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ANALYSIS OF HEAT SHOCK PROTEIN IN CULTURE MEDIA OF STAPHYLOCOCCUS AUREUS

INAS M.GAMAL[*] and A.M. NABIH [**]
[*]Immunology Unit, Animal Reproduction Research Institute (ARRI), Gizza, Egypt.
[**]Mastitis and neonatal diseases department, Animal Reproduction Research Institute (ARRI), Gizza, Egypt.

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ABSTRACT

In this study *Staphylococcus aureus* strain isolated from bovine mastitic milk was subjected to different heat treatments (25, 37 and 50ºC) for 24 and 48 hrs. Each time the heat treated strain was incubated with bovine lymphocytes *in vitro.* One dimentional SDS-PAGE and dendrogram analysis were applied on the extracted heat shock proteins (HSPs) that were expressed from *Staphylococcus aureus* strain when it was incubated at the same temperatures for the same incubation times. Transformation assay using MTT reduction showed highly significant stimulation in response to phytoheamagglutinin when incubated with 50ºC heat treated *Staphylococcus aureus* for 24 and 48 hrs. The SDS-PAGE revealed that HSPs70 were highly expressed by 22.7% when the *Staphylococcus aureus* strain was exposed to 50ºC for 48 hrs, while the lowest percentage 5.3% was found when it was exposed to 25ºC for 48 hrs. The dendrogram analysis of the HSPs showed that the highest similarity (84.21%) was found between HSPs expressed after exposure of *Staphylococcus aureus* strain to50ºC for 24 hrs (Lane1) and those exposed to 50ºC for 48 hrs. (Lane2). We concluded that, heat shock proteins have a stimulatory effect on the most important immune cells (lymphocytes) and the electrophoretic profile showed that the increasing time and degree of heat stress produced more prominent level of these heat stress proteins.

Key words: *Electrophoresis, heat shock proteins, immune response, lymphocytes transformation.*

INTRODUCTION

Staphylococcus aureus is one of the most common causes of bovine mastitis in modern dairies worldwide and the most common mastitis pathogen isolated from raw milk. It is easily transmissible and infections caused by *S. aureus* respond poorly to treatment. The economic losses due to *S. aureus* mastitis are considerable, and include decrease in milk production, reduced milk quality through contamination by bacteria and increased milk SCC, veterinary and treatment costs, premature culling of cows and loss of genetic potential. Mastitis caused by *S. aureus* also adversely affects welfare of dairy cows.

Another facet of *S. aureus* pathogenesis is the organism's ability to maintain cellular homeostasis while enduring environmental challenges, such as changes in host cell temperature or exposure to phagocyte-mediated reactive temperature species Voyich *et al.* (2005).

Heat shock proteins (HSP) are a class of functionally related proteins involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stress as infection, inflammation, exercise, exposure of the cell to toxins (ethanol, arsenic, trace metals, and ultraviolet light,. De Maio (1999). HSPs are found in virtually all living organisms, from bacteria to humans. They are named according to their molecular weight. Forexample, Hsp60, Hsp70 and Hsp90 (the most widely-studied HSPs) refer to families of heat shock proteins on the order of 60, 70, and 90 kilodaltons in size, respectively. Srivastava (2004).

The focus of the current work is to define the *S. aureus* growth temperature as a stress response factor and it's mechanisms of producing heat shock protein. This may provide better understanding of the organism's ability to adapt to an environmental challenges and further elucidiate the strategies of cellular immune response for the bacterial infection.

MATERIALS and METHODS

Bacterial strains:
Our pure research strains have been obtained from milk samples of mastitic cows from different governorates in Egypt. Samples were cultured on mannitol salt agar (specific media), blood agar (for detection of haemolysis) and nutrient agar (Oxoid media). Isolates were identified biochemically by catalase, oxidase, urease tests, phosphatase test (Quinn *et al.*, 2002), coagulase test (Koneman *et al.*,

1988), sugar fermentation, nitrate reduction test, (Cruickshank *et al.,* 1975) and Ornithine decarboxylase test (Kloos *et al.,* 1991). Final identification was done using API-Staph.kit (bioMerieux) according to manufactures instructions.

