Biological Research

versión impresa ISSN 0716-9760

Biol. Res. v.38 n.1 Santiago 2005

http://dx.doi.org/10.4067/S0716-97602005000100009

Biol Res 38: 69-82, 2005

ARTICLE

Production and immune response of recombinant Hsp60 and Hsp70 from the salmon pathogen *Piscirickettsia salmonis*

¹VIVIAN WILHELM, ¹CRISTIÁN SOZA, ¹RODRIGO MARTÍNEZ, ^{1,2,4}MARIO ROSEMBLATT, ^{1,2,4}LUIS O. BURZIO and ^{1,2,3,4}PABLO D.T. VALENZUELA

¹ Fundación Ciencia para la Vida, ² Universidad Andrés Bello, ³ Pontificia Universidad Católica de Chile e ⁴ Instituto Milenio de Biología Fundamental y Aplicada, Av. Zañartu 1482, Santiago, Chile

Dirección para Correspondencia

ABSTRACT

We have isolated and sequenced the genes encoding the heat shock proteins 60 (Hsp60) and 70 (Hsp70) of the salmon pathogen *Piscirickettsia salmonis*. The sequence analysis revealed the expected two open reading frames that encode proteins with calculated molecular weights of 60,060 and 70,400. The proteins exhibit a 70-80% homology with other known prokaryotic Hsp60 and Hsp70 sequences. The coding regions have been expressed in *E. coli* as thioredoxin fusion proteins. Both recombinant proteins were shown to elicit a humoral response when injected intraperitoneally in Atlantic salmon and also conferred protection to fish challenged with *P. salmonis*. The present data will facilitate further studies on the involvement of heat shock proteins in protective immunity of fish to infection by *P. salmonis* and their potential use in recombinants vaccines against this intracellular pathogen.

Key terms: Piscirickettsia salmonis, Hsp60, Hsp70, groEL, dnaK, immune response

INTRODUCCION

The obligate intracellular bacterium *Piscirickettsia salmonis* is the etiological agent of salmonid rickettsial septicemia (SRS), a disease responsible for extensive mortality in the Chilean salmon industry (Branson and Nieto, 1991; Fryer et al., 1990; Lannan and Fryer, 1993; Fryer and Mauel, 1997). This pathogen has also been identified in the Northern hemisphere (House et al., 1999; Brocklebank et al., 1993; Jones et al., 1998).

Recently, SRS has been partially controlled through the use of antibiotics, although they are not fully effective and have problems derived from their toxicity and generation of resistance. Vaccines have proven to work well in preventing infection. However, no effective vaccines are currently commercially available against *P. salmonis*. This pathogen grows very slowly in cell lines; it takes 15 to 20 days to develop a full cytopathic effect, making it difficult to process large quantities of cells and to separate them from host material.

In our search for potential vaccine candidates we have started the identification and study of *P. salmonis* genes coding for proteins with potential application in the production of an effective vaccine to control SRS. In addition to the membrane bound transglycosylase and transferrin binding protein (Wilhelm et al., 2004), we have directed our efforts toward the *P. salmonis* heat shock protein genes. It is well known that pathogen-derived Hsp are targets used by the host immune response to control infection. The Hsp serve as important antigens inducing very strong cellular and humoral immune responses (Kaufman, 1990, 1991; Kaufman et al., 1990). Immune responses to Hsp have been observed in infectious diseases caused by bacteria, protozoa, fungi and nematodes (Zügel and Kaufman, 1999).

We report here on the isolation, sequencing, and expression of the *P. salmonis* Hsp60 (*groEL*) and Hsp70 (*dnaK*) genes and the ability of the recombinant proteins in raising an antibody response in salmon.

MATERIAL AND METHODS

1. Cell cultures.

Inoculates of the Chinook salmon embryo cell line CHSE-214 (ATCC 1681) maintained in liquid nitrogen were thawed, pelleted, resuspended and cultured for 7 days or until confluence in 60 ml of complete MEM (Gibco BRL) supplemented with non-essential amino acids, glutamine and 5% fetal bovine serum (GIBCO BRL), in T175 flasks at 16°C.

2. Bacterial strains and plasmids.

E. coli strains NovaBlue and BL21 (DE3), used for cloning and expression respectively, were obtained from Novagen.

Inoculates of *P. salmonis* LF-89 (ATCC VR1361), each containing approximately 1×10^8 bacteria/ml, were brought to room temperature, added to flasks containing salmon cells, and incubated overnight at 16 C. The medium was then replaced by 50 ml of fresh complete MEM supplemented with non-essential amino acids, glutamine and FBS 5%, and cultured for 10-14 days at 16° C. Periodic checks of the degree of cytolysis were performed. Cultures were considered ready for harvesting or propagation when

almost 100% of the cells were cytopathic. Cells adhering to the flask walls were scraped, centrifuged twice at 150 xg, and the second supernatant was collected as the semi-purified fraction of *P. salmonis*. Further purification was performed according to Miquel et al., (2003).

The plasmids pET32a (Novagen), pET21a and pGEMT (Promega) were propagated in NovaBlue cells in medium Luria Broth (LB) with 100 μ g/ μ l ampicillin at 37°C. *E. coli* BL21(DE3) cells transformed by pET32a or pET21a were grown in LB with 100 μ g/ml ampicillin.

3. Cloning of the Hsp60 and Hsp70 coding regions.

Genomic DNA was extracted from the purified fraction of *P. salmonis* using Binder's procedure (1995) as described previously (Wilhelm et al., 2003). Coding regions of Hsp60 and Hsp70 were isolated by PCR amplification using specific primers based on information deduced from the sequence of a genomic library obtained in our laboratory (Valenzuela et al., unpublished results). The sequence of the primers is shown in Table I.

Amplified products were purified employing the Qiaquick PCR purification kit (Qiagen), ligated to pGEMT at 14°C in the presence of T4 DNA ligase and used to transform NovaBlue competent cells. Positive clones were selected by blue/white screening using lacZ a-complementation.

TABLE 1	[
---------	---

Primer	Sequence
p1	5'-atggagatataagaatg-3'
p2	5'-ccgcccatgccacccat-3'
р3	5'-atggctgaaattattggtat-3'
p4	5'-aacttcttcaaactcagcatc-3' 5'-
p5	gacggatccggagatataagaatgtcagca- 3'
p6	5'-tatgaattcttaaccgcccatgccacccat- 3'
p7	5'- tatgaattcatggctgaaattattggtattg-3'
р8	5'- gtactcgagctaaacttcttcaaactcagcatc- 3'

Oligonucleotides used as primers

4. DNA sequencing.

The Hsp60 and Hsp70 coding sequences were obtained employing Sanger's dideoxy procedure (1977) using M13 primers. Products were separated by capillary

electrophoresis in an ABI 310 Genetic Analyzer (Applied Biosystem Inc.). The *P. salmonis* Hsp60 and Hsp70 coding sequences are registered in the Gene Bank data base under accession numbers AY686756 and AY686757 respectively.

5. Protein production in E. coli.

The Hsp60 and Hsp70 coding regions previously cloned in pGEM-T were sub-cloned in pET32a or pET21a. The plasmids of the recombinant clones were used to transform *E. coli* BL21(DE3) competent cells. Expression of recombinants was induced by incubation in LB with 1 mM IPTG. Recombinant proteins were purified by a Ni-agarose column (Qiagen). Protein concentration was measured using the Micro BCA kit (Pierce). Protein analysis was performed in PAGE-SDS gels according to Laemmli (1970).

6. Monoclonal antibody production.

Two-month-old female BALB/c mice were injected intraperitoneally three times at 3week intervals with 50 µg of recombinant proteins Hsp60 or Hsp70 diluted in PBS and emulsified with Freund adjuvant. Ten days after the final injection, the animals were bled from the tail to obtain serum. The humoral response against the recombinant proteins was determined by an ELISA according to Jamett et al., (2001). To produce hybridoma, spleen cells from the immunized mice with the highest titer against the recombinant proteins were isolated and fused with NS0/2 mouse myeloma cells according to the general procedure used by Köhler and Milstein (1975) with minor modifications as described previously (Jamett et al., 2001).

7. Immunization of salmon.

A group of 104 Salmo salar with an average weight of 18 grams were tagged for group identification and injected intraperitoneally with 200 μ l of an oil-water emulsion containing 10 μ g of each recombinant Hsp60 and Hsp70. The fish were maintained at 13°C under controlled conditions of oxygenation, feeding, and water flow in 4 tanks. Non-immunized control fish were maintained in the same tanks. Serum from some of the treated and control fish was obtained 3 months after vaccination, and the immune response against the recombinant proteins was analyzed by ELISA.

