CLONING AND EXPRESSION OF DNAK GENE ISOLATED FROM LOCAL ISOLATE OF E. COLI RU-3 IN BL21 CELL

Rihab H. Zghair[1] **A.H. Ad'hiah[2]** **G.M. Aziz[2]**
Assist. Lec. **Prof.** **Prof.**
[1]Ministry of Education-Iraq **[2]College of science., University of Baghdad-Iraq**
Email: Rehab.zghair1106@sc.uobaghdad.edu.iq

ABSTRACT

A set of 60 samples were collected from different sources of multiple areas of Hyderabad (India) in order to select a local *Escherichia coli* isolates that have the ability to produce HSP70. 26 isolates were characterized after studied the morphological characteristics and microscopic properties of the isolates on macConkey agar and EMB recorded after 24hr. and grams staining was done. The isolated bacteria were screened for various biochemical tests to get 11 isolates that submitted for molecular identification, and the amplification of 16S rRNA regions using universal primer (16S rDNA) verified only 5 *E. coli* isolates which were sequenced and NCBI accession numbers were acquired. Five distinct isolates (RU-1, RU-3, RW-1, RS-4, and RF-3) of the gene dnaK and its surrounding area were successfully amplified in order to isolate the dnak gene, which encodes the DnaK protein. Amplicons' nearly identical sequences were discovered by restriction fragment length polymorphism (RFLP). The dnak gene from one representative, RU-3 isolate with accession number OL741466 isolated from Urine samples from suspected urinary tract infection (UTI) patients of Sir Ronald Ross Institute of Tropical and Communicable Diseases Nallakunta, Hyderabad, India was successfully cloned and sequenced by overlapping using 3 set of primers. The 1850 bp long dnaK gene encodes a polypeptide of 607 residues of amino acids. Using pBAD TOPO TA expression systems, the dnaK gene of RU-3 was cloned and expressed in *E. coli* BL21 (DE3). Incubating the BL21 with different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) allowed the grown *E. coli* to determine the optimum concentration which was 0.05mM to induce the production of recombinant HSP70 protein within 5 hr. which considered as the best expression time in this study.

Keywords: HSP70; isopropyl-β-D-thiogalactopyranoside; IPTG; recombinant protein.

مجلة العلوم الزراعية العراقية- 2025 :56 (3):1147-1156 زغير وآخرون

استنساخ وتعبير جين الـDnak المعزول من العزلة المحلية لبكتريا العصيات القولونية RU-3 في خلية الـ BL21

رحاب حميد زغير[1] علي حسين أدحية[2] غازي منعم عزيز[2]
مدرس مساعد أستاذ أستاذ
[1] وزارة التربية العراقية [2] كلية العلوم- جامعة بغداد

المستخلص

جمعت 60 عينة من مصادر مختلفة من مناطق مختلفة من حيدراباد- الهند لأجل الحصول على عزلات محلية لبكتريا العصيات القولونية (*E.coli*) منتجة لبروتينات الصدمة الحرارية (HSP70). شخصت 26 عزلة أعتماداً على الصفات المظهرية والمجهرية بعد زراعتها على اطباق الوسطين الماكونكي والـEMB وكونها سالبة ام موجبة لصبغة غرام. وبعد أجراء الفحوصات الكيموحيوية تم الحصول على 11 عزلة تم خضوعها للاختبارات على المستوى الجزيئي والتحري عن جين الـ 16S rRNA الخاص ببكتريا الـ*E.coli* وتم تثبيت خمس عزلات فقط عائده لبكتريا الـ *E. coli* فضلاً عن تحديد التسلسل الجيني لها وتسجيلها على قاعدة المعلومات NCBI. ولأجل التحري عن جين الـ DnaK الذي يشفر لبروتين الصدمة الحرارية (HSP70) تم تضخيم الجين بنجاح للخمس عزلات للبكتريا الـ *E.coli* قيد الدراسة ذات الرموز (RU-1, RU-3, RW-1, RS-4 and RF-3). أن أستعمال تقنية (RFLP) قد كشفت لنا ان جميع القطع المتضخمة الناتجة كانت متماثلة تقريباً في التسلسل الجيني, كما وقد تم كلونة أحد هذه الجينات التي أستخلصت من العزلة RU-3 ذات رقم الانضمام (OL741466) والمعزولة من عينات الأدرار لمرضى التهاب المسالك البولية الراقدين في معهد السير رونالد روز في نالاكونتا- حيدراباد (الهند) بنجاح كما وقد عرف التسلسل الجيني لها بواسطة تقنية الـoverlapping بأستخدام ثلاث ازواج من البواديء لأنتاج جين الـDnaK بطول مقارب لـ1805 زوج قاعدي الذي يشفر لـ607 حامض اميني. أستخدم الناقل الـpBAD ونظام الـ pBAD TOPO AT expression system. للتعبير بنجاح لهذا الجين في بكتريا الـBL21. اختبرت تراكيزمختلفة من IPGT على نمو بكتريا الـBL21 بعد نقل الناقل مع الجين لها وتحفيز انتاج بروتينات الصدمة الحرارية HSP70 وكان التركيزالأمثل للتعبير الجيني 0.05ملي مولار بعد 5 ساعات من حضن البكتريا والذي أعتبر الزمن الأمثل للتعبيرالجيني.

الكلمات مفتاحية: بروتين الصدمة الحرارية70. بروتين مؤتلف. IPGT

Received:3 /1/2022, Accepted:29/3/2022, Published:30 June.

