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Research Article

Serum-isolated exosomes from *Piscirickettsia salmonis*-infected *Salmo salar* specimens enclose bacterial DnaK, DnaJ and GrpE chaperones

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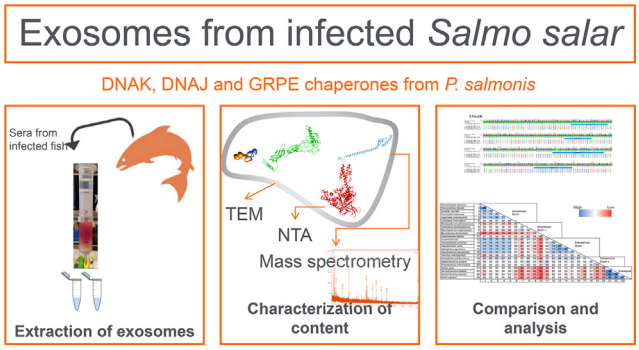
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Endosomally produced by eukaryotic cells, exosomes are microvesicles involved in cell-to-cell communication. Exosomes have shown a wide range of therapeutic potential as a drug or vaccine delivery system, and they are useful as biomarkers in several disease processes. Another biological function described is pathogen dissemination through host-derived molecules released during infection, thus modulating the immune response in the host.

Results: This work characterizes the exosomal fraction recovered from serum of *Piscirickttesia salmonis*-challenged *Salmo salar* specimens and from the corresponding non-challenged controls. Exosomes presented a spherical morphology and particle size distribution within 50–125 nm, showing similar parameters in both groups. The mass spectrometry analysis of exosomes isolated at 14 and 21 d post-challenge showed the presence of peptides corresponding to the three proteins of Hsp70/DnaK chaperone system (DnaK, DnaJ, and GrpE). BLAST search of these peptides showed the specificity to *P. salmonis*. Data are available via ProteomeXchange with identifier PXD023594.

Conclusions: The chaperones were found with >95% identity in the core genome when aligned to 73 genomes of *P. salmonis*. The proteins also showed a high degree of similarity with other microorganisms, where this system has proven to be vital for their survival under stress conditions.

The presence of these three proteins in exosomes isolated from challenged fish sera calls for further study into their potential role in bacterium pathogenicity.

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1. Introduction

Classified as Extracellular Vesicles (EVs) due to their size (30 to 100 nm diameter), origin, and mechanism of secretion, exosomes are produced and released via the endosomal pathway [1], originating as grouped multivesicular bodies (MVBs) inside cells and then released to the extracellular space upon fusion with the cell membrane. These microvesicles envelop the same content as the endosomal compartment from which they emerge [2], comprising lipids, proteins, and RNAs.

The main function of exosomes is cell-to-cell communication [3]. Exosomes have shown a wide range of therapeutic potential as a drug or vaccine delivery system [4,5,6,7] and they are useful as biomarkers in clinical diagnoses of cancer, autoimmune syndromes, and neurodegenerative conditions like Parkinson's and Alzheimer's [8,9,10,11,12]. Another biological function described is pathogen dissemination through host-derived molecules released during infection, thus modulating the immune response in the host [4]. Exosomes can be isolated from various biological fluids (serum, urine, saliva, synovial fluid) using classical techniques like ultracentrifugation, separation by density gradient, commercial kits (using polyethylene glycol precipitation), and size exclusion resins. The later have been used to great success to isolate exosomes with minimal contamination of other EVs or serum proteins, thus rendering one of the best alternatives to obtain clean exosomal fractions compared to the other methods [13,14,15].

Proteomic analysis of exosome content via LC-MS (Liquid Chromatography-Mass Spectrometry) has identified proteins of different origins and functions, including cytoplasmic, nuclear, endosomal, and membrane proteins; as well as those involved in vesicular traffic and exosomal biogenesis; tetraspanins; and, finally, heat shock proteins (HSPs) [5,6,7,8].

These heat shock proteins, also used as predictive markers or therapeutic targets for the treatment of some diseases [16], belong to the family of the chaperones, which regulate the correct folding and assembly of other proteins via its binding to newly synthesized or misfolded proteins by interacting with exposed hydrophobic segments until these proteins reach their proper functional conformation. A subgroup of adenosine 5′-triphosphate (ATP)-dependent chaperones (HSPs) assist in folding nascent proteins, refolding proteins denatured by stress factors and in assembly or disassembly of protein complexes [17,18]. HSPs and other chaperones regulate cellular homeostasis, protect cellular structures, and maintain the integrity and fluidity of lipid membranes [19].

The Hsp70/DnaK system, the most widely studied prokaryote chaperone system, is comprised of chaperone DnaK (Hsp70), co-chaperone DnaJ (Hsp40) and nucleotide-exchange factor GrpE. DnaK assists polypeptide folding, mediated by ATP hydrolysis; co-chaperone DnaJ interacts with non-folded peptides and transfers them to DnaK, concomitantly increasing the ATP hydrolysis rate; and GrpE promotes dissociation of adenosine 5′-diphosphate (ADP) bound to the nucleotide-binding domain of DnaK, thus enabling its replacement by ATP. Both GrpE and DnaJ act jointly in controlling non-folded peptide flow into and out of the substrate-binding domain of DnaK, regulating its conformation and nucleotide binding [20]. The mechanism of action is cyclic, beginning with DnaJ substrate binding, then transferred to a DnaK-ATP complex. Energy released by ATP hydrolysis properly binds the substrate to DnaK in a closed conformation, generating a DnaK-ADP-substrate complex. ADP is then released and replaced by ATP, which promotes substrate release [21]. The ADP release step is mediated by GrpE, which allows DnaK to return to an open conformation, i.e., the initial ATP-binding condition [21].

This vital chaperone system has also been identified in Gram-positive bacteria, such as *Mycoplasma sp*. Furthermore, the bacterial DnaK protein may mediate oncogenic activity through the inhibition of DNA repair and p53 functions [22]. Additionally, GrpE from *Mycobacterium tuberculosis* has been described to induce Th1 immune response via activation of dendritic cells [23]. Also, it has been reported that DnaJ has a role in *Escherichia coli* physiology and is directly involved with the formation of biofilms [24].

Another example comes from *Piscirickettsia salmonis*, a facultative, Gram-negative intracellular pathogen that causes Salmon Rickettsial Septicemia (SRS) or Piscirickettsiosis, a disease affecting the main salmonid species farmed in Chile, including Atlantic salmon (*Salmo salar*) [25]. Infectious diseases are the main causes of mortality in *S. salar* farms, responsible for up to 22.9%; and, of these deaths, Piscirickettsiosis (SRS) cases amounted to 47.6% in 2019 (Sernapesca, informe Sanitario 2019).

P. salmonis uses an arsenal of molecules during infection, including virulence effector proteins [26], small RNAs, outer membrane vesicles (OMVs) [27], and exosomes [28,29]. These latter facilitate the infectious process and allow the intracellular multiplication of the pathogen by means of modulating the host cell's behavior, specifically conferring a distinct pathogenicity and virulence to the bacterium. This characteristic makes them an ideal target for the design of novel preventive or therapeutic alternatives.

P. salmonis Hsp's proteins, GroEL (Hsp60) and DnaK (Hsp70), have been identified and then cloned and expressed as recombinants for potential use as vaccines [30]. Possessing immunogenic potential, these proteins have also been found in membrane vesicles (MVs). Several bacteria also require the DnaK system for their survival under stress conditions; during infection, Hsp's genes are activated to protect the cell machinery from the host defense mechanisms and thus strengthen virulence [31].

Based on the above, this paper reports on the identification of bacterial proteins present in the content of exosomes isolated from sera of *P. salmonis*-challenged (and non-challenged) Atlantic salmon specimens via mass spectrometry. To do so, purified exosomes at different stages of a 30-day-kinetic analysis during the infection period were obtained to determine if the Hsp70/DnaK chaperone system is selectively enriched, and therefore estimate its potential in pathogen virulence.

