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ORIGINAL ARTICLE

Expression and Dendrogram Analysis of Heat Shock Proteins in Culture Media of *Aeromonas hydrophila*

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ABSTRACT

One-dimensional SDS-PAGE of HSPs expressed from *Aeromonas hydrophila (A . hydrophila)* isolated from the fish revealed protein profiles containing 11–16 discrete bands with molecular weight of 13–105 kDa. Seven bands having molecular weight range of 13–37 kDa were present in all the isolates. A high molecular weight protein band (105 kDa) was present only when the *A. hydrophila* was incubated at 25^{O}C for 48 h. *A hydrophila* incubated at 25^{O}C for 24 h produced several proteins in increased amounts compared with amounts produced at 42^{O}C (13 bands) and 50^{O}C (12 bands). The highest level of HSP produced was when the *A. hydrophila* cells were exposed to a downshift of temperature to 25 degrees C for 48 hrs (16 bands). On the other hand, the least amount produced of some proteins was at the temperature of 42^{O}C for 48 hrs (11 bands). Some of the bands were under-expressed to the degree that the computer was unable to demonstrate its intensity. Dendrogram analysis revealed that the highest similarity (84.85%) was between HSPs group expressed after exposure of *A. hydrophila* to 42^{O}C for 48 hrs (lane 4) and those expressed after exposure of *A. hydrophila* cells to 50^{O}C for 24 hrs (Lane 5). lane 1 reflected 75.65% similarity with lanes 2-6. The one-dimensional SDS-PAGE of HSPs expressed from *A. hydrophila* isolated from the chicken revealed protein profiles containing 13–15 discrete bands with molecular weight of 14–96 kDa. Eight bands having molecular weight range of 14–43 kDa were present in all the isolates. The highest molecular weight protein band (96 kDa) was present only when the *A. hydrophila* was incubated at 42^{O}C for 24 h and the lowest (14 kDa) was when the *A. hydrophila* was incubated at 25^{O}C, 42^{O}C and 50^{O}C for 24 and 48 h. *Aeromonas hydrophila* incubated at 25^{O}C and 42^{O}C for 24 h produced several proteins in increased amounts (15 bands) compared with amounts produced at 42^{O}C for 48 h and 50^{O}C for 24 h (13 bands). The dendrogramatic analysis of the HSPs group expressed after exposure of *A. hydrophila* to 25ºC for 48 hrs (Lane 2) 72% similarity with those expressed after exposure of *A. hydrophila* cells to 42ºC for 48 hrs (Lane 4). The same percentage of similarity was noticed between Lanes 5 & 6. On the other hand, the group of HSPs produced by the *A. hydrophila* when exposed to 25^{O}C for 24 h (Lane 1), 50^{O}C for 24h and 48 h (Lanes 5 & 6) differed by 56.05% with the HSPs produced after exposure of *A. hydrophila* cells to 25ºC for 48 h, 42ºC for 24 hrs and 48 hrs (Lanes 2, 3 and 4). Dendrogram analysis revealed that the highest similarity between the expressed HSPs was between those expressed from *A. hydrophila* isolated from fish and incubated at 25^{O}C for 24 h and those expressed from *A. hydrophila* isolated from chicken and incubated at 42^{O}C for 24 h (71.54%). The least similarity recorded (56.21%) was between the HSPs expressed after the *A. hydrophila* isolated from chicken was incubated at 42^{O}C for 48 h and those expressed from *A. hydrophila* also isolated from chicken after incubation at 50^{O}C for 48 h.

Key words: Heat shock proteins; *Aeromonas hydrophila;* heat stress; dendrogram

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Introduction

Aeromonas is an environmental microorganism autochthonous of aquatic environments that can be sporadically transmitted to humans (Borrell *et al.*, 1998; Janda and Abbott, 1998). Food of animal origin, seafood and vegetables has been considered an important vehicle of *Aeromonas* spp. infections (Altwegg *et al.*, 1991; Kirov, 1993; Mattick and Donovan, 1998). *Aeromonas hydrophila*, a gram-negative motile rod, is considered one of the most important bacterial pathogens of aquatic animals in temperate areas, as it has been isolated from diseased fish, eels, frogs, and turtles (Esteve *et al.*, 1993; Kulp and Borden, 1942; Pasquale *et al.*, 1994; Thune *et al.*, 19936) causing fatal hemorrhagic septicemia (Austin, 1993; Del Corral *et al.*, 1990; Handfield *et al.*, 1996; Paniagua *et al.*, 1990).

