{10} (□) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

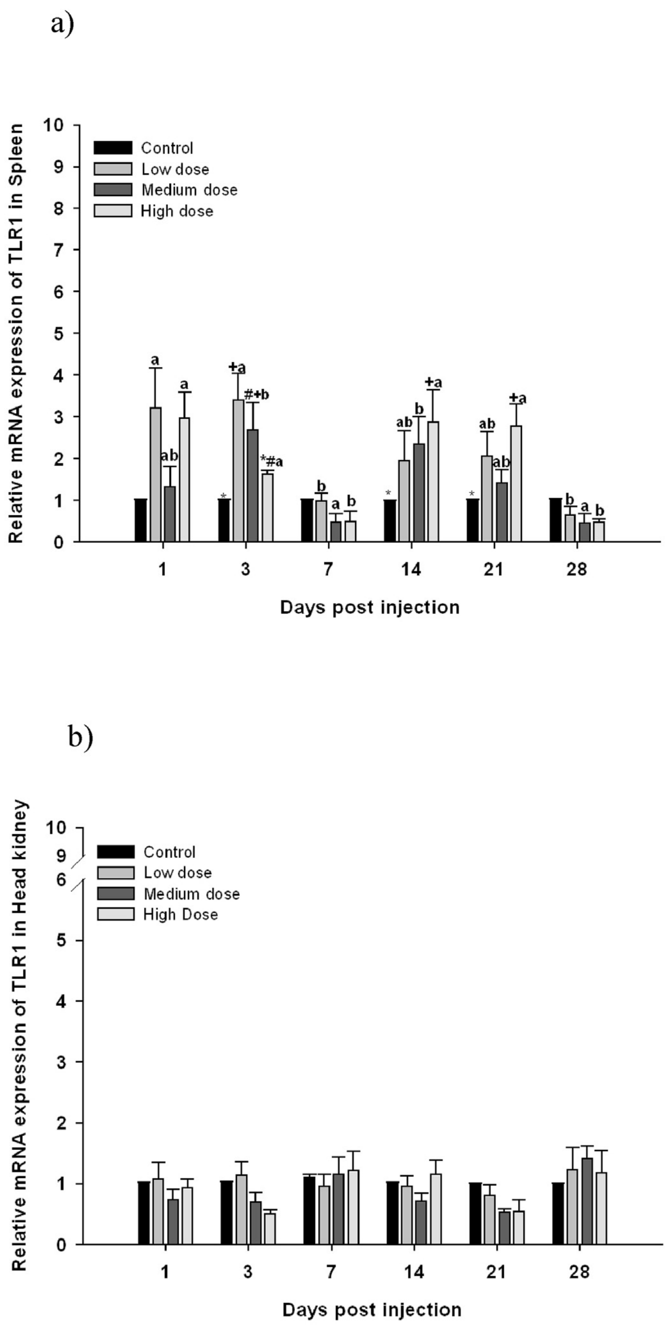

Fig. 5. TLR1 gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▢) 1,5 × 10^5 (▣), 1,5 × 10^{10} (□) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

Subsequently, MHC I expression increased in all 3 doses at day 14; the highest increase was observed in the highest dose which reached an increase of 14 fold which then decreased gradually until day 28 (Fig. 10a). The highest MHC I expression levels were observed in the anterior kidney of the lowest dose during the first three days post injection (Fig. 10b).

MHC II gene expression in the spleen increased with all 3 doses at day 1 pi. By day 3 expression significantly increased in the lowest dose, without major differences in the other doses. On day 7, values dropped to basal levels and later increased significantly in the high dose at day 21, returning to basal values at the end of the challenge (Fig. 11a). In the anterior kidney, expression significantly decreased at day 1 post injection and returned to baseline values at day 3. During the remainder of the challenge no significant differences were observed (11b).

When evaluating the expression of IgMs, overexpression was observed in all 3 doses from day 1 pi. Expression peaked at day 21 pi, observing a significant difference in the medium and high doses, increasing up to 35 times in the latter which was maintained until the end of the challenge (Fig. 12a). In the anterior kidney, the first 3 days pi

