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Expression, purification and testing of zinc metalloproteinase aureolysin as potential vaccine candidate against *Staphylococcus aureus*

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***Staphylococcus aureus* (*S. aureus*) is a versatile bacterium which exhibits multiple antibiotic resistances. To ameliorate the undesirable diseases causing potential, there is a need to design a protective vaccine capable of stimulating immune response against this pathogen. In a similar study in our laboratory, reverse vaccinology approach was used to nominate potential vaccine candidate genes against *S. aureus*. Zinc Metalloproteinase Aureolysin (aur) gene was one of the nominated genes based on that previously published in-silico study. The objective of this study is the cloning, expression, purification of aur gene and testing the aur protein reactivity with serum antibodies collected from groups of human patients with confirmed Staphylococcal disease. Cloning was done in pH6HTN His6HaloTag® vector and it was expressed in *E. coli* BL21 (*DE3*) using these conditions; 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. Purification was carried out by Immobilized Metal Affinity Chromatography (IMAC). The his-tag aur protein was detected at ~86 KDa as a single band after western blot assay and was successfully reacted with antibodies obtained from humans infected with *S. aureus*. The results encourage further testing of aur protein as a potential vaccine candidate for *S. aureus*.**

Key words: *Staphylococcus aureus,* Zinc Metalloproteinase Aureolysin (*aur),* cloning, expression.

INTRODUCTION

Staphylococcus aureus has been indicated as a causative microorganism a lot of diseases, including osteomyelitis, septic arthritis and Necrotizing pneumonia. Furthermore, Olaniyi et al. (2017) reported that *S. aureus* is responsible for most of the skin and soft tissue infections. Methicillin-Resistant *S. aureus* (MRSA) has been implicated as the cause of most nosocomial infections and is reported with high prevalence especially in the hospitals with significant mortality and morbidity as reported by Toleman et al., (2019), Marlieke et al.,(2011). In a related development, it was also reported that multidrug resistant *S. aureus* was increasingly detected globally with fewer antibiotics remaining for effective treatment as reported by Harik et al. (2016), Chong et al. (2015) and Bendary et al. (2016).

Currently, there is no effective vaccine against S. *aureus,* despite many trials have been done for example,

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during clinical developmental stage (phase III) StaphVAX (capsule glycol-conjugated vaccine) was stopped at phase III due to its low efficacy compared with placebo in patients with end-stage kidney failure as reported by Fattom et al. *(*2015)**.** A second vaccine trail termination was reported by McNeely et al. (2014) for the Merck V710 vaccine during Phase III (iron-regulated surface determinant B (IsdB)), the termination was due significant increase in mortality rate following post-cardiothoracic surgeries infections. Suggested opinions for why these vaccines failed was because they were limited to B cells and opsonic antibody initiation steps and not including T cell stimulation (Redi et al., 2018). Another opinion is that, over-reliance on rodent models and a focus on targeting cell surface components have been major contributing factors to this failure as reported by Salgado-Pabón et al. (2014).

For effective vaccines against *S. aureus* to be designed, humoral and cellular immunity should be stimulated, the vaccine should be multi-components because of the numerous *S. aureus* virulence mechanisms and the heterogeneous nature of the genome (Proctor, 2015). Conventional approaches for vaccine design which is based on pathogen culture and testing only the expressed antigens during culture are extremely time consuming, costly and they are not appropriated for non-culturable pathogens as reported by Bruno et al. (2015). Reverse vaccinology is a computer-based technique, for selection of candidate genes with potential for use as vaccines, developed by Rappuoli (2001). Reverse vaccinology is based on the analysis of whole genomes sequence data of pathogens. There are many successful trails and researches based on reverse vaccinology approach for developing vaccine against many pathogens as *Streptococcus agalactiae, Streptococcus pyogenes,* pathogenic *Escherichia coli*, *helicobacter pylori* and serogroup B *Neisseria meningitides* (Seib et al., 2012; Naz et al., 2015). In 2013, Novartis launched Bexsero® as the first vaccine based on reverse vaccinology approach against meningococcal serotype B disease as reported by Carter (2013)**.** It has been licensed in Canada, Australia, United States and United Kingdom as stated by Heinson et al. (2015).