2. Materials and methods

2.1. Ethics statement

In vivo assays and sampling were carried out for veterinarians in accordance with the standards and norms defined by the responsible entity in Chile, SERNAPESCA, and in compliance with the procedures and standards established at "The Aquatic Animal Health Code" chapter 7 of the World Organization for Animal Health (OIE) (registered with folio No. 22522 of the National Aquaculture Registry code SERNAPESCA N° 050034).

Fig. 1. Work flow for collection of exosomes. Serum is loaded onto IZON qEV size-exclusion columns (1) and eluted with sterile PBS separating large EVs (2A), exosomes (2B) and serum proteins (2C). Characterization is then performed on the exosomes and their RNA and protein content (3).

2.2. Fish husbandry and challenge

A total of 30 Salmo salar smolted individuals (average 35 g) were admitted to the Curauma Aquaculture Research Center, in hermetically closed containers with forced oxygenation (according to the Exempt Resolution No. 64 of 2003, of the Chilean National Service of Fishing and its modifications) and subjected to acclimatization for 5 d. On day six, the fish were inoculated by intraperitoneal injection in the central abdominal cavity taking care not to damage the internal organs and the anesthetic Kalmagin® 20% (Centrovet Virbac) was used as a painkiller, by submersion, according to the product specifications. Two different formulations were used: 1) 10^4 cfu/mL *Piscirickettsia salmonis* cells (genogroup EM90 Genbank access N °CP039214), in 500 μL of Marine Medium (24 individuals); and 2) 500 μL of Marine Medium (six individuals), as uninfected control. The challenge was set for 28 d; fish health and behavior were monitored daily. The fish were fed standard commercial feed pellets according to size considering a relationship of 1.5% weight/body. Of the challenged individuals, groups of six were selected at four different challenge times post-inoculation: 7 d (T_0), 14 d (T_1), 21 d (T_2) and 28 d (T_3) for collection of serum. Controls were kept until 28 d (T_3) post-inoculation. Upon each point of time and until the end of the challenge, fish are sacrificed for visualization of clinical signology in internal organs, extraction of total blood and of liver for RNA extraction (six individuals for each time point).

2.3. Collection of serum samples

Total blood was extracted from *P. salmonis*-challenged *S. salar* individuals at all four challenge times following initial inoculation with the pathogen, and from control at T_3. Blood samples were left at 4°C for 30 min to allow coagulation and then centrifuged at 500 × G for 15 min at 4°C to obtain serum. Sera from the six individuals under each condition were pooled to obtain up to 3 mL.

2.4. Exosome isolation

Serum pool (approximately 3 mL per time point) was pre-cleared in two stages: first at 1,500 × G for 10 min at 4°C and then at 10,000 × G for 10 min at 4°C, collecting supernatant after each stage. Resulting pre-cleared serum was loaded onto qEV size exclusion columns (IZON Science) pre-equilibrated with 0.22 μm filtered PBS (Gibco). Immediately, 0.5 mL fractions were collected, eluting with filtered PBS. Samples were then concentrated to 100 μL using Amicon® Ultra-0.5 mL 3 kDa MWCO centrifugal filter units (Merck Millipore). Fig. 1 summarizes this workflow.

2.5. Nanoparticle tracking analysis (NTA)

Exosome size distribution was analyzed in a Nanosight NS500 system (Nanosight Ltd.). Data were acquired using the NTA v1.3 software to calculate particle hydrodynamic diameters. From each sample, three videos, each of 30 s duration, were captured with a sCMOS camera, with a green laser shutter set at 607 ms and camera gain set at 400. Analysis was performed for control and challenged samples at 28 d.

2.6. Transmission electron microscopy (TEM)

Exosome samples were negative-stained for TEM analysis by loading 10 μL onto copper grids for 1 min and adding 10 μL of 4% (w/v) uranyl acetate in methanol for 1 min. Excess solution was removed by contacting the grid edge with filter paper. Grid was then incubated for 10 min at 37°C. The grids were examined under a Philips Tecnai 12 electron microscope (Biotwin) at a range of 12 to 80 kV. Images were acquired using FEI Software v 2.1.8 (FEI Company). Analysis was performed for control and challenged samples at 28 d.

2.7. RNA extraction

RNA extraction was performed using a mixed protocol with TRIzol® RNA isolation reagents (ThemoFisher) and the E.Z.N.A Total RNA kit (Omega Biotek). Briefly, a 10-mg portion of liver was taken from each infected fish and homogenized with 750 μl of TRIzol®, after 10 min of incubation, 250 μl of chloroform was added and centrifuged at 12,000 × g for 10 min at 4°C. Subsequently, the aqueous fraction was recovered and mixed with 700 μl of absolute ethanol and then loaded onto a column of the E.Z.N.A Total RNA kit, followed by purification according to the manufacturer's instructions. Finally, all samples were quantified in a Nanodrop-1000 spectrophotometer and kept at −80°C until use.

2.8. qRT-PCR assay

In general, a qRT-PCR analysis was performed using the methodology proposed by Flores-Herrera et al. with validated modifications to transform it in real time [32]. This methodology was aimed at the detection of the ITS gene, using the Brillant III Ultra-Fast SYBR® Green QRT-PCR Master Mix kit (according to the manufacturer's recommendations). Elongation factor (Elf) served as the housekeeping gene.

2.9. Extraction and quantification of exosome proteins

Proteins were extracted from 80 μL of EV concentrate, adding 320 μL of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% w/v SDS, 1% v/v Triton X-100, and 1x Roche Protease Inhibitor Cocktail). Samples were incubated on ice for 30 min and sonicated on ice bed using a B550 Ultrasonic Processor with 10 s pulses and 10 s rest at 20% amplitude for a total of 3 min, and then centrifuged at 10,000 × g for 30 min at 4°C. Supernatant was collected. Total protein concentration was quantified using Qubit Protein Assay Kit in a Qubit® 4.0 Fluorometer (Invitrogen) according to the manufacturer's instructions.

2.10. Western blot

Protein extract was loaded onto a 12% Criterion™ TGX™ Precast Midi Protein Gel (Bio-Rad) polyacrylamide gel under denaturing conditions (run at 300 V for 20 min) and the resulting bands were transferred to a 0.2 μm nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System (Bio-Rad) according to the manufacturer's instructions. Membrane was blocked with 5% p/v non-fat milk in PBST 0.05% for 2 h at RT. Mouse anti-salmonid MHC-II was prepared at 1:1000 dilution and was then added at 37°C for 1.5 h. Membrane was washed thrice with PBST 0.05%. Goat anti-mouse IgG (H + L) antibody (Thermo Scientific) was added in 1:7000 dilution and kept at 37°C for 1 h under mild agitation. Membrane was washed thrice with PBST 0.05%. Bands were visualized with Westar Supernova chemiluminescent substrate (Cyanagen).

2.11. Preparation of protein samples for LC-MS

Exosome protein samples were twice dialyzed against Digestion Buffer (10 mM $CaCl_2$, 100 mM Tris-HCl, pH = 8.0) using a 3.5 MWCO dialysis membrane (Thermo). Disulfide bonds were reduced with 5 mM DTT (Winkler) at 60°C for 20 min. After cooling to room temperature, free cysteinic thiols were alkylated with 15 mM Iodoacetamide (Sigma) for 15 min at RT, protected from light. Finally, 1 μg of sequencing grade chymotrypsin (Promega) was added per 200 μg of protein sample and digestion performed for 20 h at 25°C. Digested samples were desalted in Sephadex G-10 (Sigma) and lyophilized.

