الآيـــــة

(ٱلَّذِينَ قَالَ لَهُمُ ٱلنَّاسُ إِنَّ ٱلنَّاسَ قَدۡ جَمَعُواْ لَكُمۡ فَٱخۡشَوۡهُمۡ فَزَادَهُمۡ إِيمَٰنٗا وَقَالُواْ حَسۡبُنَا ٱللَّهُ وَنِعۡمَ ٱلۡوَكِيلُ (173) فَٱنقَلَبُواْ بِنِعۡمَةٖ مِّنَ ٱللَّهِ وَفَضۡلٖ لَّمۡ يَمۡسَسۡهُمۡ سُوٓءٞ وَٱتَّبَعُواْ رِضۡوَٰنَ ٱللَّهِۗ وَٱللَّهُ ذُو فَضۡلٍ عَظِيمٍ. (174)

آل عمران

DEDICATION

To the soul of my father, kind and supportive mother, to my wonderful husband, to all member of my family and to my kids.

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Abstract: This study about heat and cold shocked bacteria could be a serious problem in foods which are cooled or frozen like meat and dairy products as this could cause serious hazard for consumers. As reported by Enache *et al*. (2006) that heat treatment is one of the most widely used methods to control the rates of bacterial growth and death and is considered to be one of the most effective food processing technologies for eradicating food borne pathogens. This study aimed to gain the degree of PhD in Microbiology. The work was done in Department of Microbiology Faculty of Veterinary Medicine, University of Khartoum since November 2015. The bacteria under study were: *Staphylococcus aureus*, ATCC 25923, *E. coli*, ATCC 25922, *K. pneumoniae*, ATCC 13883 and *Ps. aeruginosa*, ATCC 27853. *Staphylococcus epidermidis, B. cereus* and *S. sonnei* were provided by National Public Health Laboratory, Khartoum as isolates. *Pasteurella multocida* was provided by Central Veterinary Research Laboratory, Khartoum. These bacteria were inoculated into nutrient broth tubes and incubated at 37°C for 24 hours. Then cultures for heat shocked were treated in a water bath at 100°C for 5 min, 90°C for 10 min, 80°C for 15 min, 70°C for 20 min and 60°C for 25 min. For cold shocked study, the cultures were treated at 0°C, -15°C and -32°C for 30 min. All biochemical tests were performed according to the methods described by Barrow and Feltham (2003). It was observed that Gram- negative bacteria were more sensitive than Gram-positive bacteria for heat. This appears clearly in *S. aureus* and *B. cereus* which were found to be resistant to

70°C and 80°C. *Staphylococcus epidermidis* cells were not affected dramatically at 0°C with a slight drop at -32 °C. On the other hand, *B. cereus* showed a remarkable drop when treated at 37°C and 0°C, while cells treated at -15°C and -32°C behaved normally. Cells treated at -32°C showed an increase in degree of positiveness towards various biochemical tests. As far as *E. coli,* there was discrepancy in the results. Cells treated at -32°C showed strong reaction towards all tests applied apart from those obtained by dulcitol, glycerol and mannitol, while cells treated at 0°C, showed a drop in all reactions. *Shigella sonnei* showed a severe drop in reaction when subjected to glycerol and mannitol tests. Cells treated at -15°C showed strong reactions towards mannitol, rhamnonse, trehalose and MR tests. *Klebsiella pneumoniae* showed strong reactions in all tests applied apart from those treated at 0°C. Like all organisms tested, *Ps. aeruginosa* showed a remarkable drop at 0°C followed by -15°C. *Pasteurella multocida* cells did not react properly when cells were treated at all temperatures. The oxidase test scored a doubtful result throughout all tests applied indicating the severe damage of the oxidase enzyme. For heat and cold shock tests, the protein profiles and their concentrations were estimated. From the chromatogram of *S. aureus*, apparent changes were noticeable upon raising of temperatures from -32°C to 100°C. The protein bands were focused between 75 kDa to 11 kDa. In case of *S. epidermidis,* unlike *S. aureus*, there was no apparent electrophoretic differernces in the protein. On the other hand, there was an increase

in protein concentrations by more than 140% as the tempreture rises from -32°C to 100°C. Protein bands were focused between 75 kDa to 11 kDa. Although there was an apparent variation in the protein composition of *B. cereus* with gradual increase of temperature, there were no clear electrophoretic bands discrepancies from -32°C to 100°C. *Escherichia coli* showed no changes till 80°C, after which sudden increase was noticed till 100°C. *Shigella sonnei*, *Ps. aeruginosa* and *P. multocida* showed more than 100% decrease in mg/ml of isolated protein as temperature was raised from 37°C to 100°C. *Klebsiella pneumoniae* incubated at 37°C showed a sharp increase in the protein content after which a sharp decline was noticed. After that, protein started to increase, which peaked at 100°C. For study of pathogenicity of *P. multocida* for rabbits, the culture was shocked at -15°C for 30 min and ten serial dilutions were done in duplicates. The dilutions**,** which did not show visible colonies on the recovery media were centrifuged and inoculated i/p in four rabbits. Rabbits inoculated with VBNC bacteria died within 24 hours. Low and high temperatures have no effect on DNA.

المستخلص: هذه الدراسة حول الباكتريا المصدومة بالبرودة والحرارة و التي قد تكون مشكلة خطيرة في الاطعمة المبردة أو المجمدة مثل اللحوم ومنتجات الألبان، وقد يتسبب ذلك في مخاطر جسيمة للمستهلكين، كما ذكرت Enache وآخرون (2006)، أن معالجة الباكتريا بالحرارة هو أحد أكثر الطرق المستخدمة على نطاق واسع للسيطرة على معدلات نمو وموت الباكتريا وتعتبر واحدة من أكثر تقنيات معالجة الأغذية فعالية والقضاء على مسببات الأمراض التي تنقلها الأغذية. تهدف هذه الدراسة للحصول على درجة الدكتوراة في علم الأحياء الدقيقة، بدأ العمل في قسم الأحياء الدقيقة بكلية الطب البيطري، جامعة الخرطوم منذ نوفمبر (2015). جميع أنواع الباكتريا تحت الدراسة (*S. aureus*, ATCC 25923, *E. coli*, ATCC 25922, *K. pneumoniae*, ATCC 13883, *Ps. aeruginosa*, ATCC 27853) (*S. epidermidis, B. cereus, S. sonnei*) تم توفيرها معزولة من مختبر الصحة العامة الوطني، الخرطوم. تم توفير *P. multocida* بواسطة معمل أبحاث البيطرة المركزي، الخرطوم. تمت زراعة هذه البكتريا في N.B وحضنت عند 37°C لمدة 24 ساعة. تمت معالجة البكتريا المصدومة بالحرارة في حمام مائي في 100°C لمدة 5 دقائق، 90°C لمدة 10 دقائق، 80°C لمدة 15 دقيقة، 70°C لمدة 20 دقيقة، 60°C لمدة 25 دقيقة. تمت معالجة البكتريا المصدومة بالبرودة في 0°C لمدة 30 دقيقة، و15°C- لمدة 30 دقيقة، و 32°C- لمدة 30 دقيقة. تم إتباع طريقة Barrow و Feltham (2003) في إجراء الإختبارات البيوكيمائية. لُوحظ أن الباكتريا سالبة الجرام أكثر حساسية للحرارة من إيجابية الجرام، هذا يظهر جلياً في *S. aureus* و *B. cereus* حيث وُجد أن مقاومتهما للحرارة حتى 70°C و 80°C. لم تتأثر خلايا *Staphylococcus epidermidis* بشكل كبير عند 0°C، خلاف الإختلاف الواضح عند 32°C-. على صعيد آخر أظهرت *Bacillus cereus* إنخفاضاً ملحوظاً عند معاجتها في 37°C و0°C، في حين أن الخلايا المعالجة في 15°C- و32°C- كانت نتائجها طبيعية. أظهرت الخلايا المعالجة زيادة في درجة الإيجابية تجاه مختلف الإختبارات البيوكيميائية. فيما يتعلق *Escherichia coli* كان هناك تناقض في النتائج

نتيجة ردة فعل قوية تجاه جميع الإختبارات و ذلك جلياً في الجليسرول والمانيتول والديسيتول. أظهرت الخلايا التي عُولجت عند 0°C إنخفاضاً حاداً في تفاعلات *Shigella sonnei* مثل الجليسرول والمانيتول. أظهرت الخلايا المعالجة عند 15°C- ردود فعل قوية تجاه إختبارات المانيتول، الراميتوز والتريهالوز و MR. كما أظهرت *Klebsiella pneumoniae* ردود فعل قوية في كل التفاعلات المطبقة خلاف التي تمت معالجتها عند 0°C. كما في العضيات الأخرى. أظهرت *Pseudomonas aeruginosa* إنخفاضاً ملحوظاً عند درجة حرارة 0°C تليها 15°C-، لم تظهر *Pasteurella multocida* تغيرات ملحوظة في التفاعلات عند تعريضها لدرجات الحرارة المختلفة. سجل إختبار الأُكسيديز نتائج مشكوك فيها خلال جميع الإختبارات المطبقة مما يشير إلي ضرر شديد في إنزيم الأُكسيديز. تم إختبار جميع البكتريات المصدومة بالحرارة والبرودة ودراسة مظاهر بروتينها وتقدير التراكيز. من كروماتوغرام *Staphylococcus aureus* لُوحظت تغيرات ظاهرة عند رفع درجة الحرارة من 32°C- إلى 100°C تم تركيز حزم البروتين بين 75 kDa إلى 11 kDa. في حالة *Staphylococcus epidermidis* على عكس *Staphylococcus aureus*، لم يكن هنالك فرق واضح في حزم البروتين. على صعيد آخر هنالك زيادة في تراكيز البروتين بأكثر من 140% عند إرتفاع درجة الحرارة من 32°C- إلى 100°C تم تركيز حزم البروتين بين 75 kDa إلى 11 kDa. لم يكن هنالك فرق واضح في حزم وتراكيز بروتين *Bacillus cereus* علي الرغم من الزيادة التدريجية في درجات الحرارة من 32-°C إلى 100°C. لم تُظهر *Escherichia coli* أي تغيرات حتى 80°C وبعد ذلك لُوحظت زيادة مفاجئة في تراكيز البروتين عند 100°C. أظهرت *Shigella sonnei*، *Pseudomonas aeruginosa* و*Pasteurella multocida* إنخفاضاً أكثر من 100% في ملغم/مل من البروتين المعزول حين إرتفعت درجة الحرارة من 37°C إلى 100°C. أظهرت *Klebsiella pneumoniae* زيادة حادة في محتوى البروتين وبعد ذلك لُوحظ إنخفاض حاد ومن ثم بدأ البروتين في الزيادة حتى بلغ زروته عند 100°C. لدراسة إمراضية *Pasteurella multocida* على الأرانب

صُــدمت هذه البكتريا عند 15°C- لمدة 30 دقيقة ثم عُملت عشرة تخفيفات. التخفيفات التي لم تُظهر مستعمرات جلية علي الوسط المغذي تم عمل طرد مركزي لها ثم حُـقِـنت بها الأرانب في التجويف البروتوني . ماتت الأرانب المحقونة بالبكتريا في غضون 24 ساعة. إرتفاع وإنخفاض درجة الحرارة ليس له تأثير على ال DNA.

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CHAPTER ONE

INTRODUCTION

Temperature plays a very important role in the composition, organization and function of biological membranes. Membranes adjust their unsaturated fatty acid composition according to the changes in the environmental temperature. If the temperature decreases, the ratio of unsaturated fatty acids increases as a function of temperature (Fulco, 1972a and 1974b).s

Heat treatment is one of the most widely used methods to control the rates of bacterial growth and death and is considered to be one of the most effective food processing technologies for eradicating food borne pathogens (Enache *et al*. 2006).

As a measure for molecular motion, temperature is one of the most important environmental factors for life as it directly influences structural and hence functional properties of cellular components (Weber and Marahiel, 2003). Temperature above optimal cause heat shock, while low temperature induce cold shock (Ivanic *et al*. 2013).

Interestingly, in contrast to the heat shock response, to date no cold-specific sigma factor has been identified. Rather, it appears that the cold shock response is organized as a complex stimulant in which post transcriptional events play an important role (Weber and Marahiel, 2003).

Shekhar (2011) stated that low temperature can influence the response of a microorganism either directly or indirectly. Direct effects include decreased growth rate, enzyme activities, alteration of cell composition and differential nutritional requirements. Indirect effects are usually observed on the solubility of solute molecules, diffusion of nutrients, osmotic effects on membranes and cell density.

Cold stress can disrupt the balance of the oxidant and antioxidant system and cause oxidative damage to several tissues by altering the enzymatic and non-enzymatic antioxidant status, protein oxidation, and lipid peroxidation (Sahin and Gumuslu, 2004).

Miller *et al*. (2000) concluded in their study that Initiation may occur through application of a sublethal stress, which results in a bacterium misinterpreting the signal from its environment. This stimulates genes that permit survival in the continued presence of the primary stress. The application of a secondary stress, such as the cold/heat shock regime described in their study, may result in enhanced lethality of the overall process. Every kind of shock can influence the functionality of the cell wall of bacteria and improve or damage their normal physiological and vital activities (Dimitrier *et al*. 2005).

Repair was defined by Hideharu (2006) as the ability of injured cells to return to normal physiological function.

Janssen and Busta (1973) reported that fast freezing and slow thawing of *Salmonella anatum* cell in no fat solid milk resulted in about 20% death and 50% injury of the cell surviving the treatment.

The objectives of this study were:

1- To study the biochemical reactions of bacteria that was shocked by heat and cold temperatures and comparing them with control bacteria that was grown at 37°C.

2- To detect protein profiles for all bacteria under study (heat, cold shocked bacteria and control).

3- Amplification of PCR for shocked and non-shocked bacteria.

4- To study the pathogenicity of cold and heat shocked bacteria using *Pasteurella multocida* and rabbit as model.

CHAPTER TWO

LITRAITEURE REVIEW

2-1. Heat shock proteins (HSP):

Heat shock proteins are families of proteins that are produced by cells in response to exposure to stressful conditions. They were first described in relation to heat shock (Ritossa, 1962), but are now known to also be expressed during other stresses including exposure to cold (El Sanousi, 1975 and Matz *et al.* 1995), UV light (Cao *et al.* 1999) and during wound healing or tissue remodeling (Laplante *et al.* 1998).

Heat-shock proteins are named according to their molecular weight. For example, Hsp60, Hsp70 and Hsp80 (the most widely-studied HSPs) refer to families of heat shock proteins in the order of 60, 70, and 80 kilodaltons in size, respectively (Li and Srivastava, 2004). The small 8-kilodalton protein ubiquitin, which marks proteins for degradation, also has features of a heat shock protein (Raboy *et al.* 1991).

As stated by Guisbert *et al.* (2008), the cellular response to heat shock includes the transcriptional up-regulation of genes encoding heat shock proteins (HSPs) as part of the cell's internal repair mechanism.

They are also called stress-proteins and respond to heat, cold and oxygen deprivation by activating several cascade pathways. HSPs are also present in cells under perfectly normal conditions. Some HSPs,

called chaperones ensure that the cell's proteins are in the right shape and in the right place at the right time. For example, HSPs help new or misfolded proteins to fold into their correct three-dimensional conformations, which is essential for their function. They also shuttle proteins from one compartment to another inside the cell, and target old or terminally misfolded proteins to proteases for degradation (Vabulas *et al.* 2010).

2-2. The effect of cold shock on bacteria:

Cold stress stabilized the secondary conformation of nucleic acid, which inhibits DNA replication, gene transcription and translation, the activity of enzymes and the rate of metabolic process, as well as membrane fluidity of compound. The intracellular formation of ice crystals damages sub cellular causing death (Vorob, 2004).

A bacterial cell has to react immediately when exposed to unfavorable external condition, through such responses as entering the stationary or sporulation phase. Cold shock occurs when bacteria are exposed to sudden down shifts in temperature, and is characterized by the expression of a well-defined set of protein which include helicase, nuclease and a plethora of nucleic acid binding protein which are so cold shock proteins (CSPs) (Wilson and Nierhaus, 2004).

Chawla *et al.* (1996) and Williams and Golden (2001) reported that food could be presumed to be safe and free from pathogenic cells, but during storage, become unsafe due to the regrowth of injured cells.

2-3. Cold shock phenomenon:

Cold shock as defined by El Sanousi (1975) comprises the injury, death or both which are caused by sudden chilling of the microorganisms. Anaerobes, for example *C. perferngens*, were shown by El Sanousi (1975) to be equally sensitive to cold shock.

As stated by El Sanousi (1975) and Jones *et al*. (1992a), the cold shock phenomenon occurs when growing bacteria are exposed to sudden temperature drop of at least 10°C leading to cold shock in susceptible microorganisms.

Collee *et al*. (1961) termed the phenomenon as **"phoenix"** due to disappearance of colonies at recovery medium and attributed this to the temperature of inoculums, a finding which was disqualified by El Sanousi (1975) who explained the phenomenon to be a mere cold shock one. This is due to cells being shocked when transferred from high temperature to low ones.

2-4. Cold shocked protein:

Bacteria express a well-defined set of proteins after a rapid decrease in temperature, which differ from those expressed under heat shock conditions. Cold shock proteins may include helicases, nucleases, and ribosome-associated components that interact with DNA and RNA. Processes such as cold signal perception, membrane adaptation, and the modification of the translation apparatus are involved (Weber and Marahiel, 2003).

These so-called 'cold shock' proteins are thought to help the cell to survive in temperatures lower than optimum growth temperature, by contrast with heat shock proteins, which help the cell to survive in temperatures greater than the optimum, possibly by condensation of the chromosome and organization of the prokaryotic nucleoid (Obokata, 1991).

The cold shock response leads to a growth block and overall repression of translation; however, there is the induction of a set of specific proteins that help to tune cell metabolism and read just it to the new conditions. For a mesophile like *E. coli*, the adaptation process takes about 4 hours (Weber and Marahiel, 2003). They also stated that taken all together, it is likely that expression of all four CSP genes is regulated essentially in the same manner. It should be mentioned, however, that the optimal temperature ranges for the induction of these genes are different (Etchegaray *et al*. 1996 and Wang *et al*. 1999).

2-5. *Staphylococcus aureus:*

Staphylococcus aureus appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates (Ryan and Ray, 2004).

Staphylococcus aureus is catalase-positive, so it is able to convert hydrogen peroxide (H_2O_2) to water and oxygen. This test is sometimes

used to distinguish *staphylococci* from *enterococci* and *streptococci*. Previously *S. aureus* was differentiated from other *staphylococci* by the coagulase test. However it is now known that not all *S. aureus* are coagulase positive and that incorrect species identification can impact effective treatment and control measures (Matthews *et al*. 1997). More than fifty species were reported by El Sanousi *et al*. (2015).