INTRODUCTION

Gram-negative bacteria from the Enterobacteriaceae family include *Escherichia coli*. They are facultative nonsporulating anaerobes. Theodor Escherich, a German-Austrian doctor, first identified these bacteria in the feces of healthy people in 1885 (2, 3, 12). Because it is found in the colon, he gave it the name *Bacterium coli commune*. Based on their structure and movement, early classifications of prokaryotes grouped these organisms into a few genera (at the time, Ernst Haeckel had categorized bacteria under the kingdom Monera). (7, 11, 14). The DNA of isolate M3 **(***Pseudomonas)* was extracted by Auda and Khalifa in 2019, and lipase gene was amplified through PCR technique, then purified and cloned into *E.coli* DH5α cells first using pTG19-T plasmid, and expressed in ***E.coli*** Bl21 with expression vector pet-28a (3) Cells create a family of proteins known as heat shock proteins (HSP) in reaction to being exposed to stressful situations. Although they were initially identified in relation to heat shock, it is now understood that they can also manifest during other stresses, including as exposure to cold, UV light, and when tissues are mending or remodeling. (26). Nearly every living thing, from bacteria to people, has HSPs. Additionally, their names are given based on their molecular weight. In terms of structure and function, HSP70s is one of these HSPs families' most heat-inducible and evolutionarily conserved proteins. (18). DnaK proteins are involved in the construction and complicated protein disassembly, membrane translocation, degrading misfolded proteins, and de novo protein folding. (17). DnaK is made up of a -helical domain, a substrate-binding motif at the COOH terminus, and a highly conserved NH2-terminal ATPase domain. They are thought to aid in the defense and recovery of cells from the negative effects of numerous physiological stressors. (25). Bacteria's dnaK gene produces a protein that is similar to Hsp 70 or DnaK. (24). Gene expression investigations at the mRNA level and deletion mutant studies have clearly demonstrated the significance of dnak in thermoregulation (30, 33). Numerous species, including Escherichia coli, have been observed to increase their thermotolerance in a heterologous system. (17) The objectives of the current study are the detection of DnaK gene of HSP70 in *E. coli* and production of recombinant HSP70 from *E. coli* through the cloning of the gene for HSP70 and express it into a carrier *E. coli* that is safe to handle during culture.

MATERIALS AND METHODS

Materials, bacteria, vectors, and growing circumstances: The media components and chemicals utilized were of analytical grade and came from Hi-Media laboratories in India, Biolife in the United States, and Merck Limited in India. The DNA markers and protein markers obtained from GCC Biotech-India. Ampicillin, kanamycin antibiotic was from Oxoid/ England, restriction enzymes came from Bio-labs/ USA, For the purpose of recombinant protein overexpression mediated by T7 RNA polymerase, Novagen's *E. coli* BL21 from Madison, Wisconsin, USA, was used. The vector pBAD comes along with pBAD TOPO TA expression kit was purchased from Invitrogen-Thermofisher/ USA (2). The local isolates of bacteria were isolated from different sources (Urine, water, decomposed fruits and soil) around Hyderabad Telangana - India. MacConkey agar medium and Eosin methylene blue (EMB) agar, were used for the isolation of *E. coli* and plates were incubated at 37±2˚C for 24hr. for the appearance of bacterial colonies. Morphological characteristics of the isolates were studied on MacConkey agar. Colony colour, colony shape, elevation, colony size, colony margin and appearance were recorded after 24hr. of incubation at 37˚C. In order to differentiate the bacteria whether it belongs to gram positive or gram negative, grams staining was done according to (1)(32). The isolated bacteria were screened for various biochemical tests such as indole, methyl red, starch hydrolysis, catalase, oxidase, Voges Proskauer, citrate utilization, triple sugar iron agar test and lysine decarboxylase as per the standard methods (28)(1)(6). Pure cultures of the isolated bacteria were cultured in nutrient broth for 24 hours until log phase in order to identify them molecularly. (29). The bacteria were streaked on LB media and incubated at 28ºC for 3 days and cells were lysed in 800µl of extraction buffer (400mM Tris, pH 8;

60mM EDTA, pH 8; 150mM NaCl; 1% sodium dodecyl sulfate, w/v) and pelleted down by 50µl of 5M potassium acetate (pH 4.8) then centrifuged at 13000rpm for 15min, Following the manufacturer guidelines, the genomic Dna was isolated using the GSure bacterial genomic DNA isolation kit (GCC Biotech). Amplification of 16S rRNA regions was done using universal primer 16S(rDNA) with sequence forward 27F5'-AGAGTTTGATCATGGCTCAG-3' and reverse 1492R 5'-ACGGATACCTTGTTTACGACTT-3' according to Pandey, *et al*., (2005). PCR amplifications were carried out in a thermocycler programmed for 30 cycles, with the first denaturation step lasting 3 minutes at 94°C, followed by denaturation for 30 seconds, primer annealing for 30 seconds at 55°C, primer extension for 2 minutes at 72°C, and the final extension for 10 minutes at 72°C. By electrophoresis on 1% agarose gel for 1 hour at a constant 80 volts, the PCR results were confirmed (8). All amplified PCR products were purified by QIAquick Gel Extraction Kit.and sequenced at Eurofins Genomics Pvt. Ltd, Bangalore, India. Using the BLAST tool, the sequences received from Eurofins Genomics India Pvt. Ltd. were compared to those from the NCBI and Ez-Taxon, aligned using Clustal X, and phylogenetic trees were generated using MEGA X.(15)(27). By using the neighbor-joining approach, the dendrogram was inferred (13). The isolated bacteria's nucleotide sequences were uploaded to GenBank, where the NCBI GenBank accession numbers were received. The concentration of the eluted genomic DNA was determined by spectrophotometry with Nanodrop2000 (35).

Genomic DNA preparation, PCR amplification, sequencing and analysis of dnaK gene: The full-length of coding region of DnaK gene of Ru-3 strain of *E. coli* (accession number: OL741466) was amplified by PCR using a set of overlapping primers shown in **table (1).** Amplification was carried out in a 25µl reaction volume containing Template DNA (100ng), PCR buffer 10x, dNTPs 2mM, HSP70 primers (100ng each), dNTPs 2mM, and Taq polymerase (2 U/l), PCR products were resolved by electrophoresis at 80V for 1h in 1% agarose gel stained with ethidume bromide.