2.12. LC-Ms

Lyophilized peptides were resuspended in 100 μL of LC-MS grade water with 0.1% trifluoroacetic acid, transferred into a glass vial and placed into a Bruker Elute UHPLC Autosampler and kept at 4°C. Peptide separation was achieved using a C18 Shim-pack GIST column (3 μm, 1 mm × 250 mm) at 55°C using a gradient of LC-MS grade water with 0.1% formic acid (Solvent A) and LC-MS grade acetonitrile with 0.1% formic acid (Solvent B). A total of 10 μL of the sample was injected into the column and subjected to a 75-min gradient (10% B for 5 min, and then a linear gradient to 50% B for 55 min) at a flow of 50 μL/min. For MS/MS data acquisition, a Bruker compact QTOF mass spectrometer with an Elute UHPLC was used in positive mode, 4 kV spray voltage, 0.9 bar nebulizer gas, 6 L/min drying gas at a drying temperature of 200°C, a spectra rate of 3 Hz, and a cycle time of 3 s.

2.13. Proteomic analysis

Trans Proteomic Pipeline (TPP, v. 5.0.0) software was used for protein identification using acquired peptide signals. Carbamidomethyl was used as a fixed modification and included Methionine Oxidation, Sodium adduct, and deamination of Asparagine and Glutamine residues as optional modifications. A BLAST [33], search using the blastp suite, was performed with the resulting MS/MS peptides corresponding to the Hsp70/DnaK chaperone system to determine specificity to the EM90 *P. salmonis* strain. Raw mass spectrometry data were analyzed using Bruker DataAnalysis 4.4, general identification analysis was performed using MaxQuant v1.6.17.0 and specific identification was performed using Trans-Proteomic Pipeline v5.0.0.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [56] partner repository with the dataset identifier PXD023594.

2.14. Data analysis

An internal database was built with the data of the 73 genomes available for *Piscirickttesia salmonis* (Table S1) with the Geneious package 9.1.8 (https://www.geneious.com), and the tblastn algorithm [33] was used to search for the corresponding identified proteins. The obtained hits were used to perform a multiple alignment for each protein, which were compared among the different defined genotypes. Sequences of Dnak, DnaJ and GrpE for different organisms were obtained from Uniprot and NCBI databases, and aligned in CLC Mainworkbench 6.9.2 to obtain pairwise comparisons, and multiple alignments.

P. salmonis protein sequences were modeled through I-Tasser server [34], and the structures were analyzed with Chimera [35].

3. Results

3.1. Exosome isolation and characterization

To assess potential differences, exosomes were isolated from sera of *P. salmonis* challenged and non-challenged *S. salar* after 28 d and then characterized (Fig. 1).

Given the small size of sampled fish, recovery of the exosomal fraction from limited serum volume was also limited. Therefore, to obtain a detectable and sufficient quantity of exosomal proteins, a pool testing approach was used: sera from six individuals in each condition were pooled for exosome isolation and the resulting exosomal fraction was concentrated. Only then was a detectable protein quantity available for further analysis. Correspondingly, mass spectrometry was chosen as the ideal technique to analyze the samples, as detection would be difficult by other standard techniques [36,37,38,39].

NTA characterization showed mean and mode size distribution values, respectively, of 124.6 nm and 106.8 nm in controls (Fig. 2A); and 126.4 nm and 99.0 nm, in challenged fish (Fig. 2B). Concentration (particles/mL) was 6.62×10^8 in controls and 7.08×10^8 in challenged fish. TEM images show spherical particles delimited by a lipid membrane at a size range of 50–125 nm (Fig. 2C and 2D).

Size distribution results as assessed by NTA confirmed that these isolated EVs are in the range of 40–125 nm supporting reports in the literature for exosomes [40,41,42] and further confirmed by TEM imaging. Between control-derived exosomes and challenged-derived exosomes at the 28-d post-infection mark, there was no relevant difference in particle size, morphology, or concentration (ranging from 4.5 to 5.5 $\times$ 10^8 particles/mL), confirming previously reported analyses [31].

3.2. Western blot

Exosomal protein extracts were resolved by denaturing SDS-PAGE and then analyzed by Western blot to detect MHC-II, one of several markers that are found in exosomes, denoting their antigen-presenting cell origin. We found a specific band around the 60 kDa marker, which corresponds approximately to the full MHC-II complex (Fig. 2E), seemingly resistant to a standard denaturation treatment with Laemmli Buffer.

3.3. qRT-PCR

To assess systemic infection of the individuals by *P. salmonis,* RNA from liver samples was extracted and the presence of the ITS gene was analyzed. The results indicate that bacterial ITS is present in all samples tested (Fig. 3) from T_0 to T_3 while pooled liver samples of non-challenged fish introduced as a negative control did not show bacterial ITS presence. This, added to clinical signology observed (Fig. 4), confirms a successful challenge and progression of the infection.

3.4. Mass spectrometry and proteomic analysis

Analysis of exosomal protein extracts collected at 7 (T_0), 14 (T_1), 21 (T_2) and 28 (T_3) d post-inoculation was carried out by ESI-MS/MS and resulting peptide sequences were compared against the CDS of 36 EM90 strains of *P. salmonis,* which is the most aggressive genogroup found in field samples [43]. Previous works have detected DnaK in this fraction along with the chaperones [27].

Indeed, the three expected proteins of the Hsp70/DnaK chaperone system, for all strains, were identified in T_1 and T_2 (14 and 21 dpi) challenged individuals in this initial search. Conversely, *P. salmonis* chaperone peptides were not found in any control sample, nor in T_0 and T_3 of the challenged samples. The peptide sequences obtained by MS/MS analysis and their corresponding protein matches are summarized in Table S1.

3.5. Core genome analysis

For further confirmation, Hsp70/DnaK system proteins were compared against those of the 73 available *P. salmonis* genomes, which are grouped into three main genogroups: LF, EM, and NC (Norway-Canada). The EM genogroup is further subdivided into three subgroups, EM1, EM2, and EM3. Based on this information, a core genome was defined (based on results from a grant project)

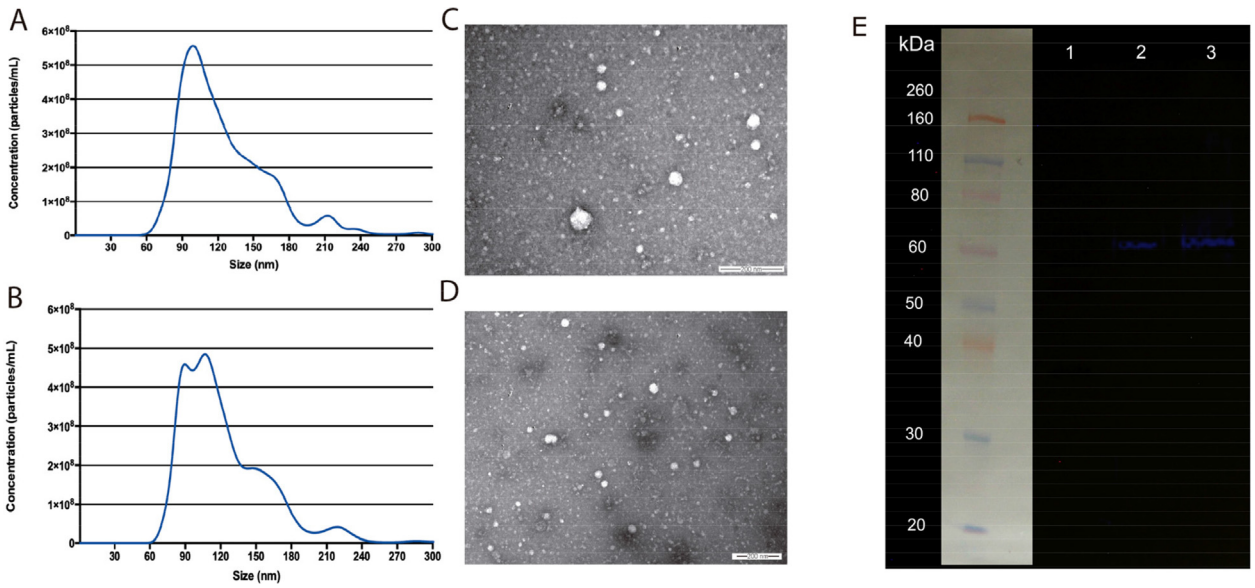

Fig. 2. Size distribution by Nanoparticle Tracking Analysis, Transmission Electron Microscopy and Western blot characterization of exosomes. (A)(C) NTA and TEM for exosomes derived from control *Salmo salar* specimens. (B)(D) NTA and TEM for exosomes derived from challenged *Salmo salar* specimens. (E) Western blotting of exosome proteins using salmon MHCII as a marker. Line 1: molecular weight markers (overlaid on the left from viewing), Line 2: exosome proteins from fishes challenged with *P. salmonis* (strain EM90), Line 3: Positive control (CP) fish serum.