As reported by Soltan et al. (2020), reverse vaccinology approach was used for the selection of potential vaccine gene candidates for *S. aureus*. Candidate genes were selected based on antigenicity score (antigenicity score > 0.45 were selected) and cellular localization (Only extracellular proteins and cell wall proteins were selected). The selected genes were subjected for screening in clinical isolates. The ones which are present in almost all isolates are selected for further cloning, expressing, purification and in-vitro and in-vivo vaccine testing. The selected aur gene shown to have high score of antigenicity and it was present in 96% of the tested clinical isolates. The aims of the current study are cloning, expression, purification of aur and demonstration of the reactivity of the purified aur against antibodies obtained from human infected with *S. aureus*.

MATERIAL AND METHODS

In silico studies

An *in-silico* study Soltan et al. (2020) was carried out in the laboratory (College of Pharmacy, Suez Canal University, Egypt), nominated aur gene to be tested for its potential use as vaccine candidate based on surface location and antigenicity score (0.7). Aur gene was tested for presence in a large panel of *S. aureus* isolates.

Isolates collection and DNA extraction

Seventy-five of S. *aureus* isolates were previously isolated by Bendary et al. (2016) were used. These isolates were cultured on Mannitol salt agar (Lab M®, UK) and their genomic DNA were extracted by QIAamp DNA Mini Kit (Qiagen, Germany) according to manufacturer's recommendation. The extracted DNA was stored at -80°C for downstream application.

Primer design for PCR assay

Primers in the current study was designed manually and the specificity of the selected primers was confirmed by blasting in nucleotide blast tool of NCBI website https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome.The designed forward primer: 5-GAGGTGACTCAAAAGAGTGAT-3 and reverse primer; 5-TGTGTAGACATCTTCACCCAT-3 were used to amplify *aur* by PCR.

The amplification was performed in 50 µl containing 1 µl of Forward primer (10 µM), 1 µl of reverse primer (10 µM), 25 µl of one PCR master mix (GenedireX, Germany), 1 µl of DNA template (50 ng/µl) and the final volume was adjusted to 50 µl PCR water (Qiagen, Germany) under the following conditions; Initial denaturation at 94°C for 5 min, followed by 35 cycle of (denaturation at 94°C for 40 seconds, annealing at 52^{o}C for 45 s and extension at 72°C for 2 min) and final extension at 72°C for 5 min. The amplified PCR products were detected by 1.5% agarose and were visualized after staining with ethidium bromide under ultraviolet trans illuminator (IFM-20 UVP, upland, USA).

Cloning primer design

Forward primer contain the started nucleotides of the inserted gene and reverse primer contain the last nucleotide sequences of the inserted gene were designed. The open reading frame (ORF) design was confirmed by EMBOSS Transeq software for sequence analysis (http://www.bioinformatics.nl/cgi-bin/emboss/transeq). The restriction sites of the selected endonuclease enzymes were added at the beginning of the primers. The selection of the endonuclease enzymes was based on their ability to cut both vector and insert at their specific sites and not able to cut inside the insert that was checked by using NEBcutter V2.0 http://nc2.neb.com/NEBcutter2/. The restriction site of *XbaI* (TCTAGA) was added at the beginning of the forward primer. While, the restriction site of *NotI* (GCGGCCGC) was added at the beginning of the reverse primer. To increase the cleavage efficiency of restriction enzymes, 5'-nucleotide extension was added at the beginning of both XbaI and NotI sites, GC nucleotides were added at the 5' of XbaI site and

TGTATC nucleotides were added at 5' of *NotI* sites.

