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Full Length Research Paper

Immunogenicity of different cellular fractions of *Vibrio parahaemolyticus* strains grown under sub-lethal heat and osmotic stress

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Vibrio parahaemolyticus **is an estuarine bacterium widely distributed in natural aquatic environments and also a well-known food-borne pathogen, and like other enteropathogenic bacteria, it survives both in its natural environment and in its host. The transition from one state to another requires a high capacity to adapt to different physico-chemical parameters, process which increases the synthesis of specific proteins, including heat-shock proteins (HSPs). In order to investigate the immunogenicity of heat shock proteins from different cellular fractions of *V. parahaemolyticus* grown under sub-lethal heat and osmotic shock, we analyzed the response of holoxenic mice to *V. parahaemolyticus* cellular fractions injected intraperitoneally by examining the anti-HSP antibodies response to stressful conditions and the immune protection installed after several immunizations. The supernatant and total cellular extract of heat or simultaneously heat and osmotically-stressed bacterial cultures induced the early occurrence of anti-HSP antibodies (after 4 immunizations), while after 8 immunizations, the anti-HSP antibodies were present in the majority of the tested batches, the most immunogenic fraction proving to be the heat-stressed culture supernatants.**

Key words: *Vibrio parahaemolyticus*, heat shock proteins, heat shock response, mice immunization.

INTRODUCTION

Vibrio parahaemolyticus, is a well-known food-borne pathogen causing gastrointestinal diseases and also an estuarine bacterium widely distributed in natural aquatic environments around the world (Colwell, 1979; Chai and pace 1993). High incidence of this pathogen undoubtedly originates from the frequent consumption of marine foods in countries like Taiwan, Japan and other Asian Pacific countries (Wong et al., 2002). *V. parahaemolyticus* is less virulent than *Vibrio vulnificus* and the disease is normally limited to a relatively mild gastroenteritis with diarrhea, abdominal cramps, vomiting, headache and fever in immunocompetent people. In people with underlying health problems, particularly liver disease, the bacteria may spread into the blood and cause septicemia. It can also cause wound infections. Some *V. parahaemolyticus* infections may require hospitalization, however the disease is rarely fatal (Hally et al., 1995).

The entry of this pathogen into the host organism involves significant environmental changes, which are expected to induce the heat shock response materialized by the induction of many proteins called heat shock proteins. Heat-shock proteins (HSPs) are commonly grouped into families based on their molecular weight. In bacteria, the major HSP_S are DnaK (70 kDa), GroEL (60 kDa), GroES (10 kDa) and DnaJ (40 kDa). In contrast to proteins expressed in bacteria, eukaryotic cells express a wide array of (HSPs) such as: small HSPs, GroES-homologue proteins or HSP10 (~10 kDa), DnaJ-homologue proteins or HSP40 (~40 kDa),

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GroEL-homologue proteins or HSP60 (~60 kDa), DnaK-homologue proteins or HSP70 (~70 kDa), HptG-homologue proteins or HSP90 (~90 kDa), and Clp ATP-dependent proteases (HSP100) (Lindquist and Craig,1988; Watson, 1990; Lathigra et al.,1991; Shinnick et al., 1988; Ellis, 1996; Ray, 1999). These include chaperones, proteases, alternative sigma factors and other regulatory and structural proteins. Some of the HSP_S are themselves virulence factors, while others affect pathogenesis indirectly, by increasing bacterial resistance to host defences or by regulating virulence genes expression (Gophna and Ron, 2003).

Even though HSP_S are ubiquitous and highly homologous among different species, they represent important antigenic targets of the cellular and humoral immune response. Several studies have proposed that these stress proteins are suitable to be antigen carriers mainly because of their ability to bind to certain peptides (Roman and Moreno, 1996).

Microbial HSP_S have been shown to be important immunogens, stimulating both T and B cells (Cohen and Jung 1991; Polla et al., 1993). The immune response to HSPs might be directed against autologous molecules and involves T-lymphocytes, if common epitopes are presented by MHC class II molecules (Aguas et al., 1990, Lo et al., 2006). Yamazaki et al. (2002) showed that HSP60-reactive T-cells accumulate in the gingival tissues of periodontitis patients and that this response is inhibited by anti-MHC class II antibodies. Lo et al. (2000) demonstrated that MHC class Ib molecules are involved in autoimmune infections with Gram-negative bacteria and identified an immunodominant epitope (GMQFDRGYL) derived from the HSP60 family that is present in *Salmonella typhimurium, Salmonella typhi, Escherichia coli*, *Yersinia enterolitica, Klebsiella pneumoniae* and *Helicobacter pylori*.