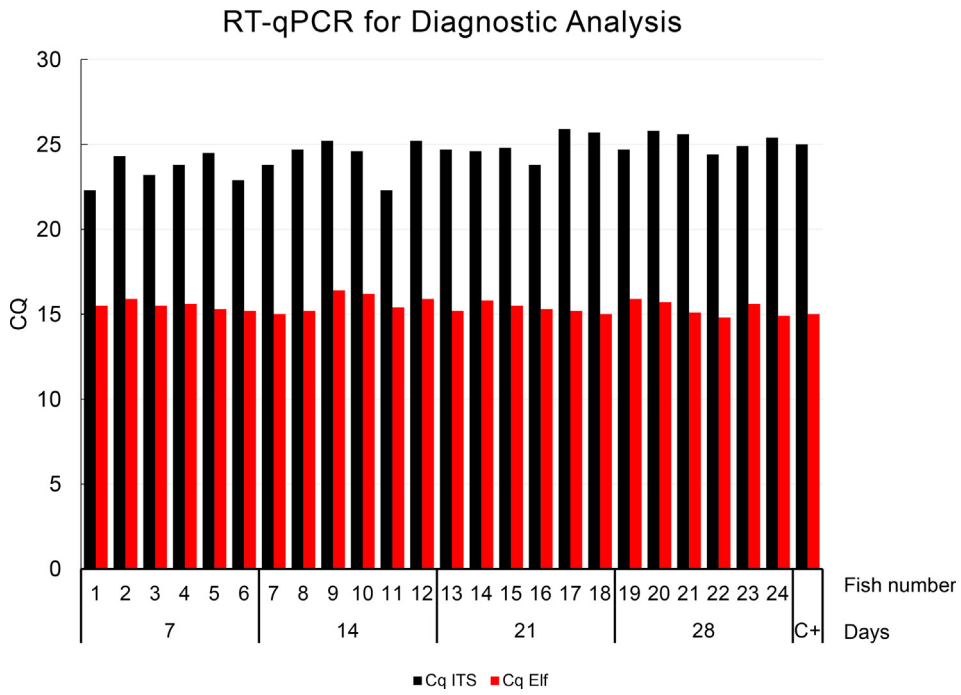

Fig. 3. qRT-PCR SybrGreen ITS *P. salmonis*. Cq for all challenged individuals from T_0 = 7 d to T_3 = 28 d compared to a housekeeping gene (Elf) and to a positive control.

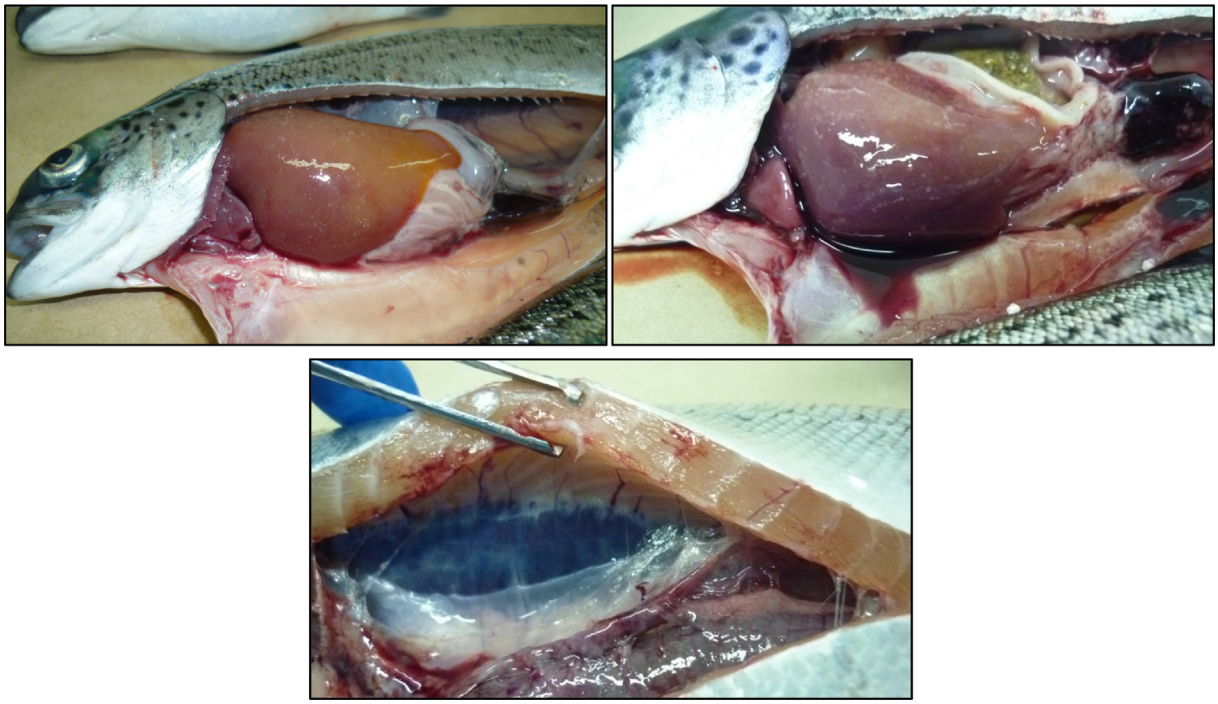

Fig. 4. Clinical signology of *P. salmonis* infection. Images show the signs of systemic *P. salmonis* infection of *Salmo salar* (A). Note white nodules in the liver (A), bloody ascites (B) and renomegaly (C).

and the DnaK system was localized in it, a schematic view is shown based on the isolate Psal104a (Genome NCBI accession CP039214) (Fig. 5).

To analyze conservation among genomes, a consensus DNA sequence generated for each main genogroup was used to construct a multiple alignment and establish identity (Fig. S1).

The BLAST search showed that the three proteins (DnaK, DnaJ, and GrpE) were present in the 73 *P. salmonis* genomes (Table S2), which means that it is part of the core genome (identity >95%, in all cases). Furthermore, as expected, identity increases to 100% for all EM90 strains. These results are summarized in Table 1.

Since most chaperone systems are ubiquitous, with constitutive proteins across species, consensus protein sequences were additionally compared to several species in order to establish conservation and active site homology. The comparison showed that among the three components, DnaK is the most conserved among all