Cloning and expression of zinc metalloproteinase aureolysin

Cloning

The aur cloning vector was PH6HTN His6HaloTag® plasmid (Promega, USA). At first, *aur* was amplified with the same conditions that were performed in screening of genes with 58°C annealing temperature and using the designed cloning primers. The forward primer was 5-GCTCTAGAATGGCAGCATTAACCTTGTTG-3 and the reverse primer was 5-TGTATCGCCGGCGTTACTCCACGCCTACTTCAT-3. After detection of *aur* band on gel electrophoresis, it was cut and purified by PCR clean-up and gel purification kit (GenedirX, Germany) according to manufacturer specifications. The purified *aur* and PH6HTN His6HaloTag® plasmid were double digested with Fast Digest *XbaI* and *NotI* restriction enzymes (Thermo- Scientific, USA) based on the manufacturer specifications. Ligation step was followed by the action of T4-DNA ligase enzyme (New England Biolabs (NEB), USA) with 3:1 ratio of insert and plasmid respectively. The transformation of the cloned plasmid into *E. coli (DH5α)* competent cells were accomplished by heat shock at 42°C for 30 s followed by immediately transferring to ice for 2 min as mentioned in Nakata et al. (1997) protocol followed by streaking the transformed cells over Luria agar (LB) (Lab M®, UK) containing 100 µg/ ml of ampicillin and incubated overnight at 37°C. The positive colonies harbored the recombinant plasmid and the negative colonies harbored the empty plasmid. These were then screened by colony PCR using confirmatory primers with forward primer: 5-GGTCTGAATCTGCTGCAAGAA-3 and reverse primer: 5-GGTTATGCTAGT TATTGCTCAG -3. The amplification condition was the same condition for screening the genes except when the annealing temperature was 52°C for 45 s. The positive colonies were determined and preserved at -80°C for further application.

Expression

Expression of aur was accomplished under the influence of T7 promoter. First, the plasmid of the positive colonies was extracted using "PureYield™ Plasmid Miniprep System" (Promega, USA) according to manufacture specifications. The extracted plasmid was transformed into *BL21* (*DE3*) Competent *E. Coli* by heat shock followed by streaking over Luria Broth (LB) (Lab M®, UK) containing 100 $\mu g.ml^{-1}$ of ampicillin and incubated overnight at 37°C. Single colony from the previous culture was inoculated in 5 ml LB medium containing 100 µg/ml ampicillin and was then incubated in a shaker incubator with 250 rpm at overnight. After incubation period, 1 ml of the previous culture was diluted in 100 ml fresh LB medium then the diluted culture was incubated on shaker incubator with 150 rpm at 37°C to an optical density 600 nm (OD_{600}) of 0.5 to 0.6. These concentrations of IPTG 0.1, 0.2, 0.5 and 1 mM were added for induction of proteins expression. Expression was accomplished under different condition for every concentration of IPTG. The applied different conditions included different temperature (20, 30 and 37°C), different incubation time intervals (3, 4, 5 and overnight) on shaker incubator at 150 rpm. The pellet of these different conditions was obtained by centrifugation at 15,000 rpm for 20 min at 4°C.

Protein analysis by SDS-PAGE

To liberate the proteins from the cultured cells, the pellets were washed with lysis buffer containing (5 mM Imidazole, 300 mM NaCl50 mM, Na2HPO4, pH at 7.4) then re-suspended in 1:10 W/V lysis buffer. Five micro liter triton-x-100, 250 µl 100 mM PMSF and 5 µl lysozyme (100mg/ml) were added to each 5 ml of lysis buffer and this suspension was incubated in refrigerator (4°C) for 15 min. Freeze-thaw technique was used to facilitate cell lysis where the previous suspension was exposed to 4 cycles of freezing (-20°C) and thawing (37°C). The cell lysate was centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was collected. To prepare the samples for SDS-PAGE, the supernatant was mixed with Laemmli sample buffer (BIO-RAD, USA) and then the mixture was boiled at 95°C for 10 min. Protein samples were separated by 12% SDS-PAGE according to Laemmli SDS-PAGE protocol mentioned in He (2011). The gel was stained by Coomassie Brilliant Blue R-250 dye (Thermofisher, USA) for 8 h followed by destaining by using mixture of 40% methanol, 20% glacial acetic acid and 40% distilled water.