Fish have low body temperature ($25^{O}C$). Therefore, it was noteworthy to hypothesize that microorganisms, such as *Aeromonas* spp., responsible for a variety of diseases in fish respond to tissue invasion with the production of heat shock proteins. It was therefore proposed that, heat shock proteins produced by bacteria affecting fish hosts maybe virulence determinants, since without the ability to produce this unique class of protein, bacteria would be incapable of producing disease (Love and hirsh, 1994). Heat shock proteins (HSPs) are a large family of proteins with different molecular weights and different intracellular localizations. These proteins undertake crucial functions in maintaining cell homeostasis. HSP Gp96/grp94, because of its peptide chaperone capacity and its ability to interact actively with professional antigen-presenting cells (APCs), is also endowed with crucial immunological functions such as natural adjuvant for priming innate and adaptive immunity (Semenova *et al.*, 2007; Strbo and podack, 2008). Therefore, it was essential to determine their production in vitro to determine whether *Aeromonas hydrophila* exhibits a heat shock response.

Materials and methods

2.1. Bacterial Strains and Growing Conditions:

Environmental strains have been isolated in our laboratory from fish and chicken samples. Presumptive *Aeromonas* spp. were isolated from 250 frozen fresh water fish (Tilapia, *Oreochromis niloticus niloticus*) purchased from the local markets in Cairo. Twenty-five grams of fish flesh were weighed aseptically and homogenized for 2 min in stomacher bags containing 225 ml of alkaline peptone water. After 18 h of incubation at $37^{O}C$, an aliquot of the enrichment was inoculated in blood agar containing 30 mg/l ampicillin and incubated for 24 h at $28^{O}C$. Five presumptive *Aeromonas* colonies were selected for further identification. Stock cultures of each strain were maintained for short periods at room temperature on blood agar base slants and for longer storage they were either frozen at $-70^{O}C$ in 20% (w/v) glycerol–Todd–Hewitt broth (Oxoid) or lyophilised in 7.5% horse glucose serum as a cryoprotector.

In addition, *Aeromonas* isolates were recovered from the liver and/or intestine of chicken from various farm sites and were included in the present study following the same procedure of identification and treatment.

2.2. Phenotypic Identification:

Before each test, all the cultures were grown on tryptose soya agar (TSA) (Oxoid) at $37^{O}C$ for 18 h. Strains were first identified as *Aeromonas* spp. and growth 6% sodium chloride was used to discriminate *Aeromonas* from *Vibrio fluvialis*. All *Aeromonas* spp. were re-identified biochemically to species level by using 14 tests chosen from those described by Altwegg (Altwegg, 1999) and were: indole, gas from glucose, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase by the Moeller's method, esculin hydrolysis, Voges–Proskauer test, acid production from L-arabinose, lactose, sucrose, salicin, m-inositol, D-manitol and the h-hemolysine.

2.3. Congo Red Uptake Test:

The Congo red uptake test was basically performed as described by Statner and George (1987). Strains were streaked on plates of TSA containing Congo red (final concentration, 50 ,ug/ml) and incubated at 28°C. Colonies were examined under obliquely reflected light on a black background. Bright orange or red colonies were considered positive. Different intensities in the dye uptake were expressed as + and + +.

2.4. Serological Testing:

All motile *Aeromonas* strains were tested by the O-serogrouping system of the Hydrobiology Department (National Research Centre) according to Eurell *et al.*, (1978). Strains were grown on TSA slants overnight at $30^{O}C$, harvested with phosphate-buffered saline ($>10^{9}$ cells/ml), and heated for 1 h at $100^{O}C$. After being

heated, 20 µl of the boiled cell suspensions (thermostable O antigen of the strains) was mixed with 20 µl of each specific rabbit antiserum (O:1 to O:30) in ceramic rings on agglutination glass slides. The mixtures were rotated for 2 min, and the degree of agglutination (0 to 2+) was recorded. Two negative controls were used, boiled cell suspensions mixed with phosphate-buffered saline and boiled cell suspensions mixed with rabbit serum obtained from nonimmunized animals.