Table1. Oligonucleotide primers used for PCR amplification to generate full-length DnaK gene

Primer	Sequence	Description
F1	**5'-CACAACCACATGATGACCGA-3'**	**overlapping primers**
R1	**5'-TATGGCAGGTTAACGTCGGT-3'**	**Product size: 895**
F2	**5'-GGTGAACCGGTAACTGAAGC-3'**	**overlapping primers**
R2	**5'-CTTGGTCGGGATAGTGGTGT-3'**	**Product size: 870**
F3	**5'-ACATCAAAGTGACTCGTGCG-3'**	**overlapping primers**
R3	**5'-CTGTGCCAGTTCCTGCATTT-3'**	**Product size: 890**

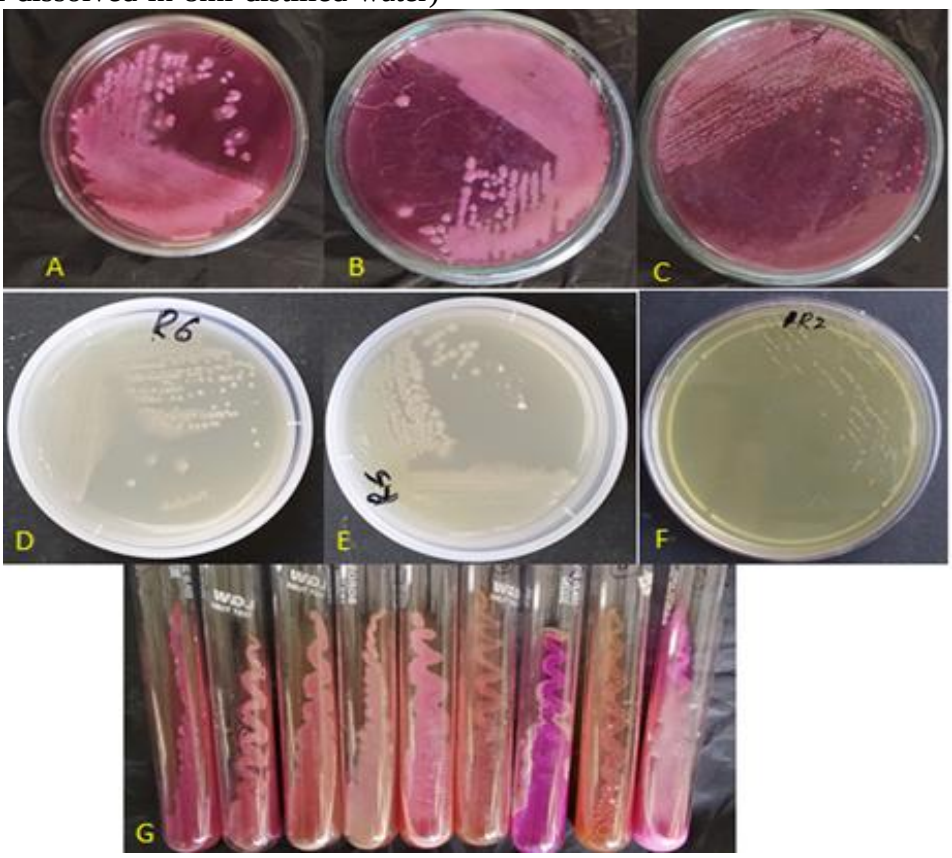
Cloning expression of dnaK of *E. coli* by BL21*:* Using Green and Sambrook's techniques, competent E. coli cells were produced (10). According to Sambrook and Russell, restriction enzyme digests and other common DNA modification techniques were used (29). pBAD-TOPO TA expression kit was used for cloning *DnaK* gene. The pBAD-TOPO vector (4.1kb) was supplied linearized with single 3'-thymidine (T) overhang for TA cloning. The open reading frame of dnaK of *E. coli* was amplified using the primers pBAD Forward 5′-ATGCCATAGCATTTTTATCC-3′ and pBAD Reverse5′-GATTTAATCTGTATCAGG-3′. The *DnaK* gene was then cloned into pBAD-TOPO vector by the TOPO cloning method following the instructions of the manufacturer and the cloned vector used for the transformation and expression in the host BL21(34). BL21 transformed with cloned pBAD plasmid was grown in LB medium with 50mg/ml of Ampicillin, the overnight grown culture was inoculated in a fresh LB broth containing ampicillin and incubated at 37°C with vigorous shaking to an OD_{600} = ~0.5. The cultures were induced with different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG 0.0mM, 0.05mM, 0.1mM, 0.5mM & 1.0mM) for over expression of *DnaK* gene, samples were collected 2hr, the optimum concentration of IPTG for protein induction was determined after purification of all tested concentrations of

IPTG on expressed protein by using rapid screening method using Ni-NTA spin column. The protein concentration in each purified sample was determined by Nanodrop2000 system, after the induction and 1ml of culture was centrifuged at 6000rpm, 4ºC for 5min. Resuspend the pellet in 50µl of 1x SDS loading buffer and heat the sample at 95ºC for 10min. spin the sample at 8000rpm for 5min., collect 10µl the supernatant and load on 10% SDS-PAGE for analysis. To determine the optimum time for recombinant protein expression, 100ml culture of the clone with the maximum expression of protein after determining the optimum IPTG concentration was incubated at 37ºC with vigorous shaking and 10ml sample was taken at hours 1, 2, 3, 4, and 5, after induction with the optimum concentration of IPTG.

SDS-PAGE analysis of expressed protein: SDS PAGE was performed by using Bio-Rad's preparative electrophoresis systems. Cell pellets harvested were suspended in SDS-PAGE loading buffer (5X) (3ml of 1M Tris-HCl pH 6.8, 1g of SDS, 5ml of glycerol, 2ml β-mercaptoethanol and 25mg of bromophenol blue were all dissolved in 8ml distilled water) and fractioned with 10% separating gel and 5% stacking gel. After electrophoresis, the gels were stained with Coomassie brilliant blue (2.5g dissolved in 500ml methanol,100ml glacial acetic acid and then destained in a 150ml methanol and 100ml glacial acetic acid solution) (9).