Stewart (2003) stated that the temperature range for growth of *Staphylococcus aureus* is 7-48°C, with an optimum of 37°C. *Staphylococcus aureus* is resistant to freezing and survives well in food stored below -20°C; however, viability is reduced at temperatures of -10 to 0°C. *Staphylococcus aureus* is readily killed during pasteurisation or cooking.

Staphylococcus aureus is responsible for many infections, but it may also occur as a commensal. The presence of *S. aureus* does not always indicate infection. It can survive from hours to weeks, or even months, on dry environmental surfaces, depending on strain (Cimolai , 2008).

The presence of small numbers of *Staphylococcus aureus* in food is not uncommon. It will occur naturally in poultry and other raw meats as a frequent competent of the skin flora. There however, been out breaks caused by milk products such as dried milk and chocolate milk, where growth and enterotoxins production occurred in the raw milk, but the organism might not survives subsequent pasteurization. Contamination by food handlers is also probably a frequent

occurrence in view of the high rate of human carriage (Adams and Moss, 2008).

Protein A is anchored to staphylococcal peptidoglycan penta-glycine bridges (chains of five glycine residues) by the trans-peptidase sortase A (Schneewind *et al*. 1995). Protein A, an IgG-binding protein, binds to the Fc region of an antibody. In fact, studies involving mutation of genes coding for protein A resulted in a lowered virulence of *S. aureus* as measured by survival in blood, which has led to speculation that protein A-contributed virulence requires binding of antibody Fc regions (Patel *et al.* 1987).

Clauditz *et al*. (2006) stated that some strains of *S. aureus* are capable of producing staphyloxanthin a golden coloured carotenoid pigment. This pigment acts as a virulence factor, primarily by being a bacterial antioxidant, which helps the microbe evade the reactive oxygen species which the host immune system uses to kill pathogens.

Staphylococcus aureus is the most common species of *S. aureus* to cause *Staphylococcus* infections and is considered as a successful pathogen due to a combination of nasal carriage and bacterial immuno-evasive strategies (Kluytmans *et al*. 1997).

It stated by Kuehnert (2005) that *S. aureus* infections can spread through contact with pus from an infected wound, skin-to-skin contact with an infected person, and contact with objects used by an infected person such as towels, sheets, clothing, or athletic equipment.

Joint replacements put a person at particular risk of septic arthritis, staphylococcal endocarditis and pneumonia.

Skin infections are the most common form of *S. aureus* infection. This can manifest in various ways, including small benign boils, folliculitis, impetigo, cellulitis, and more severe, invasive soft-tissue infections (Tong *et al*. 2015).

2-6. *Staphylococcus epidermidis*:

Staphylococcus epidermidis is a Gram- positive bacterium and one of the over 50 species belonging to the genus *Staphylococcus* ,it is part of human skin flora (commensal), and consequently part of human flora. It can also be found in the mucous membranes of animals. Due to contamination, it is probably the most common species found in laboratory tests (Queck and Otto, 2008).

Although *Staphylococcus epidermidis* is not usually pathogenic, patients with compromised immune systems are often at risk for developing an infection. These infections can be both nosocomial or community acquired, but they pose a greater threat to hospital patients. *Staphylococcus epidermidis* is also a major concern for people with catheters or other surgical implants because it is known to cause biofilms that grow on these devices (Salyers *et al.* 2002).

2-7. *Bacillus cereus:*

Bacillus cereus is an endemic, soil-dwelling, Gram-positive, rod-shaped, motile, beta hemolytic bacterium. Some strains are harmful to

humans and cause food borne illness, while other strains can be beneficial as probiotics for animals (Ryan and Ray, 2004). It is also the cause of "fried rice syndrome", as the bacteria are classically contracted from fried rice dishes that have been sitting at room temperature for hours, such as at a buffet (Asaeda *et al.* 2005 and Sana, 2011).

Cooking temperatures less than or equal to 100 °C (212 °F) allow some *B. cereus* spores to survive (Roberts *et al*., 1996). This problem is compounded when food is then improperly refrigerated, allowing the endospores to germinate (McKillip, 2000). It is stated by Kotiranta *et al.* (2000) that *B. cereus* is responsible for a minority of food borne illnesses (2–5%), causing severe nausea, vomiting, and diarrhea.

Cooked foods, not meant for either immediate consumption or rapid cooling and refrigeration, should be kept at temperatures below 10 °C or above 50 °C (50 °F and 122 °F). Germination and growth generally occur between 10 °C and 50 °C (Roberts *et al*., 1996), though some strains are psychrotrophic (McKillip, 2000).

The ability of *Bacillus cereus* to survive under adverse conditions in the environment, adapts, and eventually multiply in foods makes it a major food spoilage bacterium (Meer *et al*. 1991).

A carbohydrate-based bacterial capsule composed of hyaluronic acid surrounds the bacterium, protecting it from phagocytosis by neutrophils (Ryan and Ray, 2004). In addition, the capsule and several

factors embedded in the cell wall, including M protein, lipoteichoic acid, and protein F (SfbI) facilitate attachment to various host cells *(*Bisno *et al.* 2003).

M protein also inhibits opsonization by the alternative complement pathway by binding to host complement regulators. The M protein found on some serotypes is also able to prevent opsonization by binding to fibrinogen (Ryan and Ray, 2004).

2-8. *Escherichia coli:*

The *E. coli* is a Gram-negative, facultatively anaerobic, rod shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Singleton , 1999).

Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005).

The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 (Bentley and Meganathan, 1982) and preventing colonization of the intestine with pathogenic bacteria (Hudault *et al.* 2001 and Reid *et al*. 2001).

Eckburg *et al*. (2005) stated that fecal-oral transmission is the major route through which pathogenic strains of the bacterium can cause

disease. Cells are able to survive outside the body for limited amount of time, which makes them potential organisms to test environmental samples for fecal contamination.

Fotadar *et al*. (2005) mentioned that optimum growth of *E. coli* occurs at 37 °C (98.6 °F), but some laboratory strains can multiply at temperatures of up to 49 °C. A growing body of researchers has examined environmentally persistent *E. coli* which can survive for extended periods outside of a host (Ishii and Sadowsky, 2008).

Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide (Ingledew and Poole, 1984).

2-9. *Shigella sonnei:*

Shigella is a genus of Gram-negative, facultative anaerobic, non-spore forming, no motile, rod shaped bacteria closely related to *Salmonella*. The genus is named after Kiyoshi Shiga, who first discovered it in 1897 (Yabuuchi, 2002).

Shigella sonnei is the causative agent of human shigellosis. *Shigella* causes disease in primates, but not in other mammals (Ryan and Ray, 2004). During infection, it typically causes dysentery (Mims *et al.* 1993).

Shigella species are classified by four serogroups: Serogroup *A*: *S. dysenteriae* (15 serotypes) (Ansaruzzaman *et al*. 1995). Serogroup *B*: *S. flexneri* (six serotypes) Serogroup *C*: *S. boydii* (19 serotypes) (Yang *et al.* 1990). Serogroup *D*: *S. sonnei* (one serotype) Groups *A–C* is physiologically similar; *S. sonnei* (group *D*) can be differentiated on the basis of biochemical metabolism assays (Hale and Keusch, 1996).

Each of the *Shigella* genomes includes a virulence plasmid that encodes conserved primary virulence determinants. The *Shigella* chromosomes share most of their genes with those of *E. coli* K12 strain MG1655 (Yang *et al*. 2005).

Shigella causes dysentery which results in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. Some strains produce the enterotoxin shiga toxin, which is similar to the verotoxin of *E. coli* O157:H7 (Hale *et al*. 1996).

The diagnosis of shigellosis is made by isolating the organism from diarrheal fecal sample cultures. *Shigella* species are negative for motility and are generally not lactose fermenters apart from *S. sonnei* which ferment lactose (Ito *et al*. 1991). It was first successfully isolated from a 5 year old patient in Japan (Niyogi, 2005). It is a bacterium that is closely related to *E. coli*. It was known from the beginning that *Shigella sonnei* is related to *E. coli*, however, Shigella has evolved from many different strains of *E. coli* (Yang *et al*. 2005). In both developed and developing countries, the enteric infectious disease shigellosis, caused by *Shigella sonnei* infection has been the

most common cause of endemic diseases in those areas. *Shigella sonnei* continues to be a major food-borne threat to public health in many developed countries where the issues of sanitation are closely monitored (Shiferaw, 2004).

2-2. *Klebsiella pneumoniae* sub spp:

Klebsiella is a genus of non-motile, Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharidebased capsule (McClelland *et al.*2000). It is named after the German microbiologist Edwin Klebs (1834-1913).

The majority of human *Klebsiella* infections are caused by *K. pneumoniae*, followed by *K. oxytoca*. Infections are more common in the very young, very old, and those with other underlying diseases, such as cancer (Bagley, 1985).

It was reported by Lawlor *et al.* (2005) and Amako *et al.* (1988) that the reason for its pathogenicity is the thick capsule layer surrounding the bacterium. It is 160 nm thick of fine fibers that protrude out from the outer membrane at right angles.

Another site on the human body that these bacteria can be found is the nasopharynx. Its habitat is not limited to humans but is ubiquitous to the ecological environment. This includes surface water, sewage, and soil (Brisse and Verhoef, 2001).

Klebsiella pneumoniae contains a capsule around its cell known as K antigen; it is to protect the bacteria from phagocytosis. *Klebsiella pneumoniae* strains of serotypes 01:Kl, 01:K10, and 01:K16, which have only the K antigen exposed at the cell surface, resist complement-mediated killing by impeding complement activation. It is also clear that purified capsular polysaccharides (K antigen) from nine different serotypes (able or unable to mask the LPS) were unable to activate complement (Susana *et al.* 1992).

Brisse *et al.* (1999) reported that the diseases caused by *K. pneumoniae* can result in death for patients who are immunodeficient. Differences in the diseases are determined by the different virulence factors. For example, mucoid phenotype varies as the strains for mucoid vary (Victor *et al.* 2007), CPS and LPS O side chain are two of the most important virulence factors of *K. pneumoniae* (Cortés *et al.* 2002). They serve to protect the bacterium from phagocytosis by the host. Treatment is done by antibiotics such as clinafloxacin. But, there are an increasing amount of antibiotic-resistance strains. Ciprofloxacin is an antibiotic that is becoming less effective (Brisse *et al.* 2000)

Mundt and Daescbel (1979) concluded their study that *K. pneumoniae* is a bacterium for which the heat required for destruction of all cells is very close to that given during the canning process. Without actual chronometric and temperature monitoring in the individual kitchens it becomes impossible to assign survival to specific conditions. The

variations that do exist, may be inferred by the sporadic isolations of the bacterium during a large sampling of home-canned tomatoes and tomato juice.

2-2-1. *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa is a Gram- negative, aerobic, bacillus bacterium with unipolar motility (Ryan and Ray, 2004). *Pseudomonas aeruginosa* is not extremely virulent in comparison with other major pathogenic bacterial species for example *S. aureus* and *Streptococcus* though *Ps. aeruginosa* is capable of extensive colonization, and can aggregate into enduring biofilms (Hoiby *et al*. 2010).

It is stated by Itah and Essien (2005) that because it thrives on moist surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tarballs and oil from oil spills.

Pseudomonas aeruginosa secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown). King, Ward, and Raney developed *Pseudomonas* agar P (King A medium) for enhancing pyocyanin and pyorubin production and *Pseudomonas* agar F (King B medium) for enhancing fluorescein production (King *et al.* 1954). It creates dark, gellish mats sometimes improperly called "algae" because of their appearance. Although classified as an aerobic organism, *Ps.*

aeruginosa is considered by many as a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate as a terminal electron acceptor, and, in its absence, it is also able to ferment arginine by substrate-level phosphorylation (Palmer *et al*. 2007).

2-2-2. *Pasteurella multocida:*

Pasteurella multocida is a (Gram- negative), non-motile, penicillin - sensitive *coccobacillus*. In humans, *P. multocida* is the most common cause of infection from wound infections after dog or cat bites. The infection usually shows as soft tissue inflammation within 24 hours. High leukocyte and neutrophil counts are typically observed, leading to an inflammatory reaction at the infection site, generally a diffuse, localized cellulitis (Ryan and Ray, 2004).

Pasteurella multocida can also infect other localities, such as the respiratory tract, and is known to cause regional lymphadenopathy (swelling of the lymph nodes). In more serious cases, the bacteremia can result in, causing an osteomyelitis or endocarditis. The bacteria may also cross the blood–brain barrier and cause meningitis (Casolari and Fabio, 1988).

Diagnosis of the bacterial infection in humans was traditionally based on clinical findings, and culture and seriological testing, but false negatives have been a problem due to easy death of *P. multocida*, and

serology cannot differentiate between current infection and previous exposure. The quickest and most accurate method for confirming an active *P. multocida* infection is molecular detection using polymerase chain reaction (Miflin and Balckall, 2001).

2-2-3. Protein profiles:

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate protein by charge (Kryndushkin *et al.* 2003). Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate further than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving (Sambrook and Russel, 2001).

Gel electrophoresis as stated by Berg *et al.* (2002) can also be used for separation of nanoparticles. Gel electrophoresis uses a gel as an anticonvective medium and or sieving medium during electrophoresis, the movement of a charged particle in an electrical field. Gels suppress the thermal convection caused by application of the electric field, and can also act as a sieving medium, retarding the passage of

molecules; gels can also simply serve to maintain the finished separation, so that a post electrophoresis stain can be applied.

CHAPTER THREE

MATERIALS AND METHODS

3-1. Confirmation of identify of bacteria:

The bacterial under study were: *Staphylococcus aureus*, ATCC 25923, *E. coli*, ATCC 25922, *K. pneumoniae*, ATCC 13883 and *Ps. aeruginosa*, ATCC 27853. *Staphylococcus epidermidis*, *B. cereus* and *S. sonnei* were provided by National Public Health Laboratory, Khartoum as isolates. *Pasteurella multocida* was provided by Central Veterinary Research Laboratory, Khartoum. All biochemical tests were performed according to the methods, described by Barrow and Feltham (2003), including Gram staining reaction, micromorphology, growth characteristics and biochemical tests. All media were dispensed under aseptic conditions in an aseptic preparation room provided with ultra- violet lamp, flame, phenol disinfectant and alcohol for disinfecting the benches. All cultures on solid media were examined with naked eye for growth and colonial morphology and any visible change in the media such as colour, shape, size and haemolysis in blood agar medium. Also the liquid media were examined with naked eye for color changes and any signs of changing in the turbidity of the tubes.

3-2. Preparation and standardization of inoculum:

A full loop from each test bacterium that grown on nutrient agar was

inoculated in a tube of 5 ml nutrient broth. The inoculated tube was incubated for 24 hours at 37°C.

3-3. Methods of sterilization:

3-3-1. Flamming:

Flamming was used to sterilize needles, scalpels, glass slides, cover slips, points of scissors and mouth of culture tubes by passing them through Bunsen burner flame without allowing them becoming red hot.

3-3-2. Red heat:

Red heat was used to sterilize wire loops, points of forceps and searing spatulas by holding them over Bunsen burner flame until they become red- hot.

3-3-3. Hot air oven:

Hot air oven was used to sterilize glassware such as bottles, flasks, Petri dishes, test tubes; they were sterilized in hot air oven at 160°C for one hour.

3-3-4. Steaming (Tyndallization) at 100°C:

Steaming was used for the sterilization of sugars and media that could not be autoclaved without deteriorating effect to their constituents. Media were subjected to the steam for 1 hour for three successive days.

3-3-5. Autoclaving:

Media such as motility medium, nutrient broth, peptone water, O-F medium and nitrate broth were sterilized at 115°C for 20 minutes. Nutrient agar, blood agar, cooked meat medium, and mannitol salt agar were sterilized at 121°C for 15 minutes. Also it is used to sterilize plastic wares whereas glucose phosphate broth, gelatin agar and aesculin broth were sterilized by autoclaving at 115°C for 10 minutes.

3-4. Preparation of media:

Methods of preparation of the media applied were done according to Bridson (2006).

3-4-1. Nutrient broth:

This media was composed of 13 g nutrient broth, distilled water 1000 ml. The ingredients were dissolved and sterilized at 115°C for 20 min.

3-4-2. VP test media:

Voges-Proskuer composed of peptone 5 g, glucose 5 g, $KHPO_4$, distilled water 1000 ml. They were mixed and dissolved by gentle heating, pH 7.6 was adjusted, and distributed into tubes. Sterilized at 115°C for 20 min.

3-4-3. Arginine media:

Arginine media composed of 5 g peptone (tryptone), 5 g yeast extract,2 g K_2HPO_4, 0.5 g glucose ,3 g arginine monohydro-chloride

and 1000 ml DW, it was dissolved by heating, adjusted to pH 7.0 then boiled filtered and sterilized at 115°C for 20 min.

3-4-4. Peptone water sugar:

Peptone water composed of peptone 10 g, distilled water 1000 ml , 1% Andrade‘s indicator and sugar 1%. After mixed well and distributed in tubes, a Durham‘s was put and tubes then autoclaving was done at 115°C for 10 min.

3-4-5. Nitrate broth:

Nitrate broth composed of 0.1 g KNO_3, dissolved in 100 ml nutrient broth, distributed into tubes of 5 ml amounts, and sterilized at 115°C for 20 min.

3-4-6. Carbohydrate fermentation medium:

Carbohydrate fermentation medium was prepared according to Barrow and Feltham (2003). It contained 15 g peptone in 1L distilled water and then adding 10 g sugar and 10 ml Andrade's indicator. The pH of peptone water was adjusted to 7.1-7.3 before the addition of Andrade s indicator. The complete medium was well mixed, then distributed in aliquots of 2 ml into clean test tubes containing inverted Durham's tube. The medium was autoclaved at 121 °C for 15 minutes. The sugars used were glucose, sucrose, lactose, maltose, mannitol and sorbitol.

3-4-7. Nutrient agar:

Nutrient agar is nutrient broth gelled by the addition of 2% agar.

3-4-8. Blood agar:

Blood agar composed of 13 g nutrient broth to which 20 g agar were added and 1000 ml of DW were poured then autoclaving at 115°C for 20 min cooled till reached 55°C. After that sheep blood was added as 10%.

3-4-9. Christensen's urea agar:

Christensen's urea agar composed of 2.4 g urea base agar, 95 ml distilled water then autoclaved at 115°C for 20 min, then 5 ml sterilized DW were added to 2 g urea a crystal and mixed it well and added to the last solution. After filtration, it was mixed well and distributed in 7 ml amount into sterilized bottles and allowed to set in the slope position.

3-5. Starch agar:

Starch agar composed of 10 g potato starch, 50 ml DW and 1000 ml of nutrient agar triturated the starch with the water to a smooth cream then add to the molten nutrient agar. After mixing well sterilize at 115°C for 10 min. Distribute into Petri dishes.