Protein purification

Protein purification was performed based on IMAC technique that a profanity nickel metal charged resin (Bio-Rad, USA) was used. First, the 50% (v/v) profanity IMAC (Bio-Rad, USA) slurry was suspended by gently swirling with plastic rod. The slurry was applied to the column (2.5 cm ID x 15 cm). Then, the column was equilibrated with 5 CV of equilibrated column buffer containing 300 mM NaCl, 50 mM Na_2HPO_4, and 5 mM imidazole, pH at 7.0 followed by application of 3 to 5 column volume charging metal solution (300 mM Nickel sulphate at pH of 4). The column was washed with 5 CV equilibrated column buffer and 10 CV deionized water to remove unbound metal ions. Then 5 CV of washing buffer (50 mM Na_2HPO_4, 300 mM NaCl and 20 mM Imidazole) was added to the column. The supernatant was loaded onto column with swirl at 4°C. The 5 CV of wash buffer was used to remove unbound sample and the resulted fractures were collected for further analysis. Finally, 5 CV of elution buffer (50 mM Na_2HPO_4, 300 mM NaCl and 250 mM Imidazole) was applied to elute the protein and protein containing fractions were collected and protein was analysed using SDS-PAGE.

Demonstration of reactivity of aur with serum antibodies of *S. aureus*

Western blot technique was used for protein identification of the purified aur. Ten sera samples were collected from patients who reside at Suez Canal University Educational Hospital and suffered from pneumonia and septicemia caused by *S. aureus.* The sera were collected after the approval from ethics committee of faculty of pharmacy, Suez Canal University-Egypt (Reference number of 20166H1). After separation of protein by 12% SDS-PAGE, the gel was transferred onto 0.45 µm.30 cm×3.5 m nitrocellulose membrane (Thermo fisher, USA) (Catalog number 88018) using mini trans-blot© electrophoretic transfer cell (Bio rad, USA) under constant 300 mA for 15 min using transferring buffer contained 25 mM tris-HCL, 192 mM glycine and 20% methanol pH 8.3. The transferred nitrocellulose membrane was blocked by 3% Blocker Bovine Serum Albumin BSA (10X) (Thermo fisher, USA) diluted in PBS overnight 0.05% Tween-20 (TBST) at room temperature followed by two time washing with 0.05% Tween-20 (TBST) for 10 min. The membrane was incubated with sera (1/10 dilution) with gentle agitation on rocking shaker at 4°C overnight followed by four time washing with 0.05% Tween-20 (TBST) for 10 min to remove any residuals of primary antibodies (Sera). After washing steps, the membrane was incubated with the secondary antibody (goat antihuman IgG(H+L) conjugated with HRP) (Invitrogen, USA) (Cat number 31410) with the dilution 1:10,000 with gentle agitation on rocking shaker at room temperature for 1 h followed by three time