RESULTS AND DISCUSSIONS

isolation, morphological and biological characterization of the bacteria isolates: For the isolation of *Escherichia coli,* 60 samples from different sources (15 urine, 15 water, 15 spoiled fruits and 15 soil) were collected from various sites of Hyderabad, Telangana, India. after culturing the samples on MacConkey agar and Eosin methylene blue agar medium by streak plate method (19), 11 isolates selected to be *E. coli* due to the ability to produce bright pink colonies on MacConkey agar due to lactose fermentation, and characteristic metallic sheen colonies on the EMB agar (**figure 1)**, most species of bacterial isolate can be differentiated based on simple Gram staining technique which performed as per procedures described by Merchant and Packer (11).



First

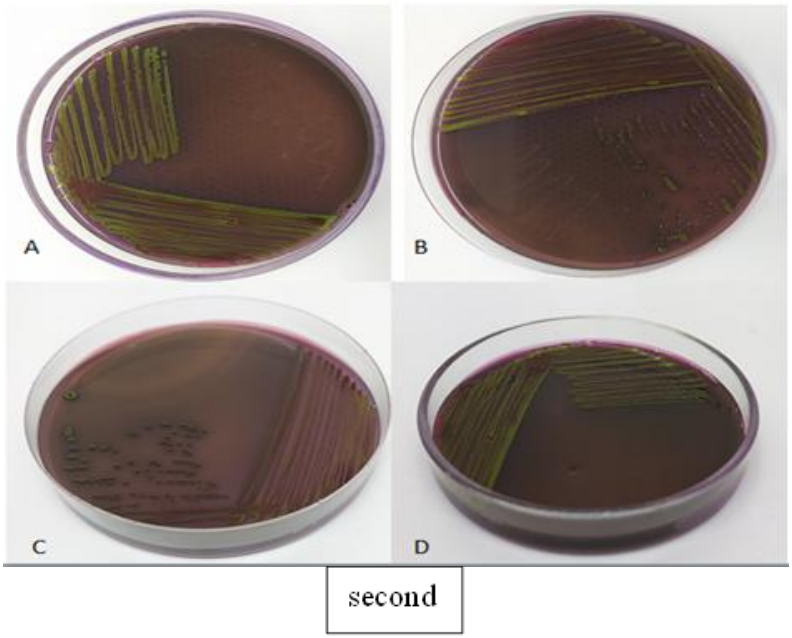


Figure1. first figures (A, B, C) Purified cultures of *Escherichia coli* maintained on MacConkey agar medium; incubation at 37^{O}C for 48hr. (D, E which are RU-1, RU-3 respectively) purified cultures of *Escherichia coli* maintained on M9 media; incubation at 37^{O}C for 72hr. (F) Purified cultures of *Escherichia coli* maintained nutrient agar medium; (G) Isolated bacterial cultures maintained on MacConkey agar slants in cooled refrigerator temperature Second figures (RU-1A, RS-4B, RF-3C, RW-1D) Purified cultures of *Escherichia coli* maintained on Eosin Methylene Blue (EMB) agar medium incubation at 37^{O}C for 48hr

Additionally, researchers looked at the biochemical traits of the various *E. coli* strains they obtained from various sources. They discovered that there was little to no variation in these biochemical traits, and they speculated that this similarity across the isolates may be caused by the presence of certain shared genetic components. The bacterial isolates were identified using biochemical and cultural characteristics as described by (31) which included indole, Methyl red, catalase, TSI agar, lysine decarboxylase, voges proskauer, starch hydrolysis, oxidase, and citrate utilization (Table 2). Out of 11 bacterial isolates, 5 isolates (RU-1, RS-4, RW-1, RF-3 and RU-3) showed the presence of heat shock protein 70 (hsp70). All the five bacterial isolates were identified at molecular level by 16S rRNA gene sequence analysis. Genomic DNA was extracted and quantified by Nanodrop2000, and was used for the amplification of 16S rRNA gene using the universal primers 27F as forward and 1492R as reverse. PCR amplification of the 16S rRNA gene yielded amplicon of approximately 1500-bp (figure 2) and were sent to Macrogen Inc. Seoul, Korea for sequencing. The nucleotide sequences of all the five isolates of *E. coli* were submitted to GenBank and NCBI accession numbers were obtained. (Table 3). Hameed *et al.,* identified and confirmed of *E.coli* isolated from arthritis in chickens and examined the isolate by culturing, VITEK test as well as molecular assay. Antimicrobial susceptibility of bacterial isolate also done. The results of the 16Sr RNA gene revealed that *E.coli* primers of the16S rRNA gene had successfully targeted the respective gene and have shown the single bands of the16S RNA gene at 1500 bp. Sequencing of this gene was performed for the isolate (11).