For a long time, HSP autoimmune responses were thought to be the result of cross-reactivity between bacterial and host HSPs. Molecular mimicry between bacterial and human HSPs has been well-documented and may allow microorganisms to avoid the host's mechanisms of defense (Dubois, 1989). A humoral response against microbial HSPs may be destructive for the host leading to an autoimmune response. Three models have been proposed to link microbial infections to subsequent autoimmune reactions involving HSPs. These models are based on molecular mimicry between microbial HSPs and HSPs or constitutive proteins from the host, inflammation-induced exposure of cryptic cell epitopes that could be a target for immune reactions, and antigen persistence in infected sites leading to chronic immunological reactions (Res, et al., 1991).

Srivastava et al. (1998) discovered that HSPs could also act as carriers of antigenic peptides derived from tumors or virus-infected cells. These HSP-peptide complexes "shuttle" antigenic peptides to the MHC class I presentation pathway of antigen-presenting cells. Wallin et al. (2002) reported that HSPs also activate the innate immune system.

The stresses of nutrient deprivation and low temperature have been shown to be the main causes of the stress response induction in some pathogenic bacteria (Wallin et al., 2002; Xu et al., 1982; Nilsson et al., 1991; Weichart et al., 1992; Wolf et al., 1992).

Therefore, it is important to understand how *V. parahaemolyticus* survives under different stress conditions and how the stress adaptation is influencing its pathogenicity and immnogenicity.

The purpose of the present study were: i) to establish the levels of heat shock proteins in different cellular fractions of *V. parahaemolyticus* in relation with a certain stress factor and ii) to investigate the *in vivo* response of holoxenic mice to different cellular fractions of *V. parahaemolyticus* grown under sub-lethal heat and osmotic shock, by examining the level of antibodies against heat shock proteins.

MATERIALS AND METHODS

Bacterial strains and cultivation

Two strains of *V. parahaemolyticus*, one hemolytic, thermostable direct hemolysin (TDH) positive (encoded strain 1) and one non-haemolytic, TDH negative strain (encoded strain 2), first isolated from acute diarrhea and the second, from Danube Delta estuarine water were used in our study. Both strains were kindly provided by the National Reference Center for Cholera and other vibrios from the National Institute of Research-Development for Microbiology and Immunology Cantacuzino. The induction of the thermal stress was obtained by incubating the bacterial cultures grown in liquid medium at 42°C for 30 min, while for the osmotic stress, bacterial strains were grown in nutrient broth supplemented with 8% NaCl.

Cellular fractions preparation

Three cellular fractions were analyzed in order to establish the level of heat proteins synthesized in normal and stress conditions. The *V. parahaemolyticus* cellular fractions studied were: Total cellular, extracellular and periplasmic fractions. The protocol of preparation for these cellular fraction was taken from an article by Wong et al. (2002).

The total cellular fraction was obtained by lysing the cell pellet in a specific buffer with the following contents: per 100 ml, 10 ml of TBS buffer (0.41M Tris, 0.4 M boric acid, 1% sodium dodecyl sulfate [SDS], pH 8.64), 5 g of glucose, 185 mg of EDTA, 5 ml of 2-mercaptoethanol, 1.9 g of SDS and 5 ml of glycerol. The extracellular fraction was obtained by centrifugation of the culture and the supernatant was concentrated 100-fold by ultrafiltration (Amicon Corp., Lexington, Mass.).

The periplasmic fraction was obtained by following the method of Callahan et al. (1995). Briefly, 40 ml of bacterial culture was pelleted by centrifugation, resuspended in 4 ml of 50 mM Tris HCl buffer (pH 8.8) at 4°C and to which 0.8 ml of chloroform was added and incubated at room temperature for 10 min. Eight milliliters of the Tris.HCl buffer was added to this mixture, mixed, centrifuged at 16,000xg for 20 min and the supernatant was collected.