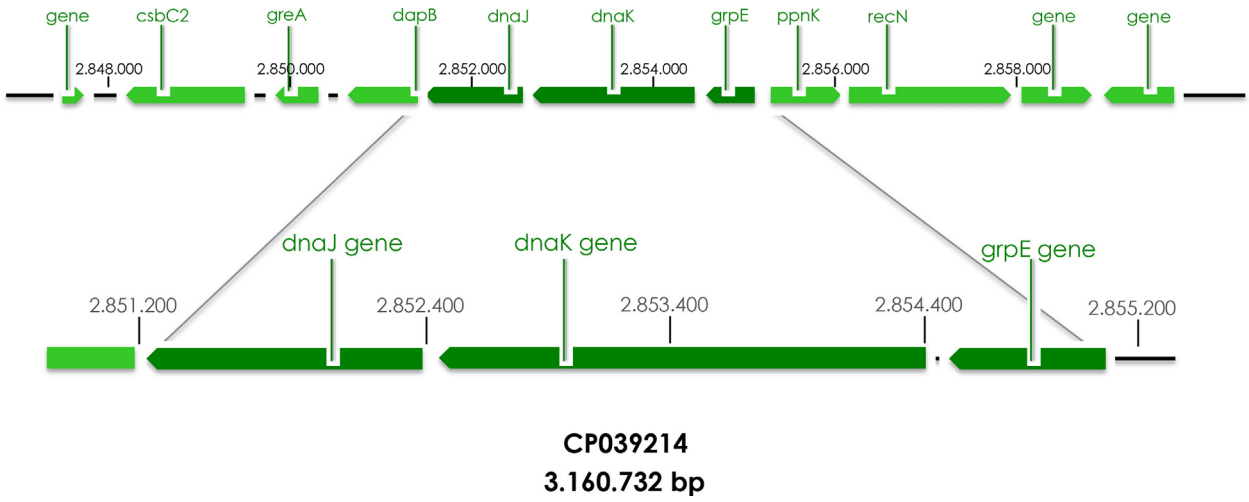

Fig. 5. Schematic view of the HSP70 system. A fragment of the genome of *P. salmonis* isolate p104a is shown, where the three genes corresponding to the HSP70 system are located.

organisms, followed by Dnaj and finally GrpE, which presents the greatest variation (Fig. 6 and Fig. S2). Table 2 summarizes the identity of the three proteins between the species compared.

This protein similarity suggests that assessing the specificity of peptides identified in the exosomal fraction is necessary. Thus, reported peptides were also searched in the blastp suite to determine whether they are specific to EM90 strains of *P. salmonis*. Peptide sequences were then classified into three categories: *P. salmonis* EM90 specific (Table 3), *Piscirickettsia spp*. specific (Table S3) and general bacterial sequences (Table S4).

The results indicate that at the T_1 and T_2 post-infection markers, two peptide sequences show a single match in *Piscirickettsia litoralis* (Table S3). Additionally, almost all peptides were found with 100% conserved sequence in all *P. salmonis* genogroups according to the 73 genomes compared. The exception is DnaK peptide 1, with differences between the EM genogroup and the other two genogroups (LF and NC) (Table 2 and Fig. S3).

4. Discussion

DnaK, the central chaperone in the Hsp70 chaperone system, shows high conservation at an evolutionary level [44], and in the case of *P. salmonis,* an identity of more than 50% is found with organisms of different classes (Fig. 6). Likewise, its cochaperones DnaJ and GrpE, with a high conservation between intracellular bacteria, both Gram- and Gram + (Fig. 5, Fig. S2-S6). Sequence analysis of DnaK shows the same domains defined for other bacteria such as *E. coli*, and structural homology modeling results in structures very similar to those experimentally determined for *E. coli* (Fig. 7). These facts together suggest that the role of these chaperones in *P. salmonis* may be similar to that of other pathogenic bacteria.

Experimentally, peptides of the Hsp70/DnaK chaperone system, specific to the EM90 *P. salmonis* strains, were detected in the exosome content of challenged *S. salar* individuals, at 14 and 21 d post-challenge, but not earlier or later (7 and 28 d). Given these results, it is clear uninfected controls are needed for 14 and 21 d to solidify these results, although bacterial Hsp70/DnaK peptides specific for the EM90 strain of *P. salmonis* should not appear in any uninfected fish. Initially, a 28-d uninfected control was considered due to infrastructure limitations for the assay.

Although proteomic analysis only showed the presence of peptides from the Hsp70/DnaK system at intermediate times of infection, analysis of liver RNA evaluated by qRT-PCR (Fig. 3), the primary target organ in Salmon Rickettsial Septicaemia (SRS), clearly detects bacterial RNA, according to the guidelines established by the laboratories of reference for the diagnosis of infection with *P. salmonis* in salmonids [32].

The interpretation of these results falls under two possible scenarios: the first one indicates that the Hsp70/DnaK chaperone system supports bacterial proteostasis under the stress conditions generated by the host cell defense mechanisms [31,45], and therefore, its export via exosomes affects bacterial survival. This would be the case when antigen-presenting cells (*e.g.*, macrophages), enriched during the middle stages of infection, are the sources of the exosomes. This alternative is supported by the presence of the MHC-II marker in the isolated exosomes (Fig. 2E). The infective process of *P. salmonis* involves the interference of the phagosome-lysosome fusion as an evasion mechanism [26,46,47], thus, exporting these proteins in exosomes may be a feasible alternative strategy of the host cell to control the infection.

Table 1
Gene location of the DnaK system proteins in *P. salmonis* genomes.

Protein	Protein name	Location	%ID by genogroup	
DnaK	Heat shock protein Hsp70	Core genome	EM	**100**
			LF	**99.5**
			NC	**99.5**
DnaJ	Heat shock protein Hsp40	Core genome	EM	**100**
			LF	**98.9**
			NC	**99.5**
GrpE	Heat shock protein	Core genome	EM	**100**
			LF	**95.1**
			NC	**98.5**

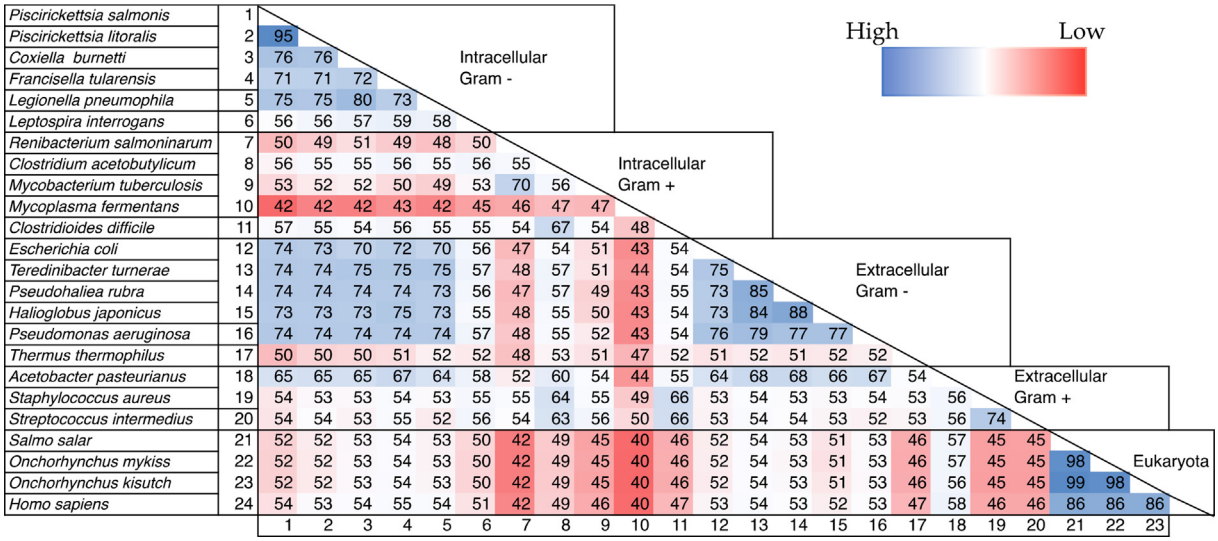

Fig. 6. Identity matrix for the DnaK protein of *P. salmonis* in comparison with the organisms in Table 2. The proteins of each species indicated in the left column are ordered numerically from 1 to 23 according to the type of organism, as indicated in the upper part of the triangular matrix. The highest percentages of identity are in blue and the lowest in red (according to the indicated scale). Values greater than 70% with respect to *P. salmonis* are indicated in bold. The highest identity with *P. salmonis* is indicated in bold.

Table 2
Comparison of the EM90 DnaK system with other organisms, expressed as percentage of identity. Accession number of the compared proteins are in Table S3.