3-5-1. Aesculin bile agar:

Aesculin bile agar composed of 1 g aesculin, 0.5 g ferric citrate, 1000 ml of nutreint broth, 15 g Agar, 40 g ox bile, dehydrated, then

dissolved all the ingredients except aesculin by heating, allowed to cool before adding the aesculin. It was dispensed in screw-capped bottles, sterilized at 115°C for 20 min and allowed to set as slopes.

3-5-2. Casein Agar (Milk Agar):

Casein agar composed of 500 ml of milk skim and 500 ml of nutrient agar double strength. The milk was sterilized at 115°C for 10 min; both of components were cooled to about 50°C then mixed together and distributed into Petri dishes or tubes.

3-5-3. Gelatin media:

Gelatin media composed of 4 g gelatin, 50 ml DW, 1000 ml nutrient agar, the gelatin was soaked in the water after softened, it was added to the melted nutrient agar mixed well then sterilized at 115°C for 10 min. Distributed into plates (Frazier, 1926).

3-5-4. Motility media:

Motility media composed of 10 g of peptone, 3 g meat extract, 5 g NaCl, 4 g agar 80 g gelatin, 1000 ml DW, the gelatin was soaked in the water for 30 min, added the other ingredients, heated to dissolve, and sterilized at 115°C for 20 min.

3-5-5. Triple sugar iron agar (TSI):

Triple sugar iron agar composed of 3 g meat agar, 3 g yeast extract, 20 g peptone, 1 g glucose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4.7H_2O$, 5 g NaCl, 0.3 g $NaS_2O_3.5H_2O$, 20 g agar, 1000 ml DW, 12 ml phenol

red 0.2% aqueous solution. The solids was heated to dissolve in the water, the indicator solution was added mixed and dispensed into tubes. It was sterilized at 115°C for 20 min, and cooled to form slopes with deep butts about 3 cm long.

3-5-6. Nutrient agar slant:

Nutrient agar slant was prepared by adding 28 g of nutrient agar to 1 L of distilled water and dissolving by boiling. The pH was adjusted to 7.4. The prepared medium was distributed in 10 ml volume into clean bottles, sterilized by autoclaving at 121°C for 15 minutes and left to solidify in inclined position.

3-5-7. MacConkey's agar medium:

About 52 g of MacConkey's agar were dissolved in 1 L distilled water. The pH was adjusted to 7.4, sterilized by autoclaving at 121°C for 15 min and then distributed in 20 ml volumes into sterile Petri dishes. The poured Petri dishes were allowed to solidify on flat surface.

3-5-8. Simmon's citrate medium:

This medium contains sodium ammonium phosphate, ammonium dihydrogen phosphate, magnesium sulphate, sodium citrate, sodium chloride, bromothymol blue as indicator and agar NO.3 (Oxoid L 13). The medium was obtained from Oxoid (Ltd). It was prepared according to manufacture instruction by dissolving 17 g of powder in 1 L of distilled water. The prepared medium was distributed in 10 ml

volume into clean bottles, sterilized by autoclaving at 121°C for 15 min and left to solidify in inclined position.

3-5-9. Oxidase test:

Oxidase test was used as a dry filter paper disc saturated with indicator solution and smears the test culture across the paper with glass rod or swab stick. The appearance of a dark purple color on the paper within 5-10 seconds denotes a positive reaction.

3-6. Catalase test:

A drop of normal saline was put on a microscope slide then a drop of culture was added and emulsified. After that drops from 3% H_2O_2 was run down the slope and examined for evolution of gas (bubbles) immediately, this indicates catalase activity.

3-6-1. Digestion of casein:

Casein agar plates was inoculated with the organism and examined for up to 14 days for clearing of the medium around the bacterial growth.

3-6-2. Gelatin liquefaction:

Nutrient gelatin was inoculated with straight wire loop and incubated at 37°C for 72 hours, then observed daily up to 30 days for growth and presence of liquefaction.

3-6-3. Sugar fermentation test:

Organisms to be tested were inoculated into the sugar tubes, incubated at 37°C and examined daily for 7 days for acid or acid and gas

production. Reversion to alkalinity was also noted. Negative tests were re-examined at regular intervals for up to 14 days.

3-6-4. Voges- Proskauer (VP) test:

Voges-proskauer medium was inoculated with the tested organism incubated for 1-2 days. One ml of the culture medium was transferred a aseptically into sterile test tube and then 0.6 ml 5% alpha- nephthol solution was added, followed by 0.2 ml 4% KOH aqueous solution; shaken well. The tubes were sloped to increase the area of the air-liquid interface, and examined after 15 min and 1 hour. A positive result was indicated by a strong red color.

3-6-5. Urease activity test:

A slope of Christensen's medium was inoculated heavily with the tested organism and examined after incubation at 37°C for 24 hours and examining daily for 5 days. Red color indicates a positive result.

3-6-6. Nitrate reduction test:

Nitrate broth was inoculated with the tested organism and incubated at 37°C for up to 5 days. One ml of nitrate reagent A was added followed by additional 1 ml of nitrate reagent B, a deep red color which shows the presence of nitrate, indicated a positive result. To tubes not showing a red color within 5 min, powdered zinc was added development of red color meant nitrate was not reduced by the organism to nitrite.

3-6-7. Motility test:

Motility medium was inoculated by stabbing with straight wire into the center of the craigie tube and then incubated at 37°C for 24 hours. The organism was considered motile if there was turbidity in the medium in and outside the craigie tube, while the growth of non-motile organism confined inside craigie tube.

3-6-8. Indole production test:

The test culture was inoculated into peptone water and incubated at 37°C for 48 hours. One ml Kovac's reagent was added to the tube. The appearance of a pink color in the reagent layer within a minute indicated positive reaction.

3-6-9. Methyl red test:

The test culture was inoculated into glucose phosphate medium and then incubated at 37°C for 48 hours. Two drops of methyl red reagent were added and shaken well. Red color indicated positive reaction. Yellow or orange color indicated negative reaction.

3-7. Citrate utilization test:

The test culture was inoculated onto Simmon's citrate medium, then incubated at 37°C and examined daily for 14 days. Blue color indicated positive reaction.

3-7-1. Hydrogen sulphide production test:

A tube of peptone water was inoculated by tested organism and lead

acetate paper was inserted between the cotton plug and the tube, then incubated at 37 °C and examined daily for a week. Blacken of the paper indicated H_2S production.

3-7-2. Coagulase test:

Two methods of coagulase test were applied: the microscopic slide, and the tube method:

A: Glass slide method:-

With minimal spreading, a colony in a drop of saline was emulsified on glass slide to produce a thick suspension. Then the bacterial suspension was stirred with a straight wire which had been dipped into undiluted rabbit plasma. A positive result was indicated by macroscopic clumping within 5 seconds. Delayed clumping does not constitute a positive reaction.

B: Tube method:

About 0.5 ml of 1-10 diluted rabbit plasma was mixed with an equal volume of an 18 -24 hours broth culture in a microtube and incubated at 37°C for 4 hours. It was examined after 2 and 4 hours for coagulation. A positive result was indicated by a definite clot formation. If negative; the tubes should be left in the incubator at 37°C overnight and then re-examined.

3-7-3. Aesculin hydrolysis:

Aesculin broth was inoculated and examined daily for up to 5 days for blackening which indicates hydrolysis of aesculin.

3-7-4. Arginine hydrolysis:

Five ml of arginine broth was inoculated and incubation for 24 hours, then 0.25 ml Nessler's reagent was added. The brown color indicated the hydrolysis of arganine.

3-7-5. Hydrogen sulphyide production:

A tube of triple sugar iron agar was inoculated by stabbing the butt and streaking the slope, it was observed daily for up 7 days for blackening of the butt, which is only due to H_2S production. Some organisms produce a dark pigment on the slope only and this was not mistaken for a positive result.

3-7-6. Novobiocin sensitivity test:

Novobiocin discs were used. After the organism was streaked lightly on nutrient agar. The disc was added gently on the surface and incubated for 24 hours, and then the area of inhibition was recorded as sensitive or resistant.

3-7-7. Sugar fermentation:

The ability of the test isolates to ferment different types of sugars was studied. Sugars used were: glucose, mannitol, maltose, lactose, sucrose, xylose, arabinose, trehalose, fructose, mannose, raffinose and galactose. Five ml of peptone water were used as a basal medium. Each test isolate was inoculated in a set of different sugars. The tubes were then incubated and examined frequently for up to 14 days before

discarding as negatives. Change of color to pink indicates positive results; gas production was indicated by bubble formation in Durham's tubes. When no acid was produced, the medium color remains unchanged.

3-7-8. Starch hydrolysis:

The plate of starch agar was inoculated with the organism then incubated at 37°C CO_2 was added for two days.

3-7-9. Heat shocked test:

The bacteria were inoculated into nutrient broth tubes and incubated at 37°C for 24 hours. Then the cultures were treated in a water bath at 100°C for 5 min, 90°C for 10 min, 80°C for 15 min, 70°C for 20 min, and 60°C for 25 min. After that, the biochemical tests were performed to detect the differences compared to the control.

3-8. Cold shocked test:

The bacteria were inoculated into nutrient broth tubes and incubated at 37°C for 24 hours. Then the cultures were subjected to 0°C, -15°C, and -32°C for 30 min. After that the biochemical tests were performed to detect the differences compared to the control.

3-8-1. Pathogenicity of *Pasteurella multocida*:

The ingredients that were used included blood agar as an enrichment medium, nutrient broth and normal saline. *Pasteurella multocida* was inoculated into nutrient broth tubes and incubated at 37°C for 24

hours. Ten serial dilutions in duplicate were performed. One of the duplicate was put in 37°C; the other was shocked at -15°C for 30 min. After incubation at 37°C for an overnight, the dilutions which did not show visible colonies on recovery media were centrifuged and inoculated i/p in four rabbits. Rabbits were postmortemed and cultures were made from heart blood by puncturing with a sterile Pasteur pipette. The same test was repeated with bacteria heated at 50°C and 40°C for 10 min.

3-8-2. Protein extraction procedure:

1-The organisms were harvested with normal saline from the plate of the culture.

2- They were centrifuged at 3000-4000 rpm for 2 min to pellet cells. The supernatant was removed leaving behind 10-20 µl residual liquid.

3- The tube was vigorously vortexed to resuspend the cells in the residual supernatant. This greatly facilitates cell lysis in step 4 below.

4- About 300 µl sample loading buffer solution were added to the resuspended cells and pippeted up and down to lyse the cells. Usually no incubation is required; however, if cell clumps were visible after mixing, the tube was incubated at 37°C until the solution was homogeneous. Samples were stable in sample loading buffer for at least 4 months at deep freezing. The protein extraction for estimation was done by adding PBS to harvest cells. The cells were treated with

the Sonicator for 5 minutes for all isolates except *S. aureus*, which was treated for 10 min.

3-8-3. Protein profile test:

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in biochemistry and molecular biology. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Proteins and nucleic acids were electrophoresed within a matrix or "gel". Most commonly, the gel was cast in the shape of a thin slab, with wells for loading the sample. The gel was immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. The gel itself was composed of either agarose or polyacrylamide.

3-8-4. SDS-PAGE typing:

The following buffers were prepared first. Acrylamide/Bis solution 29:1 is also suitable for preparation of a gel according to Schagger and Jagow (1987).

3-8-5. Polyacrylamide gel preparation (A):

The stock solution was prepared by dissolving 29 g acrylamide and 1

g of bisacrylamide in 100 ml distilled water. The solution was then stored in brown bottle covered by aluminum foil because this solution is light sensitive and refrigerated at 4 ºC.

3-8-6. Stacking gel buffer (Tris-HCl):

The stock solution was prepared by dissolving 3 g Tris-HCl (Tris-acid) in 50 ml distilled water. The volume was then completed to 100 ml using distilled water and the pH adjusted to 6.8.The stock solution was then stored at 4 ºC.

3-8-7. Resolving gel buffer (Tris-OH):

The stock solution was prepared by dissolving 18.15 g Tris-OH (Tris-base) in 50 ml distilled water. The volume was then completed to 100 ml using distilled water and the pH was adjusted to 8.8 and then stored at 4 ºC.

3-8-8. Running buffer:

The stock solution was prepared by dissolving 3.02 g Tris-OH, 14.4 g glycine and 1 g SDS in 500 ml distilled water. The volume was then completed to 1000 ml using distilled water and the pH was adjusted to 8.3 and then stored at 4 ºC.

3-8-9. Preparation of 10% Sodium Dodecyl Sulphate (SDS):

The stock solution was prepared by dissolving 10 g of SDS in 100 ml distilled water.

3-9. SDS sample loading buffer:

The stock solution was prepared by mixing 1 ml of Tirs-OH (pH 6.8), 4 ml of distilled water 0.8 ml glycerol, 1.6 SDS 10%, 400 µl mercaptoethanol, and 200 µl 0.05% bromophenol blue. This buffer was used to extract the proteins of all strains of bacteria under study.

3-9-1. Sealing gel preparation (lock of the glasses):

The sealing was prepared by mixing 1 ml of DW with 1 ml of Acrylamide, 10 µl TEMED, and 100 µl APS.

3-9-2. Resolving gel preparation (lower gel):

The resolving gel was prepared by mixing 4.2 ml of solution Acrylamide, 3.2 ml of DW, 2.5 ml solution Tris OH, 100 µl SDS, 10 µl TEMED, 100 µl ammonium presulphate (APS), used to catalyzed the polymerization of acrylamide.

3-9-3. Stacking gel preparation (upper gel):

The Stacking gel was prepared by mixing 1.3 ml of solution acrylamide, 4 ml of DW, 2.5 ml of Tris HCL, 10 µl TEMED, and 100 µl APS.

3-9-4. Staining buffer:

Staining buffer was prepared by dissolving 0.2 g coomassie blue dye in 62.5 ml of distilled water, 5.75 ml glacial acetic acid and 28.5 ml methanol.

3-9-5. De-staining buffer:

De-staining buffer was prepared by mixing 10 ml of glacial acetic acid with 50 ml of methanol and 100 ml of distilled water.

3-9-6. Mounting gels:

Glass plates were separated by spacers and stabilized using two clips to pour in gels. The resolving gel was prepared and poured to fill about 3/4 of the plate; saturated butanol was then added on top to level the gel and avoided bubbles. The gel was left to polymerize then washed by distilled water and a comb was placed to make wells. The stacking gel was then poured and left to polymerize. Polymerization of both stacking and resolving gel required up to 60 min. After 20-30 min the comb was removed and the wells were washed using distilled water.

3-9-7. Loading samples:

Ten µl of the extracted protein were heated at 90°C for 5 min in water bath to denature the protein and was then loaded into wells.

3-9-8. Applying the protein in the running gel method:

1. The wells of the gel can take up to 20 µl of the sample. The samples were all ready with stain that gained from sample loading buffer, which was used to extract the protein with it.

2. This mixture was heated in a water bath at 100°C for 5 min prior to loading.

3. The voltage was set up to 100v during the running in stacking gel then the volt was lowered to 80v in resolving gel for 2 hours till the stain reached the sealing gel after which the running was stopped.

4. The gel was allowed to stain overnight and de-stained for no time till the bands appeared, then it was washed with DW several times and the gel was preserved in DW in 4°C.

5. All prepared reagents were preserved in 4°C.

The safe stain was used instead of ethidiume bromide in DNA gel.

3-9-9. Bradford reagent recipe:

About 25 mg of coomassie brilliant blue G-250 IN 25 ml were added to 50 ml 85% (w/v) phosphoric acid (H_3PO_4). The acid solution mixture was added slowly into 425 ml of H_2O and the dye was let to dissolve completely (Note: H_2O should not add into the acid solution).

Whatman's paper type (1) filter was used to remove the precipitates just before using and stored in a dark bottle at 4°C.

3-10. Bradford assay:

The Bradford assay is a colorimetric assay based on the interaction between coomassie brilliant blue and the arginine and aromatic residues in the protein. When the dye binds to these residues, its maximum absorption shift from 470 nm to 595 nm. In general, the absorbance of a series of known concentrations of a standard curve

was measured. The standard curve was then used to calculate the concentration of the protein sample based on its absorbance.

3-10-1. Materials used:

Spectrophotometer, cuvette, blank solution, BradFord's reagent and the proteins that were needed to measure.

3-10-2. Procedure:

Blank cuvette was selected and placed in the spectrophotometer. The lid was closed. The 0 ABS 100%T button was clicked, the instrument read 0.00000A. A solution with known concentration was chosen and the absorbance was measured between the wavelengths 350 nm to 700 nm. The wavelength at the maximum absorbance value was recorded. The value of molar absorption coefficient was calculated using the equation (Y = mx+ b). Where Y = absorbance at 595 nm, X = protein concentration and b = protein concentration per mcg/ml (micrograms per microliters). The reading of protein concentrations was duplicated to avoid the error and to get the accurate means from the repetitions of the reading samples.

3-10-3. The extraction of the DNA of bacteria:-

1- About 150 ul of cell culture supernatant was transferred in the 1.5 ml micro centrifuge tube.

2- Three hundred µl of Lysis buffer was added.

3- It was mixed by vortexing for 15 second.

4- Then was incubated at room temperature (15-25°C) for 10 min.

5- Also 300 µl of binding buffer was added and completely, mixed well by gently vortexing.

6- A spin column was placed in a provided 2 ml collection tube.

7- Lysates on the column were loaded and centrifuged at 13000 rpm for 1 min.

8- Solution in collection tube was discarded and placed in column back in the same 2 ml collection tube.

9- Then 500 µl of washing buffer A were added to column and centrifuged for 1 min at 13000 rpm.

10- The solution in the collection tube was discarded and spin column was placed back in the same 2 ml collection tube.

11- Also 500 µl of washing buffer B were added to the column and centrifuged for 1min at 13000rpm.

12- The solution in collection tube was discarded and the spin column was placed back in the same 2 ml collection tube. It was centrifuge for 1 min at 13000 rpm.

13- The column was placed in a RNase-free 1.5 ml micro centrifuge tube and 30 µl of elution buffer were added directly onto the spin column membrane.

(Note: Touching of the membrane with the pipette tip should be avoide.

14- After incubation at room temperature for 1 min. The eluted buffer was centrifuged for 1 min at 13000 rpm.

15- About 2-5 µl of the eluted solution was used as template for PCR OR RT-PCR.

3-10-3. Agarose gel electrophoresis:

To check the quantity and the quality of the DNA, the samples were run in agarose gel.

3-10-4. TBE 1X preparation:

About 0.550 g of boric acid and 1.078 g of Tris-base were dissolved in 100 ml of DW, the pH was adjusted to 8.5 then 0.067 of EDETA was added and the volume was completed to 1000 ml of DW.