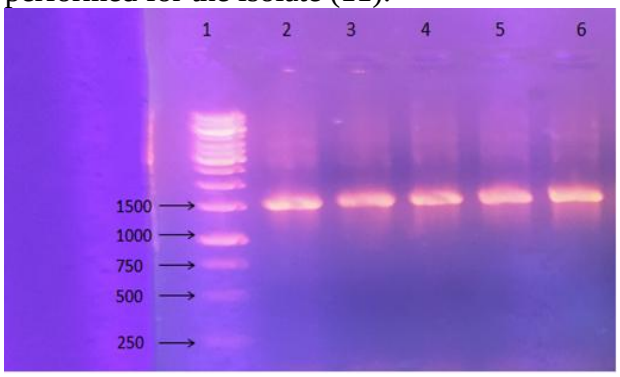


Figure2. Amplification of 16 S rDNA gene of bacterial isolates. Lane 1, 1KB ladder; Lane 2, RU-1; Lane 3, RU-3; Lane 4, RW-1: Lane 5, RS-4 and Lane 6, RF-3

Table 2 Biochemical tests of bacteria isolated from different sources

Isolate	*Indole*	*Methyl red*	*Catalase*	*TSI agar*	*Lysine decarbo-xylase*	*Voges Proskauer*	*Starch hydrolysis*	*Oxidase*	*Citrate utilization*
RU-1	+	+	+	Y/Y	+	-	-	-	-
RU-3	+	+	+	Y/Y	+	-	-	-	-
RS-1	+	+	+	Y/Y	+	-	-	-	-
RS-4	+	+	+	Y/Y	+	-	-	-	-
RF-1	+	+	+	Y/Y	+	-	-	-	-
RF-3	+	+	+	Y/Y	+	-	-	-	-
RF-5	+	+	+	Y/Y	+	-	-	-	-
RW-1	+	+	+	Y/Y	+	-	-	-	-
RW-3	+	+	+	Y/Y	+	-	-	-	-
RW-5	+	+	+	Y/Y	+	-	-	-	-
RW-7	+	+	+	Y/Y	+	-	-	-	-

(+) = positive test (-) = negative test

Table 3. Identification of bacterial isolates of *E. coli* at molecular level

Isolates	*Isolation source*	*Hit strain*	*Accession number*
RU-1	**Urine**	***Escherichia coli***	**OL739361**
RU-3	**Urine**	***Escherichia coli***	**OL741466**
RW-1	**Water**	***Escherichia coli***	**OL721964**
RS-4	**Soil**	***Escherichia coli***	**OL721963**
RF-3	**Spoiled fruits**	***Escherichia coli***	**OL724192**

sequencing, analysis, and cloning of DnaK gene : The genomic DNA that was isolated from *E. coli* RU-3 strain showed high concentration with 485ng/µl, the purified genomic DNA selected from the strain above was later subjected to DNA sequencing by designed primers used for overlapping technique to generate gene product with expected size of ≈ 1850bp which was predicted to encode a putative protein containing ≈ 607 amino acid residues **(figure 3)**. The purified PCR products of *E. coli* was compared with NCBI homologous sequence from GenBank data of *E. coli* (*Escherichia coli* str. K-12 substr. MG1655) with accession number (NC_000913.3) which shows 1850bp region of DnaK from the neucliotide (12163-14079) on the chromosomal DNA.

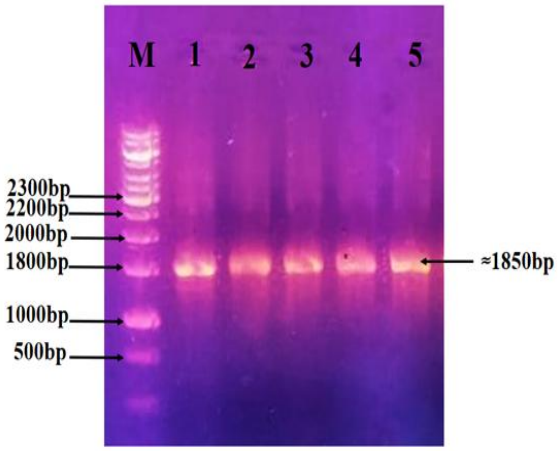


Figure 3. Amplification of HSP70 gene of bacterial isolates of *E. coli* to detect the full gene of *DnaK* on 1% Agarose gel, at 80 Volt for 1hr. Lane M, 100bp ladder; Lane 1, RU-1; Lane 2, RU-3; Lane 3, RW-1; Lane 4, RS-4; and Lane 5, RF-3

Figures (4) shown the partial sequencing using the forward primerF1. With accession number LC738622 and 769bp length

AACAGCGGTTAGATGGGTAAATAATTGGTAT
CGACCTGGGTACTACCAACTCTTGTGTAGCG
ATTATGGATGGCACCACTCCTCGTGTGCTGG
AGAACGCCGAAGGCTTCCCCCACGCCTTCTA
TCATTGCCTATACCCAGGATGGTGAAACTCT
GGTTGGTCAGCCGGCTAAACGTCAGGCAGTG
ACGAACCCGCAAAACCCCCTGTTTGCTATTA
AACGCCTGATTGGCCGCCGCTTCCAGGACGA
AGAAGTACAGCGTGATGTTTCCATCATGCCG
TTCAAAATTATTGCTGCTGATAACGGCGACG
CATGGGTCGAAGTTCAAGGGCCAGATGATGG
CACCGCCGCAGATCTCTGCTGAAGTGCTGAA
AAAAAATGAAGAAAACCGCTGAAGATTACCT
GGGTGAACCGGTAACTGAAGCTGTTATCACC
GTACCGGCATACTTTAACGATGCTCAGCGTC
AGGCAACCAAAGACGCAGGCCGTATCGCTGG
TCTGGAAGTAAAACGTATCATCAACGAACCG
ACCGCAGCTGCGCTGGCTTTACGGTCTGGAC
AAAGGTACTGGCAACCGTACTATCGCGGTTT
ATGACCTGGGTGGTGGTACTTTCGATATTTC
TATTATCGAAATCGACGAAGTTGACGGCGAA
AAAACCCTTCGAAGTTCTGGCAACCAACGTG
ATACCCCACCTGGTTGGTGAGACTTCGACAG
CCGTCTGATCAACTACCTGGTTGAGAGTTCA
GAAAGATCAGGCATTGACCTGCGCA

Figure 4. Partial sequencing of *DnaK* gene isolated from *E. coli* bacteria using the forward primer (F1). Signal G:131 A:291 C:534T:241, Sample: RU-3_F1, Lane: 27, Base spacing: 13.853784, 769 bases in 9729 scans