Stress Induction:
Field strain of ***S. aureus*** was grown in 250 ml of brain heart infusion broth. Heat shock stress conditions were imposed according to the following procedures:

- The culture broth was incubated at 25, 37 and 50 ºC respectively.
- 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. The density of bacterial cells was measured spectophotometrically (540 nm) and the cells number was calculated by using previously determined standard curves (based on CFU counts) C L S I (2005). Bacterial culture concentration was adjusted to 5×10^{6} bacterial cells for each treatment using 0.5 McFarland tube.

Lymphocytes transformation:
Blood samples: blood samples were taken during the experiment on heparin for separation of T-lymphocyte by aseptically collection, centrifugation at 2500 rpm at 4c° for 30 minutes, the leukocytic layer transferred on to sterilized tissue culture tube, overlayed on ficol (1.077) in sterilized tissue culture tube, centrifuged at 4000 rpm for 30 minutes at room temperature. The mononuclear interphase layer was taken for separation of T-lymphocyte for lymphocyte transformation assay using MTT reduction assay according to Chin *et al.* (2000). Briefly the cells washed twice by (Hank's Balanced Salt Solution (HBSS), the cells suspended in RPMI in number $5X10^{6}$/ml and cultivated in sterilized 96 well tissue culture plate with phytohaemagglutinin (15ug/ml) and 10% fetal calf serum and incubated for 72 hour in 10% CO_2 incubator at 37 ºC, then we add MTT 5mg/ml PBS for 4 hour then lyses by lysis buffer, the lysate then measured spectrophotometrically at 570nm.

S. aureus **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 70,000 rpm 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 70,000 rpm 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).

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).

Statistical analysis:
Data were subjected to statistical analysis according to Snedecor and Cochram (1982) by one way ANOVA employing a completely randomized design. eukocytic layer present in the interface was separated to obtain lymphocytes.

RESULTS

S. aureus strain was exposed to different heat treatments for 24 and 48 hrs, the first treatment at 25ºc showed non significant effect on lymphocytes transformation in response to phytoheamagglutinin as in table (1). When *S. aureus* was incubated with the lymphocytes at 37 ºC for 24hrs, there was no significant stimulation in response to phytoheamagglutinin, but there was significant stimulation after its incubation for 48 hrs when compared with the control. Table (1).

Lymphocytes transformation showed high significant stimulation in response to phytoheamagglutinin when incubated with 50ºC heat treated *S. aureus* for 24 and 48 hrs. Table (1).

By comparing the results of lymphocytes incubated with ***S. aureus*** exposed to different temperatures and times (50 ºc for both 24 and 48 hrs), we found that there were significant stimulation of lymphocytes transformation in response to phytoheamagglutinin when it was incubated at50 ºc for both 24 and 48 hrs in comparison to the other groups. Table (2,3).

Table1: Lymphocyte transformation activeties in the presence and absence of *S. aureus* at different incubation tempretures and time.

Incubation temp. / incubation times	25ºC		37 ºC		50 ºC	
	Control	**Cells with bacteria**	**Control**	**Cells with bacteria**	**Control**	**Cells with bacteria**
24hrs	0.741±0.081	0.494±0.060	0.679±0.008	0.834±0.033	0.628±0.048	1.808±0.050***
48hrs	0.677±0.058	0.699±0.130	1.55±0.139	2.326±0.129***	.0.638±0.048	3.196±0.297***

*** significant at P< 0.05

Table 2: Effect of different heat treatments of *S. aureus* on lymphocyte transoformation activity at 24 hrs. incubation temperature.

Temperatures	**25°C**	**37°C**	**50°C**
OD	0.494 ± 0.060	0.834±0.033	1.808±0.050 ***

*** significant at P<0.05
OD:reading in optical density

Table 3: Effect of *S. aureus* heat shock protein on lymphocyte transoformation activity at 48 hrs. incubation temperature.