2.5. Molecular Typing of Aeromonas hydrophila:
2.5.1. Bacterial DNA Extraction for PCR:

Approximately 100 µl of a Tryptone soya broth (Oxoid) culture grown for 16 h at 28°C was used for DNA extraction using the InstaGene matrix (Bio-Rad Laboratories AG, Glattbrugg, Switzerland) in accordance with the manufacturer's instructions. Subsequently, 5 µl of the DNA solution was used as a template for PCR amplification.

2.5.2. Molecular Identification:

All strains were re-identified on the basis of the restriction fragment length polymorphism patterns (RFLP) obtained from the 16S rDNA following the method described by Borrell *et al.,* (1997) and Figueras *et al.,* (2000).

2.6. Stress Induction:

Field strain of *A. hydrophila* was grown in 250 ml of L broth containing 0.3 M NaCl. Heat shock stress conditions were imposed according to the following procedures:
******* the culture was transferred from 25^{O}C to temperatures of 42^{O}C and 50^{O}C.
*******At each temperature, samples were taken at intervals after heat shock, and crude cell extracts were prepared. Culture samples were taken after 24h and 48h.

2.7. Analysis of A. hydrophila culture supernatant proteins by SDS-PAGE electrophoresis:

Samples were taken at intervals after heat shock, and cellfree supernatant proteins were prepared using the protocol of Love and Hirsh (1994). Bacterial cells were removed from cultures by centrifugation at 7,000 xg for 20 min and subsequent filtration through a 0.22-mm-pore-size filter. Proteins from the cellfree culture supernatants were then precipitated by adding 10% (v/v) trichloroacetic acid and recovered by centrifugation at 7,000 xg for 20 min. Pellets were resuspended in 4 ml of phosphate-buffered saline (PBS), and proteins were precipitated again by adding 20 ml of cold acetone. After centrifugation at 7,000 xg for 20 min, the pellets were washed once with cold acetone, dried, and resuspended in 250 ml of PBS. Polyacrylamide gel electrophoresis, Coomassie blue staining analysis of proteins was carried out by standard protocols (Maniatis *et al.,* 1982).

2.8. Computer-aided Analysis of the Gels:

Images of the gels were captured using a Sharp JX-330 flat-bed scanner, and image analysis of the protein profiles was performed using Amersham Pharmacia Biotech ImageMaster 2-D Elite software. The relative amount of each protein spot was calculated and expressed by the software as the percentage of the spot volume and represented the intensity of each individual spot compared to the intensity of the whole gel. The genetic similarity coefficient between two genotypes was estimated according to Dice. The similarity-derived dissimilarity matrix was used in the cluster analysis by using the unweighted pair-group method with arithmetic averages (UPGMA).

Results and Discussion

3.1. Pheno- and Genotyping:

All strains of the isolated *Aeromonas hydrophila* in the present investigation, displayed the following characteristics typical of the genus *Aeromonas*: Gram-negative straight motile rods, chemo-organotrophic with both oxidative and fermentative metabolism, and cytochrome oxidase and catalase are positive. Optimal growth occurs after 24 h at 25^{O}C on TSA medium. No brown water-soluble pigment is produced on TSA medium.

Indole is positive and Voges–Proskauer-positive. Urease and H_2S are not produced. The following substrates are used as the sole carbon and energy sources by *A. hydrophila*: D-mannitol and sucrose. None of the species use m-inositol. Acid is uniformly produced from D-mannitol and sucrose, and salicin but not from lactose, inositol. *Aeromonas hydrophila* was able to hydrolyse aesculin. These isolates were tentatively confirmed as *A. hydrophila* by molecular tests.