Expression of RU-3 DnaK

The cloning and expression vector pBAD-TOPO with 4.1kb size single 3'- Thymidine(T) overhangs at both ends that creates a sticky end for the PCR product that was generated with non-proofreading enzyme such as *Taq* DNA polymerase. Fifty µl of transformation mixture cultured on the LB agar plates with 100µg/ml ampicillin produced approximately a hundred of Transformant colonies and there are no colonies on the LB agar plate with 50µg/ml kanamycin **(figure5).**

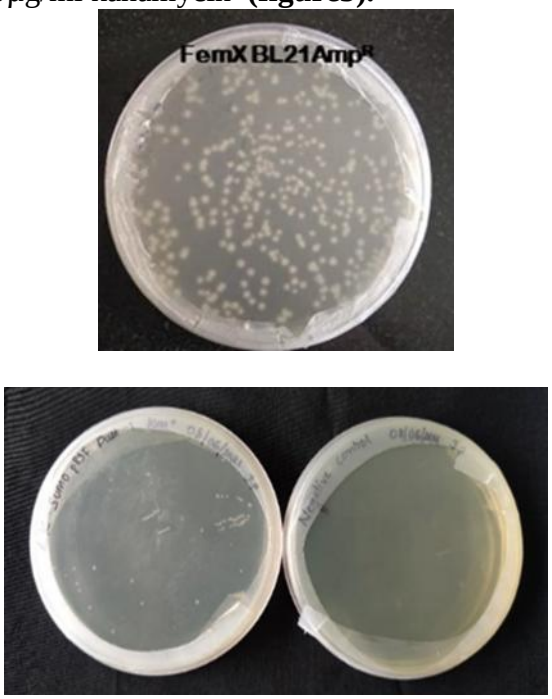


Figure 5. Recombinant colonies by transformation: upper plate 100µg/ml ampicillin; lower plates 50µg/ml kanamycin

All the colonies were subjected to analyzing by colony PCR by using the gene designed set of primers, they all showed a positive result for the presence of the gene into the vector. Positive colonies result for five selected colonies shown in (**figure 6**)

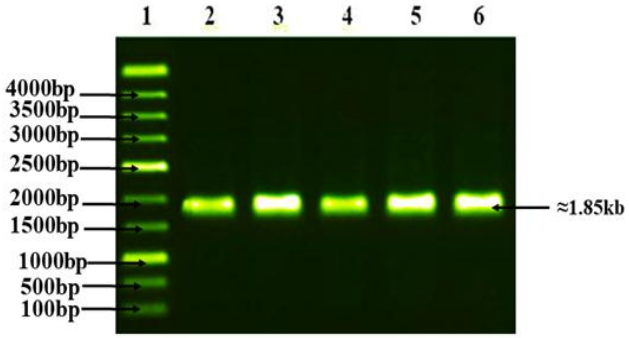


Figure 6. Agarose gel electrophoresis showing the conformation of positive transformant colonies by using colony PCR and screening on 1% agarose gel at 80 volts for 1hr.Lane1: DNA marker, Lanes (2-6) the positive results for the presence of the gene with 1.85kb.

The recombinant plasmids were isolated and purified from transformed clones with Alkaline lysis method first describe by Birnboim and Doly in 1979 (5) by inoculating single colony in LB broth medium under aseptic conditions. By using Thermo Scientific GeneJET Plasmid Miniprep Kit, pure recombinant plasmid was isolated. The plasmids had an approximate concentration of 820-850 ng/µl with high purity. After digest the vectors, they were religated again by using Quick ligation kit and transformed to a new BL21 competent cells. The result of this transformation gave hundreds of colonies on the LB agar plated mixed with 100µg/ml ampicillin, the colony PCR was used to screen the positive transformants, the PCR product produced by using the forward primer of pBAD vector and reverse primer of *DnaK* gene (16) **(figure 7).** all the tested colonies gave positive results with an expected size of approximately ≅ 1.9kb (the *DnaK* gene + region of the vector).

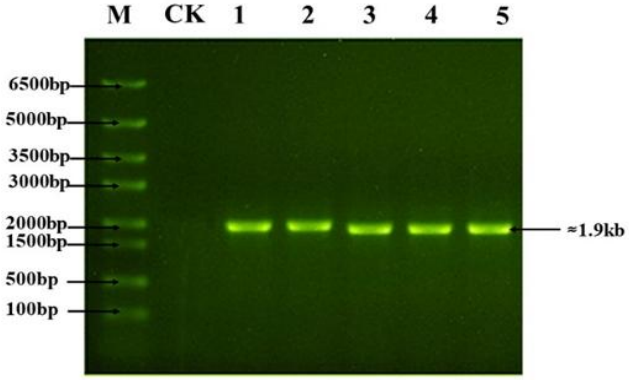


Figure7. Agarose gel electrophoresis of the PCR product from the recombinant clones' after digested with *Nco*1-HF® and relegation, on 1% agarose gel, at 80volts, for 1hr

M: DNA Markers

CK: Negative control

Lane 1-5: PCR product (*DnaK* gene + region of the vector).