Temperatures	**25°C**	**37°C**	**50°C**
OD	0.699 ± 0.130	2.326 ±0.129	3.196 ±0.297***

***highly significant at P<0.05
OD: reading in optical density

Heat shock proteins pattern of *S. aureus* isolates:
The one-dimensional SDS-PAGE of HSPs revealed protein profiles containing 16-18 discrete bands with molecular weight of 13-176 kDa. (Fig.1). The highest molecular weight protein band (176 kDa) was present only when the *S. aureus* was incubated at 37ºC for 48 hrs and the lowest (13 kDa) was when the *S. aureus* was incubated at 25ºC for 48 hrs.

HSPs70 were highly expressed by 22.7% when the *S. aureus* exposed to 50ºC for 48 hrs, while the lowest percentage 5.3% was found when *S. aureus* exposed to 25ºC for 48 hrs.

The dendrogramatic analysis of the HSPs (Fig. 2) group expressed after exposure of *S. aureus* cells to 50ºC for 24 hrs (Lane 1) showed the highest similarity (84.21%) with those expressed after exposure of *S. aureus* to 50ºC for48hrs (Lane2).

On the other hand, the group of HSPs produced by the *S. aureus* when exposed to 50ºC for 24 hrs (Lane 1), 50ºC for 48 hrs (Lane 2) differed by 75.36% with the HSPs produced after exposure of *S. aureus* cells to 37ºC for 24 hrs, 25ºC for 24 hrs and 48 hrs (Lanes 3,5 and 6). While the group of HSPs produced by *S. aureus* when exposed to37 ºC for 48 hrs (Lanes 4) differed by 74.58% with the HSPs produced from the rest of the groups (Lanes1,2,3,5 and 6).

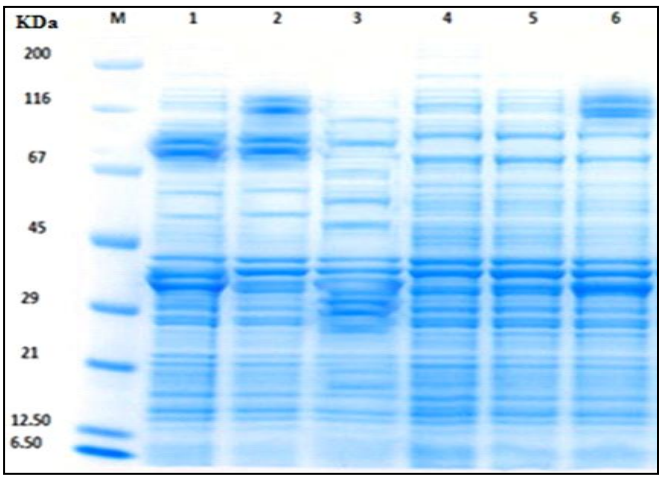

Fig (1): Effect of heat shock treatment on the pattern of protein synthesis in ***S. aureus*** isolates.
Marker lane (M) (200, 116, 67, 45, 29, 21, 12.50 and 6.50 KDa.).
Lane1. ***S. aureus*** incubated at 50ºc for 24 hrs.
Lane2. ***S. aureus*** incubated at 50ºc for 48 hrs.
Lane3. ***S. aureus*** incubated at 37ºc for 24 hrs.
Lane4. ***S. aureus*** incubated at 37ºc for 48 hrs.
Lane5. ***S. aureus*** incubated at 25ºc for 24 hrs.
Lane6. ***S. aureus*** incubated at 25ºc for 48 hrs.

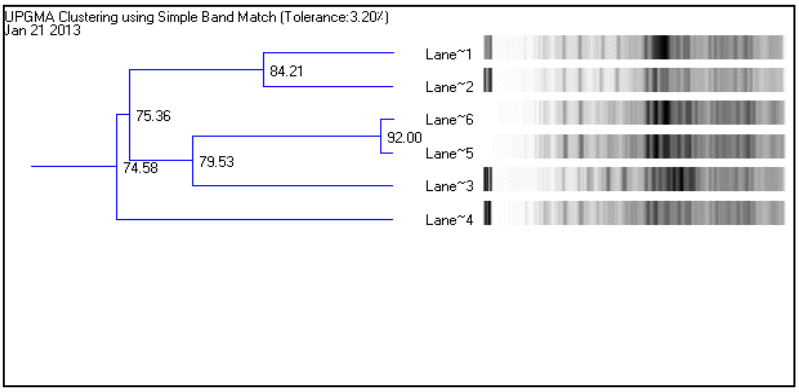

Fig (2): Dendrogram analysis of the expressed HSPs bands of ***S. aureus*** when subjected to different conditions of temperatures and times.