These cellular fractions were analyzed in order to establish the level of heat proteins synthesized in normal and stress conditions (Callahan et al., 1995). The contamination of cytoplasmic proteins

into the periplasmic and extracellular fractions was monitored by determining the presence of cytoplasmic glucose-6-phosphate dehydrogenase using a spectrophotometric enzyme assay kit (Sigma Co., St. Louis, MO) (Taubert et al., 2000). Protein concentration in the samples was determined by Coomassie blue dye binding method (Dai et al., 1992).

Western immunoblot

Proteins from different fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel. In order to quantify the protein levels, 20 µg of total proteins extract were migrated per lane. Separated proteins were electro-transferred to a PDVF (Invitrogen) membrane for 1 h in a buffer containing 20 mM Tris-HCl, (pH 7.4), 150 mM glycine and 20% methanol. The membrane containing transferred proteins were blocked and further treated according to WesternBreeze® Chromogenic Kit (Invitrogen). Briefly, the membrane were incubated with first antibodies [rabbit anti-GroES-10kD (Sigma-G8909); rabbit anti-GroEL-60kD (Sigma-G6532); mouse monoclonal anti-heat shock protein 70 (Sigma-H5147)] over night at 4°C. Each membrane slice was then washed and treated with alcaline phosphatase marked goat anti-rabbit immunoglobulin G (for HSP10 and HSP60) or goat anti-mouse immunoglobulin G (for HSP70) and finally reacted with BCIP/ NBT.

Mouse immunization

Conventional (holoxenic) mice, *Mus musculus*-Balb/c were used in the experiments. The number of laboratory animals to be used was minimal and all measures were taken so that the discomfort, suffering and pain would be minimal. For this experiment, the approval of the University of Bucharest Ethics Commission was obtained. The mice were separated in batches of five animals.

The total cellular, extracellular and periplasmic fractions of *V. parahaemolyticus* cultures were used in immunization studies. Each batch of 5 animals was immunized by intraperitoneal (i.p.) administration of 0.1 ml of *V. parahaemolyticus* culture fractions. The immunization was repeated once a week for 10 weeks. Ten mice kept in the same experimental conditions and injected with PBS were used as controls. All the experiments were performed in duplicates. The course of infection was determined by daily examination of each batch (Chifiriuc et al., 2007).

The serum from the analyzed holoxenic mice was collected at four and respectively, eight weeks of immunization by retroorbital puncture, a method which has the benefits such as the absence of marked hemolysis, a short manipulation time and the possibility of repeated collection at the same site.

Evaluation of anti-HSP antibodies response using ELISA

The evaluation of immune response induced by different fractions of the stressed bacterial cultures (soluble fraction located in the supernatant or cell fraction located in the bacterial cytoplasm or periplasmic compartment) was achieved by ELISA assay the anti-HSP antibodies (anti-GroES, anti-GroEL and anti-DnaK) in animal sera harvested after four and respectively eight weeks of immunization (Pierce Endogen kit, respecting the producer's indications).

The assay principle was based on indirect ELISA. Briefly, the MaxiSorp microtiter plate (Nalge Nunc International, NY, U.S.A) was coated at 4°C overnight with 2 ng/ml heat shock protein (Sigma) in phosphate buffered saline (PBS), and washed with phosphate buffered saline Tween (PBST). The microtiter plate was washed 5 times with 300 µl/well of PBST and soaked for 1 min each cycle. Residual moisture was thoroughly tap-dried from the wells onto absorbent papers before the plate was subjected to the next step. The remaining unbound plate surfaces were then blocked for 2 h at room temperature by floating each well with 300 µ1 of blocking buffer (0,1% BSA, 0,05% Tween 20 in PBS). The plate was then washed and thoroughly tap-dried before it was reacted for l h at room temperature with 100 µl/well sera (samples or control). After another washing cycle, the presence of HSP antibodies were detected by incubation at room temperature for 1 h with l00 µl/well of anti-mouse IgG-HRP (2 pg/ml) in dilution buffer.

Finally, 100 µl/well of freshly prepared substrate buffer was added, and incubation was carried out for another 20 min at room temperature in the dark. The reaction was then terminated by acidification with 100 µl/well of stopping solution before the absorbance was measured spectrophotometrically. Absorbance was read in microplate reader (Apollo LB911, Berthold Technologies) at dual-wavelength 450/630 nm. The cut-off was calculated as mean OD of negative (control) + 3 standard deviation. Samples above the grey zone (15% over cut-off) were positive. The levels of anti-HSP antibodies were expressed as the ratio between the sample and control absorbance average value.