8. Challenge.

After 625 degree days, a tank containing 13 vaccinated fish, 13 adjuvant injected fish, and 13 control non-vaccinated fish was challenged with a dose of *P. salmonis* equivalent to 2XLD50. Mortality of fish was registered periodically and dead fish were analyzed to confirm that SRS was the cause of mortality. LD50 was previously determined by analyzing cumulative mortality of intraperitonally injected fish with serial dilution of *P. salmonis*. The effectiveness of the vaccine was determined by the relative percent survival (RPS), which was calculated as follows: RPS = [1-(% mortality of test group ÷ % mortality of control group)] x 100%.

9. Western blot analysis.

Recombinant Hsp60 and Hsp70 or whole-cell protein obtained from *P. salmonis* were separated by PAGE-SDS gel electrophoresis, transferred to nitrocellulose (Towbin et al., 1979) and analyzed with a dilution of anti-*P. salmonis* rabbit polyclonal serum,

specific monoclonal antibodies or with a 1:200 dilution of serum obtained from immunized salmon. The blots were developed with an anti-mouse IgG conjugated to alkaline phosphatase, or an anti-rabbit IgG conjugated to alkaline phosphatase. Incubation with a monoclonal antibody against salmon IgM was included in the analysis of salmon serum.

10. ELISA.

ELISA assays were carried out according to Aguayo et al. (2002) with minor modifications, using recombinant Hsp60 and Hsp70 as antigens and dilutions of serum from immunized salmon. Incubation with an anti-salmon IgM monoclonal was included before developing the reaction with anti-mouse IgG conjugated to alkaline phosphatase.

RESULTS AND DISCUSSION

1. Isolation of Hsp60 and Hsp70 coding regions of P. salmonis.

Our laboratory has sequenced approximately 90% of the *P. salmonis* genome. The sequences of the contigs has allowed us to identify about 1,500 genes that are being arranged in a circular genome of about 2x10⁶ base pairs. Genes corresponding to heat shock proteins Hsp60 and Hsp70 were identified by comparison to sequences of genomes of phylogenetically related organisms such as *Pseudoalteromonas haloplankitis* for Hsp60 and *Legionella pneumophila* for Hsp70. Figure 1 shows that the region coding for Hsp60 is found in contigs 291 (1,072 base pairs) and 750 (566 base pairs). Contig 291 includes from the 5' end up to nucleotide 1004 and contig 750 comprises the coding region for the carboxyl end of the protein, from nucleotide 1070 to nucleotide 1,636.



Figure 1. Isolation of the *P. salmonis* Hsp60 coding region by PCR from genomic *P. salmonis* DNA. The DNA MW ladder is a mixture of Hind III-digested Lambda DNA and Hae III digested ØX174 DNA.

This was confirmed by amplifying a fragment of 1,651 base pairs (Fig. 1) with primers p1 and p2 (Table I). This fragment was cloned into pGEM-T and sequenced. An open reading frame of 1,638 base pairs codifies a protein of 546 amino acids (Fig. 2) corresponding to a protein of 60,060 daltons. A high degree of homology was found when comparing this protein with Hsp60 of other microorganisms (Fig. 2). Comparative analysis of Hsp60 of *P. salmonis* with that of phylogenetically-related microorganisms shows that the first

lacks a sequence of about 5 amino acids in the carboxyl end, due probably to the incomplete data available of the genomic library.