DISCUSSION

Staphylococcus aureus is a common and important cause of farm animal diseases including bovine mastitis, tick pyemia (enzootic staphylococcosis), abscesses, dermatitis, furunculosis, meningitis, osteomyelitis, food poisoning, and wound suppuration. Staphylococci (peptidoglycan, lipoprotein, or teichoic acid), triggering innate host immune responses that escape killing replicate in infected tissues and generate proinflammatory responses mediated by the release of cytokines and chemokines from macrophages, neutrophils, and other immune cells Jonsson *et al.* (2003).

Innate immune responses limit the establishment of infectious foci and thereby reduce the severity of staphylococcal infections. These early events culminate in the activation of adaptive immune responses, during which T and B cells capable of specific antigen recognition lead to the eradication of *Staphylococci*.Wardenburg *et al.* (2006).

Heat shock proteins (HSP) are intracellular molecular chaperones with many immunological functions. They are high immunogens with further important role in vaccine development against infectious diseases. They have an anti-inflammatory effect on various inflammatory conditions such as infection, ischemia injury, and cardiovascular diseases. They also have recently shown to mediate a range of powerful effects in neuronal cells and immune cells Chen (2007) and Calderwood *et al.* (2007). HSP act as adjuvant that can augment the immunogenicity of weak antigens and can stimulate antigen presenting cells, lymphocytes and macrophages. Holakuyee *et al.* (2012) and Noort *et al.* (2013).

Lymphocyte proliferation assay (LPA) measures the ability of lymphocytes to undergo clonal proliferation when stimulated by a foreign molecule, antigen or mitogen in vitro so we studied the effect of heat treated *S. aureus* on lymphocytes function and proliferation.

The incubation of lymphocytes with *S. aureus* at 37ºC for 2 different incubation periods demonstrated significant stimulation in lymphocytes proliferation at ($p<0.05$) (Table 1,2). At the same time there was high significant stimulation when *S. aureus* strain was incubated at 50ºC for 24 and 48 hrs. This finding was supported by MEI-TENG LOH. (1995) who reported that Staphylococcal enterotoxins (SE) bind to major histocompatibility complex (MHC) class II molecules and the Vβ region of T cell receptors (TCR) and subsequently induces T cell proliferation and Taylor *et al.* (2012) who said that the bacterial superantigen exotoxins of Staphylococcus aureus and Streptococcus pyogenes are potent stimulators of polyclonal T-cell proliferation.

These results may be attributed to the production of different group of heat shock protein which affect the lymphocyte proliferation that run in parallel with the results of Tsan and Baochong (2009) who mentioned that Heat shock proteins (HSPs) such asHSP 60, Hsp70, Hsp90, and gp96, have been reported to play important roles in antigen presentation and cross-presentation, activation of macrophages and lymphocytes.

In this study the heat treated *Staphylococcus aureus* was investigated using high-resolution SDS-PAGE electrophoresis. 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 50ºC. The HSP70 family was the most prominantly expressed. 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 newly synthesized or overexpressed in *Staphylococcus aureus* cells specifically in response to heatshock. These findings look like those of Osman *et al.* (2009).

Dendrogram analysis revealed that the highest degree of similarity (84.21%) expressed after exposure of *S. aureus* to 50ºC for 24 hrs (Lane 1) with those expressed after exposure of *S. aureus* cells to 50 ºC for 48 hrs (Lane 2). Our data is coincided with Mita *et al.* (1997) who subjected Sunflower suspension cell cultures to different heat treatments and the electrophoretic patterns of heat-induced endocellular and secreted proteins were analyzed, he found that two major polypeptides with specific molecular weights were strongly induced.