3.2. Generation of Heat Shock Proteins:

3.2.1. Hsps Pattern of the Aeromonas Hydrophila Isolated from Fish:

One-dimensional SDS-PAGE of HSPs revealed protein profiles containing 11–16 discrete bands with molecular weight of 13–105 kDa. Seven bands having molecular weight range of 13–37 kDa were present in all the isolates (Fig. 1). A high molecular weight protein band (105 kDa) was present only when the *A. hydrophila* was incubated at 25^{O}C for 48 h.

Aeromonas hydrophila incubated at 25^{O}C for 24 h produced several proteins in increased amounts compared with amounts produced at 42^{O}C (13 bands) and 50^{O}C (12 bands) (Fig. 1). The highest level of HSP produced was when the *A. hydrophila* cells were exposed to a downshift of temperature to 25 degrees C for 48 hrs (16 bands). On the other hand, the least amount produced of some proteins was at the temperature of 42^{O}C for 48 hrs (11 bands). Some of the bands were under-expressed to the degree that the computer was unable to demonstrate its intensity.

The dendrogram derived following numerical analysis of the protein electrophoregrams is shown in Fig. 2. Dendrogram analysis revealed that the highest similarity (84.85%) was between HSPs group expressed after exposure of *A. hydrophila* to 42^{O}C for 48 hrs (lane 4) and those expressed after exposure of *A. hydrophila* cells to 50^{O}C for 24 hrs (Lane 5). lane 1 reflected 75.65% similarity with lanes 2-6.

3.2.2. Hsps Pattern of the Aeromonas Hydrophila Isolated from Chicken:

The one-dimensional SDS-PAGE of HSPs revealed protein profiles containing 13–15 discrete bands with molecular weight of 14–96 kDa. Eight bands having molecular weight range of 14–43 kDa were present in all the isolates (Fig. 3). The highest molecular weight protein band (96 kDa) was present only when the *A. hydrophila* was incubated at 42^{O}C for 24 h and the lowest (14 kDa) was when the *A. hydrophila* was incubated at 25^{O}C, 42^{O}C and 50^{O}C for 24 and 48 h, in other words under the experimental conditions the *A. hydrophila* was subjected to.

Aeromonas hydrophila incubated at 25^{O}C and 42^{O}C for 24 h produced several proteins in increased amounts (15 bands) compared with amounts produced at 42^{O}C for 48 h and 50^{O}C for 24 h (13 bands) (Fig. 3). Again, some of the bands were under-expressed to the degree that the computer was unable to demonstrate its intensity.

The dendrogramatic analysis of the HSPs (Fig. 4) group expressed after exposure of *A. hydrophila* to 25ºC for 48 hrs (Lane 2) 72% similarity with those expressed after exposure of *A. hydrophila* cells to 42ºC for 48 hrs (Lane 4). The same percentage of similarity was noticed between Lanes 5 & 6.

On the other hand, the group of HSPs produced by the *A. hydrophila* when exposed to 25^{O}C for 24 h (Lane 1), 50^{O}C for 24h and 48 h (Lanes 5 & 6) differed by 56.05% with the HSPs produced after exposure of *A. hydrophila* cells to 25ºC for 48 h, 42ºC for 24 hrs and 48 hrs (Lanes 2, 3 and 4).

3.3. Dendrogram Hsps Similarity of Aeromonas Hydrophila Isolated from Fish and Chicken:

The dendrogram derived following numerical analysis of the protein electrophoregrams between the different treatments in fish vs chicken is shown in Fig. 5. Dendrogram analysis revealed that the highest similarity between the expressed HSPs was between those expressed from *A. hydrophila* isolated from fish and incubated at 25^{O}C for 24 h and those expressed from *A. hydrophila* isolated from chicken and incubated at 42^{O}C for 24 h (71.54%). The least similarity recorded (56.21%) was between the HSPs expressed after the *A. hydrophila* isolated from chicken was incubated at 42^{O}C for 48 h and those expressed from *A. hydrophila* also isolated from chicken after incubation at 50^{O}C for 48 h.