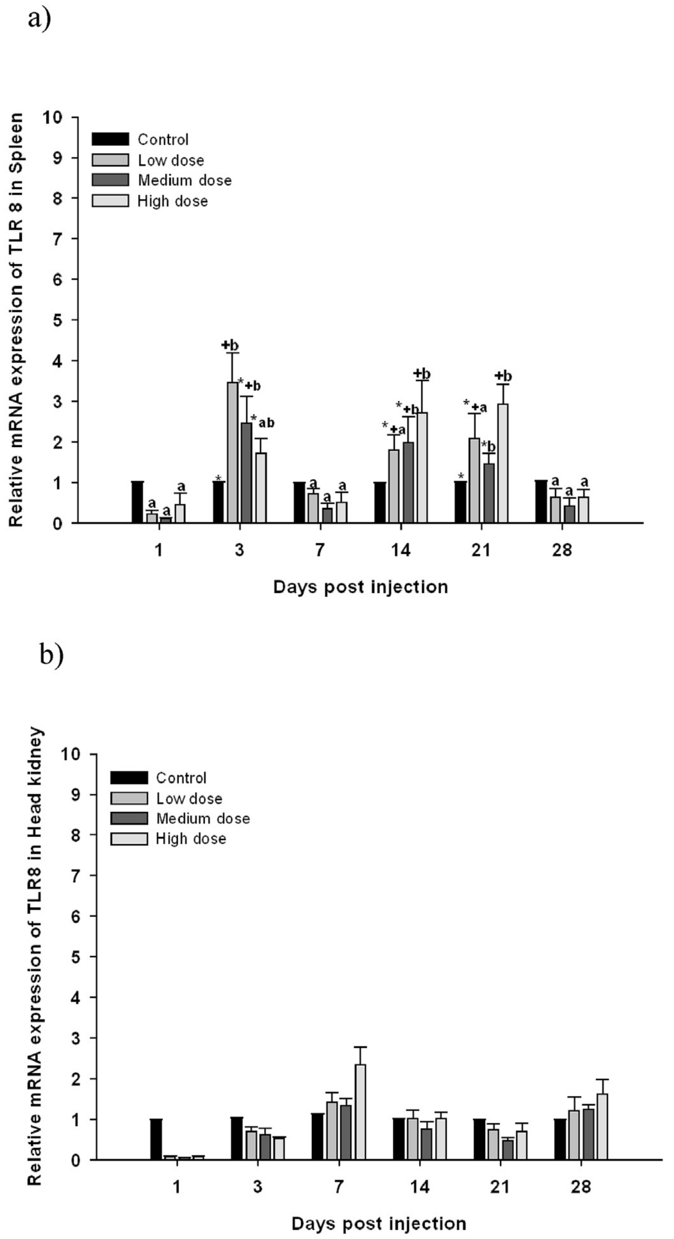

Fig. 6. TLR8 gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* $1,5 \times 10^1$ (▨) $1,5 \times 10^5$ (▩), $1,5 \times 10^{10}$ (☐) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

presented significant differences which were not maintained throughout the challenge (Fig. 12b).

3.5. Plasma IgM levels

Anti-Francisella IgM levels were not significantly different on the first days post injection in all the experimental groups compared to the control group. The lowest dose did not present significant differences throughout the challenge ($P > 0.05$). In the medium dose, IgM levels increased from day 14 post injection, with the highest peak observed on this day, and then gradually fell until day 28; without presenting a significant differences. Meanwhile, fish injected with the highest dose of *Francisella* spp showed an increase in IgM levels from day 7 and presented the highest levels at day 28 post injection (Fig. 13).