We concluded that, heat shock proteins have a stimulatory effect on the most important immune cells (lymphocytes) and the electrophoretic profile showed that the increasing time and degree of heat stress produced more prominent level of these heat stress proteins.

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تحليل بروتينات الصدمة الحرارية فى المنابت البكتيرية للبكتيريا العنقودية الذهبية

إيناس جمال ، أشرف نبيه

في الآونة الآخيرة بدأ العلماء فى دراسة تأثير درجات الحرارة المنخفضة والمرتفعة على افراز بروتينات الصدمة الحرارية من الخلايا البكتيرية بمختلف أنواعها. ونحن في هذه الدراسة قد قمنا بتتبع تأثيردرجات الحرارة المختلفة على نمو الميكروب العنقودي الذهبى وكذلك افرازه لبروتينات الصدمة الحرارية ، وتأثير انتاج هذا البروتين على قدرة الخلايا المناعية. آملين أن تكون بداية للاستفادة من خواص هذا الميكروب فى الوقاية والعلاج لبعض الامراض البكتيرية. في هذا العمل البحثى تم عزل وتصنيف سلالة الميكروب العنقودى الذهبى من ألبان أبقار مصابة بألتهاب ضرع ظاهرى ، ثم تم تعريض الميكروب لدرجات حرارة وفترات زمنية مختلفة وهى 25، 37 و 50 درجة مئوية لمدة 24 و 48 ساعة على التوالى . عند كل معاملة حرارية للميكروب ، كان يتم تحضينه مع خلايا ليمفاوية سبق استخلاصها من من دم أبقار معمليا لدراسة مدى تأثير الميكروب على القدرة المناعية للخلايا. تم تطبيق اختبار ال (SDS – PAGE) وأيضا تحليل الدندروجرام البيانى على بروتينات الصدمة الحرارية المنتجة من الميكروب العنقودى الذهبى عند تعرضه لكل درجة حرارة ووقت تحضين على حدة. جاءت نتائج اختبار ال (MTT) لتحول الخلايا الليمفاوية موضحة للتأثير الايجابي العالي لاستجابة الخلايا للفيتوهيمأجلوتينين عند تحضين الميكروب لدرجة 50 درجة مئوية ولمدة 24 ثم 48 ساعة. كما تم تحديد مجموعة بروتينات الصدمة الحرارية 70 (HSPs70) كأعلى نسبة انتاج لنوع بروتين (22,7%) عند تحضين الميكروب لدرجة 50 درجة مئوية لمدة 48 ساعة ، فى حين كانت أقل نسبة لانتاج البروتين من نفس المجموعة (5,3%) عند التعرض لدرجة حرارة 25 درجة مئوية لمدة 48 ساعة. أكد التحليل البياني لاختبار الدندروجرام لبروتينات الصدمة الحرارية مدى التشابه فى النوع والكم بين تعرض الميكروب لدرجة 50 درجة مئوية لمدة 24 ساعة والممثل فى خط الفصل الكهربائى رقم (1) وبين خط الفصل الكهربائى رقم (2) والموضح لتعرض الميكروب لدرجة 50 درجة مئوية لمدة 48 ساعة ، وقد وصلت نسبة التشابه الى 84,21% . من هذه الدراسة نستطيع أن نستنتج أن بروتينات الصدمة الحرارية المستخلصة من الميكروب العنقودى الذهبى لها تأثير مناعى منشط على واحدة من أهم الخلايا المناعية وهى الخلايا الليمفاوية ، وأن انتاجية بروتينات الصدمة الحرارية تتزايد كميتها مع ارتفاع درجة الحرارة وكذلك مع طول الفترة الزمنية التى يتعرض لها الميكروب العنقودى الذهبى معمليا. وهى نتائج يمكن الاستفادة منها والزيادة عليها للوصول الى منتج بروتينى مناعى يمكن الاستعانة به فى الوقاية وكذلك فى العلاج لبعض الامراض التى قد يسببها هذا الميكروب لحيوانات المزرعة المختلفة.