Discussion:

All the *A. hydrophila* strains produced and secreted cytotoxins at 37^{O}C (Granum *et al.*, 1998). It has been speculated that *A. hydrophila* virulence could involve several extracellular enzymes including proteases, hemolysins, enterotoxins, and acetylcholinesterase. The two major extracellular proteolytic activities of *A.*

hydrophila that have been described so far, a 38-kDa thermostable metalloprotease (Rivero et al., 1990) and a 68-kDa temperature-labile serine protease (Rivero *et al.,* 1991), are present in most *A. hydrophila* culture supernatants. In addition, a 19-kDa zinc proteinase was found in the growth medium of a strain of *A. hydrophila* isolated from the intestinal tract of the leech Hirudo medicinalis (Loewy *et al.,* 1993), and a 22-kDa serine proteinase, which is stable at 56^{O}C for 10 min, was purified from A. hydrophila strain B_{32} culture supernatant (Rodriguez *et al.,* 1992). Most A. hydrophila strains secrete two proteases into the culture medium, a thermostable metalloprotease (Cascon *et al.,* 2000). The S layers of motile O:11 *Aeromonas* strains are composed of subunits of a single surface array protein of around 52 to 55 kDa molecular mass (Kokka *et al.,* 1990).

The effect of heat shock on the pattern of protein synthesis in *A. hydrophila* was investigated using high-resolution SDS-PAGE electrophoresis. In this study we found that the same heat-specific proteins were induced in response to heat shock but that the level of induction varied with the temperature and time and was most pronounced after 48 hrs exposure to 25^{O}C with the maximum increase in new proteins apparent (16 bands). The HSPs six different families according to their respective molecular weights were expressed. They are the HSP100 family, the HSP90 family, the HSP70 family, the HSP60 family, the HSP40 family, and the small heat shock family (sHSPs) including HSP27. They were present in increased amounts or were newly synthesized after heat shock (Fig. 1). They were designated heat-specific stress proteins because they were either synthesized de novo or overexpressed in *A. hydrophila* cells specifically in response to heat shock.

Dendrogram analysis revealed that the highest degree of similatity exceeded 80% in fish and 70% in chicken. When the dendrogram analysis was then applied to the expressed HSPs from *A. hydrophila* isolated from animals (fish vs chicken) the similarity between the HSPs did not exceed 72%. Although the different HSP families are genetically and biochemically unrelated (Javid *et al.,* 2007) yet, whole-cell proteins were used to distinguish *Weissella soli* from all previously described *Weissella* species by electrophoretic analysis deriving the dendrogram from the (unweighted pair group arithmetic average-linkage algorithm) clustering of correlation coefficients of the SDS-PAGE protein patterns (Magnusson *et al.,* 2002).

The stress-specific proteins induced by heat may act by counteracting the damage, adapting to the stress action, or repairing damage induced by the stress. *In vitro* as well as *in vivo* chaperonins, either alone or with other chaperones and ATP, have been shown to orchestrate the re-folding of partially denatured proteins. In addition to their prominent role as molecular chaperones members of the GroEL and Hsp60 families have long been recognized as highly immunogenic proteins and consequently have attracted much attention from immunologists.

Heat shock proteins (HSPs) have been implicated in the stimulation and generation of both innate and adaptive immunity (Javid *et al.*,2007). They represent dominant antigens in numerous microbial infections, suggesting a potential use of pathogen-derived HSPs for vaccination (Sagi *et al.,* 2006; Paliwal *et al.,* 2008). LaFrentz *et al.,* (2004) and Sudhees *et al.,* (2007) demonstrated that the 70-100 and 41-49 kDa regions elicited significant protection following subcutaneous challenge with 2 doses of a virulent strain of *F. psychrophilum*. Immunization with the 70-100 kDa region resulted in near complete protection in fish. This suggests that these antigens may be involved in eliciting a highly protective immune response, and could serve as vaccine candidates against CWD and RTFS and other fish diseases.