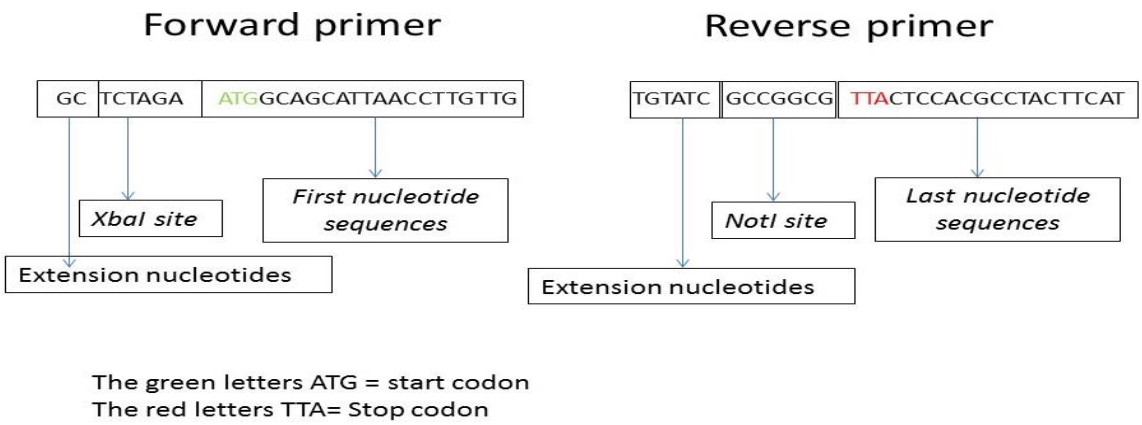

Figure 1. Cloning primer analysis.

washing with 0.05% Tween-20 (TBST) for 10 min. Finally, Super Signal West Pico PLUS substrate (Thermo scientific™, USA) was applied to the membrane and incubated at room temperature (30°C) for 5 min. The signal was captured.

RESULTS

Primer design

All the designed primers (screening and cloning primers) in this study were specific to their templates with low undesired secondary structure annealing. The structure of the designed cloning primers was illustrated in Figure1.

Cloning and screening of the correctly inserted *aur* in MCS of plasmid

The insert was cloned in PH6HTN His6HaloTag® plasmid between *XbaI* site and *NotI* site of multiple cloning sites (Figure 2). After screening of positive colonies that harbored the recombinant plasmid and negative colonies that harbored the empty plasmid by colony PCR technique using confirmatory primers previously mentioned, the amplicon size of positive colonies was detected at ~ 1500 bp (Figure 3). While, negative colonies were detected at ~245 bp.

Expression and purification of aur

After application of different expression conditions (different concentration of IPTG, incubation times and temperatures) and analysis of these conditions by SDS-PAGE, the optimum condition for Aur expression was 0.5 mM IPTG at 37°C after 4 h that showed heavy band at ~86 KDa (the molecular weight of the expressed Aur + His6 tag) (Figure 4). The supernatant of this condition was purified by IMAC and analyzed by SDS-PAGE showed single band at the expected molecular weight (86 KDa).

Demonstration of reactivity with serum antibodies

The reactivity of aur was demonstrated by using western blot assay. Positive reaction of the purified aur band was showed at ~86 KDa that was the molecular weight of the purified aur protein (his6halo tagged). Each human serum sample was tested individually and the positive reaction of aur against patient's serum is illustrated in Figure 5.

DISCUSSION

The increasing rate of prevalence of antibiotic resistance in *S. aureus* necessitated the research into the development of a protective vaccine against the organism, but unfortunately all the clinical trials that were carried out to date failed as reported by Proctor (2015).

The new vaccines will be targeted at preventing infections by reducing biofilm formation and / or adhesion to the foreign body. This criterion has already been tested in some clinical trials where anti-capsular polysaccharide (types 5 and 8), anti-SdrG and anti-Clamping factor A (ClfA) were tested either for passive or active immunity (Rupp et al., 2007) but, all of them have failed in human clinical trials.

Clinical trial failures can be attributed to many reasons, some of which includes: Many of the trial targeted B-cell activation rather than T-cell activation especially Th17 as suggested by Proctor (2012). These are essential for activation and mobilization of neutrophils as reported by Lin et al (2009). Furthermore, *S. aureus* causes variety of diseases therefore there is a need for more than a single vaccine that will be potent against many of the strains as stated by Anderson et al. (2012). Another