	1 60
P. salmonis	MEAKEVEFSTO BROKMLDGVVELANAVKVIIGLEGRNVII SKSEGAPTITHDGVSVAREI
C. burnetii	MAAKVEN FSHEVLHAMSRGVENLANAVKVTLGPRGRNVVLDKSFGAPTETKDGVSVAKE I
F. tularensis	MRAROUL VSTRANMEDOUNT LANAVEVTEOPRORNVYEDRSHOAPTTTRDGVSVARET
L. pneumophila	-MAKELE CHORAELONIAGUNALA DAVOUTEGERGENUUTERSEGAPTUTEDGUNVARET
P. aeruginosa	REAR OF THE SERVENT OF THE AVENT OF CONVERTING AND THE SERVER
ar avauganoos	Charles and a sub-share a set of the second of the sub-second second second second second second second second
SE CONSTRUCTIONS	61 120
P. salmonis	ELSDEFENMGAQMVKEVASKENDDAGDGTTTATVLAQAT OEGAKEVAAGMNPMDLKRGI
C. burnetii	KIEDKFENMGAQMVKEVASETEDDAGDGTTTATVLAQATEVEGEKAVIAGNNPMDLKRGI
F. tularensis	ELECKFENNGAQIVKEVASETAOVAGDGTTTATVLAQALITEGEKAVTAGMNPMOLKEGI
L. pneumophila	EFEHREMNHGAONVKEVASKTEDTÅGDGTTTATVLARETEVEGHKAVAAGMNPHDLKRGI
P. aeruginosa	ELKOKFENMGACOVKOVASKANDAAGDGTTTATVLACATVNEGEKAVAAGMNPMDLKRGT
PERMIT PROVIDE RECEIPTING	101
D. an Imania	
F. Saimonias	
C. Durnetii	
F. LUIAFensis	DIALARIVELLATION SUPPORT LEVISION AND A TABABAN UNE SVITVERS
L. pneumophila	DEAVEAVIANTER, QAMSEPURUSEA LAUVOTISANSULATUAT LATAMERVOME OVITVEDO
P. aeruginosa	DKA VATVAQLEELAK PCADTKA LAOVOTISANSDESI QQLIARAMEKVIKKEVITVERG
	181
P. salmonis	SSLENELDVVEGMOEDRGYLSPYENNKORXHIAR SPITLLVDKKTSN (PRILIPTLESU
C. burnetii	SOLENAL EVVEGNOFERGYL SPYFTHNOCH MEASTERN POTT VOKKT SHTPT TOTT FUT
E fularancie	PREPARE INTERNATION OF STATE AND THE NEW TRUNKS SHITTENED AND THE STATE OF STATE
t pnaumonhila	MARKEN AND AN ADDRESS TO AN ADDRESS AND ADDRESS
D approximate	THE PLACE PROPERTY AND
P. aeruginosa	Sanotes not a position of the same same same should be a subsection of the same sources of the same source
	241 300
P. salmonis	AKSOKPLFITAEDVEGEALATLVVNNTRGIVKVCAVKAPOFODRRKAMLEDIAILTOGTV
C. burnetii	AKSGRELLA LAED SGEALATLVVNN ROVVVA VKAPGFODRRKAMLODIAULTOGRV
F. tularensis	SKNGRALI LAEDVESEALATLVVNNNRGVVKVCTVKAPGFGDRRKAMEEDIATLIGA F
L. pneumophila	AKSGRPLIIIAEDVEGEALATLVVNNMRGTVKVCAVRAPGFGDRRKAMLODIAILTKGV
P. aeruginosa	AKAGEPLLIVAEDVECEALATLVVNNMRGIVKVAAVKAPGFGDERKAMLODIATLTCGTV
the second	
ar assayshes	
	301 360
P. salmonis	360 SEEVEDDERATLERLOWARR VVTKIMTT TOGAGEQTATEARVTOTRACMEETESDY
P. salmonis C. burnetii	360 SEEVELDLERATLERLOTAKETVVTKIMITTUIDGAGEQTATEARVTOTRAGVEESSOY SEEVELSLEAASLEDLOSAKEVVVTKODTTUIDGSGDAGEIKNRVEDTREESSOY
P. salmonis C. burnetii F. tularensis	360 SEEVELDLERATLERLOCARRIVVITRINITIUTDCAGEOTATEARVIOTRAOVERSISDY SEEVELSLEAASLIDLOSARRVVVITRIDITUTOCSGDAGEINNRVEDTREETSBSSOY VSEDLSRKLEETSBEILGCASRVVVITRIDITUTDGAGEREATARPINVITRANTAAASDY
P. salmonis C. burnetii F. tularensis L. pneumophila	301 SERVED LEKATLEH LOTAKRIVVIKIMITT I IDGAGEQTALEARVTOI RACVHETS SDY SERVELS LEASUNDLOS AKRVVVIKID TT I IDGAGE (INNEXE) IKE IEN SSSDY SEDUS RKLEETING II LOTASR VOVIKIMITT I IDGAGE KEALAKRINVIKANI ALAN SDY SEDIS RKLEETING II LOTASR VOVIKIMITT I IDGAGE KEALAKRINVIKANI ALAN SDY SEDIS RKLEETING II LOTASR VOVIKIMITT I IDGAGE KEALAKRINVIKANI ALAN SDY
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa	301 SERVED LEKATLEN LOTAKRI VUR DI TU IDO GEOTALEA DUTO I RAVETI SDY SELVELS LEA SU DELOS AKRIVUT KODTU IDO SEDAGE INNEVED I KE IEN SS SDY VII DI SEKLEETINGI LOTA SEVOUTIUNTI I DEA GEREA. AKHINVI KANI ARAN SDY SEDI GR SLEGATLEN LOTA SEVOUTIUNTI I IDGEGRATE INAPIACI RAVE DI SDY SEDI GR SLEGATLEN LONAREVINKENTI I IDGA SVOADIEN RULO FROIDET SDY
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa	301 360 SELVED LEKATLEN LOTAKRI VITRIDI TI IDO GEOTALEA VITI RAVETESSIY ISELVELS LEAST DELESAKRIVITRIDI TI IDO GEOTALEA VITI RAVETESSIY VIEDI SEX LEETING ILLETASE VOT FUNTTI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY ISEDI GRELEGATLED LOGARRIVITRINITI IDGA GEREA. AKE NIVI KAN TA FANSIY
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis	301 360 SEEVED LEKATLEN LOTAKRI VUR DUTTI LOGAGAQTA PARTI RAVATED SOY SEEVELSLEAS LOD LOSAKRIVVIK DOTTI LOGAGAQTA PARTI RAVATED SOY SEEVELSLEAS LOD LOSAKRIVVIK DOTTI LOGAGARA AKPINVIKAI LA PARSOY SEEVELSLEAS LOD LOSAKRIVVIK DITI LOGAGARA AKPINVIKAI LA PARSOY SEEVELSLEGATLEN LOTA SRIVVIK DITI LOGAGARA AKPINVIKAI LA PARSOY SEEVELSLEGATLEN LOVAKRIVVIK DITI LOGAGAVQADI PARILO RAVELO SOY 361 420
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii	301 360 STEVED DEKATION COAKS, WITHOUTT IDESCRATA TEAM TO RADUCTSON SERVELSIES UD LOSAKS, WITHOUTT IDESCRATE INNUES IKE INNESS OF SERVELSIES LEASTON DE STANDART IDESCRATE AND VERNING AND AND AND SERVELSIES THE DESCRATE VERNIT IDESCRATE INNESS RECEIPTION SERVELSIES THE DE STANDART VERNIT IDESCRATE INNESS RECEIPTION SERVELSIES AND VERNIT IDESCRATE INTO A DESCRATE INTO A DESCRATE IN SERVELSIES AND AND AND AND AND A DESCRATE INTO A DESCRATE IN SERVELSIES AND AND AND A DESCRATE INTO A DESCRATE IN SERVELSIES AND AND AND AND A DESCRATE IN A DESCRATE IN SERVELSIES AND AND AND AND AND A DESCRATE IN SERVELSIES AND AND AND AND AND AND AND A DESCRATE IN SERVELSIES AND AND AND AND A DESCRATE IN SERVELSIES AND
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii	301 360 SEEVELD LEASE OF LOCAKE WITHOUTT ID SECONAL PART OF RAVELED SOY SEEVELD LEASE OF LOCAKE WITHOUTT ID SECONAL PART OF RAVELED SOY SEEVELD LEASE OF LOCAKE WITHOUTT ID SECONAL PART OF RAVELED SOY SEEVELD LEASE OF LOCAKE WITHOUTT ID SECONAL PART OF RAVELED SOY SEEVELD LEASE OF LOCAKE WITHOUTT ID SECONAL PART OF RAVELED SOY SEEVELD LEASE OF LOCAKE WITHOUTT ID SECONAL PART OF RAVELED SOY SEEVELD LEGAT LEASE WITHOUT TO ID SECONAL PART OF RAVELED SOY 361 420 DREKLOER VALLAGE WAVE KNOWN KOAT PART OF RAVELED SOY 361 420 DREKLOER VALLAGE WAV INVGAAT PART ENERKK DRV DALHAT RAVEEG VEGGVAL DREKLOER AND AG ON VINT WAAT PART OF RAVELOR AVEC OVER GOVAL
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis	301 360 SELVCED LEKATLEH LOTAKREV VIRIOTTI I DORGACIA TERRITO I RAVIETE SDY SELVCED LEKATLEH LOTAKREV VIRIOTTI I DORGACIA TERRITO I RAVIETE SDY SELVCES LEASUDEL OS AKREV VIRIOTTI I DORGACIA. INNEROTI KE TENSISSINY SELVCES LEGATLED LOGA KREV VIRIOTTI I DORGEKATI INNEROTI KE TENSISSINY SELVCES LEGATLEH LONAKREV VIRIOTTI I DORGEKATI INNERATI RAVIETI SDY 361 VIRIOTI KE GOVANT KVOATETE ENKEKKDRVD DALHATRAAVEEGVIR GOGVALI DEEKLOERTAKLING VIRVAATETE ENKEKKDRVD DALHATRAAVEEGVIR GOGVALI DEEKLOERTAKLING VIRVAATETE ENKEKKDRVD DALHATRAAVEEGVIR GOGVALI DEEKLOERTAKLING VIRVAATETE ENKEKKDRVD DALHATRAAVEEGVIR GOGVALI
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila	301 360 SEEVEL SEATTH LOTAR INTERNET TO DESERVE SEATTH S
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa	301 360 SERVICE SEATCH LOTAKEN VERION THE DESCRIPTION ADDRESS SON SREVICE SEATCH LOTAKEN VERION THE DESCRIPTION ADDRESS SON SERVICE SEATCH LOTA SREVING FOR DESCRIPTION ADDRESS SON SECURIS LEGATER LOTAS REVENTED TO DESCRIPTION ADDRESS SON SECURIS LEGATER LONAR POLINEED TO DESCRIPTION ADDRESS SON 361 420 SECURIS LEGATER LONAR POLINEED TO DESCRIPTION ADDRESS SON 361 420 DESCRIPTION AND SON ADDRESS SON ADDRESS SON ADDRESS SON 361 420 DESCRIPTION AND SON ADDRESS SON ADDRESS SON ADDRESS SON ADDRESS SON 361 420 DESCRIPTION AND SON ADDRESS SON ADD
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa	301 360 SEEVED LEKATLEN LOTAKRI VIR DITTI LOGGEQUALEN TO LADORES DY SEEVELS LEAST DELOSAKRI VIR DITTI LOGGEQUALEN TO LADORES DY SEEVELS LEAST DELOSAKRI VIR DITTI LOGGERATION RECTARE INSERTION SEET GKSLEGATER LOGA SR VOUTENT TI LOGGERATION RECTARE INSERTION SEET GKSLEGATER LOGA SR VOUTENT TI LOGGERATION RECTARE INSERTION SEET GKSLEGATER LOGA SR VOUTENT TI LOGGERATION REACTARE INSERTION SEET GKSLEGATER LOGA SR VOUTENT TI LOGA SVOAD EN FULLER OF SOY SEEVELS LEGATER LOGA RATION REACTARE INFERDING TO LOGA SVOAD EN FULL SROTED SOY 361 420 SEKLOER LAKI SGOVAVI KVGAATEVENKEKK RVUDALIATRAAVEG VEGGVAL DEKKLOER LAKI SGOVAVI KVGAATEVENKEKK RVUDALIATRAAVEG VEGGVAL DEKLOER LAKI SGOVAVI KVGAATEVENKEKK RVUDALIATRAAVEG VEGGVAL DEKLOER LAKI SGOVAVI KVGATEVENKERK RVUDALIATRAAVEG VEGGVAL DEKLOER LAKI SGOVAVI KVGATEVENKERK RVUDALIATRAAVEG VEGGVAL DEKKLOER LAKI SGOVAVI KVGATEVENKERK RVUDALIATRAAVEG VEGGVAL DEKLOER LAKI SGOVAVI KVGATEVENKERK RVUDALIATRAAVEG VEGGVAL DEKLOER LAKI SGVAVI KVGATEVENKERK RVUDALIATRAAVEG VEGGVAL DEKKLOER LAKL SGVAVI KVGATEVENKERK RVUDALIATRAAVEG VEGGVAL 12
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis	301 360 SELVED LEKATLEN LOTAKRI, VIR DITTI DEGEGATALEN TO LADOREDS DY SELVEL SLEAST DELEGAKRI, VIR DITTI DEGEGATEN NEVEL RETENSS DY SELVEL SLEAST DELEGAKRI, VIR DITTI DEGEGATEN NEVEL RETENSS DY SELVEL SLEAST DELEGAKRI, VIR DITTI DEGEGATEN NEVEL RETENSS DY SELVEL SLEAST DELEGAKRI, VIR DITTI DEGEGATEN NEVEL RADOREDS DY SELVEL SLEATTER LOTAS ROUTENT TI DEGEGATEN NEVELATER SLEATES DY SELVEL SLEATTER LOTAS ROUTENT TI DEGEGATEN NEVELATER SDY SELVEL SLEATTER LOTAS ROUTENT TI DEGEGATEN NEVELATER SDY SELVEL SLEATTER LOTAS ROUTENT TI DEGEGATEN NEVELATER SDY SELVEL SLEATTER LOTAS ROUTENENT TI DEGEGATEN NEVELATER SDY SELVEL SLEATTER LONAR ROUTENENT TI DEGEGATEN NEVEL RADOREDS DY SELVER SLEATTER LONAR ROUTENENT TI DEGEGATEN NEVEL RADOREDS DY SELVER SLEAT SECON NEVEL SAN TRUE SECON SEC
P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii	301 360 SELVED LEKATLEL COARRENTER DITT I DOGG OTALEART I ROVELS SON SELVED LEKATLEL COARRENTER DITT I DOGG OTALEART I ROVELS SON SELVEL SLEAST DELGA KRYNTER DITT I DOGG OTALEART I RETENS SON SELVEL SLEAST DELGA KRYNTER DITT I DOGG OTALEART I RETENS SON SELVEL SLEAST DELGA KRYNTER DITT I DOGG KAAT I NER AG I ROVELED SON SELVEL SLEGATEE D. COARRENT RETT I DOGG GERAT I NER AG I ROVELED SON SELVEL SLEGATEE D. COARRENT RETT I DOGG KAT I NER AG I ROVELED SON SELVEL SLEGATEE D. COARRENT RETT I DOG GERAT I NER AG I ROVELED SON 361 420 DEEN GER AKLING GWAN I RUGATEE EMREKK DEV DALHATRAAVEEG VEGGVAL I REKLOER AKLING GWAN I RUGATEE EMREKK REVE DALHATRAAVEEG VEGGVAL I REKLOER AKLING GWAN I RUGATEE EMREKK REVE DALHATRAAVEEG VEGGVAL I REKLOER AKLING GWAN I RUGATEE EMREKK REVE DALHATRAAVEEG VEGGVAL I REKLOER AKLING GWAN I RUGATEE EMREKK REVE DALHATRAAVEEG VEGGVAL I REKLOER AKLING GWAN I RUGATEE EMREKK REVE DALHATRAAVEEG VEGGVAL I REKLOER AKLING GOVAN I RUGATEE EMREKK REVE DALHATRAAVEEG VEGGVAL I REKLOER AKLING GOVAN I RUGATEE EMREKK REVE DALHATRAAVEG I VEGGVAL 1 REKLOER AKLING GOVAN I RUGATEE EMREKK REVE DALHATRAAVEG I VEGGVAL 1 REKLOER AKLING GOVAN I RUGATEE EMREKK REVE DALHATRAAVEG I VEGGVAL 21 480 1 RUGATEE AKLING GOVAN I RUGATEE EMREKK REVE DALHATRAAVEG I VEGGVAL 24 480 1 RUGATEE AKLING OVAN I RUGATEE EMREKK REVE DALHATRA