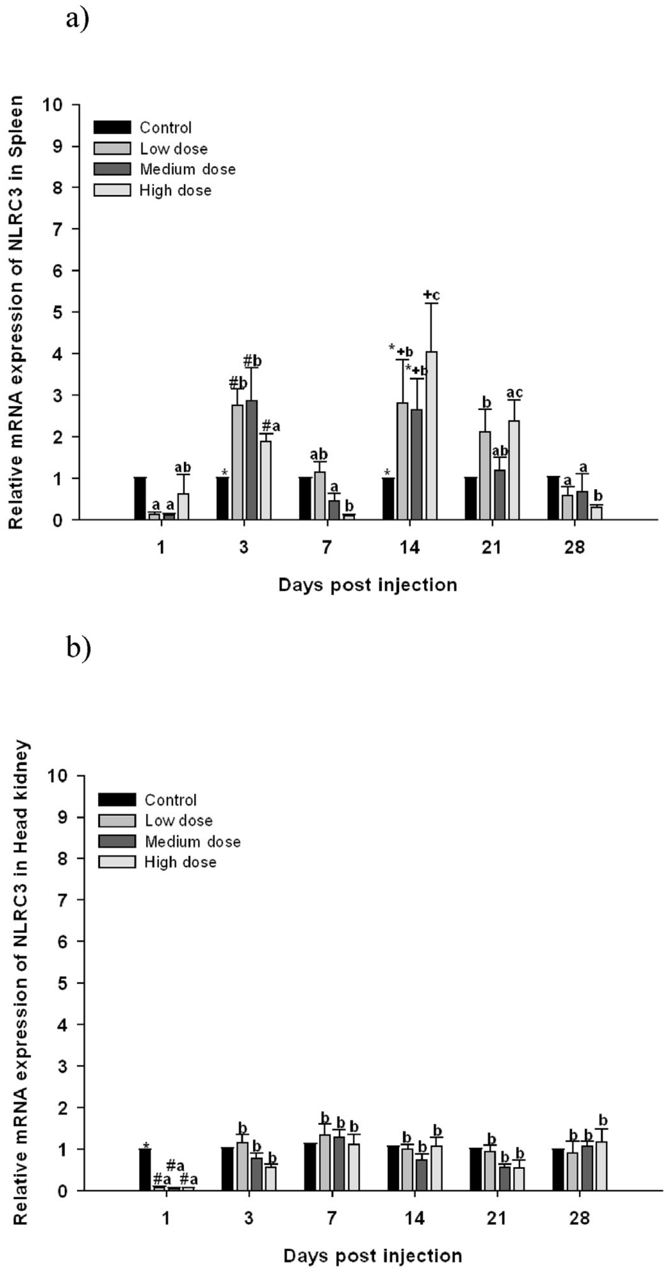

Fig. 7. NLRC3 gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* $1,5 \times 10^1$ (▨) $1,5 \times 10^5$ (▩), $1,5 \times 10^{10}$ (☐) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

4. Discussion

Nowadays, environmental and farming conditions create a propitious scene for the establishment of infectious outbreaks, added to the fact that pathogens like *Francisella* are capable of infecting a great variety of species in multiple latitudes. In this scenario, not only are the farmed species harmed, but also the native adjoining fauna. According to the literature of genes and gene sequences, the presence of *Francisella*

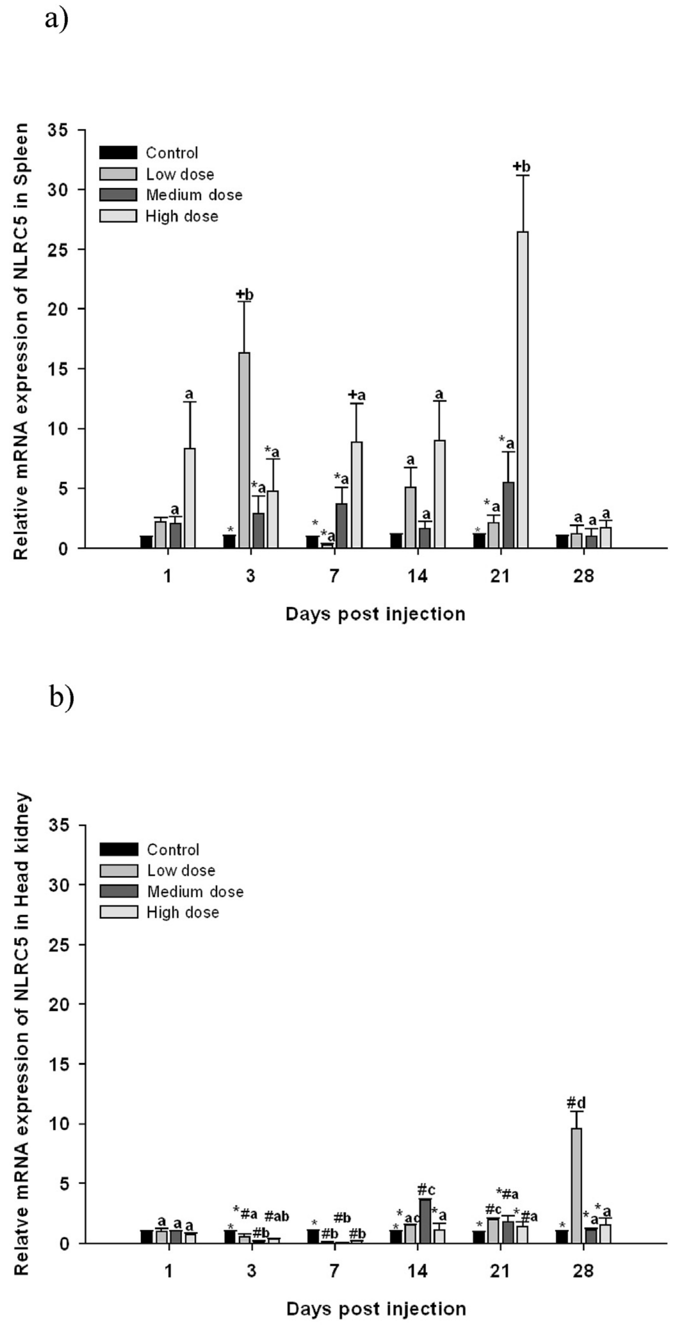

Fig. 8. NLRC5 gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▨) 1,5 × 10^5 (▨), 1,5 × 10^{10} (☐) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