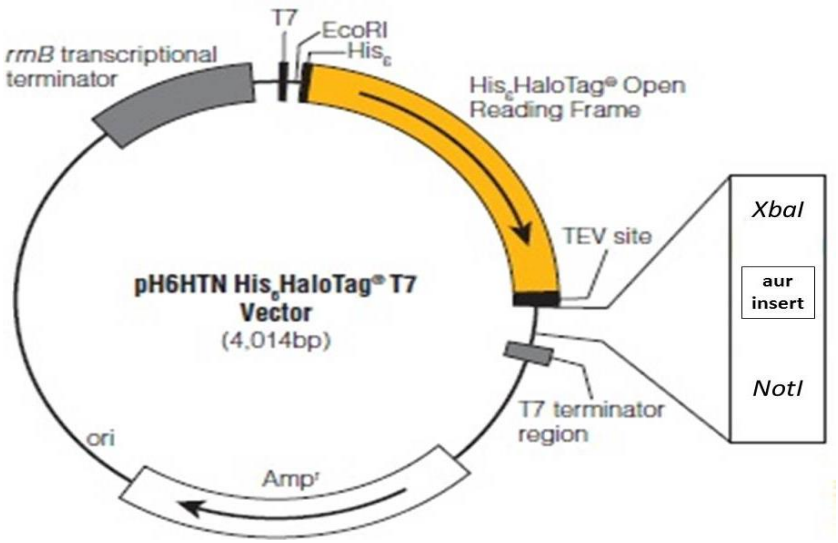

Figure 2. Recombinant PH6HTN His6HaloTag®-*aur* plasmid structure. *aur* was cloned between *XbaI* and *NotI* sites of plasmid. The PH6HTN His6HaloTag® plasmid contains many features such as T7 promoter that promote the expression of the recombinant plasmid in *E. coli* strains having T7 RNA polymerase, The N-terminal His6HaloTag® region that facilitate simple purification of protein via 6 histidine tag. A TEV protease site that cleaves the expressed protein form His6HaloTag® region by the action of Halo TEV protease and ampicillin resistant gene (Ampr) that facilitates the selection of the transformed colonies. This figure was modified from the Promega pH6HTN His6HaloTag®T7 Vector Certificate analysis part no. 9PIG797, printed 2/2017.

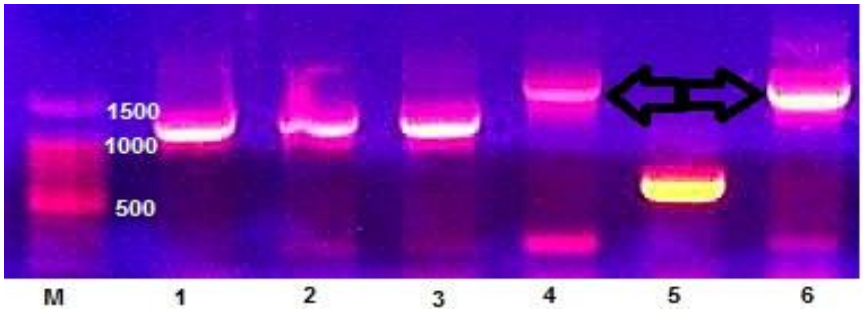

Figure 3. Gel electrophoresis of the amplified positive colonies after transformation of recombinant PH6HTN His6HaloTag®-aur plasmid into *E. coli* (*DH5α*) strain. M was 100 bp DNA Ladder RTU (GendireX, Germany). Lane 1, 2 and 3 was the amplified recombinant PH6HTN His6HaloTag®-*plc* plasmid that detected at 1232 bp while lane 5 was non-specific amplification. Lane 4 and 6 was the amplified recombinant PH6HTN His6HaloTag®-aur plasmid that detected at 1500 bp.

important factor is the variability between isolates which may give rise to triggering of inappropriate immune response, and may cause organs failure as reported by Lloyd et al. (2020). Finally, *S. aureus* is one of the normal flora and has the ability to develop several mechanisms for escaping from host immunity particularly opsonophagocytic processes (Fowler et al., 2013; Van Kessel et al., 2014).