3-10-5. Gel preparation:

About 0.4 g of agarose was dissolved in 20 ml of 1 X TBE and heated at 70°C until the mixture was clear .One µl of ethidium bromide was added and the mixture was poured in the trail with the comb in it to make the wells. The gel was left for 20 min to polymerize then the comb was removed. While the gel was solidifying, the DNA mixtures were prepared by electrophoresis as follows: One µl of each DNA was transferred to a clean eppendorf tube and 3 µl loading dye was added to DNA sample. The mixture was mixed several times using a

micropipette, and then was put in the wells using a plastic-tipped micro-pipette. The gel was then immersed in 1 X TBE buffer. The buffer was added until it reached the level of approximately 3-5 mm above the gel surface the apparatus was closed and the power was turned on, the voltage was adjusted to 80V (100 mA) and the running was continued without cooling for 90 minutes. Then the power supply was removed, visualized in a gel documentation system (Biosystematica, Mo 33874. Power 25W), and photo-graphed using a digital camera, Nikon, 45000.

CHAPTER FOUR

RESULTS

4-1. Morphology and staining:

Staphylococcus aureus appeared in stained smears as Gram- positive *cocci* arranged in clusters, and *Bacillus cereus* appeared as rod shape. *Shigella sonnei*, *Escherichia coli*, *Pseudomonas aeruginosa* and *P. multocida* appeared as coccobacillus. Coccoid appeared in stained smears as Gram- negative, which appear singly, in pairs or as short chains.

4-2. Blood agar medium:

Staphylococcus aureus: colonies were circular, smooth, opaque, low convex, soft and easily emulsified when touched by the wire loop, with large zone of Alfa hemolysis in blood agar.

Bacillus cereus colonies were dull gray and opaque with a rough matted surface. Colony perimeters were irregular and represented the configuration of swarming from the site of initial inoculation, perhaps due to *B. cereus* swarming motility. Zones of beta-hemolysis surround and conform to the colony morphology.

4-3. MacConkey's medium:

Escherichia coli showed mucoid colonies which have entire margins and are slightly raised. Older colonies often have a darker center. *Pasteurella multocida*: small mucoid or dry colonies. No hemolysis on

blood agar, but some strains gave a greenish decolorization. *Shigella sonnei:* showed colorless colonies. *Pseudomonas aeruginosa* grown on nutrient agar showed colonies surrounded by bluish green coloration.

For the pathogenicity only, cold-shocked cells showed their effect on the experimentally inoculated rabbits. The rabbits died within 24 hours. They showed typical lesions of haemorrhagic septicemia and *Pasteurella multocida* was isolated.

The characters and biochemical properties of *S. aureus, S. epidermidis, B. cereus, E. coli, S. sonnei, K. pneumoniae, Ps. aeruginosa* and *P. multocida* are shown in Tables (1, 2, 3, 4, 5, 6(a-b), 7(a-b) and 8).

Table (1): Characters and biochemical properties of *S. aureus* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock			Heat shock
	37°C	**0°C/ 30 min**	**-15°C/ 30 min**	**-32°C/ 30 min**	**60°C / 25 min**
VP	++++	+++++	+++	+++++	+++
Coagulase	++++	++++	+++	+++++	+++
Novobiocin	s	S	s	s	S
Urease	+++	++++	++	++++	+++++
Arganine	++++	+++	++	++++	++
Lactose	+++++	+++	+++	+++++	+++++
Maltose	++++	++	+++	++++	++++
Mannitol	+++	+++	+++	++++	++
Fructose	++++	++	+++	++++	+++
Sucrose	+++	++++	++	++++	+++
Trehalose	++++	+++++	+++	+++++	++
Mannose	++++	+++++	+++	+++++	+++++
Nitrate	++++	++++	+++	+++++	++++

Key: +++++ Very strong. ++++ Strong. +++ Moderate. ++ Week.

Oxidase, xylose, cellobiose and raffinose were negative and catalase was strong positive till 100°C. For the cold shocked, *Staphylococcus aureus* the reaction seemed to be the same as at 0°C and -32°C, while it was also found equal at -15°C and 37°C and a little different when temperature was decreased at -15°C. Biochemical properties of *S. aureus* which were subjected to, 80°C, 90°C and 100°C showed negative results. The reaction appeared till 70°C but it varies in the degree of concentrations: VP and arginine seemed to be doubtful, while fructose, nitrate were weak and urease was moderate. The rest of the tests were negative.

Table (2): Characters and biochemical properties of *S. epidermidis* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	0°C/ 30 min	-15°C/ 30 min	-32°C/ 30 min
VP	++++	+++++	++++	++++
Novobiocin	S	S	S	S
Urease	++++	+++++	+++	+++
Arganine	++++	+++++	+++	+++
Lactose	+++++	+++++	++++	+++
Maltose	++++	+++++	++++	++++
Fructose	++++	+++++	++++	+++
Sucrose	++++	+++++	++++	+++
Mannose	+++	+++++	++++	+++
Nitrate	++++	++++	++++	+++

Key: +++++ Very strong. ++++ Strong. +++ Moderate.

Oxidase, coagulase, mannitol, trehalose, xylose, cellobiose and raffinose were negative. For cold shocked *S. epidermidis* the reaction seemed to be the same as at 37°C, -15°C, and -32°C, while it was increased at 0°C. Biochemical properties of *Staphylococcus epidermidis* that subjected to 60°C, 70°C, 80°C, 90°C and 100°C. All tests were negative to these temperatures with exception to lactose, maltose, fructose, sucrose, mannose that were doubtful at 60°C.

Table (3): Characters and biochemical properties of *B. cereus* grown at 37°C and subjected to 0°C, -15°C and-32°C

Temperature / Test	Control	Cold shock		
	37°C	0°C /30min	-15°C/30 min	-32°C/30 min
Motility	+++	+++	++++	+++++
Glucose	+++	+++	++++	++++
Cellobiose	+++	+++	++++	++++
Salacin	+++	+++	+++	++++
VP	+++	+++	++++	+++++
Nitrate reduction	+++	+++	++++	++++
Casein hydrolysis	+++	++	+++	++++
Starch hydrolysis	+++	+++	++++	++++
Oxidase	+++	+++	++++	+++++

Key: +++++ Very strong. ++++ Strong. +++ Moderate. ++ Weak.

Galactose, mannose, melibiose, raffinose, xylose, citrate, urease and indole were negative for all temperatures used. For cold shocked *B. cereus*, the reaction seemed to be the same as at 37°C and 0°C, while it was gradually increased at -15°C and -32°C. Biochemical properties of *Bacillus cereus* that were subjected to 60°C, 70°C, 80°C, 90°C and 100°C, all the tests were negative with the exception of glucose, salacin, VP and nitrate reduction. Casein hydrolysis and starch hydrolysis were moderate in the reaction at 60°C; also motility was moderate at 60°C 70°C but weak at 80°C. Salacin, VP, nitrate reduction, casein hydrolysis and starch hydrolysis were weak in the reaction at 70°C, also glucose was weak at 70°C and 80°C. Cellobiose was strong at 60°C and 70°C. Oxidase was strong till 100°C. Nitrate reduction was doubtful from 80°C till 100°C.

Table(4): Characters and biochemical properties of *E. coli* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	0°C/ 30 min	-15°C/ 30 min	-32°C/ 30 min
MacConkey's growth	++++	++++	+++	++++
Arganine	+++	++++	++	++++
Glucose(Gas)	+++	++++	+++	++++
Arabinose	+++	++++	+++	++++
Dulcitol	+++	++	++	++
Glycerol	++	++	++	++
Lactose	++++	++	+++	++++
Maltose	++++	++	++	++++
Mannitol	++++	++	+++	+++
Rhamenose	+++	++++	+++	++++
Sorbitol	+++	++++	+++	++++
Trehalose	+++	++++	+++	++++
Xylose	+++	+++	++	++++
MR	+++	++++	+++	++++
Indole	+++	++++	+++	++++

Key: ++++ Strong. +++ Moderate. ++ Week.

Simmon's citrate**,** urease, gelatin hydrolysis, H_2S from TSI, malonate, selenite reduction and casein hydrolysis, were negative in all cold shock temperatures. For cold shocked bacteria the reaction seemed to be the same as at 0°C and -32°C as in *S. aureus*, while it decreased at 37°C and -15°C. For the biochemical properties of *Escherichia coli* that were subjected to 60°C, 70°C, 80°C, 90°C and 100°C, all the tests were negative except on MacConkey's, which showed weak growth at 60°C and 70°C.

Table (5): Characters and biochemical properties of *S. sonnei* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	0°C/30 min	-15°C/30 min	-32°C/30 min
MacConkey's Growth	++++	++	++++	+++
Arabinose	+++	++	++++	+++
Glycerol	+	+	+	+
Maltose	+++	+	-	-
Mannitol	+++	+++	++++	+++
Rhamenose	+++	+++	++++	+++
Trehalose	++++	++	+++++	+++
MR	++++	+++	+++++	++++

Key: +++++ Very strong. ++++ Strong. +++ Moderate. ++ Week. + Doubtful. - Negative.

Motility, simmon's citrate, urease, gelatin hydrolysis, H_2S from TSI, malonate, arganine, selenite reduction, casein hydrolysis, glucose (Gas), adonitol, dulcitol, inistol, lactose, raffinose, sorbitol, sucrose, xylose, starch, VP and indole were negative when subjected to the temperatures mentioned above.

For cold shocked *S. sonnei* the reaction seemed to be the same as 37°C and -15°C, while it was decreased at -32°C and 0°C. Biochemical properties of *Shigella sonnei* that subjected to 60°C, 70°C, 80°C, 90°C and 100°C, all tests showed negative results to these temperatures.

Table (6a): Characters and biochemical properties of *K. pneumoniae* grown at 37°C and subjected to 0°C, -15°C and -32°C:-

Temperature / Test	Control	Cold shock		
	37°C	0°C/ 30 min	-15°C/ 30 min	-32°C/ 30 min
MacConkey's growth	++++	++	+++	++++
Simmon's citrate	++++	++	+++	++++
Urease	++++	++	+++	++++
Glucose(Gas)	++++	++	+++	++++
Adonitol	++++	++	+++	++++
Arabinose	++++	++	++	++++
Dulcitol	+++++	+++	++++	+++++
Glycerol	+++	++	++	++
Inoistol	++++	+++	+++	++++
Lactose	++++	++++	++++	++++
Maltose	+++++	+++	+++	+++++
Mannitol	++++	++	+++	++++

Key: ++++ Strong. +++ Moderate. ++ Week.

Motility, gelatin hydrolysis, H_2S from TSI, H_2S from Pb paper, malonate, arganine, selenite reduction, casein hydrolysis, adonitol, dulcitol, inistol, lactose, raffinose, sorbitol, sucrose, xylose, starch, VP and indole were negative to cold shock temperatures.

Table (6b): Characters and biochemical properties of *K. pneumoniae* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	**0°C/ 30 min**	**-15°C/ 30 min**	**-32°C/ 30 min**
Raffinose	++++	++	+++	++++
Rhamenose	+++++	+++	++++	+++++
Salicin	++++	+++	+++	++++
Sorbitol	++++	++	+++	++++
Sucrose	+++++	+++	++++	+++++
Trehalose	+++++	+++	+++	+++++
Glycerol	++++	+++	+++	++++
Xylose	++	++	++	++
Starch	+++++	+++	++++	+++++
MR	++++	+++	+++	++++

Key: +++++ Very strong. ++++ Strong. +++ Moderate. ++ Week.

VP and indole were negative.

For cold shock *K. pneumoniae* the reaction seemed to be the same as 37°C and -32°C, while it was decreased at -15°C and 0°C. The properties of *Klebsiella pneumoniae* that was subjected to 60°C, 70°C, 80°C, 90°C and 100°C showed negative results except MR which was weak at 60°C, MacConkey's and urease were doubtful at 60°C, also urease at 70°C.

Table (7a): Characters and biochemical properties of *Ps. aeruginosa* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	0°C/ 30 min	-15°C/ 30 min	-32°C/ 30 min
Motility	+++	++	+++	+++
Green pigment	++++	+++	+++	++++
MacConkey's growth	++++	+++	+++	++++
Oxidase	++++	+++	+++	++++
O-F Medium	++++	++	+++	++++
Nitrate reduction	++++	+++	+++	++++
Simmon's citrate	++++	+++	+++	++++
Urease	++++	+++	+++	++++
Gelatin hydrolysis	++++	+++	++++	++++
Malonate	++++	+++	++++	++++
Adonitol	++++	+++	+++	++++
Arabinose	++++	+++	+++	++++
Dulcitol	++++	+++	+++	++++

Key: ++++ Strong. +++ Moderate. ++ Week.

Table (7b): Characters and biochemical properties of *Ps. aeruginosa* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	0°C/ 30 min	-15°C/ 30 min	-32°C/ 30 min
Fructose	++++	+++	+++	++++
Glycerol	++++	+++	+++	++++
Inositol	++++	++	+++	++++
Lactose	++++	+++	+++	++++
Maltose	++++	++	+++	++++
Mannitol	++++	+++	+++	++++
Salicin	++++	+++	+++	++++
Sorbitol	++++	+++	+++	++++
Sucrose	++++	++	+++	++++
Trehalose	++++	+++	+++	++++
Xylose	++++	++	+++	++++
Arginine hydrolysis	++++	++	+++	++++
Casein hydrolysis	++++	++	+++	++++
Aesculin hydrolysis	++++	++	+++	++++

Key: ++++ Strong. +++ Moderate. ++ Week.

Raffinose, starch hydrolysis, and glucose (Gas) were negative in the temperatures mentioned above. For cold shocked bacteria the reaction for *P. aeruginosa* was on line with *K. pneumoniae.* Biochemical properties of *P. aeruginosa* that were subjected to 60°C, 70°C, 80°C, 90°C and 100°C showed negative results except the green pigmentation which appeared till 80°C. Fructose was weak at 60 °C and doubtful at 70°C.

Table (8): Characters and biochemical properties of *P. multocida* grown at 37°C and subjected to 0°C, -15°C and -32°C

Temperature / Test	Control	Cold shock		
	37°C	**0°C/ 30 min**	**-15°C/ 30 min**	**-32°C/ 30 min**
Catalase	++++	++++	++++	++++
Oxidase	+	+	+	+
Lactose	++++	++	+++	++++
Maltose	+++	++	++	+++
Mannitol	++++	++	+++	+++
Sorbitol	+++	++	+++	++
Sucrose	++++	++	+++	++++
Nitrate reduction	++++	++	++	+++
Indole	+++++	+++	++++	+++++

Key: ++++ Strong. +++ Moderate. ++ Weak. + Doubtful.

All tests were positive but vary at the degree of the color. MacConkey's, Arabinose, raffinose, salicin, trehalose, xylose, aesculin, gelatin hydrolysis and urease were negative to the temperatures mentioned above.

For cold shocked *P. multocida* the reaction seemed to be the same as 37°C and -32°C, while it was decreased at -15°C and 0°C. Biochemical properties of *P. multocida* that was subjected to 60°C, 70°C, 80°C, 90°C and 100°C, showed negative results except on catalase which was strong positive till 100°C. Nitrate reduction was weak, sucrose and indole were doubtful at 60°C.

4-5. Estimation of protein concentrations:-

Upon quantifying the amount of protein of *Staphylococcus aureus* obtained upon lysis of cell, there was clear increase in protein expression (mg/ml) from -32°C to 0°C. At 0°C, the content of protein remained almost unchanged.

Shigella sonnei, and *Pseudomonas aeruginosa* expressed gradual protein decreased from 37°C to 100°C. Almost 400% protein loss was noticed as the temperatures raised to high as 100°C (Figure, 5B).

There was a gradual, though not very significant, decrease in the protein content as the tempreature increase from -32°C to 0°C. In raised *Klebsiella pneumoniae*, however, at 37 °C showed a sharp and sudden increased in the protein content was observed after which sharp declination was also noticed after which protein started to increase again which peaked at 100°C.

The electrophoretic pattern of proteins profiles and the estimation of the protein concentrations are shown in figures (1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B, 8A and 8B).