Species	DnaK	DnaJ	GrpE	Class	Type	Lifestyle
Piscirickettsia salmonis	100	100	100	Gammaproteobacteria	Gram –	Intracellular
Piscirickettsia litoralis	94.9	92.7	80.9	Gammaproteobacteria	Gram –	Intracellular
Coxiella burnetti	75.7	59.8	32.1	Gammaproteobacteria	Gram –	Intracellular
Francisella tularensis	71.0	53.9	34.4	Gammaproteobacteria	Gram –	Intracellular
Legionella pneumophila	74.9	62.7	36.0	Gammaproteobacteria	Gram –	Intracellular
Leptospira interrogans	56.1	48.2	4.2	Spirochaetia	Gram –	Intracellular
Renibacterium salmoninarum	48.9	38.1	5.5	Actinomycetia	Gram +	Intracellular
Clostridium acetobutylicum	54.7	44.3	6.0	Clostridia	Gram +	Intracellular
Mycobacterium tuberculosis	51.6	37.0	6.8	Actinobacteria	Gram +	Intracellular
Mycoplasma fermentans	42.2	14.3	7.2	Mollicutes	Gram +	Intracellular
Clostridioides difficile	55.4	49.4	7.3	Clostridia	Gram +	Intracellular
Escherichia coli	72.9	62.1	7.6	Gammaproteobacteria	Gram –	Extracellular
Teredinibacter turenrae	74.2	65.5	6.4	Gammaproteobacteria	Gram –	Extracellular
Pseudohaliea rubra	74.2	63.9	7.9	Gammaproteobacteria	Gram –	Extracellular
Halioglobus japonicus	73.4	63.7	9.0	Gammaproteobacteria	Gram –	Extracellular
Pseudomona aeruginosa	73.9	65.3	6.9	Gammaproteobacteria	Gram –	Extracellular
Thermus thermophilus	49.6	14.3	14.9	Deinococci	Gram –	Extracellular
Acetobacter pasteurianus	64.8	48.2	6.2	Alphaproteobacteria	Gram +	Extracellular
Staphylococcus aureus	53.0	43.4	8.1	Bacilli	Gram +	Extracellular
Streptococcus intermedius	53.6	43.9	6.9	Bacilli	Gram +	Extracellular
Salmo salar	51.8	12.4	8.5	Actinopteri	–	–
Onchorhynchus mykiss	51.7	12.4	5.7	Actinopteri	–	–
Onchorhynchus kisutch	51.7	12.9	8.1	Actinopteri	–	–
Homo sapiens	53.2	12.4	8.5	Mammalia	–	–

Table 3
P. salmonis EM-specific peptides for DnaK system proteins (DnaK, DnaJ and GrpE).

	#	T_1	T_2
DnaK	1	SVSDVTDVILVGGQTRMPKAQEVVKNFF	SVSDVTDVILVGGQTRMPKAQEVVKNF
	2		QELVGARNNAEAMIHATEKGL
	3		APPQISAQVLAKMKKTAEDYL
DnaJ	1	HPDRNPGDTATEAKFKEAKEAYEVL	HPDRNPGDTATEAKFKEAKEAYEVL
GrpE	1	TTMHQGVELTLKML	
	2		RAKAESENARRRFEREAIQARQF

The second scenario may involve *P. salmonis* hijacking fish cell exosomes as a potential signaling tool to aid in its survival and dissemination [4]. This is supported, first, by our finding of all three proteins of the system in exosomes, rather than one or two of them separately. Indeed, in other Gram-negative facultative bacteria related to *P. salmonis*, these three proteins are well described [48,49,50]. Second, it has been reported that *Oncorhynchus mykiss* exosomes are enriched with salmonid Hsp70 under heat stress conditions [51]; since Hsp70 lacks the classic export signal peptide [52], this suggests a signaling role in other cell targets [51]. Given

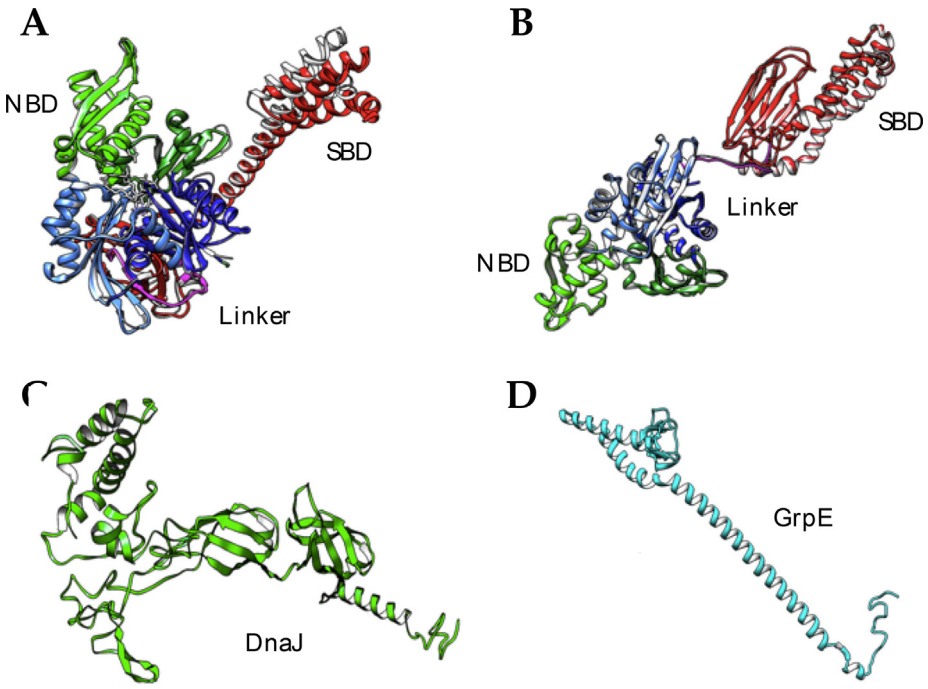

Fig. 7. Homology models for the HSP70/DnaK system of P. salmonis. A. Open form of DnaK superposed with E. coli DnaK PDB ID 4B9Q in white. B. Closed form of DnaK superposed with E. coli DnaK PDB ID 2KHO. C. DnaJ model. D. GrpE model.

that bacterial DnaK is a functional equivalent to Hsp70, *P. salmonis* may adopt a similar survival strategy and perhaps facilitate dissemination to other organs [4]. Indeed, the confinement of these proteins within the exosomal structure protects them from immune detection and IgM neutralization, an advantage for the pathogen. Furthermore, other sequences with high identity to marine gamma proteobacteria such as *Teredinibacter turnerae*, *Pseudohaliea rubra* and *Halioglobus japonicus* were also found, which could indicate a conserved survival mechanism for these organisms under stress conditions.

While specific sequences allow us to identify *P. salmonis* DnaK in the exosome content, other sequences found are common bacterial sequences. Interestingly, one sequence identified, MAKII-GIDLGTTNSCVAVL, corresponds to a conserved N-terminal portion of the protein, containing the TTN ATP-binding motif. This amino terminal segment is highly conserved in gammaproteobacteria and other widely studied pathogens, such as *Escherichia coli* and *Staphyloccocus aureus* (Fig. 6 and Fig. S4), which is a potential indication that we could find similar roles for *P. salmonis* DnaK as those described for these pathogens. While no studies have been conducted to assess the role of the Hsp70/Dnak system in *P. salmonis* survival and pathogenicity, previous work can be considered to support our proposal. First, the role of the Hsp70/DnaK system is very well described in *E. coli* as central in its chaperone network [53]. Under stress conditions, it is essential for bacterial survival. *E. coli* provides a useful starting point as a Gram-negative model. In addition, we established a 75% identity between *P. salmonis* EM90 and *E. coli* DnaK (Fig. 6, Fig. 7, Fig. S4 and Table 2) which is conducive to such a proposal. Furthermore, while not a Gram-negative model, it has been described that the Hsp70/DnaK system is crucial for *Staphylococcus aureus* survival to oxidative, temperature and antibiotic stress conditions in hosts [54]. Finally, DnaK from a closely related Gram-negative intracellular facultative pathogen, *Francisella tularensis,* has been shown to bind and inhibit alkaline phosphatase (AP) in human infections [55]. This is relevant for bacterial survival against host defenses, as AP has been shown to neutralize LPS [56,57]. DnaK sequences show more than 70% identity between *C. burnetii* and *F. tularensis* and *P. salmonis* DnaK (Fig. 6), which could imply similar functions. It will be interesting to conduct further experimentation to establish the role of the Hsp70/Dnak system in the survival and pathogenicity of *P. salmonis* and if it participates in the same mechanisms of bacterial survival as described for other pathogens.