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis 	301 360 SEEVELD LEKATLEH LOTAKRIVY KIDITTI I DEGEGATERATO I RAVIETES BY SEEVELS LEASUDELES AKRYVYK DETTI I DEGEGATERATO I RAVIETES BY SEEVELS LEASUDELES AKRYVYK DETTI I DEGEGATERATO I RAVIETES BY SEEVELS LEGATLED LOGA KRYVYK DETTI I DEGEGATERATO I NAPLACI KALAN SEY SEEVELS LEGATLED LOGA KRYVYK DETTI I DEGEGATERATO I NAPLACI KAVE SEY SEEVELS LEGATLED LOGA KRYVYK DETTI I DEGEGATERATO I NAPLACI KAVE SEY 361 420 SEEVELS LEGATLER LOVAKRYVK VERATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELS LEGATLER LOVAKRYVK VERATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELS LEGATLER LOVAKRY VERATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELS LORAKRA GOVAVI KVGATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELS LORAKRA GOVAVI KVGATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELS SEVENSE OF AVIENDATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELSE OF AKLA GOVAVI KVGATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELSE OF AVIENDATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELSE OF AKLA GOVAVI KVGATER ENKEKK BY DALHATRAAVE SY PEGGVAL SEEVELSE OF AVIENDATER ENKERN BY DALHATRAAVE SY PEGGVAL 421 480 SEEVELSE OF SOLVEN ENKER SET SY SEGENATION VERSEN I NEVENDER SET SY PEGENATION VERSEN I NEVENDER SET SY PEROVENCEN AND VERSEN I NEVENDER SET SY PEGEN I NEVENDER SE
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila F. tularensis L. pneumophila L. pneumophila 	301 360 SEEVEL DEKATER LOTAKEN VERDET TODAGE OTATER FOTO FROM TO SERVEL 360 SEEVEL DEKATER LOTAKEN VERDET TODAGE OTATER FOTO FROM TO SERVEL 360 SEEVEL DEKATER VERDET TODAGE OTATER FOTO FROM TO SERVEL 361 SEEVEL DEKATER VERDET TODAGE ON TOTAL SERVED 361 SEEVEL SEGURATE ON THE SERVER AND TODAL HAT TRAVESORY DEGEVAL 361 SERVEL SEGURATING ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SEGURATING ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SEGURATING ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SEGURATING ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SEGURATING ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SERVE ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SERVE ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 362 SERVEL SERVE ON THE SERVE ON THE SERVER AND DAL HAT TRAVESORY DEGEVAL 360 SERVEL SERVE ON THE SERVE ON THE SERVER AND DEGEVE ON THE SERVER AND THE SERVER AND THE SERVER AND DAL HAT TRAVESORY DEGEVE ON THE SERVER AND THE
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	301 360 SEEVEL SEATTH LOAKEN VERDET LOAGQUALENET DEADERS 360 SEEVEL SEATTH LOAKEN VERDET LOAGQUADENERS 360 SEEVEL SEATTH LOAKEN VERDET LOAGQUADENERS 360 SEEVEL SEATTH LOAKEN VERDET LOAGQUADENERS 360 SEEVEL SEATTH LOAKEN VERDET LOAGGUADENERS 361 SEEVEL SEATTH LOAKEN VERDET LOAGGUADENERS 361 SEEVEL SEATTH LOAKEN VERDET LOAGGUADENERS 361 SEEVEL SEATHER LOAKEN VERDET E SEATHER
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	301 360 SEEVEL SEATCH COARD VERDET FOR SECTION FOR THE SET OF S
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	301 360 SEE NOLD LEKATLEH LOTAKEN VER DET ELDERSONGALERENT ELANERSEN POLISION 100 SECOND LEKATLEH LOTAKEN VER DET ELDERSONGALERENT ELANERSEN POLISION SEE NOLS LEAS ELDELOS AKREV VER DET ELDERSONGALERENT ELANERSEN 100 SECOND LEKATLEH LOTAKEN VER DET ELDERSONGALERENT ELANERSEN SEE ELENTER LOTAKEN VER DET ELDERSONGALERENT ELANERSEN 100 SECOND LEGATLEH LOTAKEN VER DET ELDERSONGALERENT ELANERSEN SEE ELENTER LOTAKEN VER DET ELDERSONGALERENT ELANERSEN 100 SECOND LEGATLEH LOTAKEN VER DET ELDERSONGALERENT ELENTERSEN SEE ELENTER LEGATLEH LOTAKEN VER DET ELDERSONGALERENT ELENTERSEN 100 SECOND ELENTER LOTAKEN VER DET ELDERSONGALERENT ELENTERSEN 301 420 303 420 304 420 305 420 306 420 307 420 308 420 309 420 309 421 421 480 302 421 421 480 303 420 304 421 421 480 302 421 303 420 304 421 305 420 306 421 307 420 308 421 309 420 300 <t< td=""></t<>
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis L. pneumophila P. salmonis P. salmonis P. salmonis 	301 360 SELVED LEKATLEL CLARREN VER DET ELDERGEQTATERET TO FROM ESSENCE 100 SELVET SUBJECT SELVER VER DET ELDERGEQTATERET ELDERGES SELVET SUBJECT SELVER VER DET ELDERGEQTATERET ELDERGES 100 SELVET SUBJECT SELVER VER DET ELDERGEQTATERET ELDERGES SELVET SUBJECT SUBJECT SELVER VER DET ELDERGEQTATERET ELDERGES 100 SELVET SUBJECT SUBJECT SELVET SUBJECT SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT SELVET SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT SELVET SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT 361 420 SELVET SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT 361 420 SELVER SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT 361 420 420 SELVER SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT 361 420 420 SELVER SUBJECT 100 SELVET SUBJECT 100 SELVET SUBJECT 421 420 420 421 420 SELVEN SUBJECT 100 SELVET SUBJECT SELVEN SUBJECT 100 SELVEN SUBJECT 100 SELVEN SUBJECT 421 420 SELVEN SUBJECT 100 SELVEN SUBJECT 100 SELVEN SUBJECT 421 420 SELVEN SUBJECT 100 SELVEN SU
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. salmonis C. burnetii 	301 360 SELVED LEKATLEL COARS 10 SELVED LEKATLEL COARS SELVED SEAS DE LEAS DE LES ARRY VERDETTE DESEGACE INNELSE I RETENESSEY SELVEL SLEAS DE LES ARRY VERDETTE DESEGACE INNELSE I RETENESSEY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE I RETENESSEY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE I RADIE ES DY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE I RADIE ES DY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE I RADIE ES DY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE I RADIE ES DY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE I RADIE ES DY SELVELS LEGATE EL COARSE VERDETTE DESEGACE INNELSE SELVER LEGATE EL COARSE VERDETTE DESEGACE INNELSE REKLOER AKLAGOVAVI KVGAATE VERMEKK DRV DALHATRAAVEEG VEGGVAL DEKKLOER AKLAGOVAVI KVGAATE VERMEKK RVE DALHATRAAVEEG VEGGVAL DEKKLOER AKLAGOVAVI KVGAATE VERMEKK RVE DALHATRAAVEGT VEGGVAL DEKKLOER AKLAGOVAVI KVGAATE VERMEKK RVE DALHATRAAVEGT VEGGVAL VERMER AKLAGOVAVI KVGAATE VERMEKK RVE DALHATRAAVENE VEGGVAL VERMER AKLAGOVAVI KVGAATE VERMEKK RVE DALHATRAAVENE VEGGVAL VERMER AKLAGOVAVI KVGAATE VERMEKK RVE DALHATRAAVENE VEGGVAN </td
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. salmonis C. burnetii F. aeruginosa P. salmonis C. burnetii F. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis C. burnetii F. tularensis	301 360 SEEVELD LEKATLEH LOAKRIVYK KUTT I DEGEGATER FYTT I ROVERES SDY SEEVELS LEAS UDDEG AKRYVYK KUTT DEGEGAGE. KNN KET KENSSON SEEVELS LEAS UDDEG AKRYVYK KUTT DEGEGAGE. KNN KET KENSSON SEEVELS LEGATLEN DE GASKRYVYK KUTT DEGEGKATE. NN FEG KKE IN SSON SEEVELS LEGATLEN DE GASKRYVYK KUTT DEGEGKATE. NN FEG KKE IN SSON SEEVELS LEGATLEN DE GASKRYVYK KUTT DEGEGKATE. NN FEG KKE IN SSON 361 420 SEEVELS LEGATLEN LOVAK KUTT DEGEGKATE. NN FEG KKE IN SON 361 420 SEEVELS LEGATLEN LOVAK KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL SEEVELS LEGATLEN LOVAK KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL SEEVELS LEGATLEN LOVAK KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL SEEVELS LORAL AKL SEGVAVI KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL SEEVELS LORAL AKL SEGVAVI KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL SEEVELS DER AKL SEGVAVI KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL SEEVELSEN AKL SEGVAVI KUGATEE ENKEKK DEV DALHATRAAVEEGVE GEGVAL <
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila F. tularensis L. pneumophila 	301 360 SEEVELD LEKATLEH LOAKRIVYK KUTT I DEGEGATER FYTT I RAVEES SPY SEEVELS LEAS UDLES AKRYVYK KUTT I DEGEGAATER FYTT I RAVEES SPY SEEVELS LEAS UDLES AKRYVYK KUTT I DEGEGKATER FYTT I ALARS DY SEEVELS LEAS UDLES AKRYVYK KUTT I DEGEGKATER FYTT I ALARS DY SEEVELS LEGATLEH LOVAKRYVKKENT I DEGEGKATER FYTT I ALARS DY SEEVELS LEGATLEH LOVAKRYVKKENT I DEGEGKATER FYTT I ALARS DY SEEVELS LEGATLEH LOVAKRYVKKENT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKKENT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKKENT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKEN FYTT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKEN FYTT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKEN FYTT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKEN FYTT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LEGATLEH LOVAKRYVKEN FYTT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELS LOGAT FYTT RAVE FYTT I DEGEGKATER FYTT I RAVEES VEGGVAL SEEVELSE RAVE GOVAVI KVGATTER ENKEKK RYT DALHATRAVES OF VAGGVAL SEEVELSE RAVE GOVAVI KVGATTER ENKEKK RYT DALHATRAVES OF VAGGVAL SEEVELSE RAVE GOVAVI KVGATTER ENKEKK RYT DALHATRAVES OF VAGGVAL SEEVELSE RAVE GOVAVI KVGATTER ENKEKK RYT DALHATRAVES OF VAGGVAL SEEVELSE RAVE GOVAVI KVGATTER ENKEKK RYT DALHATRAVES OF VAGGVAL
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	301 360 SEEVEL SERVICE SERVICE SERVICE SET TO DESCRIPTION TO THE SERVICE SERVI
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	301 360 SEE NOLD LEKATLEH LOAKRIV TRIDITI DOGOQATES FITTI LANDES SIN BENOLS DEASUDE OSAKRIV TRIDITI DOGOQAGO INDEEO I KE TENSON SEE OKSLEANDE OSAKRIV TRIDITI DOGOQAGO INDEEO INDEE SEE OKSLEANDE OSAKRIV TRIDITI DOGOQAGO INDEE OSAKRIV SEE OKSLEANDE OSAKRIV TRIDITI SEE OSAKRIV TOTOTON SEE OKSLEANDE OSAKRIV TRIDITI SEE OSAKRIV TOTOTON SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TOTOTON SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TOTOTON SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV SEE OSAKRIV SEE OSAKRIV TRIDITI SEE OSAKRIV TRIDITI SEE OSAKRIV
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. aeruginosa 	301 360 313 310 314 310 315 310 316 310 317 310 318 310 319 310 310 310 310 310 310 310 310 310 311 3
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	301 300 302 301 303 301 304 301 305 301 306 301 307 301 308 301 309 301 301 301 302 301 303 301 304 301 305 301 306 301 307 301 308 301 309 301 301 301 302 301 303 301 304 301 305 301 306 301 307 301 308 301 301 301 302 301 303 301 304 301 305 301 306 301 301 301 302 301 303 301 304 3
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis E. purnetii F. tularensis 	101 100
 P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila L. pneumophila J. pneumophila 	101
 P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. salmonis C. burnetii F. tularensis L. pneumophila P. aeruginosa 	101