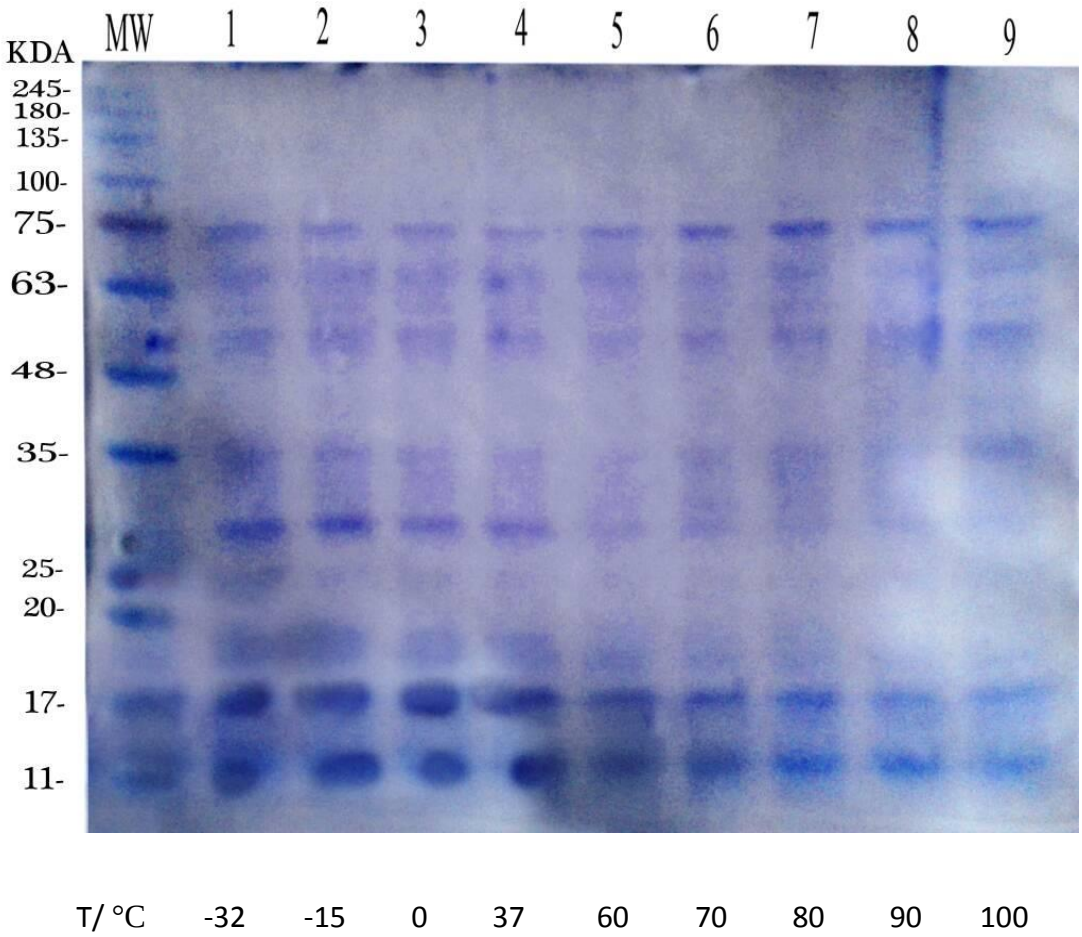


Figure (1A): Electrophoretic patterns of proteins of *S. aureus* isolate which were stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

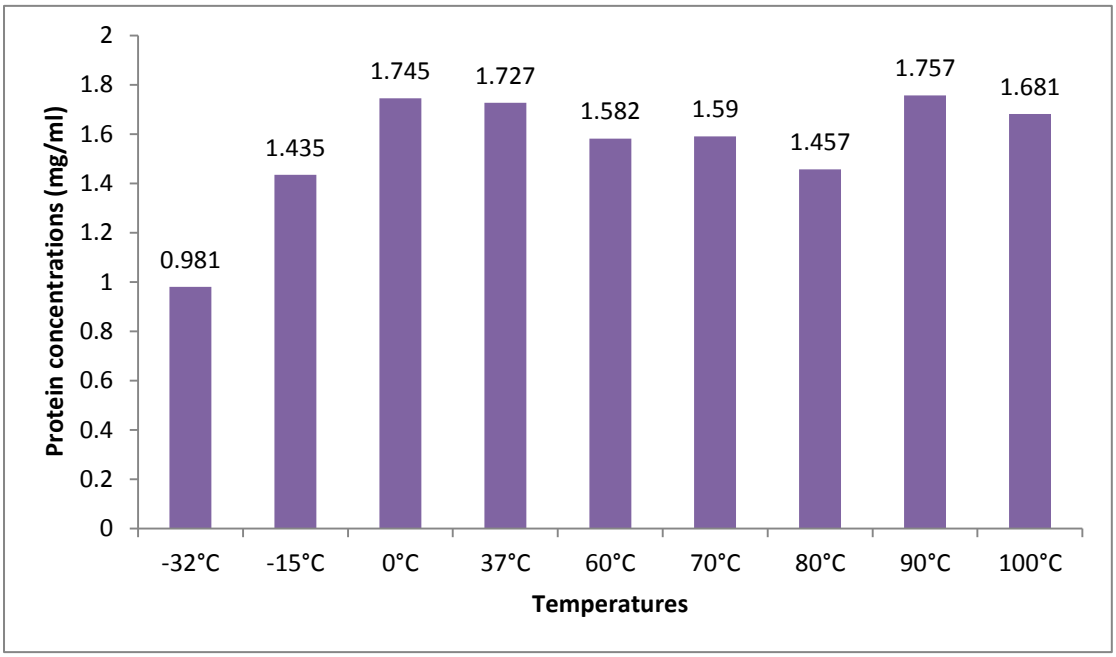


Figure (1B): Estimation of protein concentration of *S. aureus,* which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.

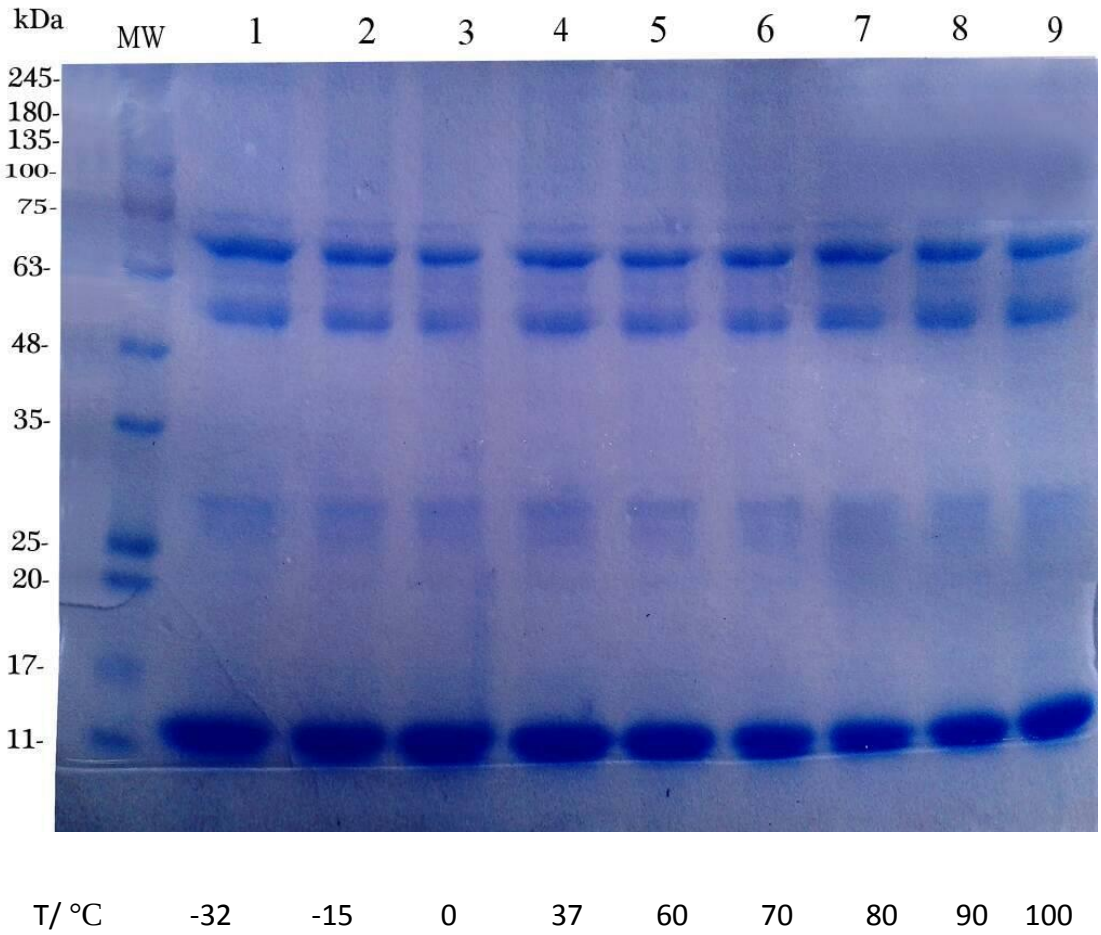
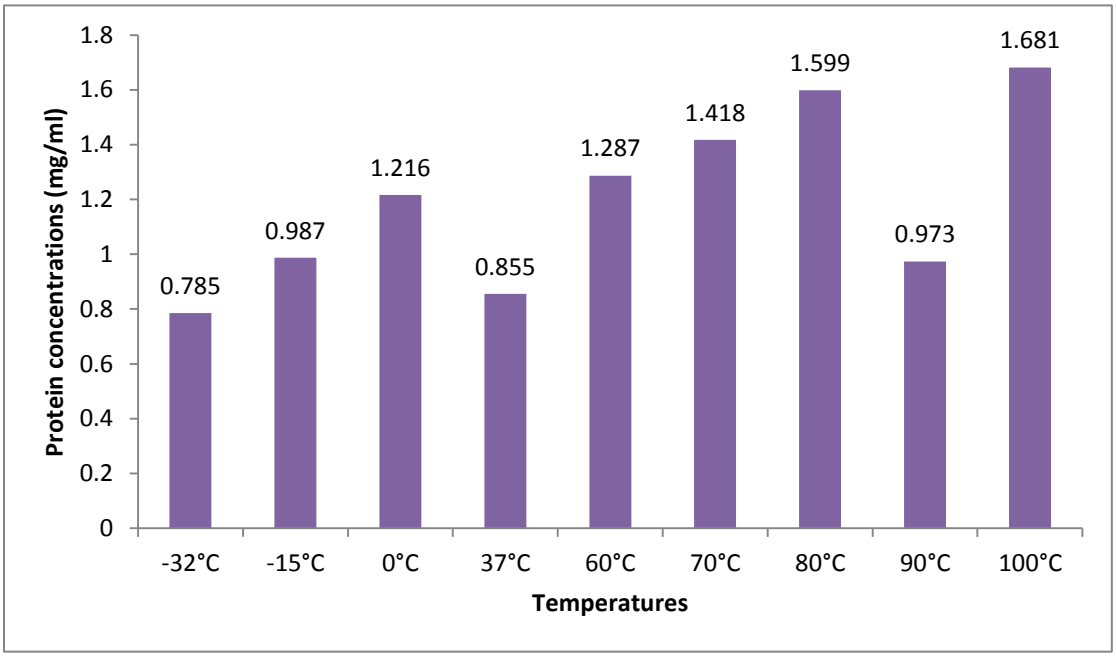


Figure (2A): Electrophoretic patterns of proteins of *S. epidermidis* isolate, which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.



Fiugre (2B): Estimation of protein concentration of *S. epidermidis,* which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.

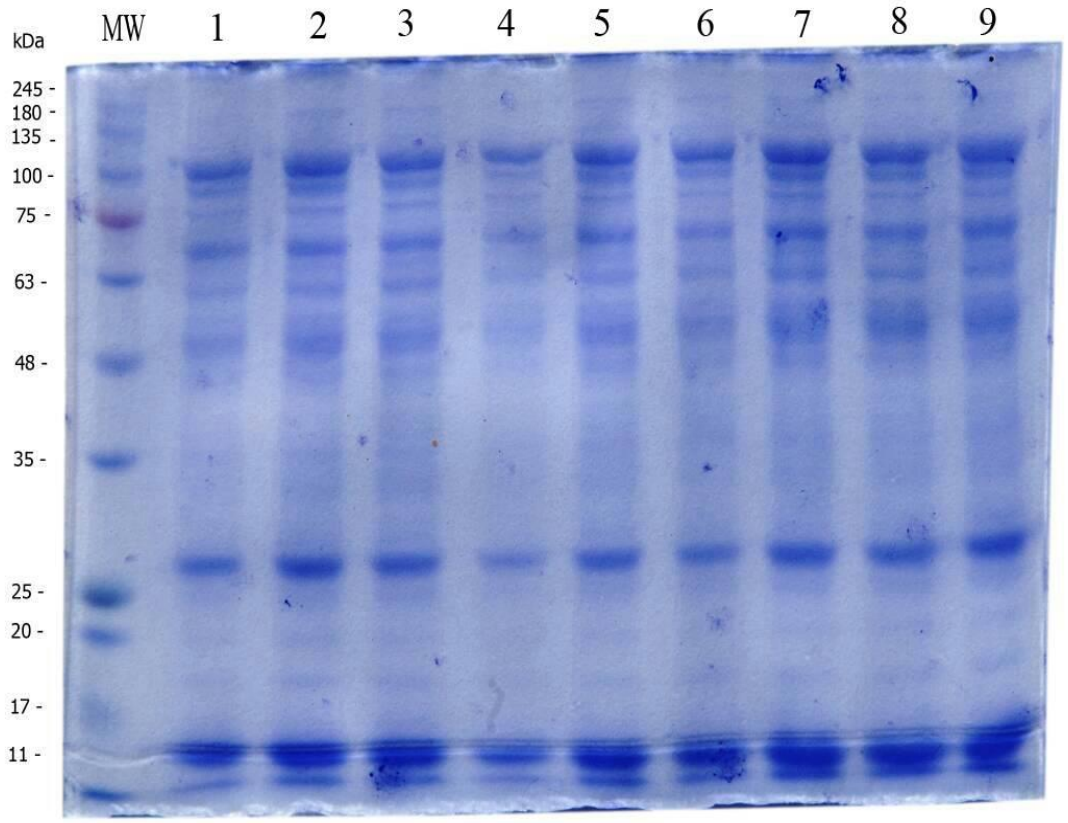


Figure (3A): Electrophoretic patterns of protein of *B. cereus* isolate which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

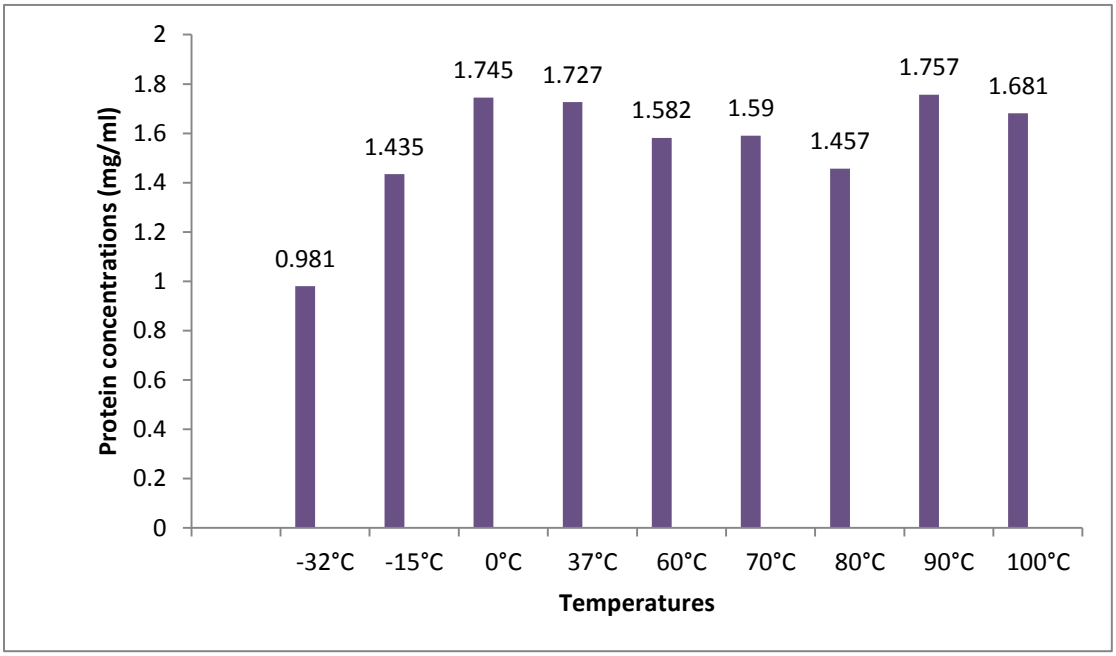


Figure (3B): Estimation of protein concentration of *B. cereus* which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.

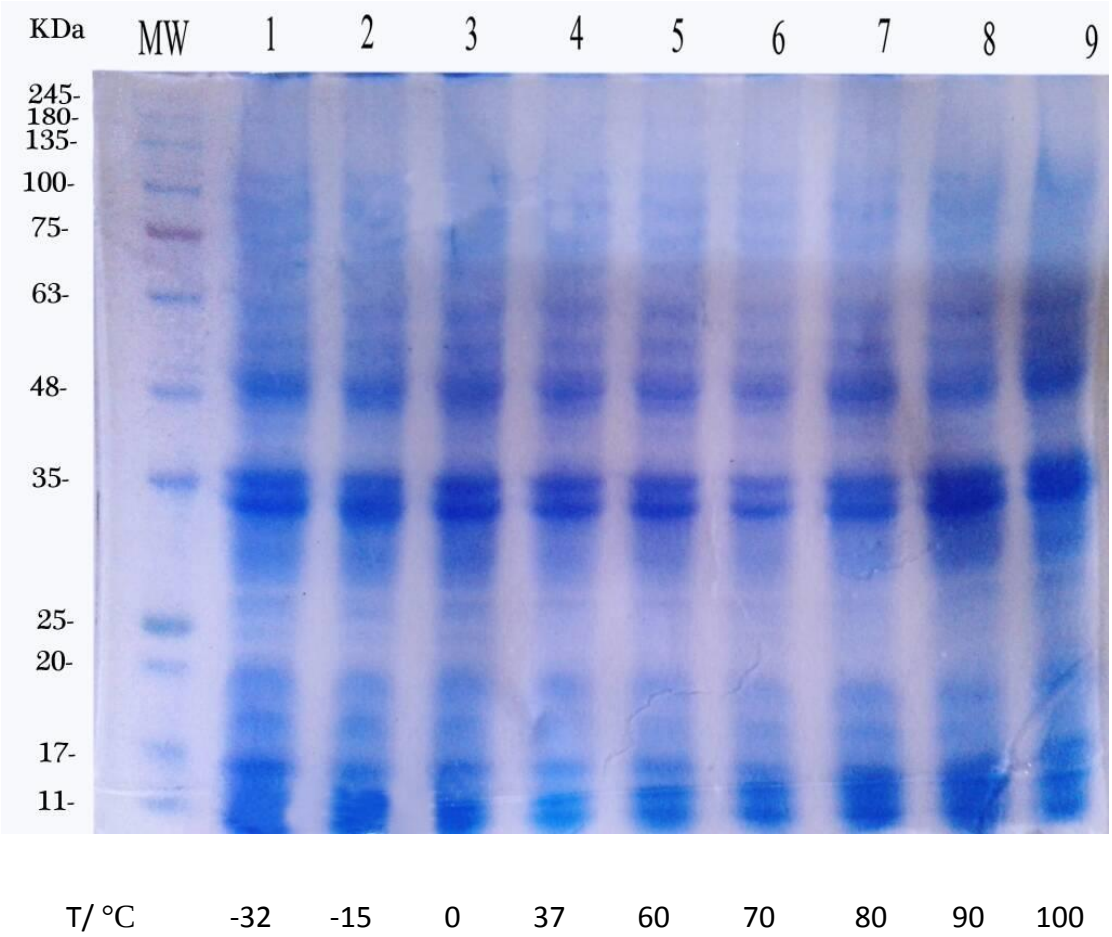


Figure (4A): Electrophoretic patterns of protein of *E. coli* isolate, which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

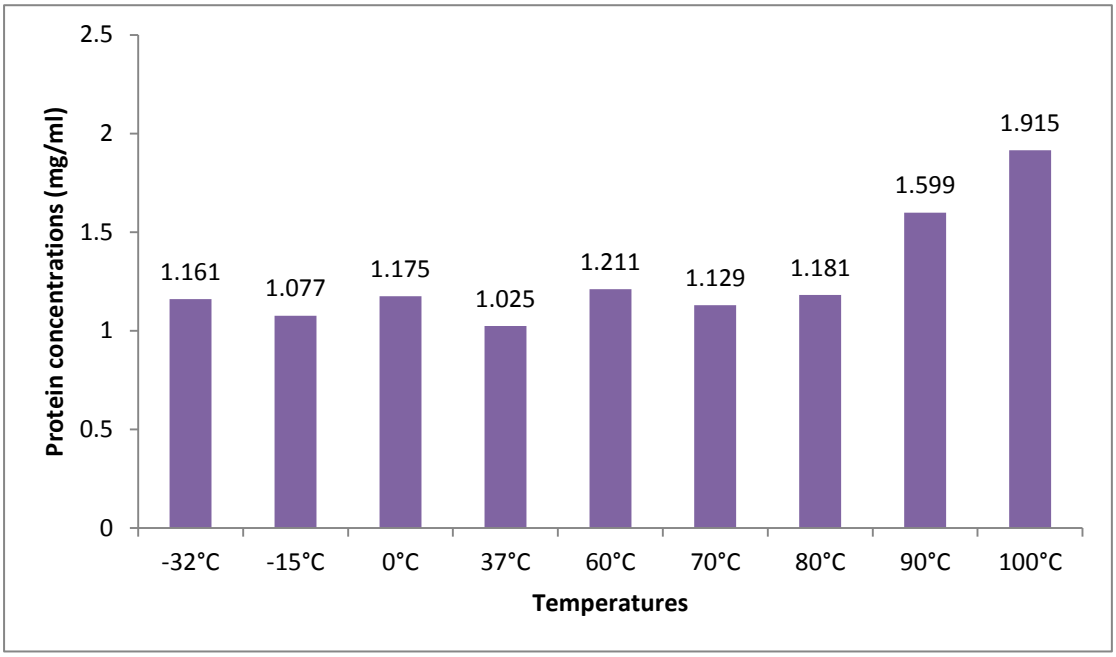


Figure (4B): Estimation of protein concentration of *E. coli* which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.