RESULTS

Detection of heat shock proteins in *V. parahaemolyticus* was investigated by SDS-PAGE and Western immunoblotting procedure using anti-HSP 10, anti-HSP 60 and anti-HSP 70 antibodies. Our results confirmed the presence of heat shock proteins GroES (HSP 10kDa), GroEL (HSP 60kDa) and DnaK (HSP 70kDa).

In our study, the amount of these proteins varied depending on the strain, the analyzed cellular fraction and the different stressful conditions applied to the bacterial cultures (Figures 1 to 3). Among the 3 types of proteins, GroEL was expressed regardless of the type of experimental conditions (in the presence or absence of heat or osmotic stress) in all cellular fractions. As expected, both strains showed an increased protein synthesis (both GroEL and DnaK) after heat or simultaneous heat and osmotic shock. This is well observed in case of GroEL levels for KP supernatant fractions exposed to stress conditions (Figure 1).

In case of the KP strain, DnaK levels were high for periplasmic and total cellular fractions exposed to simultaneous heat and osmotic stress conditions. For the KN strain, both GroEL and DnaK were expressed in high concentrations in all cellular fractions exposed to heat shock.

Concerning the GroES expression in different stress conditions, the KP and respectively KN strain responded differently, GroES accumulated preferentially in the supernatant of simulatenously heat and osmotically-stressed KP strain cells, while in the case of KN strain, GroES was evidenced in the periplasmic and total lysate fraction of non-stressed or heat-stressed cells (Figure 3).

Overall, the highest levels of expression were observed for the KN strain (isolated from the environment) exposed to heat shock conditions (Figures 2 and 3: strain encoded2/42). The antibodies against heat shock protein GroES could not be detected in our ELISA assay in any

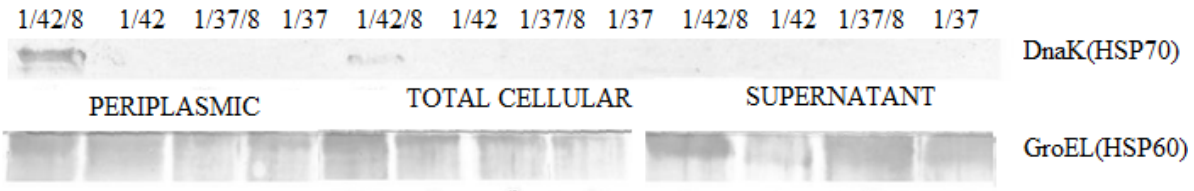

Figure 1. Levels of heat shock proteins (DnaK and GroEL) for different fractions of Kanagawa positive *V. parahaemolyticus* strain (encoded 1); 42/8-concomitant stress, 42-heat shock, 37/8-osmotic stress and 37-normal condition.

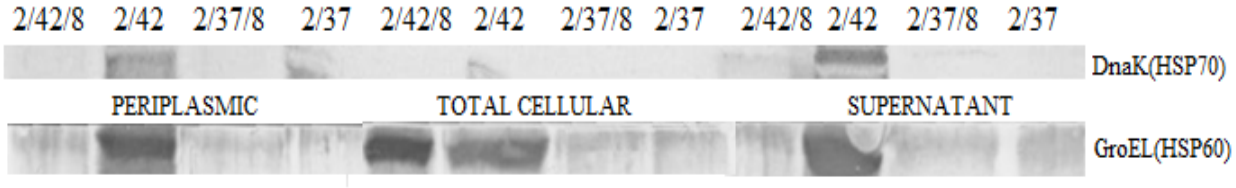

Figure 2. Levels of heat shock proteins (DnaK and GroEL) for different fractions of Kanagawa negative *V. parahaemolyticus* strain (encoded 2); 42/8-concomitant stress, 42-heat shock, 37/8-osmotic stress and 37-normal condition.