Soltan et al. (2020) reported the use of reverse vaccinology to nominate genes that are surface localized and showed with high antigenicity scores, the nominated genes (16 genes) were screened for presence in a large panel of *S. aureus* clinical isolates. The genes present in almost all the tested isolated were used further to test their vaccine potential. Phosphatidylinositol phosphodiesterase (PI) was selected for cloning, expression and B cell and T cell epitope mapping. PI was shown to be highly reactive with antibodies obtained from

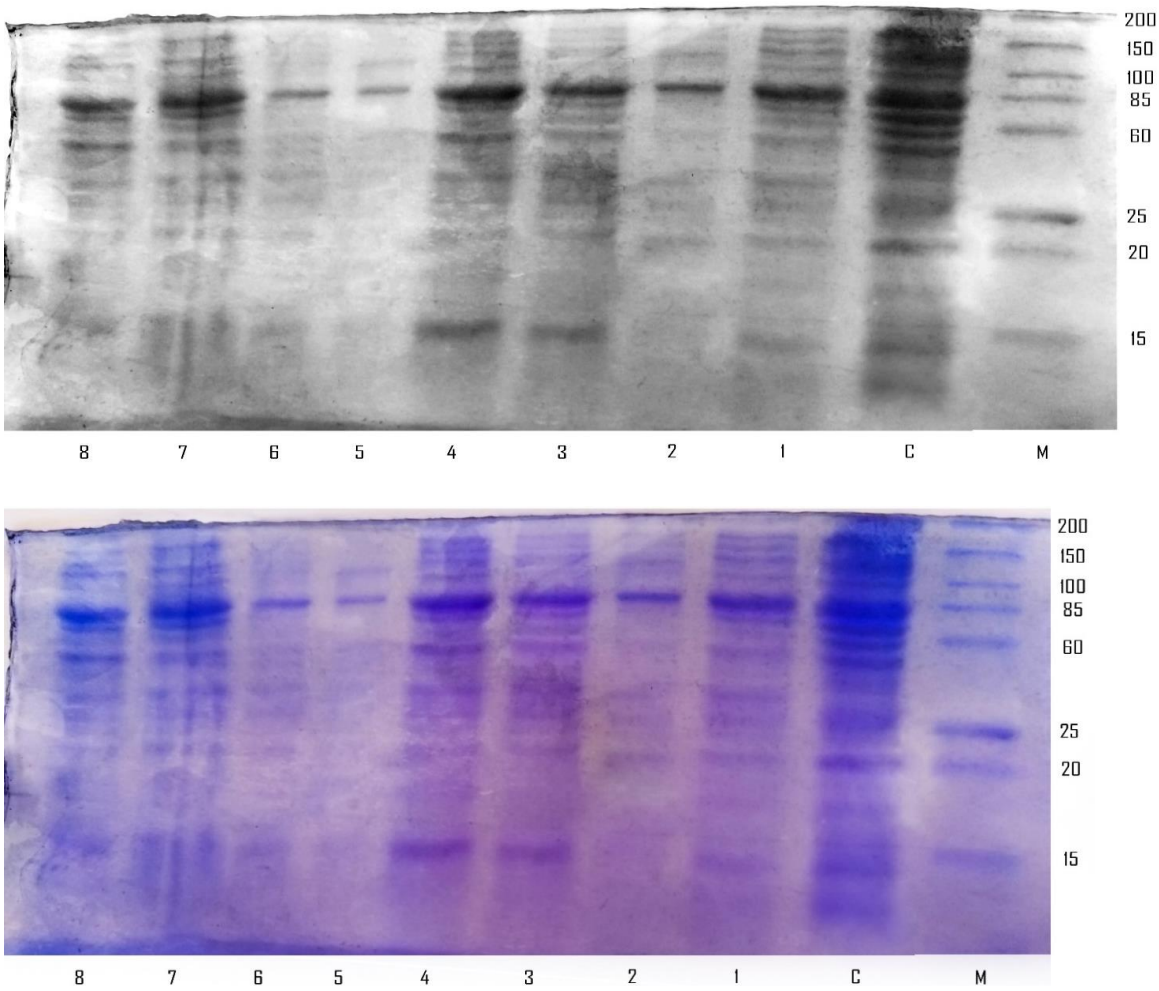

Figure 4. SDS-PAGE analysis of the optimum condition of His6HaloTag®-aur expression under the induction of IPTG. Lane M: Unstained Protein Standard (10-200 kDa) (NEB, USA). Lane C: The culture condition without the addition of IPTG. Lane 1-4: Culture growth under 0.1, 0.2, 0.5 and 1 mM IPTG respectively at 37°C after 3 h. Lane 5-8: Culture growth under 0.1, 0.2, 0.5 and 1 mM IPTG respectively at 37°C after 4 hours. Lane 7: The best condition of recombinant His6HaloTag®-Aur expression.

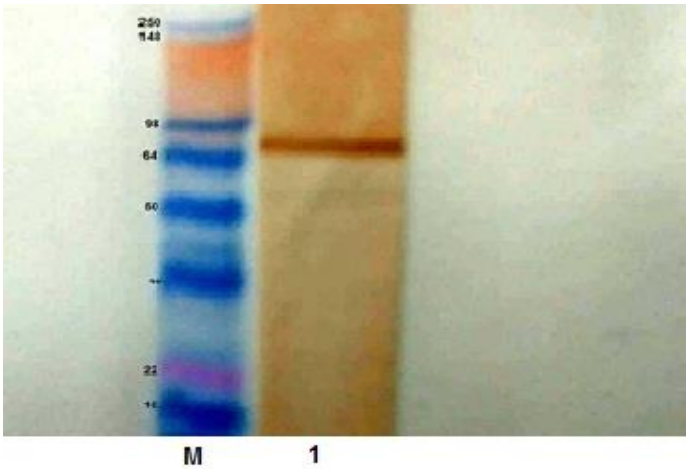

Figure 5. Western blot analysis of the purified His6HaloTag®-Aur. Lane M: Protein marker; lane1: Purified recombinant Aur at 86 KDa.

serum of humans infected with *S. aureus*. The in-vivo induction of immune response against PI in a bacteremia mice model resulted in protection of the mice injected with B cell and T cell epitope mixture.

The second nominated gene by Soltan et al. (2020) was aur gene; it was detected in large number of *S. aureus* isolated (96%). In addition to its critical role in *S. aureus* pathogenesis, *aur* has a role in *S. aureus* resistance to innate immunity because it degrades the antimicrobial peptide