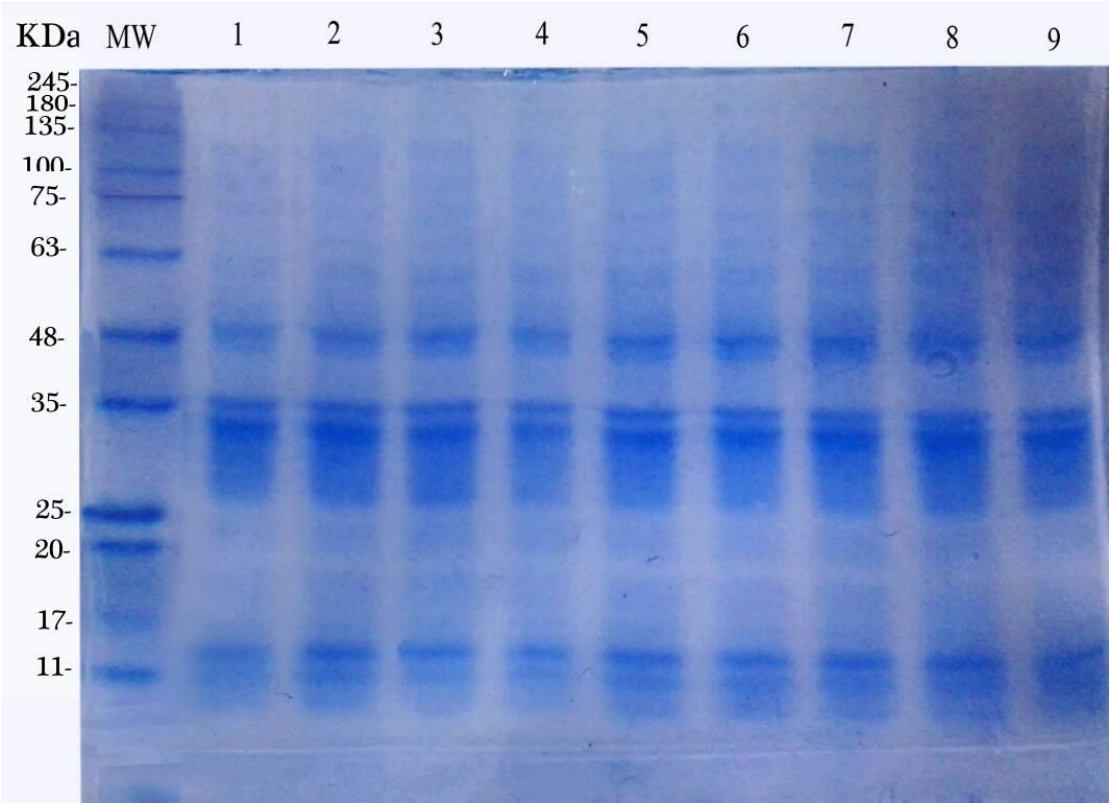


Figure (5A): Electrophoretic patterns of protein of *S. sonnei* isolate, which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

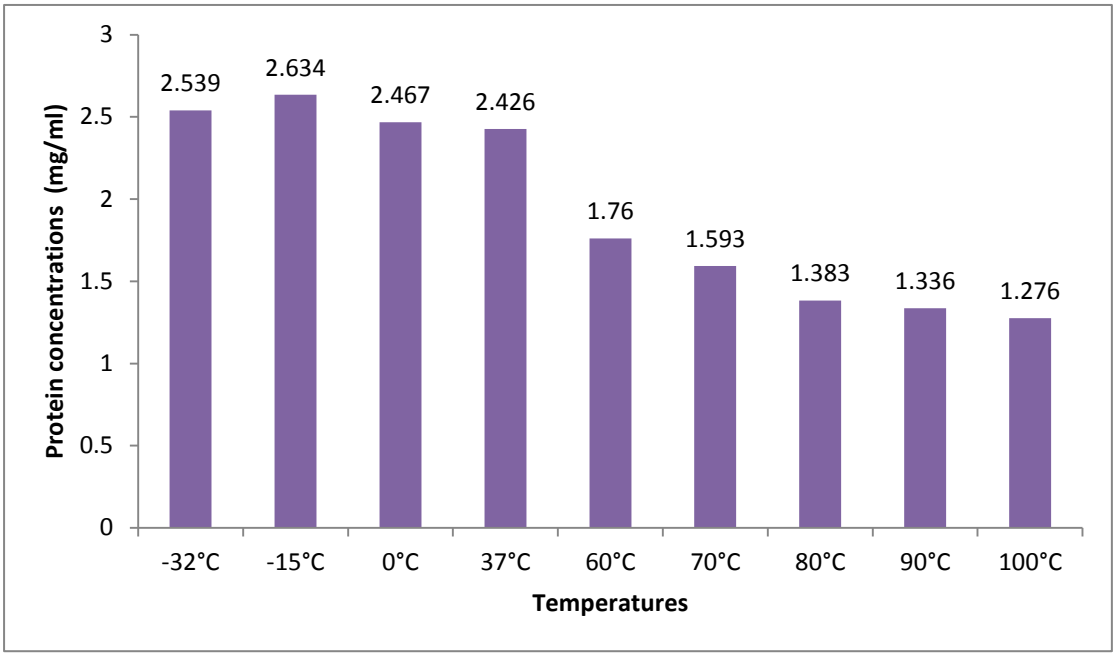


Figure (5B): Estimation of protein concentration of *S. sonnei*, which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.

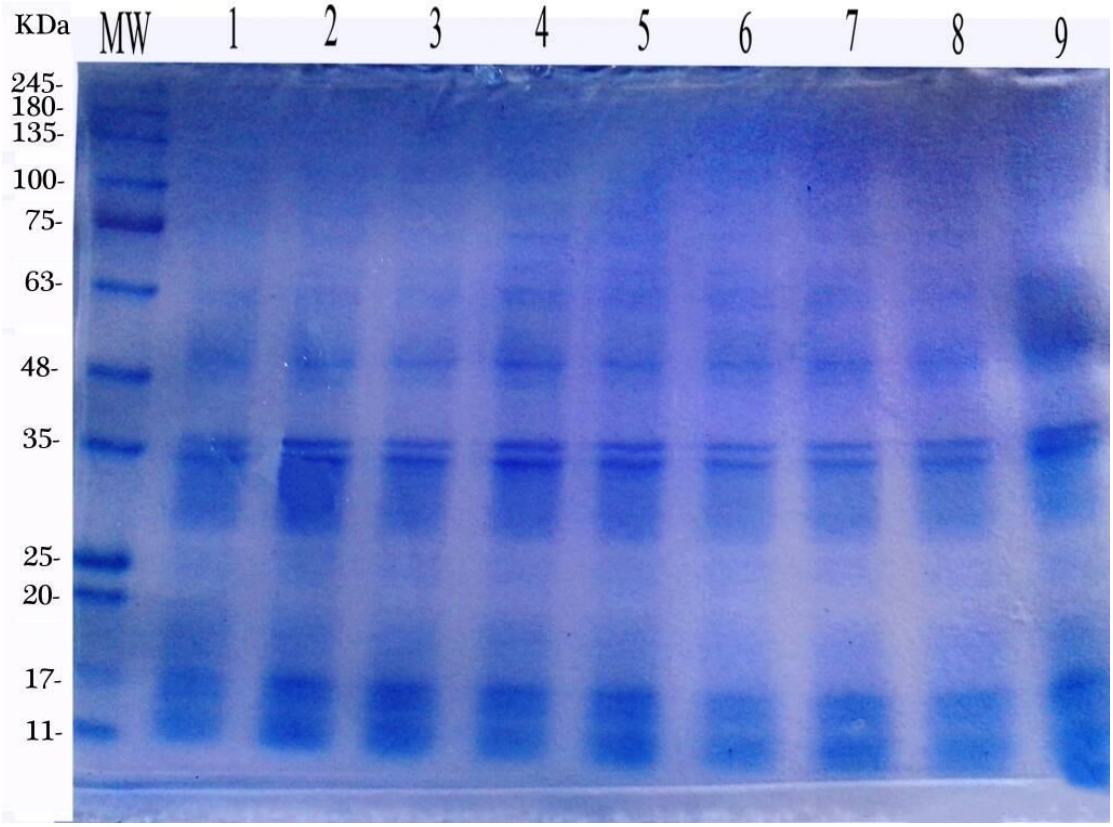


Figure (6A): Electrophoretic patterns of protein of *K. pneumoniae* isolate, which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

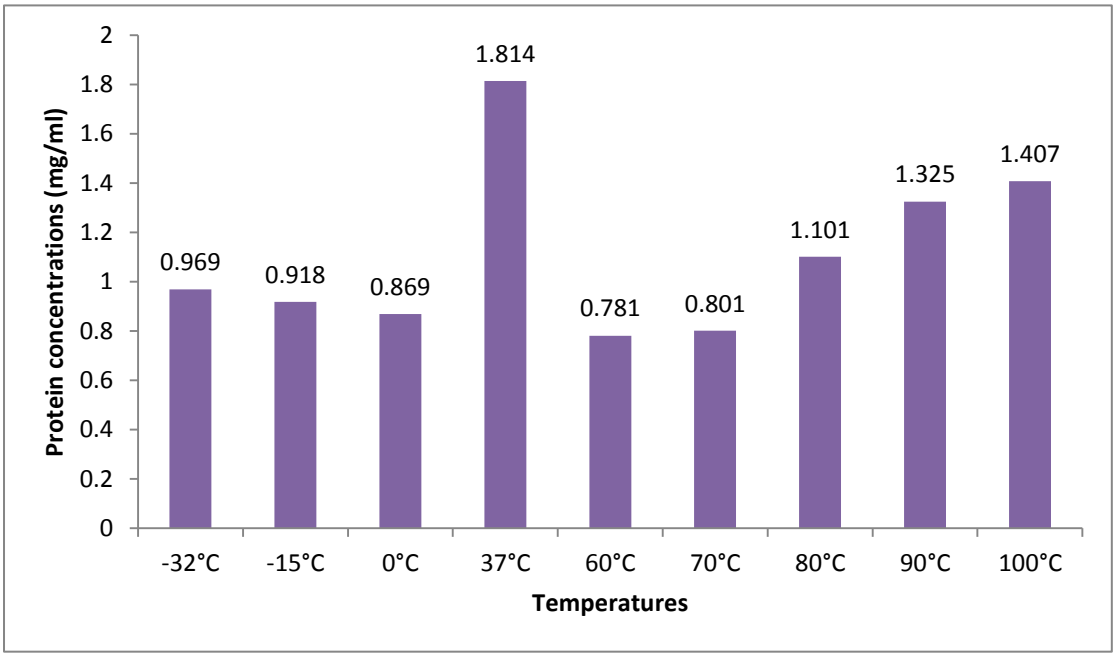


Figure (6B): Estimation of protein concentration of *K. pneumoniae*, which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.

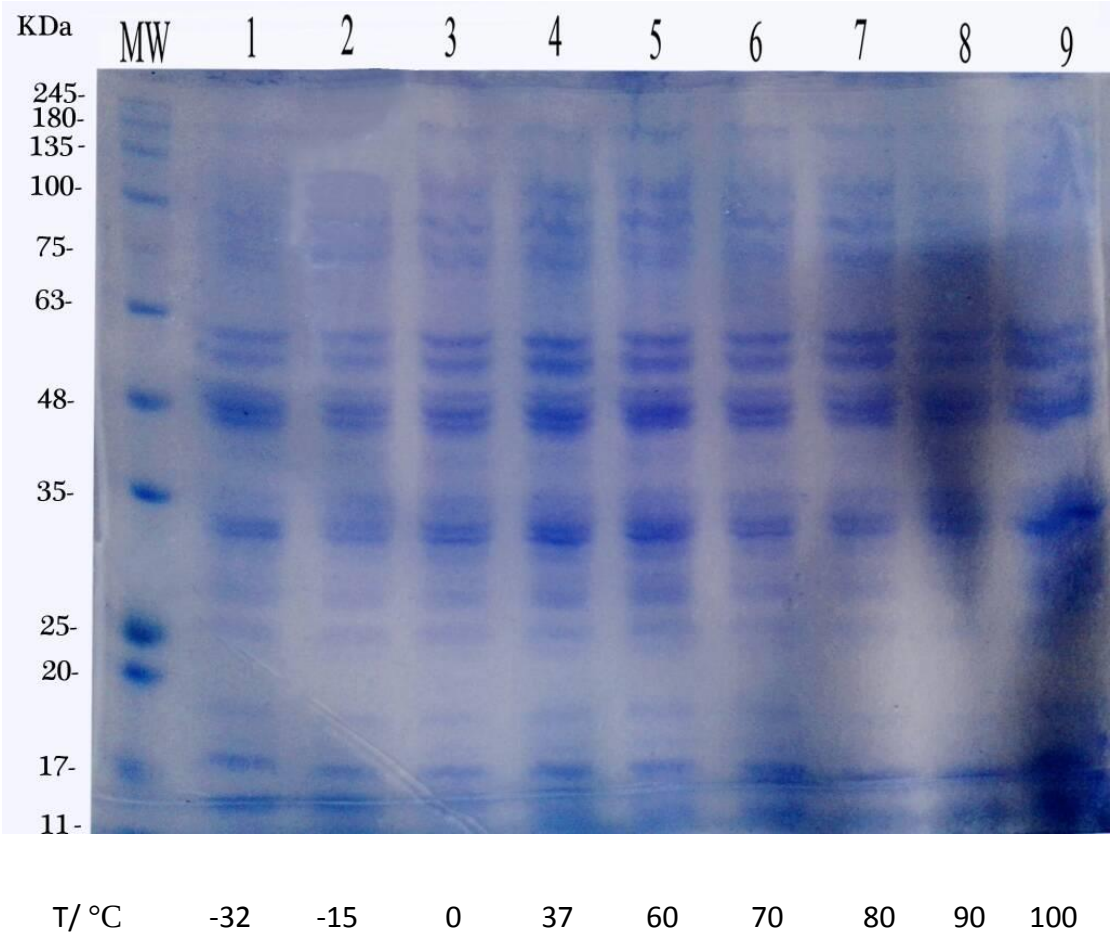


Figure (7A): Electrophoretic patterns of protein of *Ps. aeruginosa* isolate, which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

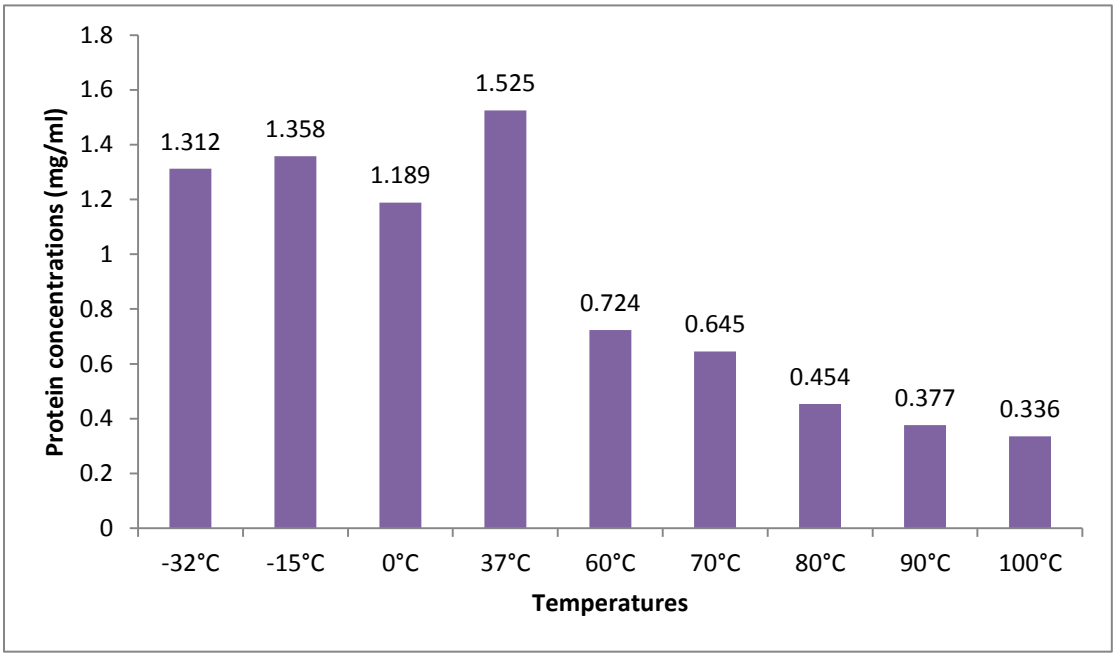
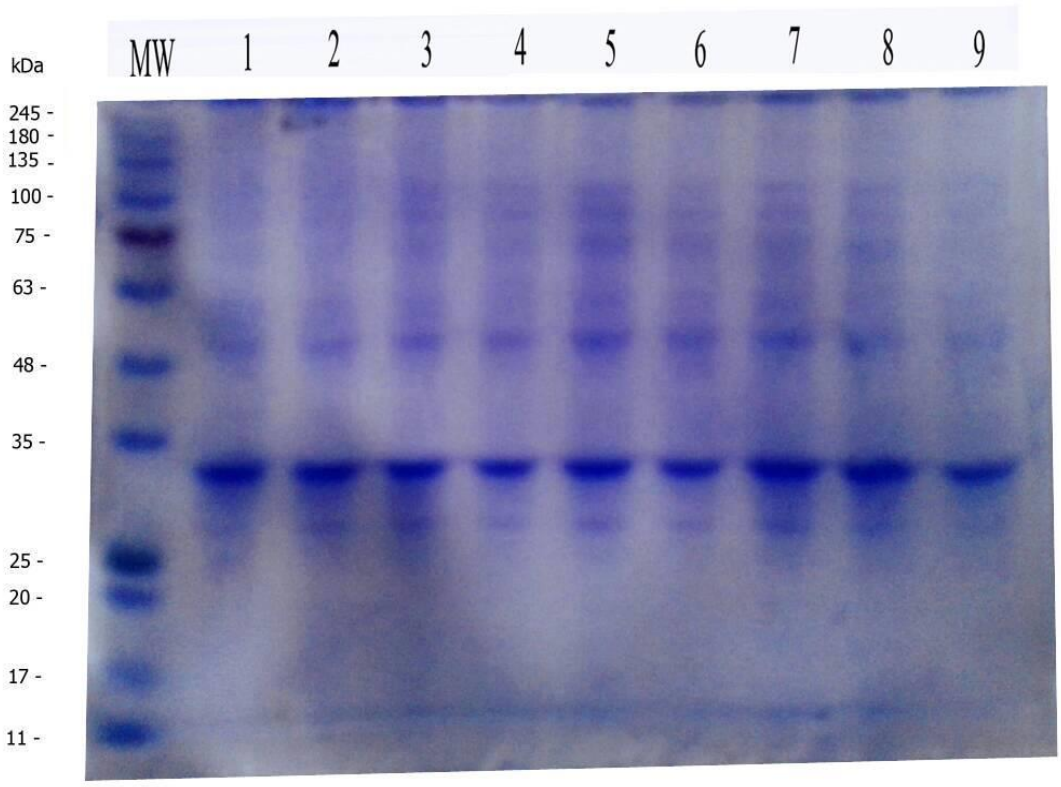


Figure (7B): Estimation of protein concentration of *Ps. aeruginosa*, which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.