Many other studies were made for isolation and cloning of *DnaK* gene from different organisms in order to study the immunogenicity and the protective efficiency properties of the gene product. Bogado and his co-workers (4) discovered that the incomplete hsp70 gene was successfully expressed in *E. coli*, resulting in the production of a 23-kDa

protein under insoluble circumstances. Additionally, the antigen properties predicted by hydrophilicity analysis point to the potential development of an avian coccidiosis vaccine. Miao *et al.,* in 2020 (21) analyzed the effects of various heat treatments on the activities of superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), and Glutathione-S-transferases (GST) and discovered four induced LbHsp70 genes and one induced LbHsp110 gene. Expression of recombinant protein in *E. coli* on the pBAD vector was tested by inoculated the isolated clones that carries the recombinant vector in a fresh LB broth containing ampicillin and incubated at 37°C with vigorous shaking to an OD_{600} = ~0.5. The cultures were induced with different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) in order to induce the production of the recombinant protein, the results showed in 10% SDS-PAGE with an expected molecular weight ≈ 67KDa (22) **(figure 8)** after staining the gel with Coomassie brilliant blue G-250; The results for the IPTG of (0.0mM, 0.05mM, 0.1mM, 0.5mM & 1.0mM) were 48, 18, 8.2, & 4.8µg/ml respectively, keeping the 0.0mM was used as un-induced clone for the negative control, and found that the optimum concentration of IPTG was 0.05mM, which was agreed with many studies before such as (23). Maksum used the IPTG as inducer to protein expression. The concentration should be slightly above the threshold at which the promotor is activated, but it came almost close to this research results. IPTG is a commonly used inducer because of its high expression rate, and it is an allolactose that that *E. coli* cannot metabolize. However, in particular cases, IPTG usage offers drawbacks such as the metabolic burden on the cell due to the exacerbation of haloalkane. The expression of heterologous proteins sometimes becomes an issue when the protein expressed is toxic (20) When they employed various optimal induction settings, temperatures of 28, 30, 34, and 37°C were attained. They discovered that the best inducer concentrations were found to be between 0.05 and 0.1mM IPTG for all temperatures studied.

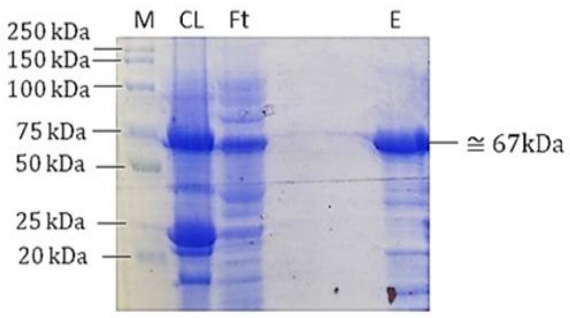


Figure 8. SDS-PAGE (10% w/v) analysis of the optimum IPTG concentration (0.05mM) used to induced the expression of the recombinant *DnaK* purification by using Ni-NTA spin column (affinity chromatography) at 100 volts, for 1hr. M: Protein marker, CL: Crude cell lysate induced with 0.05mM IPTG for 2hrs at 37°C, Ft: Flow-through, E: Elution.

The optimum time for the protein expression was granted by using the 0.05mM IPTG in different periods of time (1, 2, 3, 4, & 5) hr. after purified the protein with Ni-NTA spin column and measuring the protein concentration with NP-2000 and giving the results (38,50, 64, 87, 95) µg/ml respectively. thus, the 5 hr. was indicated as the best expression time in this study, avoiding long time was because of it may lead to protein degradation.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

REFERENCES

1. Ajulo, H.O, M.O. Ajulo, and N.U. Ekereumoh, 2020. Isolation and identification of Salmonella and E. coli from raw goat meat in Uyo Metropolis, Akwaibom state. Asian Food Science Journal, 50-60.

2. Abdullah, T., J. M. Rutz, S. P. Singh, V. I. Kalve, and P. E. Klebba, 1991. Evolution of the ferric enterobactin receptor in gram-negative bacteria. Journal of bacteriology, 173(19), 5964-5974.

https://doi.org/10.1128/jb.173.19.5964-5974.1991

3. Angelom D, T. James Peeler, and E. Gary Rodrick. 1995. Effect of oxytetracycline-medicated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. Applied and environmental microbiology, 61(6), 2335-2340. https://doi.org/10.1128/aem.61.6.2335-2340.1995

4. Bogado P., M. Hostens, P. Sys, P. Vercauteren, and G. Opsomer, 2017. Cytological endometritis at artificial insemination in dairy cows: Prevalence and effect on pregnancy outcome, Journal of Dairy Science, Volume 100, Issue 1, Pages 588-597,

5. Bossi F.N., R. Balbontín, and L. Bossi. 2022. Preparing plasmid DNA from bacteria. Cold Spring Harbor Protocols, 2022(10), pdb-prot107852.

6. De Oliveira A. N, L. A. de Oliveira, J. S. Andrade, and J.A.F. Chagas. 2007. Rhizobia amylase production using various starchy substances as carbon substrates. Braz. J. Microbiol. 38: 208-216

7. Farrar, J., P. Hotez, T. Junghanss, G. Kang, D. Lalloo, and N.J. White. 2013. Manson's Tropical Diseases E-Book. (23rd ed.). Oxford: Elsevier health sciences.

8. Fellahi, S., M.E. Harrak, J.H. Kuhn, et al. 2016. Comparison of SYBR green I real-time RT-PCR with conventional agarose gel-based RT-PCR for the diagnosis of infectious bronchitis virus infection in chickens in Morocco. BMC Res Notes **9**, 231.

9. Gabe, C.M., S.J. Brookes, and J. Kirkham 2017. Preparative SDS PAGE as an Alternative to His-Tag Purification of Recombinant Amelogenin. Frontiers in physiology, 8,424.

10. Green M.R. and J. Sambrook. 2020. ‘’The Inoue Method for Preparation and Transformation of Competent E. coli: “Ultracompetent” Cells Cold Spring Harbor Laboratory Press pp:210.

11. Hameed, S.S., A. H. Ulaiwi, and S.M. Hamad. 2022. Diagnosis of E.coli isolated from arthritis in chickens by VITEK and molecular methods. Iraqi Journal of Agricultural Sciences, 53(1), 141-146. https://doi.org/10.36103/ijas.v53i1.1518

12. Hwang, J., L.M. Mattei, L.G. VanArendonk, P.M. Meneely, and I.N. Okeke. 2010. A pathoadaptive deletion in an enteroaggregative Escherichia coli outbreak strain enhances virulence in a Caenorhabditis elegans model. Infection and immunity, 78(9), 4068–4076.