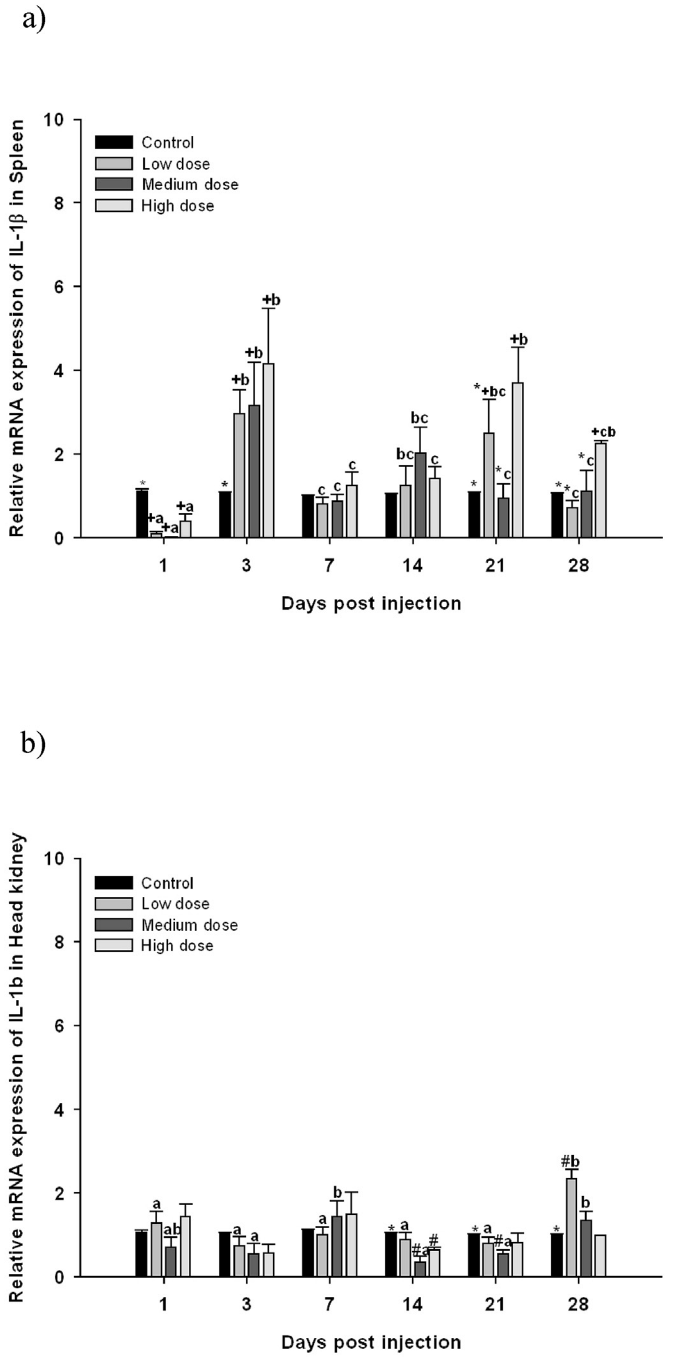

Fig. 9. IL-1b gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▨) 1,5 × 10^5 (▨), 1,5 × 10^{10} (☐) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

and bacterial replication have been detected [39]. Additionally, the immune gene response of *Eleginops* was evaluated to determine if it could respond and eventually eliminate the pathogen in response to infection. Also, external and internal clinical signs were evaluated and the *anti-Francisella* antibody titter was measured. Our results showed increased gene expression in the spleen, with evident repression of IL-1b, NLRC3, and TLR8 at day 1 pi, and the rest of the genes were overexpressed. However in the anterior kidney, the host immune response when faced with *Francisella* infection was poor. No external clinical signs were observed, however the haematocrit showed anaemia only in the high infection dose. Finally, when determining the antibody titre in serum, the lowest dose was not able to generate antibodies and the highest titter was observed in the high dose at day 28 post-infection.