T/ °C	-32	-15	0	37	60	70	80	90	100

Figure (8A): Electrophoretic patterns of protein of *P. multocida* isolate, which was stored at different temperatures showing similar results. Molecular mass markers are indicated. Equal quantities of protein were loaded.

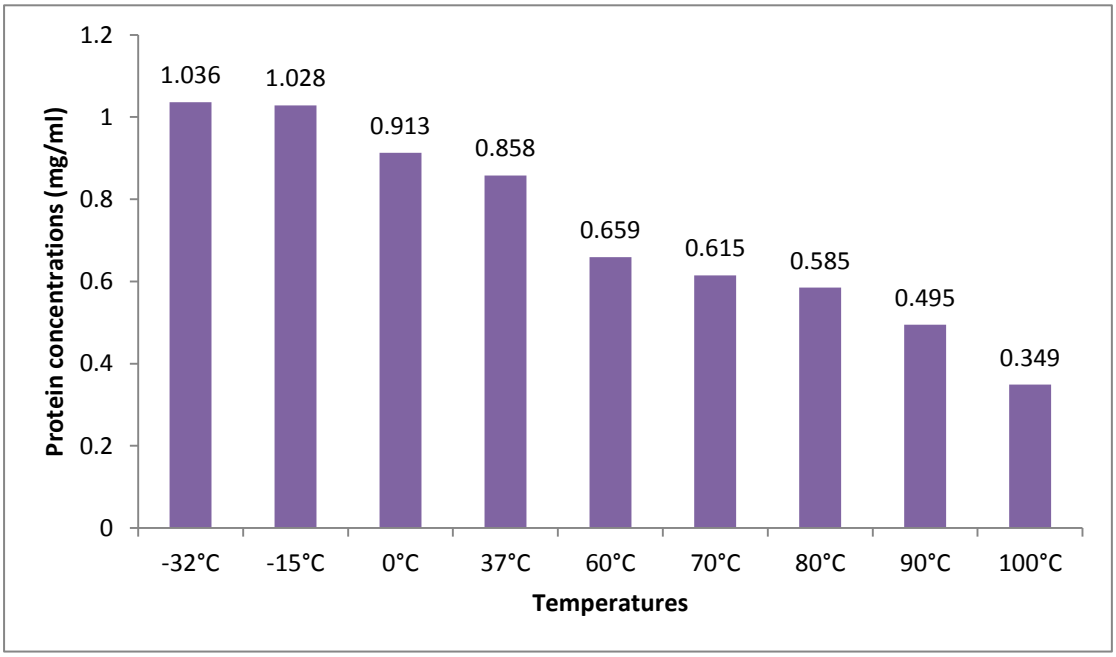
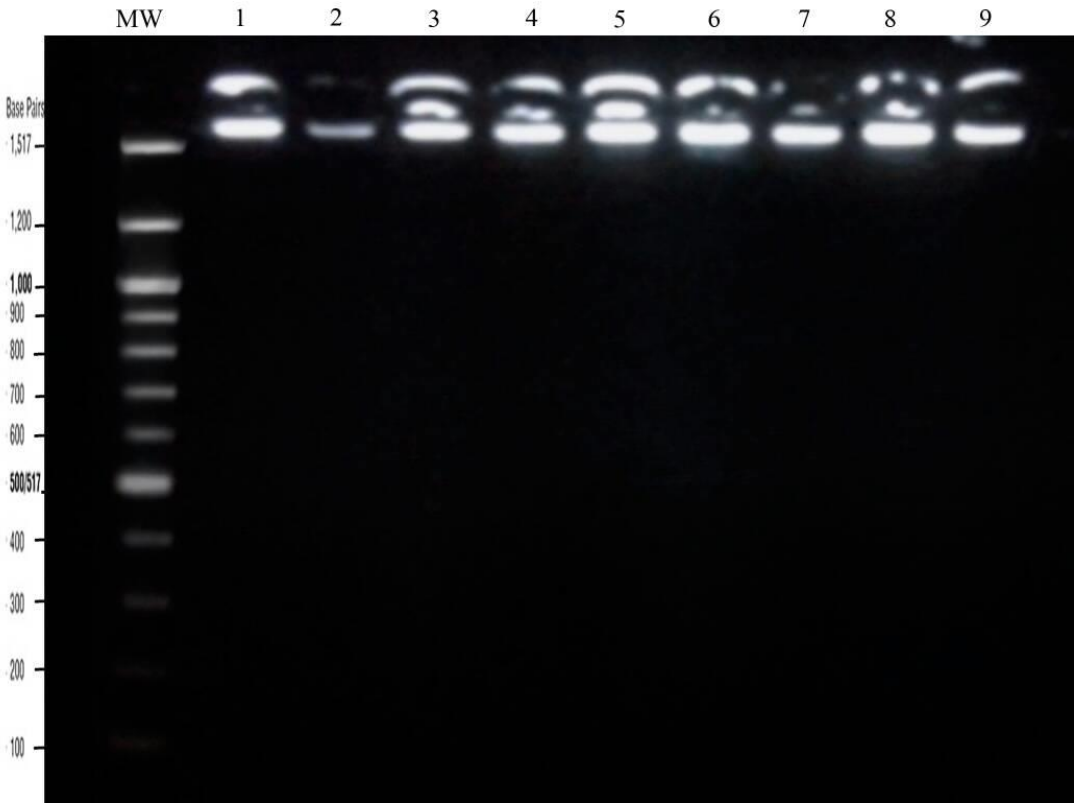


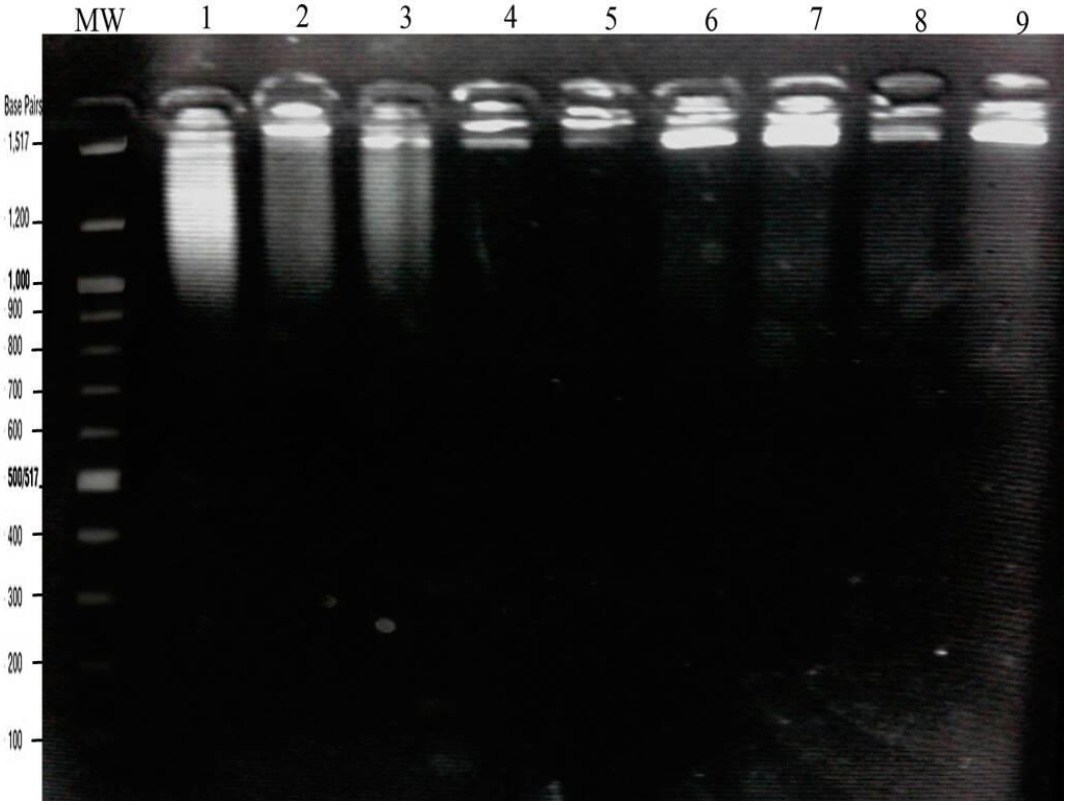
Figure (8B): Estimation of protein concentration of *P. multocida*, which was stored at differernt temperatures (-32°C- 100°C) using standard graph of BSA.



T/ °C	-32	-15	0	37	60	70	80	90	100

Pulsed field gel electrophoresis (PFGE)

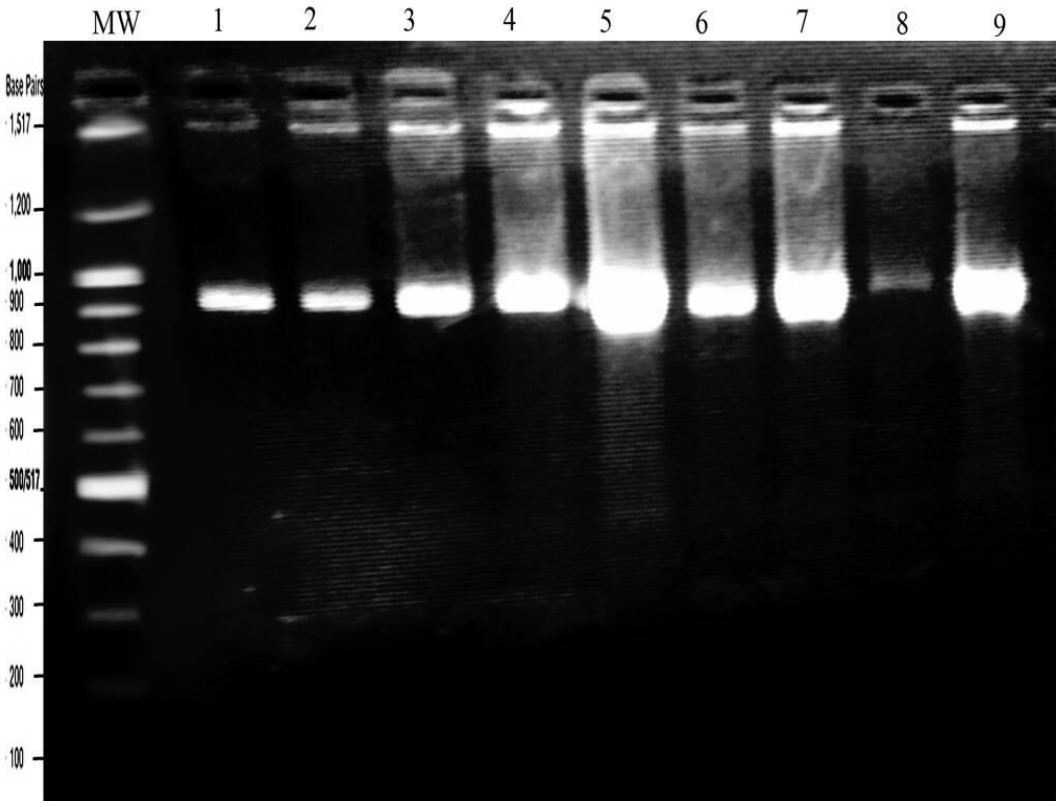
Figure (9): Electrophoretic patterns of DNA of *S. aureus* isolate, which was stored at different temperatures showing similar results.



T/ °C	-32	-15	0	37	60	70	80	90	100

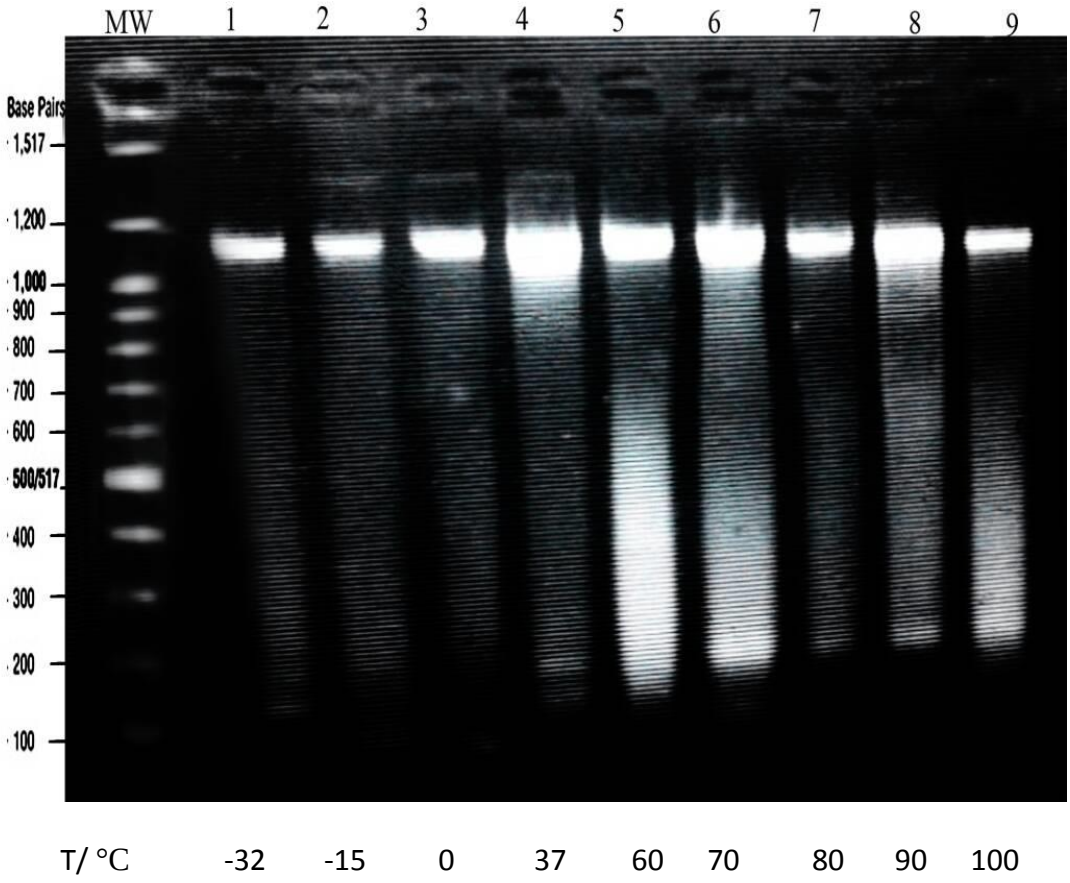
Pulsed field gel electrophoresis (PFGE)

Figure (10): Electrophoretic patterns of DNA of *S. epidermidis* isolate , which was stored at different temperatures showing similar results.



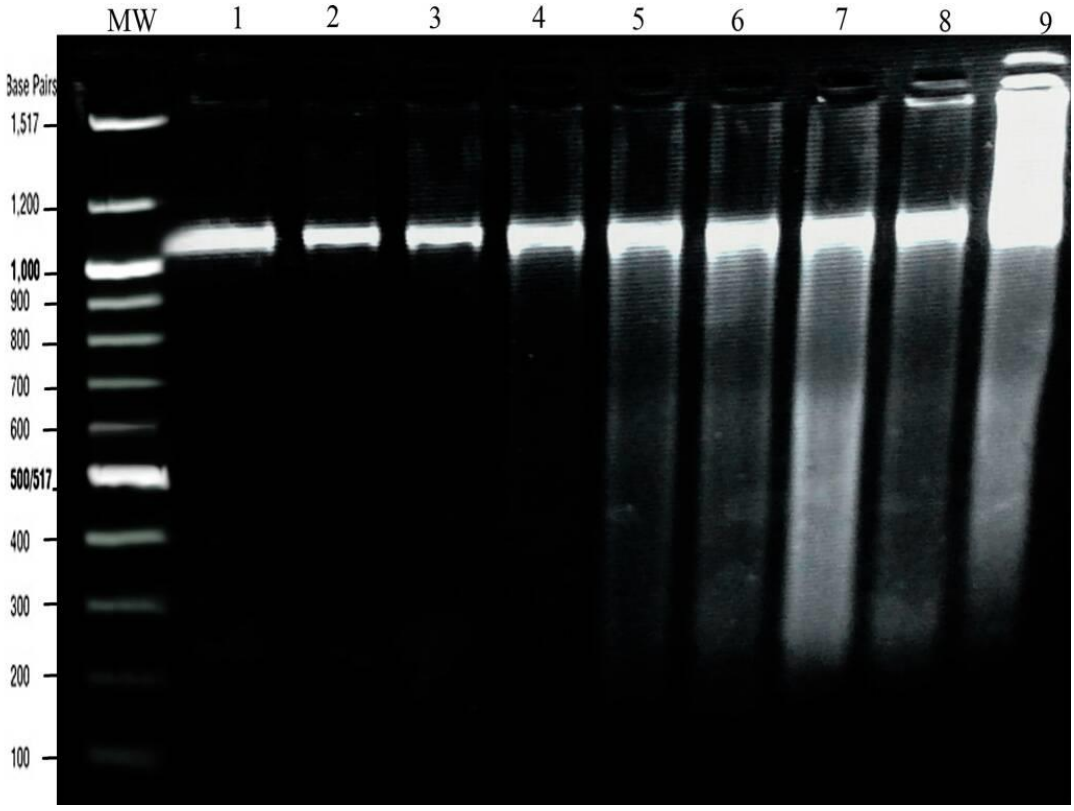
Pulsed field gel electrophoresis (PFGE)

Figure (11): Electrophoretic patterns for DNA of *B. cerues* isolate, which was stored at different temperatures showing similar results.