13. Kapli, P., Z. Yang, and M.J. Telford, 2020. Phylogenetic tree building in the genomic age. Nature Reviews Genetics, 21(7), 428-444.

14. Karim, S. J. I., M. Islam, T. Sikder, R. Rubaya, J. Halder, and J. Alam. 2020. Multidrug-resistant Escherichia coli and Salmonella spp. isolated from pigeons. Veterinary world, 13(10), 2156.

15. Kumar, S., G. L. Stecher, C. Knyaz, and K. Tamura. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and Evolution, 35(6), 1547.

16. Lü, R., D. Yang, Z. Feng, Y. Li, and G. Zhang, 2024. Cloning and functional analysis of heat shock protein Hsp70 from Sclerotinia sclerotiorum. Sheng wu gong cheng xue bao= Chinese journal of Biotechnology, 40(10), 3677-3688.

17. Liang, W., X. Wang, M. Lin, and L. Lin, 2009. A 70-kDa molecular chaperone, DnaK, from the industrial bacterium Bacillus licheniformis: gene cloning, purification and molecular characterization of the recombinant protein. Indian J. Microbiol. 49, 151–160

18. Macošek Jakub, Mas Guillaume, Hiller Sebastian 2021. Redefining Molecular Chaperones as Chaotropes.Frontiers in Molecular Biosciences. vol8

19. Mahadeo Mandal, M., S. Mehdi, and P.K. Mishra. 2022. Evaluation of bacterial profile and antimicrobial susceptibility pattern for urinary tract infection in prefnancy. Int J Acad Med Pharm, 4(4), 235-237.

20. Maksum I. P, F. P. U. Latifah, A. Nabiel, N. F. Lestari, S. Sriwidodo, M. I. Azizah, et al. 2023. An Overview of Culture Conditions for Recombinant Protein Expression in Escherichia coli. J Appl Biotechnol Rep.2023;10(1):864-75. doi:10.30491/JABR.2022.349844.1547

21. Miao, Z. Q., Y. Q. Tu, P. Y. Guo, W. He, T. X. Jing, J. J. Wang, and D. D. Wei, 2020. Antioxidant Enzymes and Heat Shock Protein Genes from Liposcelis bostrychophila Are

Involved in Stress Defense upon Heat Shock. Insects, 11(12), 839.
22. Mital, S., Christie, G. and Dikicioglu, D. 2021. Recombinant expression of insoluble enzymes in Escherichia coli: a systematic review of experimental design and its manufacturing implications. Microb Cell Fact 20, 208.
23. Mühlmann, M., E. Forsten, S. Noack, and J. Büchs. 2017. Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures. Microbial cell factories, 16(1), 1-12.
24. Murugan Kumar, Radha Prasanna, Showkat Lone, Jasdeep Chatrath Padaria, Anil Kumar Saxena 2014. Cloning and expression of dnaK gene from Bacillus pumilus of hot water spring origin. Applied & Translational Genomics, Vol3, Issue 1, Pages 14-20,
25. Ono K, T. Hibino, T. Kohinata, S. Suzuki, Y. Tanaka, T. Nakamura, and T. Takabe. 2001. Overexpression of DnaK from halotolerant C. Aphanothece halophytica enhances the high-temperature tolerance of tobacco during germination and early growth. Plant Sci. 160, 455-461
26. Rao, J. Venkateshwara. 2019. Insilico characterization, phylogenetic analysis and homology based 3d structural modeling of hspa4 heat shock protein family a (Hsp70) Member 4 from Homo Sapiens (Human). International Journal of Engineering and Advanced Technology. 9. 3086-3091.
27. Riggs A. D. 2021. Making, Cloning, and the Expression of Human Insulin Genes in Bacteria: The Path to Humulin. Endocrine reviews, 42(3), 374–380.
28. Saadi Al-Baer, A., and A.A. Hussein. 2017. Isolation and Identification of Escherichia coli Producing Cytosine Deaminase from Iraqi patients. Int. J. Adv. Res. Biol. Sci, 4(11), 1-6.
29. Sambrook J, and D. W. Russell. 2001. Molecular Cloning: a Laboratory Manual.3rd edn. New York: Cold Spring Harbor Laboratory Press
30. Singh, V.K., S. Utaida, L.S. Jackson, R.K. Jayaswal, B.J. Wilkinson, and N.R. Chamberlain. 2007. Role for dnaK locus in tolerance of multiple stresses in Staphylococcus aureus. Microbiology 153, 3162–3173.
31. Tabar, R. H., E. Ghavanloo, and S.A. Fazelzadeh.2016. Nonlocal continuum-based modeling of mechanical characteristics of nanoscopic structures. Physics Reports, 638, 1-97.
32. Tu, Z., L. Tang, M. H. Abo-Raya, M. Sun, H. Shen, and Y. Wang, 2024. Cloning and characterization of heat shock transcription factor 1 and its functional role for Hsp70 production in the sea slug Onchidium reevesii. Gene, 893, 147945.
33. Wetzstein, M., U. Volker, J. Dedio, F. Lobau, U. Zuber, M. Schiesswohl, C. Herget, M. Hecker, and W. Schumann. 1992. Cloning, sequencing, and molecular analysis of the dnaK Locus from Bacillus subtilis. J. Bacteriol. 174, 3300–3310
34. Zhang, J., Hu, Y., Feng, J., He, N., Li, X., Liyi, Q., ... and Yang, W. 2025. Hsp70-mediated manipulation of antimicrobial peptide-related genes promotes BmNPV proliferation in Bombyx mori at relatively high temperatures. International Journal of Biological Macromolecules, 143426.
35. Zhang, J., J. Huang, C. Fang, W. Li, H. Zhao, F. Kong, and Q. Wang, 2022. Molecular cloning of heat shock protein 60 (Sp HSP60) from Schizothorax prenanti and the gene expressions of four Sp HSPs during lipopolysa ccharide (LPS) infection. fishes, 7(3), 139.