Conclusion:

The heat-shock response involves the induction of many proteins - called heat-shock proteins, or Hsp's - in response to elevation of temperature (Neidhardt and Vanbogelen, 1987). The bacterial heat-shock response is not limited to changes in temperature and is a general stress response, as many of the heat-shock proteins are induced by interaction with eukaryotic hosts (Van bogelen *et al.,* 1987; Blom *et al.,* 1992; Van Dyk *et al.,* 1995; Hecker *et al.,* 1996; Muffler *et al.,* 1997). The heat-shock proteins include chaperones and proteases that are presumably essential for overcoming changes that involve protein denaturation. Induction of this response improves thermotolerance (Kusukawa and Yura, 1988; Larossa and Dyk, 1991; Volker *et al.,* 1992; Inbar and Ron, 1993; Menzel *et al.,* 1996). Moreover, in several bacterial species heat shock proteins have been shown to play an important role in pathogenesis (Lathigra *et al.,* 1991; Mckay and Lu, 1991; Kaufmann, 1992; Bohne *et al.,* 1994; Brunham and Peeling, 1994; Mauchline *et al.,* 1994; Sheehan *et al.,* 1994; Macario, 1995; Mager and De Kruijff, 1995; Schurr and Deretic, 1997; Karunasagar *et al.,* 1997) and survival within macrophages (Baum;er *et al.,* 1994).

The article deals with *Aeromonas,* an environmental microorganism autochthonous of aquatic environments that can be sporadically transmitted to humans. Food of animal origin, seafood and vegetables have been considered an important vehicle of *Aeromonas* spp. infections. *Aeromonas hydrophila* is considered one of the most important bacterial pathogens of aquatic animals in temperate areas, as it has been isolated from diseased fish, eels, frogs, and turtles causing fatal hemorrhagic septicemia.

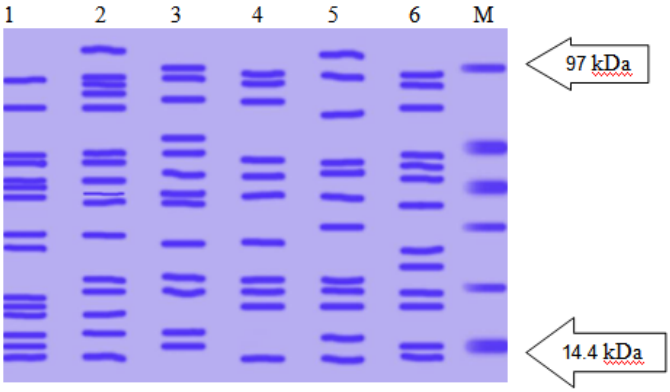

Fig. 1: Effect of heat shock treatment on the pattern of protein synthesis in *Aeromonas hydrophila* (CR+ ve) isolated from fish.

Lane 1. *A. hydrophila* incubated at 25oC for 24 hr
Lane 2. *A. hydrophila* incubated at 25oC for 48 hr
Lane 3. *A. hydrophila* incubated at 42oC for 24 hr
Lane 4. *A. hydrophila* incubated at 42oC for 48 hr
Lane 5. *A. hydrophila* incubated at 50oC for 24 hr
Lane 6. *A. hydrophila* incubated at 50oC for 48 hr
Lane 7. Marker (M) (97, 66, 45, 30, 20.1 and 14.4 kDa).

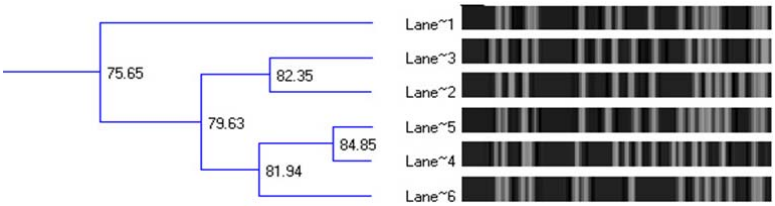

Fig. 2: Dendrogram analysis of the expressed HSPs bands of *Aeromonas hydrophila*, isolated from fish when subjected to different conditions of temperature and time.