We detected bacterial DNA in the three doses, but only the high bacterial dose induced replication at day 28 in the liver. It is possible that bacterial DNA may have been eliminated in the low and medium doses, or it may have entered a dormant state [40] in which *Francisella*

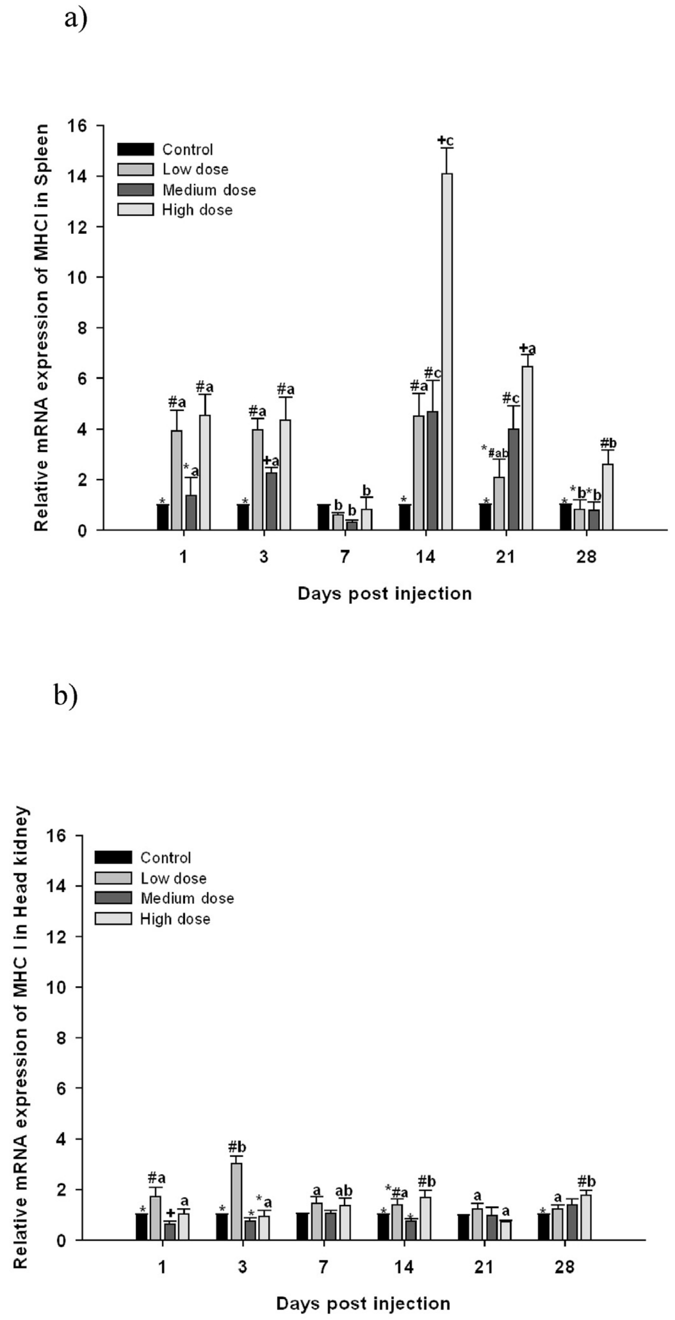

Fig. 10. MHC I gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▤) 1,5 × 10^5 (▩), 1,5 × 10^{10} (□) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, P < 0.05; n = 6).

is no longer virulent [41]; suggesting a direct relationship with the results observed in the haematocrit measurement, where only the high dose generated anaemia. This indicates that this species could be a vector of the infection, since when they are challenged with *Francisella* the bacteria was detected but the pathology was not developed; this has been observed in other species such as the common carp and striped catfish [42].