Figure 2. Protein sequence comparison of *P. salmonis* Hsp60 with the corresponding proteins from other bacteria. Multi-alignment was developed by the Vector NTI Suite 6 program.

Approximately 70% of the region coding for Hsp70 was found in contigs 2130 (595 base pairs), 1687 (619 base pairs) and 1748 (581 base pairs) based on an alignment with *Legionella pneumophila* (Fig. 3). The Hsp70 gene was amplified using specific primers designed based on the 5' and 3' end sequences of the gene. Primer p3 (Table I) contains the sequence of the first 20 nucleotides of the gene found in contig 2130. The 3' end was not found in the genomic sequence; as a consequence, primer p4 (Table I) was designed using a consensus sequence based on a highly conserved domain in phylogenetically-related species (see Fig. 4). PCR amplification resulted in a fragment of 1920 base pairs, which corresponds to the size of this gene in other microorganisms (Fig. 3). The sequence of the 1920 base pairs fragment codifies a 70,400 dalton protein of 640 amino acids (Fig. 4). The protein shows a high degree of homology with Hsp70 of other microorganisms.



Figure 3. Isolation of the *P. salmonis* Hsp70 coding region by PCR from genomic *P. salmonis* DNA. The DNA MW ladder is a mixture of Hind III digested Lambda DNA and Hae III digested ØX174 DNA.