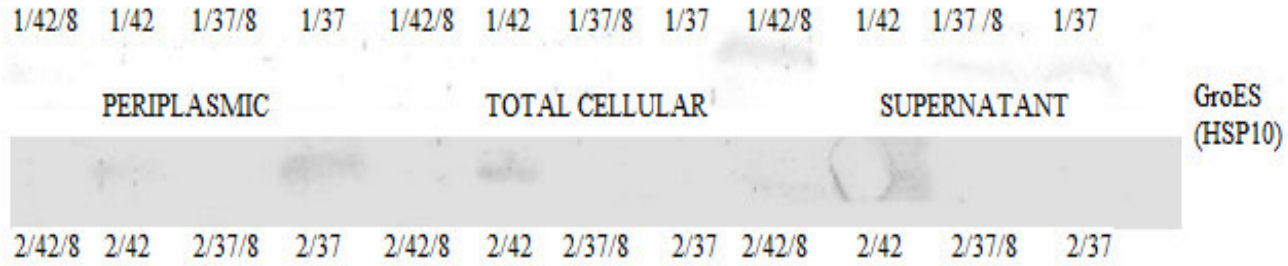

Figure 3. Levels of GroES for different fractions of *V. parahaemolyticus* strains (KP-1; KN-2); 42/8-concomitant stress, 42-heat shock, 37/8-osmotic stress and 37-normal condition.

animal batches. The levels of anti-GroEL and anti-DnaK antibodies exhibited an ascendant temporal dynamic for all analyzed cellular fractions (Figures 4 to 9).

The bacterial supernatants used for mice immunization induced a high level of antibodies in case of the KP strain exposed to heat shock and simultaneous shock. Thus, we can observe an elevated antibody response oriented against HSPs (GroEL, DnaK) in case of the KP strain heat and osmotically stressed, response which is well corellated with the Western blot analysis. For the KN strain, the supernatant fraction exposed to heat stress induced high levels of antibodies after 8 weeks of immunization.

The immunization with periplasmic fractions obtained from the KP strain led to the rapid appearance (after 4 immunizations) of anti-DnaK antibodies. The periplasmic fractions of KP strain exposed to simultaneous stress and KN strain heat stressed induced high levels of anti-DnaK and anti-GroEL antibodies (Figures 6 and 7).

The early occurrence of these antibodies was observed for the total cellular fraction of the KN strain exposed to heat shock. This result was expected due to the high levels of corresponding HSPs accumulated in this fraction (as revealed by the Western blot analysis). An elevated antibody immune response was also highlighted in case of immunization with KP strain exposed to heat and heat and osmotic shock (Figures 8 and 9).

The interest in the beneficial effects of these thermotolerant proteins includes their possible contribution to protective immunity (Polla et al., 1996). Given the fact that HSPs are considered dominant antigens in numerous infections, several studies have demonstrated the

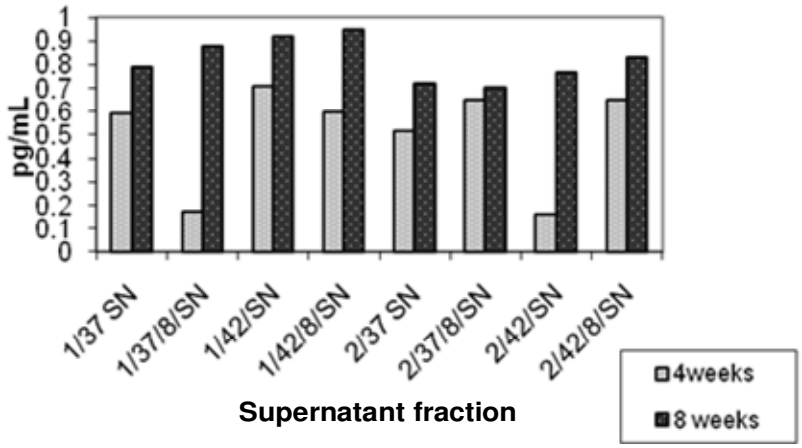

Figure 4. The levels of anti-DnaK antibodies obtained after mice immunization with supernatant fractions and thereafter measured using ELISA method; serum was collected 4 and 8 weeks after immunization.