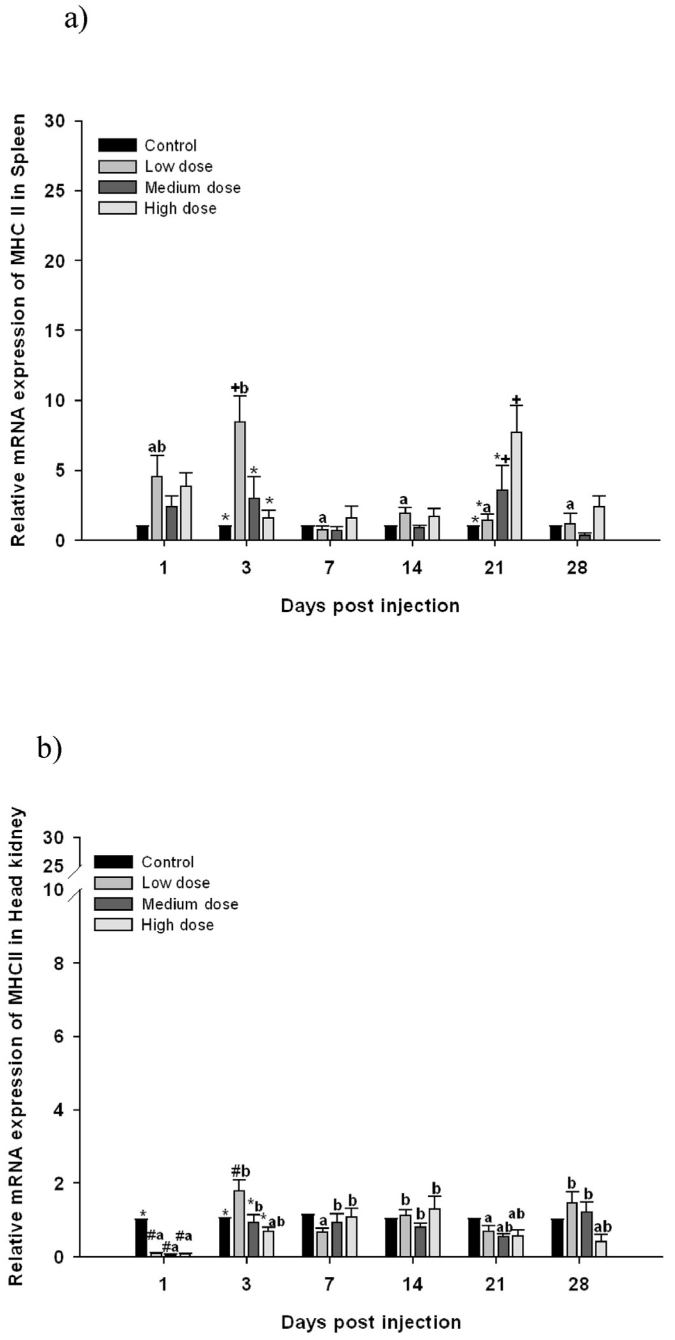

Fig. 11. MHC II gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▤) 1,5 × 10^5 (▩), 1,5 × 10^{10} (□) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, P < 0.05; n = 6).

4.1. *Innate immune response*

C3 is a protein involved in the complement cascade, the beginning of the common complement pathway begins with the cleavage of C3 by a convertase into two fragments (C3a and C3b), thus triggering the membrane attack complex [27,44]. In the spleen, a significant increase in C3 gene expression was observed in all 3 doses on day 1 pi. A gradual increase was observed in the highest dose which could be due to