		1 60
۶.	salmonis	MAEIIGIDLGTTNSCVAVLDGDKPRVIESAEGDRTTPSTVAYTND-GVTVGDPAKRQAVT
2.	burnetii	MAEIIGIDLGTINSCVAVMEGEKVEVIENAEGSETTESIVAYTEDGEVUVGASAKKOAVT
**	pneumophila	MARTIGIDLYTTNSCVAV MEGDER VIENSEGHRTTPSTVATTODNET VGOS AKROSVT
;	aeruginosa	MORT IGIDLGTINSCVALUENGNV VIENAEGARTIPSTATINDGETLVGOPAKKOAVT
•	cholerae	TIGHT THE TROP AND TRANSPORTED
		61 120
٥.	salmonis	NENNTIFATKRLIGERFSEDTVORDIEREPYTTAAADNGDAWTDVNGEKLAPPOT
2.	burnetii	NADRTINAI KRLIGHTEDDNYVOKDIKMY PYKII KADNGDAWVEVKDKEGKSOKLAPPOI
	pneumophila	NPEKTLEAIKRLIGREFDDPIVQKDIKMVPYKIMKADNGDAWVEVKDDDKAPPQI
•	aeruginosa	NFONTLY AV KELIGER FEENVOLDIONVPYSIVKADNGDAWVEVKGOKMAPPOI
	cholerae	NEORILEAR KRUIGREERDEEVORDIREMPIKINAUNGDAWVEARGORMAAESY
		121 180
P	salmonis	SAQVLAKMIKTAEDYLG <mark>EDVKE</mark> AVITVPAYFNE <mark>A</mark> QRQATKDAG <mark>R</mark> IAGL <mark>O</mark> VKRIIDEPTAA
÷.	burnetii	SAQVLIKMKKTAEDYLGH EVHD AVITVPAYFND <mark>B</mark> ORQATKDAG <mark>K</mark> IAGLNVKRIIN <mark>EPTA</mark> A
	pneumophila	SAEVLEKMEKTAEDYLGEEVKEAVITVPAYFNDE ORQATEDAGE IAGLEVERIINEPTAA
ŀ	aeruginosa	SAEVLEKMEETAEDY LGEPVTEAVITVPAYFNDS GROATEDAGE IAGLEVEET IN EPTAA
	choierae	SALVLERMENTALDE DU LY TRAVITY PATENDE CRUMCEDAUE INCLEVENT INCLEVENT
		181 240
۶.	salmonis	ALAYGNDKKRGDGVIGVYDLGGGTFDISILEIAEVDGEHOFEVLAINGDIHLGGEDFDLR
	burnetii	ALAYONDEKEGÜRKTAVYDLGOGTFÜISTIETAEVDGENGFEVLATNGDTELGGEDFÜLR
4.	pneumophila	ALAYG <mark>MDKKEGDSVIA</mark> VYDLGGGTFD <mark>ISI</mark> IEIAEVD <mark>GEHOFEVLATNGDTFLGGEDFDL</mark> A
•	aeruginosa	ALAYGEDKAKGDHTVIVYDLGGGTFDVSVIEIAEVDGE <mark>HO</mark> FEVLATNGDTVLGGEDFDT
	cholerae	ALAYO DECEMBERT AVYDIGEGTED STIEIDEVERETEVILTNEDTHIAGEDEDN
		241 300
é.,	salmonis	LISYLVDEFKREOGTDENNDPLALORIKEASEKAKIELSSTOOTDVNLPYTADATGPRH
2.	burnetii	LIDYLAGEFKKIEG <mark>VOL</mark> HNDPLAL <mark>O</mark> RLKEA <mark>A</mark> EKAKIELSS <mark>S</mark> OOTDVNLPY <mark>I</mark> TADASGPKH
٠.	pneumophila	LIZYLASEFKRUTGIULHNUPLALORLKEALEKAKIELSSAQQTUVNLPYTADASGPKH
۶.	aeruginosa	LIDYLVDEFKKESGINLKGDPLANORLKEAAEKANIELSSIOQTDVNLPYNTADASGPKH
۰.	cholerae	INYL MUEFEKDOGIIL ER <mark>DPLANORLEEAAEKAKIELSSAQQTUVNLEYI</mark> TADA <mark>I</mark> GPKH
		301 360
ė.,	salmonis	NTRVTRAKFESLVEDLVEGT EFERVALKDAGLEVNEVT LVGGOTRMPKAGAVVKN
2.	burnetii	INTRETRAKEESIVEDIVERTTEPCKVALKDAGIKVSEIDDVILVGGQTRMPKVQEAVKN
.	pneumophila	LNTRETRAKERSLVER LVERTTEPCKTALKDAGLEVSØINEVTLVGGØTRMPLVØKTVER
۶,	aeruginosa	LNXKVSRAKLESLVEDLVORTTEPCKTALKOAGLUVEDIHEVILVGGQTEMPDVQKTVAE
1.	cholerae	NIKVIRAKLEALVEDUVORSTEPLAVALADADLEVNDITEVILVOGOTEMPAVOKVAL
		361 420
٥,	salmonis	FFGKERROVNPDEAVAVGAATOCOVLAGDVKOVLLLOVTPLELGIETMGGVMTKLIEKN
Ξ.	burnetii	FFGKEARKDVNPDEAVALGAALCEAVLSGEVKDVLLLDVTPLELGIETEGGVMTKLIEKN
ι.	pneumophila	EFGKERKDVNPDEAVAVGAAIONAVLEGEVKDILLDVTPLELGIETMGGVMTKLIEKN
۶.	aeruginosa	FFGKEARKDVNPDEAVAVGAAIOCAVLAGDVKDVLLLDVTPLELGIETEGGVMTGLIEKN
1.	cholerae	FPGKERKDVNPDEAVAVGAAVQGAVLAGKVKDVLLLDVTFLSLGIETMGGVMTKLIEKN
		421 480
۰.	salmonis	TELPTKASQTFSTAQDNQTAVTVHVLQGEREVATGNKSLGREDLEDIPAPRGNPOVEVT
2.	burnetii	TTIPTKANOVFSTADDNOTAVTVHVLQGEREMASANKSLGREDLEDIPAPROVPOTEVT
••	pneumophila	TTIPTKATOVESTADINGTAVTVNVLQGERROASANKSLGREDLED PAPRGVPQTEVT
٩.	aeruginosa	TTIPTKKOVFSTADDNOGAVTINVLOGERKOAAONKSLOKPDIADIPAPROVPOTEV
· .	cholerae	TTIFTMINOVFSTAKDNOVAVTTHVLQGERKOAMYNKSLGQFNLEGINFAPRGMPQTEVI
		481 540
۶.	salmonis	EDI DANGI LNVSAKOKOTGKE OSIVI KASSGLSDORVOAMIKOAEDHADDORKE OEDVGA
3.	burnetii	EDIDANGILHVSAKDKATCKEOSIVIKASSGLSDEEVEKMVKDAEAHROSDEKFHELVDA
ι.	pneumophila	FDIDANGILNVSAKDKATGKA <mark>QSIVIKASS</mark> GLS <mark>EKEM</mark> AA <mark>MVKD</mark> AQ <mark>SHAENDK</mark> KEKEMAEL
•	aeruginosa	FDIDANGILHVSAKDKATCKOOBIVIKASSGLEDCTOOMVRDAEANAEEDKKFEBLAAA
	cholerae	CURINGELLIVSAKOKOTOKEOKITIOASEGUSIAETEKMVOEADAKKPEETATA
		541 600
ĕ.	salmonis	ENNALANT HATERO KEALD KRAADOKTALEKAI SELATVISUNDKAVI DERVEALTOAS
	burnetii	ENCADAMI HAARRSWEDLESEVSADE KSALEKAVNEL KEARRSNDE DALEAR TKALTERS
	pneumophila	ENOADSLIHSCERS <mark>MEDLADELGELEKROLETAL</mark> SELKEAVOOTDKARIEDKEKVLIDAS
۴.	aeruginosa	RNOGDALMHATIRKMI TENGDKANAEDKAT LEKALGELEAAVKGDDKAE I EAKMNALSQAS
/.	cholerae	ENDADONI HATEKOTTEASEALEADEKAKTETAINELETAKKSEUKAETDAKYOALMAAA
		601 656
ē.,	salmonis	AMALVI YANQGAE AEAAAAAGAEQAQSQTDEKHODDVVDAEFILEV
2.	burnetii	SKEAERVYAKK <mark>O</mark> GA <mark>A</mark> GAPP <mark>OGEA</mark> EGEP <mark>O</mark> AQAGG <mark>KKEDVVDAEFEEVKDENK</mark> KDEDK
÷.,	pneumophila	AFMAERTYAKKSSECOAACCOTQSCESTKPAEECVVDAEFEEVKEEDKK
۰.	aeruginosa	TPLAOKMYAEQAQQGEDAPQGEQAKAADDVVDAEFEEVKBNK
Z	cholerae	I ACCOACTION AND A AND A A A A A A A A A A A A A A

Figure 4. Protein sequence comparison of *P. salmonis* Hsp70 with the corresponding proteins from other bacteria. Multi-alignment was developed by the Vector NTI Suite 6 program.