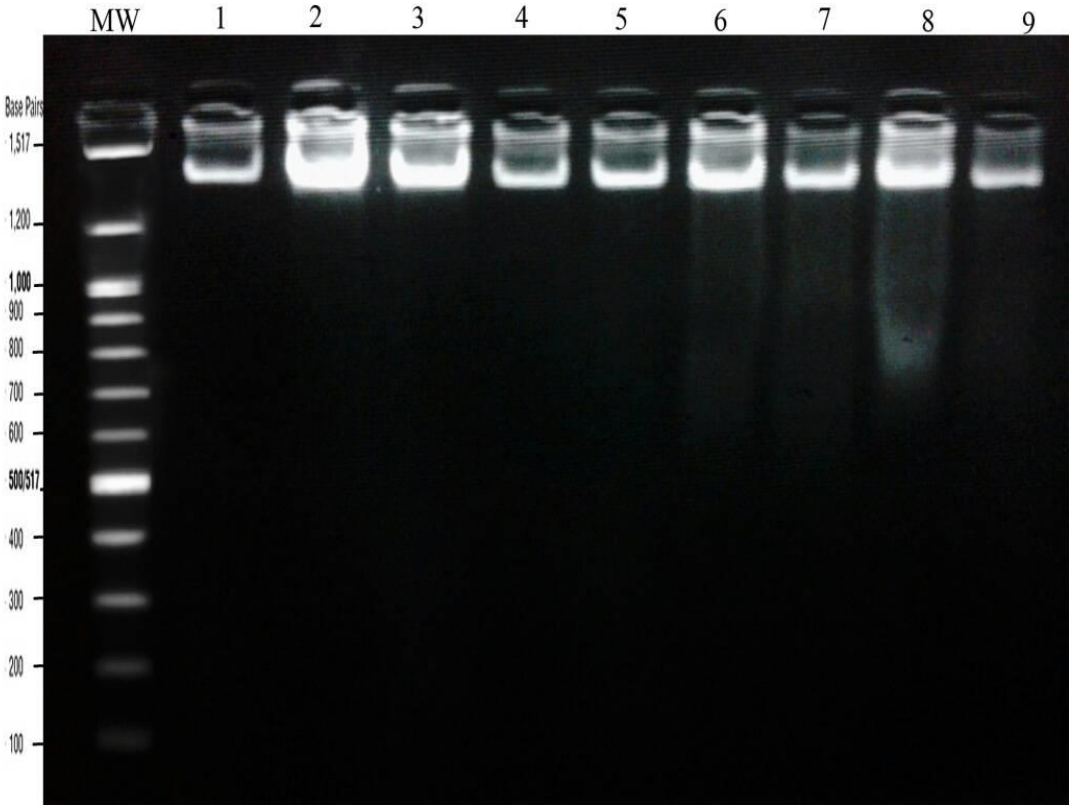
Pulsed field gel electrophoresis (PFGE)

Figure (12): Electrophoretic patterns of DNA of *E. coli* isolate, which was stored at different temperatures showing similar results.



Pulsed field gel electrophoresis (PFGE)

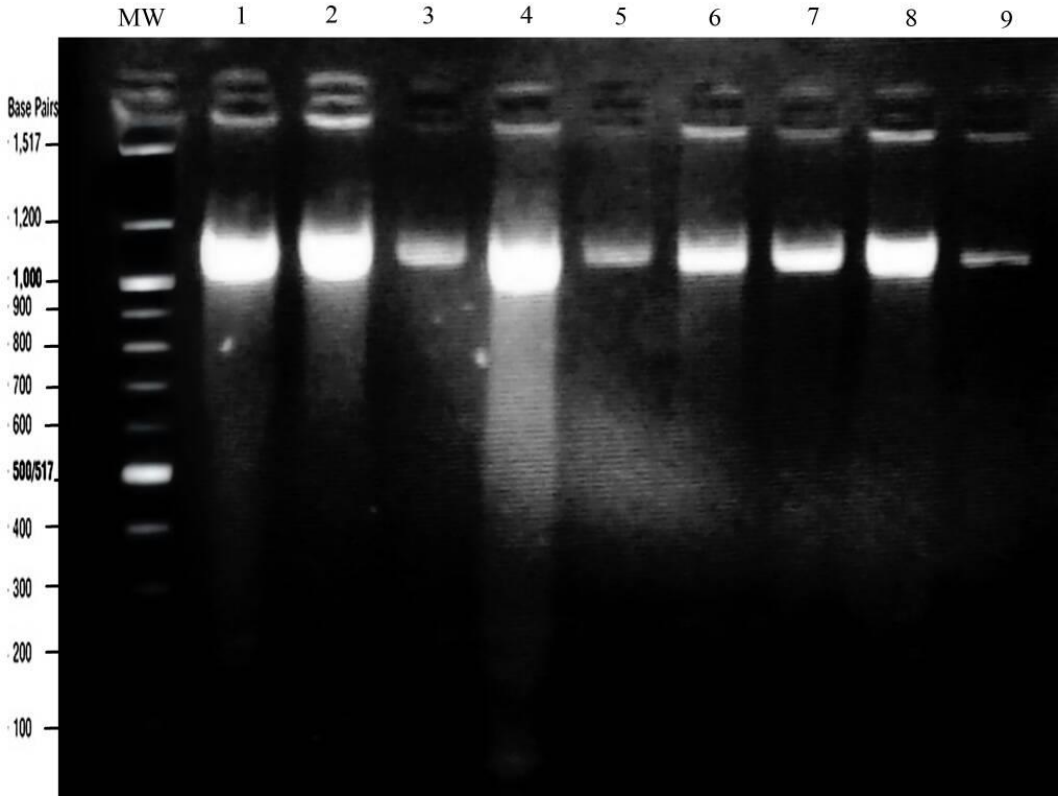
Figure (13): Electrophoretic patterns of DNA of *S. sonnei* isolate, which was stored at different temperatures showing similar results.



T/ °C	-32	-15	0	37	60	70	80	90	100

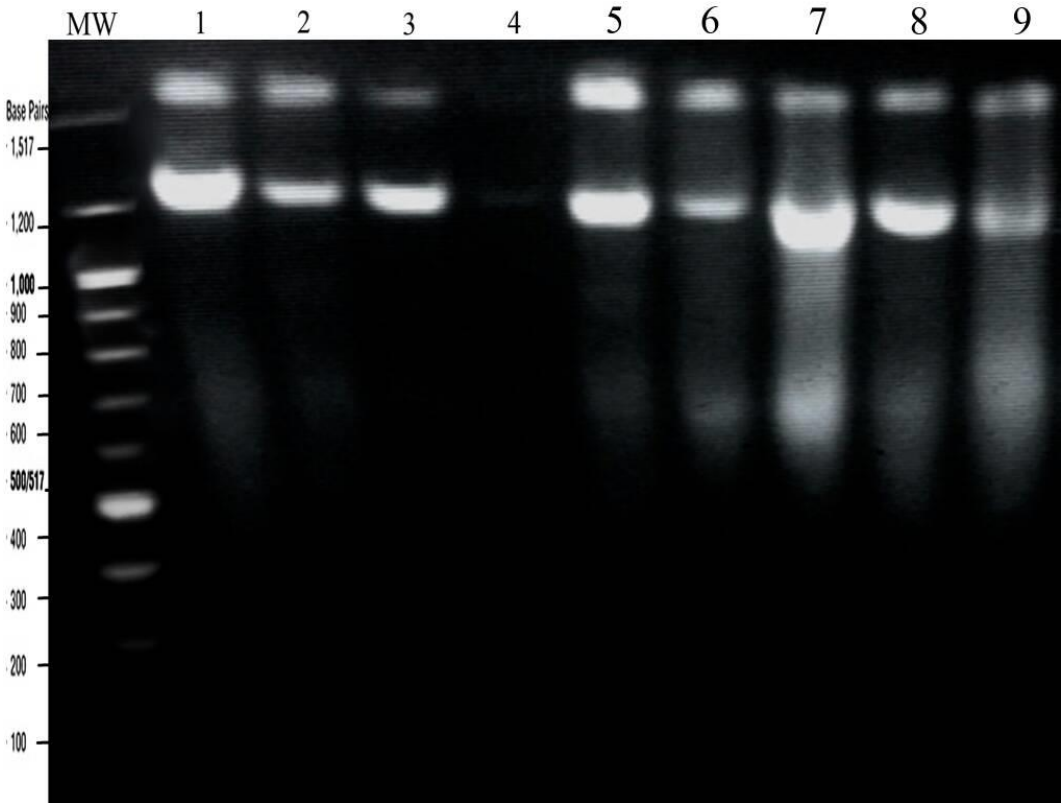
Pulsed field gel electrophoresis (PFGE)

Figure (14): Electrophoretic patterns of DNA of *K. pneumoniae* isolate, which was stored at different temperatures showing similar results.



Pulsed field gel electrophoresis (PFGE)

Figure (15): Electrophoretic patterns of DNA of *Ps. aeruginosa* isolate, which was stored at different temperatures showing similar results.



Pulsed field gel electrophoresis (PFGE)

Figure (16): Electrophoretic patterns of DNA of *P. multocida* isolate, which was stored at different temperatures showing similar results.

CHAPTER FIVE

DISCUSSION

It is evident from the results obtained in this study, that *S. aureus* treated at 0°C, -15°C, -32°C and 60°C showed little differences when subjected to various biochemical tests and sugars fermentation. However, and apart from cells subjected to VP, coagulase, lactose and to some extend maltose, they showed a sharp drop of count at 0 °C followed by -15°C. Also it is interesting to note that cells treated at 60°C failed to give a degree of reaction more than strong indicated by four positives. Contrary to this, *S. epidermidis* cells were not affected dramatically at 0°C with a slight drop at -32°C. On the other hand, *B. cereus* showed a remarkable drop in reaction when cells were treated at 37°C and 0°C, while cells treated at -15°C and -32°C behaved normally compared with the control; though cells treated at -32°C showed an increase in reaction towards various biochemical tests.

As far as *E. coli* results obtained, we observed discrepancy in the results. For example, cells treated at -32°C showed strong reaction towards all tests applied apart from those obtained by dulcitol, glycerol and mannitol tests. Cells treated at 0°C apart from those obtained in dulcitol, glycerol, lactose, maltose and mannitol that showed a drop in the reaction.

Shigella sonnei showed a severe drop in reaction when cells were subjected to glycerol and mannitol. Cells treated at -15°C showed

strong reactions towards mannitol, rhamnonse, trehalose and MR tests.

Klebsiella pneumoniae showed strong reactions in all tests applied apart from those treated at 0°C. However, testing glycerol and xylose were severely affected.

Like all organisms tested against biochemical and sugar fermentation tests, *Ps. saeruginosa* showed a remarkable drop in reaction at 0°C followed by -15°C.

Pasteurella multocida cells did not react properly when cells treated at all temperatures. The oxidase test scored a doubtful result throughout all tests applied indicating the severe damaging of the oxidase enzyme. This was followed by the reactions towards the nitrate test.

The biochemical tests applied for the bacteria treated at different temperatures were closely similar to those obtained by Sahar (2016).

These results are similar to Jones *et al*. (1992a), who reported that the cold shock response and the heat shock response may have an inverse relationship on cells. Also our results substantiate the work of Fotadar *et al*. (2005) in that the optimum growth of *E. coli* occurred at 37 °C, but some laboratory strains can multiply at temperatures of up to 49 °C.

These results go in line with Roberts *et al*. (1996) and McKillip (2000) in that cooking temperatures, less than or equal to 100 °C

(212 °F), allow some *B. cereus* spores to survive. This problem is compounded when food is then improperly refrigerated, allowing the endospores to germinate.

Pasteurella multocida is an enigmatic pathogen. It is remarkable both for the number and range of specific disease syndromes with which it is associated, and the wide range of host species affected. The pathogenic mechanisms involved in causing the different syndromes are poorly understood, or completely unknown for the most part. The biochemical and serological properties of some organisms responsible for quite different syndromes appear to be similar (Wilkie *et al*. 2012).

Cold-shocked *P. multocida* was able to resuscitate and cause death of inoculated rabbits. Such results are similar to those obtained by Nagla (2010) using *P. multocida, E. coli, Salmonella* spp and *Staphylococcus aureus* bacteria in that cold-shocked *P. multocida* could retain its pathogenicity for rabbits. Effective monitoring of microbial pathogen is essential for a successful preventive food safety and hygiene strategy. However, as most monitoring strategies are growth- based, these tests fail to detect pathogenic bacteria that have entered the viable but non-culturable (VBNC) state (Christian *et al*. 2018).

Cold-shocked *Pasteurella multocida* seemed to regain all pathogenic factors when they were resuscitated in the inoculated rabbits.

From chromatogram of *Stasphylococcus aureus*, it is evident that apparent change is noticeable upon raising of temperatures from -32°C to 100°C. Though the difference appeard closely associated with the pattern of protein expression rather than presence or absence of the protein. The protein band appeared at molecular weight 70 kDa seems to be up regulated; whereas the one above 25 kDa and below 35 kDa were down regulated as the temperature rised up. The protein bands were focused between 75 kDa to 11 kDa.

Sheridan and McDowell (1998) mentioned that chilling did not enhance the resistance of *S. aureus* to subsequent thermal inactivation. Pathogenic bacteria can develop systems that assist them to survive and adapt to environmental stresses such as heat and cold in a variety of ways. These responses entail the production of protective proteins, some of which offer protection to more than one type of stress.

In case of *Staphylococcus epidermidis*, and unlike *Staphylococcus aureus*, there is no apparent electrophoretic differernce in the protein expression from low to high temperature. On the other hand, on protein estimation, there was an increase of an increament in protein by more than 140% as the tempreture rised from -32°C to 100°C. Protein bands were focused between 75 kDa to 11 kDa.

Cordwell *et al*. (2002) reported that *Staphylococcus aureus* is known to produce these cold shock proteins, although the ability to produce

and intensification of production may vary from strain to strain. Thus pasteurization at a time-temperatures combination of anything less than 70°C for 20 min might result in the survival of *S. aureus* allowing for subsequent growth and toxin production if the product was not chilled after cooking. Reheating would have little effect on the heat stable (Fung *et al.* 1973).

Though there was apparent variation in the protein composition of *Bacillus cereus* with gradual increase of protein concentrations, there were no clear electrophoretic bands discrepancies from -32°C to 100°C. McKillip (2000) was on the opinion that cooking temperatures, less than or equal to 100 °C, allow some *B. cereus* spores to survive. This problem is compounded when food is then improperly refrigerated, allowing the endospores to germinate. It is recommended by Roberts *et al*. (1996) that cooked foods not meant for either immediate consumption or rapid cooling and refrigeration should be kept at temperatures below 10°C or above 50°C. Germination and growth generally occur between 10°C and 50°C, though some strains are psychrotrophic thus supporting the work of McKillip (2000).

The electrophoretic patterns of *Escherichia coli* did not show a very clear difference of the bacterial protein of the isolates at different temperatures. These results were consistent with the protein estimation as protein content remained almost unchanged till 80°C, after which sudden increase was noticed till 100°C. Weber and

Marahiel (2003) stated that the cold shock response leads to a growth block and overall repression of translation; however, there is the induction of a set of specific proteins that help to tune cell metabolism and readjust it to the new conditions. For a mesophile, like *E. coli*, the adaptation process takes about 4 hours.

For *Shigella sonnei*, interesting observations were noticed from 37°C till 80°C there were almost no very clear bands detected above 63 kDa. However, above 80°C several bands were noticed above 63 kDa. Upon protein quantification of the isolate in different temperatures, it was interesting to record an apparent drop in the protein content (mg/ml) as the temperature raised above 37°C. In overall results, more than 100% decrease in mg/ml of isolated protein was demonstrated as temperature raised from 37°C to 100°C. These results are similar to Enache *et al*. (2006) who reported that heat treatment is one of the most widely used methods to control the rates of bacterial growth and death and is considered to be one of the most effective food processing technologies for eradicating food borne pathogens.

The SDS- PAGE of *Pasteurella multocida* revealed that almost all protein bands were centered between 100 kDa to around 25 kDa, with almost no or little protein bands below 25 kDa. Estimation of protein content of isolates by biuret showed consistent gradual protein degradation pattern from around 1.0 (mg/ml) to as low as 0.3 mg/ml. This result agrees with Jones *et al*. (1992b) in that the cold

shock response and the heat shock response may have an inverse relationship. After cold shock, the cold shock protein synthesis increases, but heat shock protein synthesis decreases.

Schindler *et al*. (1999) reported that in some bacteria, a group of proteins which can be induced at low temperatures were chemically identified and called (cold-induced proteins) CIPs or (cold shock proteins) CSPs.

As stated by Shekhar (2011) low temperature can influence the response of a microorganism either directly or indirectly. Direct effects include decreased growth rate, enzyme activities, alteration of cell composition and differential nutritional requirements. Indirect effects are usually observed on the solubility of solute molecules, diffusion of nutrients, osmotic effects on membranes and cell density.

It is suggested from these results that non-culturable bacterial cells due to cold-shock could be pathogenic. They will resuscitate when inoculated in rabbits and cause their death. Also such observation about the pathogenicity of LBCB could be a serious problem in cold foods which are cooled or frozen like meat and dairy products as it could cause serious hazard for consumers. Also it worth mentioning that the zero temperature can create damage to the stored bacteria more serious than -15°C and -32°C. Thus such temperature i:e 0°C must be avoided when storing bacteria. While this is so, more

studies are needed, using more types of bacteria, to study their behavior towards heating and cooling

CONCLUSION AND RECOMMENDATIONS

It is concluded from this study that Gram- negative bacteria are more sensitive than Gram-positive bacteria to heat. This appears clearly in *S. aureus* and *B. cereus* which were found to be resistant to 70°C and 80°C.

All bacteria in this study showed only mild changes in their biochemical behaviors when exposed to cold and heat-shock.

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE) showed similar results in protein profiles both in cold and heat shocked bacteria, but the variations of estimations of protein concentration are obvious.

Low and high temperatures have no effect on DNA, this will recommend that food should be heated to suitable temperature to ensure death of bacteria.

LBNCB could be a serious problem for estimation of bacterial load. Under estimation of bacterial load could lead to passing food as suitable for human consumption while it is not.

Proper media should be used to help counting living but not culturable bacteria.

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