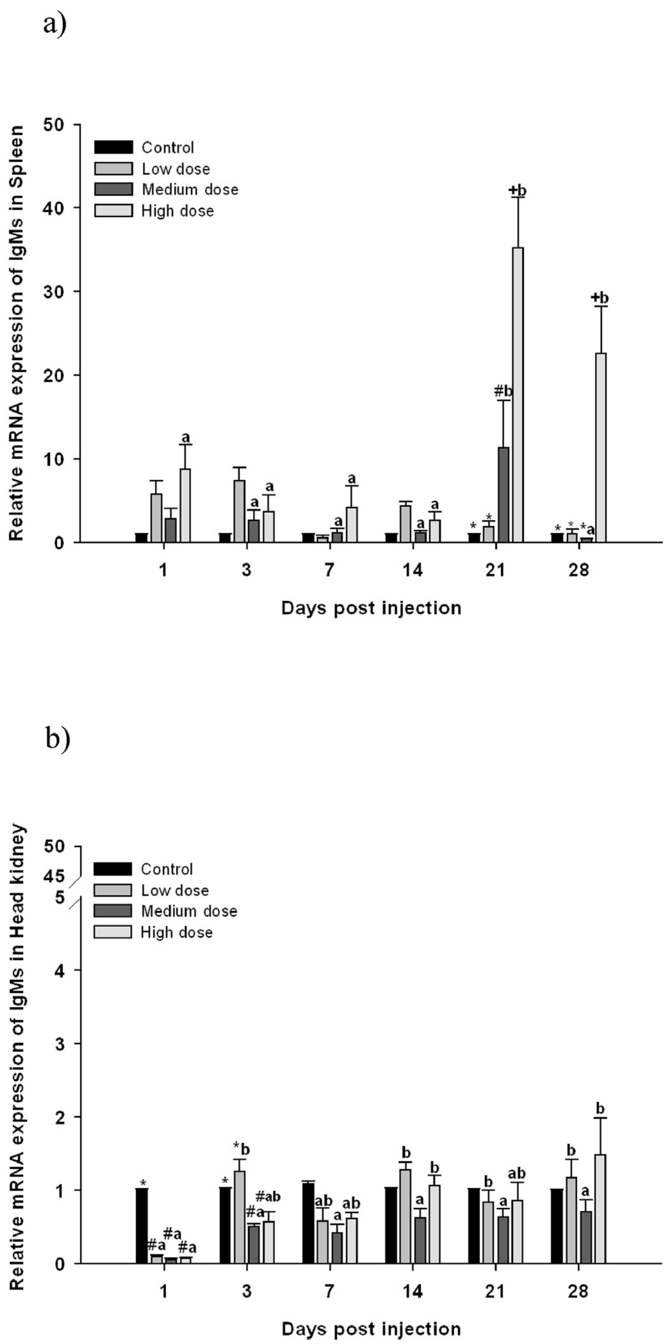

Fig. 12. IgM gene expression analysis in the spleen (a) and head kidney (b) of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▨) 1,5 × 10^5 (▩), 1,5 × 10^{10} (□) for 28 days. Expression analysis of mRNA was performed by real-time qPCR and 18S was used for normalization. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

ongoing bacterial replication (Fig. 3), and therefore, a greater amount of bacteria being opsonized-phagocytized. In the anterior kidney the response takes longer, this may be due to the large number of cortisol receptors, therefore majority of the evaluated genes presented decreased expression [47].

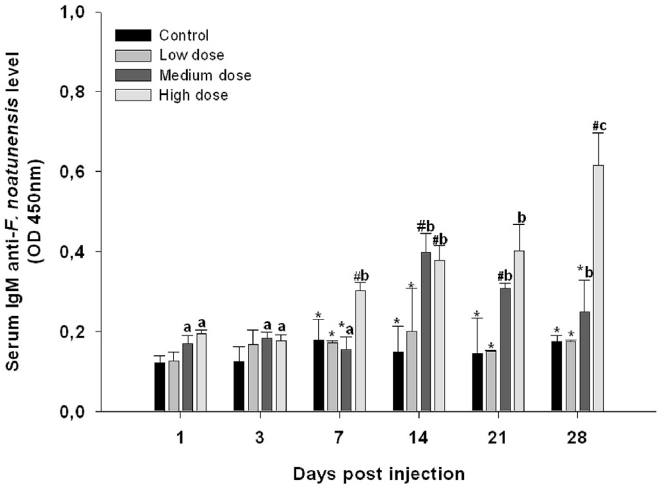

Fig. 13. Total serum IgM levels in of *Eleginops maclovinus* specimens challenged with control (■) and three different dose of *Francisella noatunensis* 1,5 × 10^1 (▨) 1,5 × 10^5 (▩), 1,5 × 10^{10} (□) for 28 days. Symbols over the bars indicate statistical differences between the different treatments at the same time point. Different letters indicate statistical differences in the same treatment at different times (two-way ANOVA, $P < 0.05$; n = 6).

Toll-like receptors are membrane receptors or endosomes which recognize molecular patterns present in pathogens (PAMP) [27]. TLR1 recognizes bacterial lipopeptides and LPS [48], meanwhile TLR8 recognizes single-stranded RNA [49,50]. Our results showed a similar expression pattern that only differed in TLR1 expression, which was overexpressed in the low and high doses on day 1. Since TLR1 is found on the cell membrane and is not endosomic, like TLR8, this could make it difficult for the bacteria to avoid recognition via TLR1; therefore the bacteria can only evade recognition by being inside the cell. In addition, TLR8 and NLRC3 showed a very similar expression pattern, confirming the above.

The Nod-like receptors are intracellular receptors capable of recognizing PAMPs inside the cell. According to our transcriptomic analyses, two transcripts for NOD receptors, NLRC3 and NLRC5 [38,51] were identified in the described species. NLRC3 can regulate the immune response by inhibiting the NFkB transcription factor [52,53], whereas NLRC5 regulates MHCI expression [54,55]. In the spleen, NLRC3 showed an evident expression pattern from day 14, which then gradually decreased until the end of the experiment. Meanwhile NLRC5 was highly increased in high dose, unlike the other doses throughout the challenge, showing early transcription at day 1 after injection. However, NLRC3 expression in the anterior kidney did not show any major difference, whereas NLRC5 did not show any evident expression pattern. Our results are different to those observed in catfish, where the highest NLRC expression levels were observed in the anterior kidney, but not in the spleen [56]. Additionally, little is known about the role of NLRC3 and 5 in the immune response, even in mammals. Additionally, another pro-inflammatory component of the inflammosome to be studied for a more complete understanding is the AIM2 complex, which recognizes dsDNA [57].