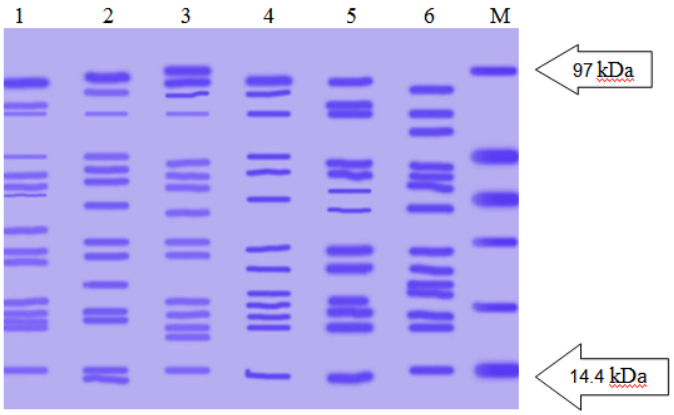

Fig. 3: Effect of heat shock treatment on the pattern of protein synthesis in *Aeromonas hydrophila* (CR+ ve) isolated from chicken.

Lane 1. *A. hydrophila* incubated at 25oC for 24 hr
Lane 2. *A. hydrophila* incubated at 25oC for 48 hr
Lane 3. *A. hydrophila* incubated at 42oC for 24 hr
Lane 4. *A. hydrophila* incubated at 42oC for 48 hr
Lane 5. *A. hydrophila* incubated at 50oC for 24 hr
Lane 6. *A. hydrophila* incubated at 50oC for 48 hr
Lane 7. Marker (M) (97, 66, 45, 30, 20.1 and 14.4 kDa).

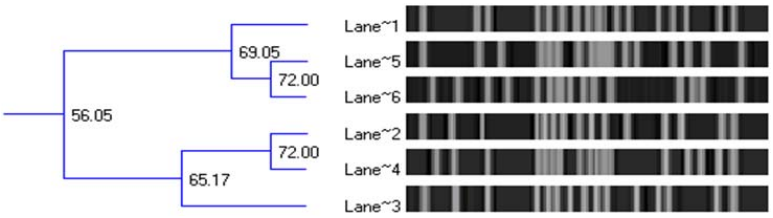

Fig. 4: Dendrogram analysis of the expressed HSPs bands of *Aeromonas hydrophila* (CR +ve), isolated from chicken when subjected to different conditions of temperature and time.

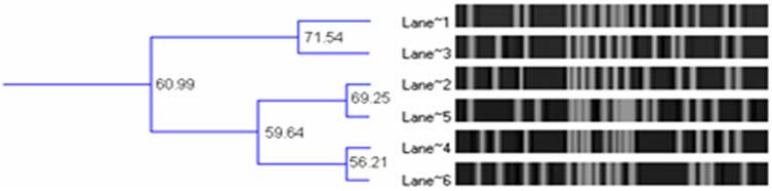

Fig. 5: Dendrogram showing HSPs similarity expressed by *Aeromonas hydrophila* isolated from fish and chicken when subjected to different conditions of temperature and time determined by the SDS-PAGE HSPs pattern analysis using Dice similarity coefficient and UPGMA cluster method.

Lane 1. *A. hydrophila* isolated from fish and incubated at 25oC for 24 hr
Lane 2. *A. hydrophila* isolated from chicken and incubated at 25oC for 24 hr
Lane 3. *A. hydrophila* isolated from fish and incubated at 42oC for 24 hr
Lane 4. *A. hydrophila* isolated from chicken and incubated at 42oC for 48 hr
Lane 5. *A. hydrophila* isolated from fish and incubated at 50oC for 24 hr
Lane 6. *A. hydrophila* isolated from chicken and incubated at 50oC for 48 hr

Heat shock proteins (HSPs) have been implicated in the stimulation and generation of both innate and adaptive immunity (Javid *et al.*, 2007). Therefore, it was essential to determine their production in vitro to determine whether *Aeromonas hydrophila* exhibits a heat shock response. They represent dominant antigens in numerous microbial infections, suggesting a potential use of pathogen-derived HSPs for vaccination. This approach for antigen identification may provide a basis for targeted vaccine development for *Aeromonas* infections or Motile *Aeromonas* Septicemia (MAS) and other fish diseases.

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