Author contributions

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-Revision of the results and approved the final version of the manuscript: FA Gómez, C Cárdenas, R Belmonte, SH Marshall.

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Conflicts of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Cristian F. Munoz Bravo reports financial support was provided by MSD Animal Health GmbH. Marisela Carmona reports financial support and equipment, drugs, or supplies were provided by Sequencing and Omics Technologies Facility UC-Santiago de Chile. CONICYT FONDEQUIP EQM170172.]

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Supplementary material

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References

[1] Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. J Cell Biol 2013;200(4):373–83. https://doi.org/10.1083/jcb.201211138. PMid: 23420871.
[2] Doyle L, Wang MZ. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. Cells 2019;8(7):727. https://doi.org/10.3390/cells8070727. PMid: 31311206.
[3] Momen-Heravi F, Getting SJ, Moschos SA. Extracellular vesicles and their nucleic acids for biomarker discovery. Pharmacol Ther 2018;192:170–87. https://doi.org/10.1016/j.pharmthera.2018.08.002. PMid: 30081050.
[4] Schorey JS, Cheng Y, Singh PP, et al. Exosomes and other extracellular vesicles in host–pathogen interactions. EMBO Rep 2015;16:24–43. https://doi.org/10.15252/embr.201439363. PMid: 25488940.
[5] Kalra H, Adda CG, Liem M, et al. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. Proteomics 2013;13(22):3354–64. https://doi.org/10.1002/pmic.201300282. PMid: 24115447.
[6] Pietrowska M, Funk S, Gawin M, et al. Isolation of exosomes for the purpose of protein cargo analysis with the use of mass spectrometry. In: Kaufmann M, Klinger C, Savelsbergh A, editors. Functional Genomics, Methods in Molecular Biology, vol. 1654. New York, NY: Humana Press; 2017. p. 291–307. https://doi.org/10.1007/978-1-4939-7231-9_22.
[7] Kreimer S, Belov AM, Ghiran I, et al. Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. J Proteome Res 2015;14(6):2367–84. https://doi.org/10.1021/pr501279t. PMid: 25927954.
[8] Maravillas-Montero JL, Martínez-Cortés I. Los exosomas de las células presentadoras de antígeno y su papel en la regulación de las respuestas inmunológicas. Rev Alerg México 2017;64(4):463–76. https://doi.org/10.29262/ram.v64i4.285. PMid: 29249108.
[9] Xu H, Jia S, Xu H. Potential therapeutic applications of exosomes in different autoimmune diseases. Clin Immunol 2019;205:116–24. https://doi.org/10.1016/j.clim.2019.06.006. PMid: 31228581.
[10] Osaki M, Okada F. Exosomes and Their Role in Cancer Progression. Yonago Acta Med 2019;62(2):182–90. https://doi.org/10.33160/yam.2019.06.002. PMid: 31320822.
[11] Yu H, Sun T, An J, et al. Potential roles of exosomes in Parkinson's disease: From pathogenesis, diagnosis, and treatment to prognosis. Front Cell Dev Biol 2020;8:86. https://doi.org/10.3389/fcell.2020.00086. PMid: 32154247.
[12] Cai Z-Y, Xiao M, Quazi S, et al. Exosomes: A novel therapeutic target for Alzheimer's disease? Neural Regen Res 2018;13(5):930–5. https://doi.org/10.4103/1673-5374.232490. PMid: 29863025.
[13] Brennan K, Martin K, FitzGerald SP, et al. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. Sci Rep 2020;10:1039. https://doi.org/10.1038/s41598-020-57497-7. PMid: 31974468.
[14] Lobb RJ, Becker M, Wen SW, et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. J Extracellul Vesicles 2015;4(1):27031. https://doi.org/10.3402/jev.v4.27031. PMid: 26194179.
[15] Bordas M, Genard G, Ohl S, et al. Optimized Protocol for Isolation of Small Extracellular Vesicles from Human and Murine Lymphoid Tissues. Int J Mol Sci 2020;21(15):5586. https://doi.org/10.3390/ijms21155586. PMid: 32759826.
[16] Monfared H, Jahangard Y, Nikkhah M, et al. Potential therapeutic effects of exosomes packed with a miR-21-sponge construct in a rat model of glioblastoma. Front Oncol 2019;9:782. https://doi.org/10.3389/fonc.2019.00782. PMid: 31482067.
[17] Young JC. Mechanisms of the Hsp70 chaperone system. Biochem Cell Biol 2010;88(2):291–300. https://doi.org/10.1139/O09-175. PMid: 20453930.
[18] Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. Nat Rev Mol Cell Biol 2013;14:630–42. https://doi.org/10.1038/nrm3658. PMid: 24026055.
[19] Imamoglu R, Balchin D, Hayer-Hartl M, et al. Bacterial Hsp70 resolves misfolded states and accelerates productive folding of a multi-domain protein. Nat Commun 2020;11:365. https://doi.org/10.1038/s41467-019-14245-4. PMid: 31953415.
[20] GrpE HC. a nucleotide exchange factor for DnaK. Cell Stress Chaperones 2003;8(3):218. PMid: 14984054.
[21] Chen Y, Wang B, Liu D, et al. Hsp90 chaperone inhibitor 17-AAG attenuates Aβ-induced synaptic toxicity and memory impairment. J Neurosci 2014;34(7):2464–70. https://doi.org/10.1523/JNEUROSCI.0151-13.2014. PMid: 24523537.
[22] Benedetti F, Cocchi F, Latinovic OS, et al. Role of mycoplasma chaperone DnaK in cellular transformation. Int J Mol Sci 2020;21(4):1311. https://doi.org/10.3390/ijms21041311. PMid: 32075244.
[23] Kim WS, Jung ID, Kim J-S, et al. *Mycobacterium tuberculosis* GrpE, A Heat-Shock Stress Responsive Chaperone, Promotes Th1-Biased T Cell Immune Response via TLR4-Mediated Activation of Dendritic Cells. Front Cell Infect Microbiol 2018;8:95. https://doi.org/10.3389/fcimb.2018.00095. PMid: 29637049.
[24] Grudniak AM, Markowska K, Wolska KI. Interactions of *Escherichia coli* molecular chaperone HtpG with DnaA replication initiator DNA. Cell Stress Chaperones 2015;20:951–7. https://doi.org/10.1007/s12192-015-0623-y. PMid: 26246199.
[25] Garcés LH, Larenas JJ, Smith PA, et al. Infectivity of a rickettsia isolated from coho salmon *Oncorhynchus kisutch*. Dis Aquat Organ 1991;11:93–7. https://doi.org/10.3354/dao011093.
[26] Gómez FA, Tobar JA, Henríquez V, et al. Evidence of the Presence of a Functional Dot/Icm Type IV-B Secretion System in the Fish Bacterial Pathogen *Piscirickettsia salmonis*. PLoS ONE 2013;8(1):e54934. https://doi.org/10.1371/journal.pone.0054934. PMid: 23383004.
[27] Oliver C, Valenzuela K, Hernández M, et al. Characterization and pathogenic role of outer membrane vesicles produced by the fish pathogen *Piscirickettsia salmonis* under *in vitro* conditions. Vet Microbiol 2016;184:94–101. https://doi.org/10.1016/j.vetmic.2015.09.012. PMid: 26854350.
[28] Tandberg JI, Lagos LX, Langlete P, et al. Comparative analysis of membrane vesicles from three *Piscirickettsia salmonis* isolates reveals differences in vesicle characteristics. PLoS ONE 2016;11(10):e0165099. https://doi.org/10.1371/journal.pone.0165099. PMid: 27764198.
[29] Lagos L, Tandberg J, Kashulin-Bekkelund A, et al. Isolation and characterization of serum Extracellular Vesicles (EVs) from Atlantic salmon infected with *Piscirickettsia salmonis*. Proteomes 2017;5(4):34. https://doi.org/10.3390/proteomes5040034. PMid: 29194379.
[30] Wilhelm V, Soza C, Martínez R, et al. Production and immune response of recombinant Hsp60 and Hsp70 from the salmon pathogen *Piscirickettsia salmonis*. Biol Res 2005;38(1):69–82. https://doi.org/10.4067/S0716-97602005000100009. PMid: 15977412.
[31] Ghazaei C. Role and mechanism of the Hsp70 molecular chaperone machines in bacterial pathogens. J Med Microbiol 2017;66(3):259–65. https://doi.org/10.1099/jmm.0.000429. PMid: 28086078.
[32] Flores-Herrera P, Arredondo-Zelada O, Marshall SH, et al. Selection and validation of reliable housekeeping genes to evaluate *Piscirickettsia salmonis* gene expression. Infect Genet Evol 2018;63:151–7. https://doi.org/10.1016/j.meegid.2018.05.031. PMid: 29860100.
[33] Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. J Mol Biol 1990;215(3):403–10. https://doi.org/10.1016/S0022-2836(05)80360-2.
[34] Yang J, Yan R, Roy A, et al. The I-TASSER Suite: Protein structure and function prediction. Nat Methods 2015;12:7–8. https://doi.org/10.1038/nmeth.3213. PMid: 25549265.
[35] Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera–A visualization system for exploratory research and analysis. J Comput Chem 2004;25(13):1605–12. https://doi.org/10.1002/jcc.20084. PMid: 15264254.
[36] Pocsfalvi G, Stanly C, Fiume I, et al. Chromatography and its hyphenation to mass spectrometry for extracellular vesicle analysis. J Chromatogr A 2016;1439:26–41. https://doi.org/10.1016/j.chroma.2016.01.017. PMid: 26830636.
[37] Abramowicz A, Marczak L, Wojakowska A, et al. Harmonization of exosome isolation from culture supernatants for optimized proteomics analysis. PLoS ONE 2018;13(10):e0205496. https://doi.org/10.1371/journal.pone.0205496. PMid: 30379855.
[38] Patel GK, Khan MA, Zubair H, et al. Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications. Sci Rep 2019;9:5335. https://doi.org/10.1038/s41598-019-41800-2. PMid: 30926864.
[39] Helwa I, Cai J, Drewry MD, et al. A Comparative study of serum exosome isolation using differential ultracentrifugation and three commercial reagents. PLoS ONE 2017;12(1):e0170628. https://doi.org/10.1371/journal.pone.0170628. PMid: 28114422.
[40] Sokolova V, Ludwig A-K, Hornung S, et al. Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids Surfaces B Biointerfaces 2011;87(1):146–50. https://doi.org/10.1016/j.colsurfb.2011.05.013. PMid: 21640565.
[41] Lopez-Verrilli MA, Court FA. Exosomes: mediators of communication in eukaryotes. Biol Res 2013;46(1):5–11. https://doi.org/10.4067/S0716-97602013000100001. PMid: 23760408.
[42] Paulaitis M, Agarwal K, Nana-Sinkam P. Dynamic scaling of exosome sizes. Langmuir 2018;34(32):9387–93. https://doi.org/10.1021/acs.langmuir.7b04080. PMid: 29542322.
[43] Rozas M, Enríquez R. Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review. J Fish Dis 2014;37(3):163–88. https://doi.org/10.1111/jfd.12211. PMid: 24279295.
[44] Kityk R, Kopp J, Mayer MP. Molecular mechanism of J-Domain-Triggered ATP hydrolysis by Hsp70 chaperones. Mol Cell 2018;69(2):227–237.e4. https://doi.org/10.1016/j.molcel.2017.12.003. PMid: 29290615.