Interleukin 1-beta is a proinflammatory cytokine which is key in the innate immune response where it mediates inflammation, coagulation, and synthesis of acute phase proteins. This cytokine is expressed after the recognition of PAMPs by Toll-like receptors and NOD [27]. The immune system is highly influenced by the neuroendocrine system [58], for example, in mammalian osteoblast-like cells there is an inverse relationship between cortisol synthesis and IL-1b expression [59]. In our study, we observed a high amount of plasma cortisol in the high dose throughout the challenge (data not shown), however this declined from day 14 post injection. In the spleen, IL-1b gene expression was significantly different at 21–28 dpi in the high dose. This late expression of IL-1b was described by Mares [60] during infection with *Francisella* in mammals; observing this delay in expression not only in IL-1b but in several immune genes. As suggested by Bencurova et al. [61], *Francisella* has the ability to inhibit NFkB as an evasion mechanism.

4.2. Adaptative immune response

The major histocompatibility complex I (MHCI) is a component of antigen presentation to CD8 + lymphocytes, from intracellular sources (viruses, bacteria) [62]. This receptor is present in all the nucleated cells of the organism [63]. Our results in spleen showed a significant increase in all three doses from 14 dpi, followed by a gradual decrease in expression. However, at day 28 there was still a significant difference in the high dose compared to the control group. Studies in mice challenged with *Coxiella* sp indicate that signalling of the MHCI-CD8 + component critical for controlling bacterial replication during infection [64]. This could explain why we still observe expression of this gene at 28 dpi, since bacteria was still replicating in this dose.

The major histocompatibility complex II (MHC II) is key for presenting antigens to TCD4 + lymphocytes. These antigens are derived from extracellular proteins that are internalized by antigen presenting cells [27]. MHC II is important for the destruction of Extracellular bacteria, its expression is restricted to the surface of macrophages, dendritic cells, and B cells [65,66]. Our results showed an increase in MHC II expression in spleen on day 21, which would be late activation of the adaptive immune system, which subsequently decreased to basal levels. In the anterior kidney no significant differences were observed. Studies indicate that *Francisella* can send the histocompatibility II system to ubiquitination in order to avoid recognition by the immune system [33]; which could be the case in this experimental condition, since MHC II expression decreases in an abruptly and no significant increase is observed.

Teleosts present 3 types of immunoglobulins [22], with IgM being the most prevalent immunoglobulin in plasma [67]. Unlike MHC complexes, B lymphocyte receptors can bind to antigens of varied nature [27], allowing them to recognize various components of the bacterial surface. In the high dose, the increase in IgM expression in the spleen was observed from day 7 and reached a significant increase on days 21 and 28; this was not significant in the anterior kidney. Meanwhile, maximum levels in the medium dose were observed on day 14, decreasing gradually over the remainder of the challenge. This increase on days 7–14 is related to the activation of the adaptive immune system [38,68]. Since there is no active replicating-circulating bacteria (Fig. 2), it is possible that antibodies are not required; therefore the Ac titre decreases during the course of the experimental challenge in the medium dose. Additionally, studies in mice have indicated an increase in the titre of antibodies from 7 dpi, reaching its peak at 7 weeks pi, similar to that observed in the high dose [69]. These antibodies are mainly reactive against LPS [69,70]. Finally, it is important to indicate that when mRNA expression and plasmatic IgM are quantified, there is often an evident discrepancy in the results, since authors such as Vogel & Marcotte [71] indicate that, mistakenly, absolute and relative concentrations are compared. Therefore, we propose that curves or trends should be compared in these cases, in order to obtain a more global view of what is happening in the secondary immune response.

5. Conclusion

In conclusion this is the first study that evaluates the survival of *Eleginops maclovinus* challenged with *Francisella*. According to our results, we can conclude that *Eleginops* is susceptible to infection with *Francisella* since the bacteria was able to replicate and induce initial clinical signs, however, this was only possible with a high dose. More cohabitation tests must be performed to determine if there is horizontal transmission of *Eleginops maclovinus* to *Salmo salar* and vice versa; since this is the most farmed salmonid species in Chile.

Conflicts of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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