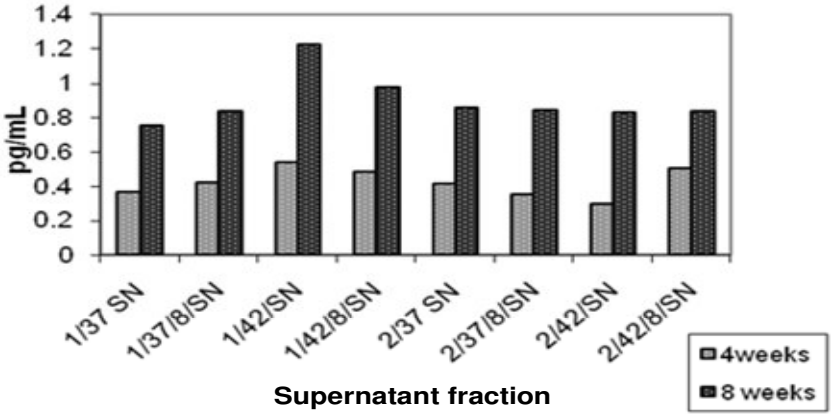

Figure 5. The levels of anti-DnaK antibodies obtained after mice immunization with supernatant fractions and thereafter measured using ELISA method; serum was collected 4 and 8 weeks after immunization.

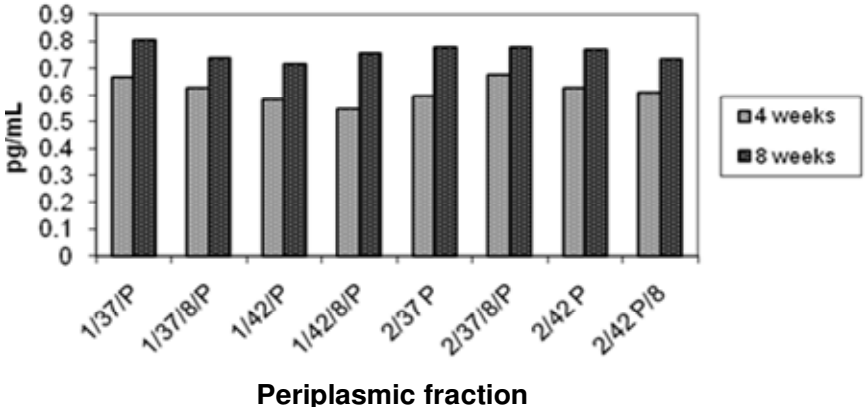

Figure 6. The levels of anti-DnaK antibodies obtained after mice immunization with periplasmic fractions and thereafter measured using ELISA method; serum was collected 4 and 8 weeks after immunization.

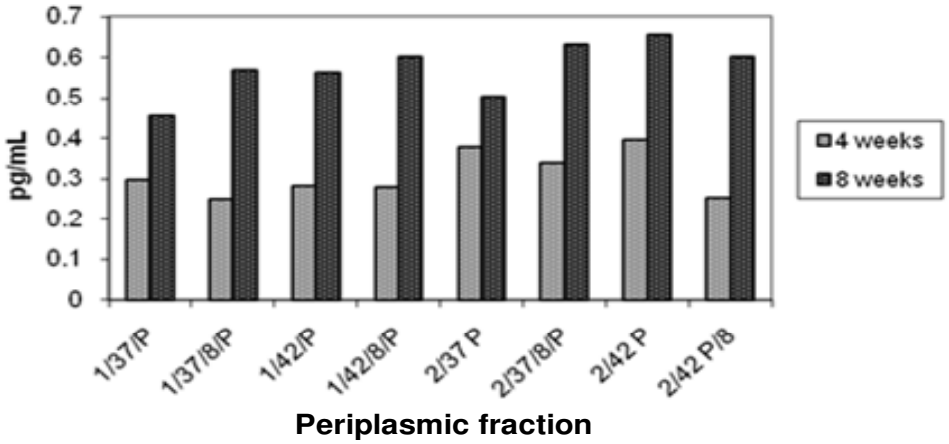

Figure 7. The levels of anti-DnaK antibodies obtained after mice immunization with periplasmic fractions and thereafter measured using ELISA method; serum was collected 4 and 8 weeks after immunization.