2. Synthesis and characterization of P. salmonis Hsp60 and Hsp70.

In order to characterize Hsp60 and Hsp70 from *P. salmonis* and study their immunogenic properties we proceeded to express in E. coli the corresponding recombinant proteins using vector pET32a, in which the cloned genes are expressed as a fusion protein with *E. coli* thioredoxin. For this purpose, the Hsp60 coding region was amplified with primers complementary to the ends of the gene (primers 5 and 6 in Table I) containing the restriction sites BamHI and EcoRI. Primer p6 also contained an extra nucleotide to complete the glycine codon inferred from the consensus sequence for Hsp60 (Fig. 2) and a stop codon. The amplified fragment that codifies an Hsp60 protein 5 amino acids shorter at the carboxyl end in comparison with its homologue in phylogenetically-related species (Fig. 2) was cloned into pGEM-T and digested with EcoRI and BamH I. The fragment released was ligated to vector pET32a, which was previously digested with the same restriction enzymes. Positive clones were identified by enzymatic digestion. BL21(DE3) cells were transformed with the recombinant vector pET32a-Hsp60 to express the recombinant protein. After a 3-hour induction the recombinant protein was analyzed in a 12.5% PAGE-SDS gel. The protein of the expected MW (80 KDa) is found in both, the insoluble and the soluble fractions (Fig. 5). When necessary, the insoluble fraction was solubilized in 4M urea and the recombinant Hsp60 purified in an affinity column with Ni-agarose resin under denaturating conditions (Fig. 5).

The procedure followed for the gene encoding Hsp70 is in essence the same one used for Hsp60. The Hsp70 coding region was amplified from the genome of *P. salmonis* with primers 7 and 8 (Table I), complementary to the ends of the gene and containing the restriction sites EcoRI and XhoI respectively and a stop codon. The amplified fragment was cloned into pGEM-T and later subcloned in pET32a. *E. coli* BL21 (DE3) cells transformed with pET32a-Hsp70 were induced to express the recombinant protein for 3 hours, and the recombinant protein was analyzed in a 12.5 PAGE-SDS gel. The protein is primarily found in the soluble protein fraction. The recombinant protein was purified using a nickel-agarose resin to approximately 85% of purity (Fig. 5).



Figure 5. SDS-PAGE analysis of the expression in *E. coli* and purification of *P. salmonis* Hsp60 and Hsp70 fusion proteins.

A: Lane 1 : Molecular weight markers. Lane 2: 10 μ g of insoluble protein fraction of recombinant BL21(DE3)/ pET32-Hsp60 cells after 3 hours of induction with IPTG. Lane 3: 10 μ g of soluble protein fractions of recombinant BL21(DE3)/pET32-Hsp60 cells after 3 hours of induction with IPTG. Lane 4: Purified Trx-Hsp60 recombinant protein after Ni-agarosa purification.

B: Lane 1 : Molecular weight markers. Lane 2: 10 μ g of insoluble protein fraction of recombinant BL21(DE3)/pET32-Hsp70 cells after 3 hours of induction with IPTG. Lane 3: 10 μ g of soluble protein fraction of recombinant BL21(DE3)/pET32-Hsp70 cells after 3 hours of induction with IPTG. Lane 4: Purified Trx-Hsp70 recombinant protein after Ni-agarosa purification.

3. Detection of Hsp60 and Hsp70 in P. salmonis cultured in cell lines.

A good vaccine candidate is expected to be expressed during host infection by the pathogen. In an attempt to determine whether the genes coding for Hsp60 and Hsp70 were expressed during the replication of *P. salmonis* inside the host cells, CHSE cells infected with *P. salmonis* were cultured until a 90% cytopathic effect was achieved; the bacteria was then harvested as described in Methods. Whole-cell P. salmonis protein was analyzed by Western blot with monoclonal antibodies produced in mice against the recombinant proteins Trx-Hsp60 and Trx-Hsp70. The specificity of the monoclonal antibodies for Hsp60 and Hsp70 was previously confirmed by ELISA and Western blot against each recombinant protein respectively (data not shown). These antibodies recognized the recombinants excised from the thioredoxin fusion peptide with enterokinase, confirming their specific interaction with the target. The Western blot in Figure 6 shows the presence of Hsp60 and Hsp70 in *P. salmonis* cultured in the CHSE cell line. The detected proteins migrate according to the expected size of the native proteins. The mouse antibodies elicited by the recombinant proteins appear specific for the *P. salmonis* heat shock proteins and has no cross reactivity with the eukaryotic counterparts since the monoclonal antibodies do not react with proteins of non-infected CHSE cells (data not shown).





Gel was loaded with 30 µg of whole-cell *P. salmonis* protein and the blotted membrane was analyzed with a 1:200 dilution of each monoclonal antibody. 1: Coomasie blue staining of *P. salmonis* lysate. 2 and 3: Molecular weight markers. 4: Membrane analyzed with a monoclonal antibody against Hsp60. 5: Membrane analyzed with a monoclonal antibody against Hsp70.

The high expression of Hsp60 and Hsp70 detected in *P. salmonis* is coincident with the strong production of these proteins described for other intracellular bacteria during infection (Fernández et al, 1997). In contrast to their cytoplasmic location in most bacteria and eukaryotes, Hsp60 and Hsp70 have been detected in pathogenic bacteria in the periplasm, on bacterial surfaces, and sometimes as extracellular secreted proteins during host infection (Garduño et al., 1998; Scorpio et al., 1994; Gillis et al., 1985). No leader sequences or other recognizable motif that suggest a secretory role are present in the chaperonin proteins, raising the possibility of a unique specific transport mechanism for Hsp in these pathogens. The surface location of Hsp has been linked to a significant role in mediating attachment and invasion of host cells as well as immune modulation activities (Ensgraber and Loos, 1992; Huesca et al., 1996; Retzlaff et al., 1994; Hoffman and Garduño, 1999).

4. Immunogenic properties of the P. salmonis Hsp60 and Hsp70.

Immunogenic reactivity of *P. salmonis* Hsp60 and Hsp70 was studied by the Western Blot technique using a polyclonal rabbit serum developed against an extract of *P. salmonis* obtained from a cell culture. Figure 7 shows that both recombinant proteins are recognized by the antiserum against *P. salmonis*. This result confirms that native Hsp60 and Hsp70 expressed during the intracellular life cycle of *P. salmonis* are immunogenic in rabbits. We also found that serum from Atlantic salmon that had been immunized three months earlier with a formulation containing the two recombinant Hsp reacted with the fusion Trx-Hsp60 and Trx-Hsp70. This was analyzed by ELISA (Fig. 8) and Western blot (Fig. 9A). To confirm that fish serum recognized epitopes of the Hsp and was not merely reacting against the thioredoxin fusion protein, the serum was also tested by Western blot against recombinant Hsp60 and Hsp70 not fused to thioredoxin obtained by expression with vector pET21a (Fig. 9B and 9C). The recognition of both recombinants confirmed that *P. salmonis* Hsp60 and Hsp70 are immunogenic in salmon and thus are bonafide antigens to be included in an experimental vaccine to study their ability to protect against *P. salmonis* infection.



Figure 7. Western blot analysis of recombinant Hsp60 and Hsp70 with

anti P. salmonis serum.

A: Trx-Hsp60 tested with a 1:1000 dilution of *P. salmonis* polyclonal serum.

B: Trx-Hsp70 tested with a 1:1000 dilution of *P. salmonis* polyclonal serum.



Figure 8. Measurement of antibodies to Trx-Hsp60 and Trx-Hsp70 in serum of salmon immunized with both recombinant proteins.

A: ELISA analysis of antibodies against Hsp60 in immunized (O) and control fish (Δ).

B: ELISA analysis of antibodies against Hsp70 in immunized (O) and control fish (Δ).



Figure 9. Western blot analysis of serum obtained from salmon immunized with recombinant Hsp60 and Hsp70.

A: Lane 1 : Electrophoretic pattern of a mixture of purified Trx-Hsp60 and Trx-Hsp70; Lanes 2-6: blots tested with serum of immunized fish; Lane 7: blot tested with serum of non-immunized fish.

B: Lane 1 : Electrophoretic pattern of whole-cell extract containing recombinant T7-Hsp70; Lanes 2-6: blots tested with serum of immunized fish; Lane 7: blot tested with serum of non-immunized fish.

C: Lane 1 : Electrophoretic pattern of whole-cell extract containing recombinant T7-Hsp60; Lanes 2-6: blots tested with serum of immunized fish; Lane 7: blot tested with serum of non-immunized fish.

5. Protection against P. salmonis in vaccinated Atlantic salmon.

One tank containing vaccinated and control non-injected fish was challenged with *P.salmonis* 625 degree days after immunization with the formulation that contained recombinant Hsp60 and Hsp70. A group of adjuvantinjected fish was also included in the trial as control. At day 21 after *P. salmonis* infection, control fish started to die and cumulative mortality increased dramatically during the next 20 days. In contrast, a reduced number of vaccinated fish died during this period, achieving a relative percent survival (RPS) of 89.6% at the end of the challenge (<u>Table II</u>). The strong protective response elicited by the recombinant formulation contrast with the weak protection that has been described for bacterins against *P. salmonis* (Smith et al., 1997) and is coincident with the protective effect elicited by Hsp60 and Hsp70 in other animal models (Ferrero et al., 1995; Gómez et al., 1995). In conclusion our data strongly

supports the use of Hsp60 and Hsp70 as antigens in a recombinant vaccine against the intracellular pathogen *Piscirickettsia salmonis*.