[45] Ghazaei C, El Helou ML. Beyond proteostasis: Roles of type I chaperonins in bacterial pathogenesis. J Med Microbiol 2018;67(9):1203–11. https://doi.org/10.1099/jmm.0.000811. PMid: 30074472.
[46] Álvarez CA, Gómez FA, Mercado L, et al. *Piscirickettsia salmonis* imbalances the innate immune response to succeed in a productive infection in a salmonid cell line model. PLoS ONE 2016;11(10):e0163943. https://doi.org/10.1371/journal.pone.0163943. PMid: 27723816.
[47] Pérez-Stuardo D, Morales-Reyes J, Tapia S, et al. Non-lysosomal activation in macrophages of Atlantic Salmon (*Salmo salar*) after infection with *Piscirickettsia salmonis*. Front Immunol 2019;10:434. https://doi.org/10.3389/fimmu.2019.00434. PMid: 30941123.
[48] Zuber M, Hoover TA, Court DL. Analysis of a *Coxiella burnetti* gene product that activates capsule synthesis in *Escherichia coli*: requirement for the heat shock chaperone DnaK and the two-component regulator RcsC. J Bacteriol 1995;177(15):4238–44. https://doi.org/10.1128/jb.177.15.4238-4244.1995. PMid: 7635811.
[49] Tomoyasu T, Tabata A, Imaki H, et al. Role of *Streptococcus intermedius* DnaK chaperone system in stress tolerance and pathogenicity. Cell Stress Chaperones 2012;17:41–55. https://doi.org/10.1007/s12192-011-0284-4. PMid: 21822788.
[50] Zuber M, Hoover TA, Dertzbaugh MT, Court DL. Analysis of the DnaK molecular chaperone system of *Francisella tularensis*. Gene 1995;164(1):149–52. https://doi.org/10.1016/0378-1119(95)00489-S.
[51] Faught E, Henrickson L, Vijayan MM. Plasma exosomes are enriched in Hsp70 and modulated by stress and cortisol in rainbow trout. J Endocrinol 2017;232(2):237–46. https://doi.org/10.1530/JOE-16-0427. PMid: 27872197.
[52] Mambula SS, Stevenson MA, Ogawa K, et al. Mechanisms for Hsp70 secretion: Crossing membranes without a leader. Methods 2007;43(3):168–75. https://doi.org/10.1016/j.ymeth.2007.06.009. PMid: 17920512.
[53] Calloni G, Chen T, Schermann SM, et al. DnaK functions as a Central Hub in the *E. coli* Chaperone Network. Cell Rep 2012;1(3):251–64. https://doi.org/10.1016/j.celrep.2011.12.007. PMid: 22832197.
[54] Singh VK, Syring M, Singh A, et al. An insight into the significance of the DnaK heat shock system in *Staphylococcus aureus*. Int J Med Microbiol 2012;302(6):242–52. https://doi.org/10.1016/j.ijmm.2012.05.001. PMid: 22748508.
[55] Arulanandam BP, Chetty SL, Yu J-J, et al. *Francisella* DnaK inhibits tissue-nonspecific alkaline phosphatase. J Biol Chem 2012;287(44):37185–94. https://doi.org/10.1074/jbc.M112.404400. PMid: 22923614.
[56] Beumer C, Wulferink M, Raaben W, et al. Calf intestinal alkaline phosphatase, a novel therapeutic drug for Lipopolysaccharide (LPS)-mediated diseases, attenuates lps toxicity in mice and piglets. J Pharmacol Exp Ther 2003;307:737–44. https://doi.org/10.1124/jpet.103.056606. PMid: 12970380.
[57] Koyama I, Matsunaga T, Harada T, et al. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and *in vitro* through dephosphorylation. Clin Biochem 2002;35(6):455–61. https://doi.org/10.1016/S0009-9120(02)00330-2.