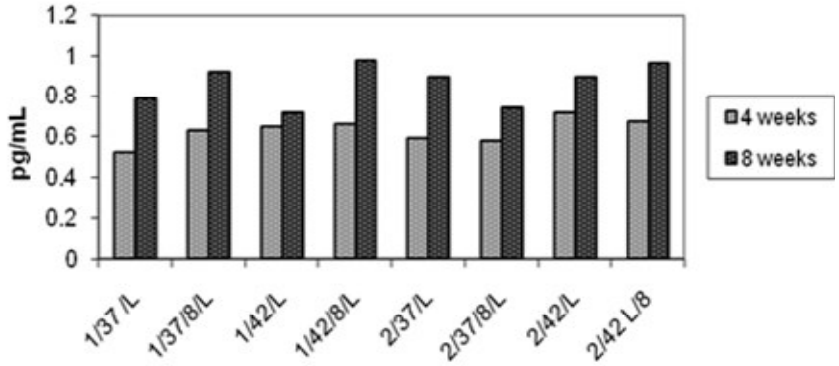

Figure 8. The levels of anti-DnaK antibodies obtained after mice immunization with cellular lysate fractions and then measured using ELISA method; serum was collected 4 and 8 weeks after immunization.

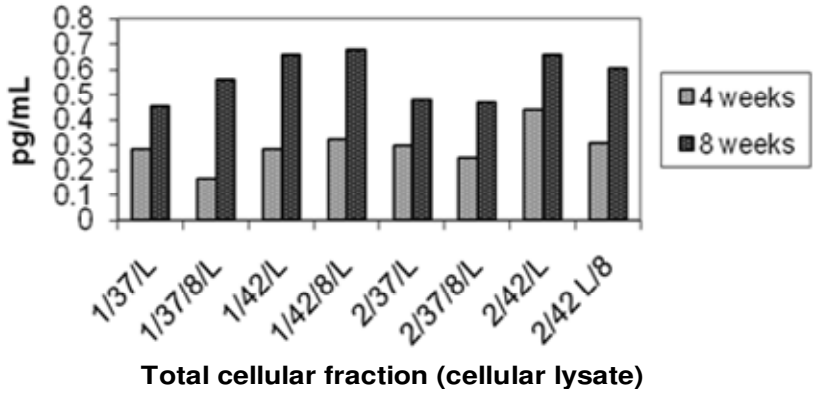

Figure 9. The levels of anti-DnaK antibodies obtained after mice immunization with cellular lysate fractions and then measured using ELISA method; serum was collected 4 and 8 weeks after immunization.

protective role induced by the vaccination using HSPs from *H. pylori* (Ferrero et al., 1995), *Histoplasma capsulatum* (Gomez et al., 1995), *Y. enterocolitica* (Noll and Autenrieth, 1996) and *Mycobacterium tuberculosis* (Silva and Lowrie, 1994).

Previous studies have shown that HSPs activate the immune inflammation network. It was demonstrated that GroEL stimulates the production of both interleukin-6 (IL-6) and interleukin-8 (IL-8) by human gingival fibroblasts without affecting their viability and also stimulates the production of IL-6 by a confluent monolayer of human gingival epithelial cells and is cytotoxic when used at high concentrations.

HSPs are potent molecules that signal tissue damage and cell stress to the immune system (Yamaguchi et al., 1992, 2002). HSP60 seems to be the best activator of human monocytes and dendritic cells (Bethke et al., 2002).

The level of anti-HSP antibodies was correlated with the presence of high amounts of the respective HSP in a certain bacterial fraction, as demonstrated by the immunoblotting *in vitro* assay. The most illustrative example was the correlation between the presence of DnaK in the supernatant fraction of the heat-stressed *V. parahaemolyticus* cells and the generation of an early anti-DnaK (HSP 70) immune response in the animals immunized with this fraction. Also, the undetectable levels of anti-GroES antibodies have been well correlated with the lowest expression of this protein in the analyzed fractions of bacterial strains submitted to different limiting conditions.

Conclusion

The immunization of holoxenic mice with different *V. parahaemolyticus* cellular fractions submitted to heat and/or osmotic stress demonstrated that these fractions are immunogenic, inducing after repeated mice immunizations, the occurrence of different protective antibodies, including anti- GroEL (~60 kDa) and anti-DnaK (~70 kDa), proving that heat shock proteins may play a regulatory role in the patho-physiological infectious processes. However, further studies are necessary in order to clarify if these antibodies are exhibiting any protective role in experimental infection challenge if this role could be exclusively correlated with the presence of anti-HSP antibodies or there is a cumulative effect due to the presence of different bacterial components in the analyzed fractions, acting as immunogenic substances or adjuvants.

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