TAB	LE	Π

Protective response of immunized salmon to <i>P. salmonis</i>						
Group		Challenge				
Group	30 days Mortalit	s ty RPS	50 days Mortality RPS			
V1	8%	82.6	8%	89.6		
V0	58%	-26	69%	10.4		
Control	46%		77%			
RPS	=	Relative percent survival				
Control	=	Non-inje	Non-injected fish			
V0	=	Adjuvan	Adjuvant-injected fish			
V1	=	Fish inje proteins	Fish injected with recombinant proteins			

REFERENCES

AGUAYO J, MIQUEL A, ARANKI N, JAMETT A, VALENZUELA PD, BURZIO LO (2002) Detection of *Piscirickettsia salmonis* in fish tissues by an enzyme-linked immunosorbent assay using specific monoclonal antibodies. Dis Aquat Organ 49: 33-38 [Links]

BINDER S (1995) Mitochondrial nucleic acid purification and analysis. Methods Mol Biol 49: 383-389 [Links]

BRANSON EJ, NIETO DÍAZ-MUÑOZ D (1991) Description of a new disease condition occurring in farmed coho salmon, *Oncorhynchus kisutch* (Walbaum) in South America. J Fish Dis 14: 147-156 [Links]

BROCKLEBANK JR, EVELYN TPT, SPEARE DJ, ARMSTRONG RD (1993) Rickettsial septicaemia in farmed Atlantic and Chinook salmon in British Columbia: Clinical presentation and experimental transmission. Can Vet J 34: 745-748 [Links]

ENSGRABER M, LOOS M (1992) A 66-kilodalton heat shock protein of *Salmonella typhimurium* is responsible for binding of the bacterium to intestinal mucus. Infect Immun 60: 3072-3078 [Links]

FERNÁNDEZ RC, LOGAN SM, LEE SH, HOFFMAN PS (1997) Elevated levels of *Legionella pneumophila* stress protein Hsp60 early in infection of human monocytes and L929 cells correlate with virulence. Infect Immun 64: 1968-1976 [Links]

FERRERO RL, THILBERGE JM, KANSAU I, WUSCHER N, HUERRE M, LABIGNE A (1995) The GroES homolog of *Helicobacter pylori* confers protectiveimmunity against mucosal infection in mice. Proc Natl Acad Sci USA 92: 6499-6503 [Links]

FRYER JL, LANNAN CN, GARCÉS LH, LARENAS JJ, SMITH PA (1990) Isolation of a rickettsia-like organism from diseased coho salmon (*Oncorhynchus kisutch*) in Chile. Fish Pathol 25: 107-114 [Links]

FRYER JL, MAUEL MJ (1997) The Rickettsia: an emerging group of pathogens in fish. Emerg Infec Dis 3: 137-144 [Links]

GARDUÑO R, FAULKNER G, TREVORS MA, VATS N, HOFFMAN P (1998) Immunolocalization of Hsp60 in *Legionella pneumophila*. J Bacteriol 180: 505-513 [<u>Links</u>]

GILLIS TP, MILLER RA, YOUNG DB, KHANOLKAR DR, BUCHANAN TM (1985) Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. Infect Immun 49: 371-377 [Links]

GÓMEZ FJ, ALLENDOERFER R, DEEPE GS JR (1995) Vaccination with recombinant heat shock protein 60 from *Histoplasma capsulatum* protects mice against pulmonary histoplasmosis. Infect Immun 63: 2587-2595 [Links]

HOFFMAN PS, GARDUÑO RA (1999) Surface-associated heat shock proteins of *Legionella pneumophila* and *Helicobacter pylori*: roles in pathogenesis and immunity. Infect Dis Obstet Gynecol 7: 58-63 [Links]

HOUSE ML, BARTHOLOMEW JL, WINTON JR, FRYER JL (1999) Relative virulence of three isolates of *Piscirickettsia salmonis* for coho salmon *Oncorhynchus kisutch*. Dis Aquat Organ 35: 107-113 [Links]

HUESCA M, BORGIA S, HOFFMAN P, LINGWOOD CA (1996) Acidic pH changes receptor binding specificity of *Helicobacter pylori*: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. Infect Immun 64: 2643-2648 [Links]

JAMETT A, AGUAYO J, MIQUEL A, MÜLLER I, ARRIAGADA R, BECKER MI, VALENZUELA P, BURZIO LO (2001) Characteristics of monoclonal antibodies against *Piscirickettsia salmonis.* J Fish Dis 24: 205-215 [Links]

JONES SR, MACHAM RJ, GROMAN DB, CUSACK RR (1998) Virulence and antigenic characteristics of a cultured Rickettsia-like organism isolated in farmed Atlantic salmon *Salmo salar* in eastern Canada. Dis Aquat Organ 33: 25-31 [Links]

KAUFMAN SHE (1990) Heat shock proteins and the immune response. Immunol Today11: 129-136[Links]

KAUFMAN SHE (1991) Heat shock proteins and pathogenesis of bacterial infection. Springer Semin. Immunopathol 13: 25-36 [Links]

KAUFMAN SHE, SCHOOL B, WAND-WÜRTTENBERGER A, STEINHOFF U, MUNK ME, KOGA T (1990) T-cells, stress proteins and pathogenesis of mycobacterial infections. Curr Top Microbiol Immunol 155: 125-141 [Links]

KÖHLER G, MILSTEIN C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) 256: 495-497 [Links]

LAEMMLI UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685 [Links]

LANNAN CN, FRYER JL (1993) *Piscirickettsia salmonis*, a major pathogen of salmonid fish in Chile. Fish Res 17: 115-121 [Links]

MIQUEL A, MÜLLER I, FERRER P, VALENZUELA PDT AND BURZIO LO (2003) Immunoresponse of Coho salmon immunized with a gene expression library from *Piscirickettsia salmonis*. Biol Res 36: 313-323 [Links]

RETZLAFF C, YAMAMOTO Y, HOFFMAN PS, FRIEDMAN H., KLEIN TW (1994) Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures. Infect Immun 62: 5689-5693 [Links]

SANGER F, NICKLEN S, COULSON AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467 [Links]

SCORPIO A, JOHNSON P, LAQUERRE A, NELSON DR (1994) Subcellular localization and chaperone activities of *Borrelia burgdorferi* Hsp60 and Hsp70. J Bacteriol 176: 6449-6456 [Links]

SMITH PA, CONTRERAS JR, LARENAS JJ, AGUILLÓN JC, GARCES LH, PÉREZ B, FRYER JL (1997) Immunization with bacterial antigens: Piscirickettsiosis. Dev Biol Stand 90: 161-166 [Links]

TOWBIN H, STAEHELIN T, GORDON J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedureand some application. Proc Natl Acad Sci USA 76: 4350-4354 [Links]

WILHELM V, VILLEGAS J, MIQUEL A, ENGEL E, BERNALES S, VALENZUELA PDT, BURZIO LO (2003) The complete sequence of the mitochondrial genome of the Chinook salmon, *Oncorhynchus tshawystscha*. Biol Res 36: 223-231 [Links]

WILHELM V, MORALES C, MARTÍNEZ R, ROSEMBLATT M, BURZIO LO, VALENZUELA PDT (2004) Isolation and expression of the genes coding for the membrane bound transglycosylase B (MItB) and the transferring binding protein B (TbpB) of the salmon pathogen *Piscirickettsia salmonis*. Biol Res 37: 783-793 [Links]

ZÜGEL U, KAUFMAN SHE (1999) Role of heat shock proteins in protection and pathogenesis of infectious diseases. Clin Microbiol Rev 12: 19-

39 [Links]Corresponding Author: Pablo D. T. Valenzuela, Address: Fundación Ciencia

para la Vida, Av. Zañartu 1482 Ñuñoa, Santiago, Chile. Tel: (56-2) 239-8969, Fax: (56-2) 237-2259. E-mail: <u>pvalenzu@bionova.cl</u>

Received: December 7, 2004. Accepted: February 28, 2005

Todo el contenido de esta revista, excepto dónde está identificado, está bajo una Licencia Creative Commons

Sociedad de Biología de Chile

Canadá 253, piso 3º, Dpto. F.

PO Box 16164

Santiago - Chile

Tel.: (56-2) 22093503

Fax: (56-2) 22258427

Mail <u>socbiol@biologiachile.cl</u>