dermicidin and cathelicidin LL-37 (Beaufort et al., 2008). Also aur has a role in transition of *S. aureus* form adherent to invasive stage by cleavage of staphylococcal surface- associated proteins (Stach et al., 2018). Therefore, immunization against aur antigen can affect *S. aureus* invasiveness.

For proper cloning and expression of different proteins of *S. aureus*, many researches use two vectors (one for cloning and other for expression) as mentioned in Das and Biswas (2019) who use pGEM-T Easy cloning vector and pET28a expression vector for Phi11 *gp07,* however, this approach is expensive. On the other hand, other studies employ one vector for cloning and expression as mentioned by Chen et al (2019), in which pJET1.2 vector in serine acetyltransferase cloning was used. In this study, *aur* gene was cloned and expressed in pH6HTN His6HaloTag® T7.

As reported in some of the previous research works, different IPTG concentrations ranged from 0.1 to 1 mM and different induction periods (2 to overnight) at different temperature (20 to 37°C) were used for optimization of the protein expression. But in this study, the optimal IPTG concentration was 0.5 mM. In contrast, Nickerson et al. (2008) study has induced aur by using 1 mM IPTG.

There was a little increment in the yield of aur expression between overnight induction and after 4 h. Therefore, induction after 4 h is economical compared with overnight as it saves energy that was been consumed by the shaker incubator. This 4 h conditioning was consistent with Nickerson et al (2008) where they induced aur after 4 h.

Many affinity tags can be used to purify the expressed protein and in this study, the His-tag was selected as it is highly specific in target protein purification and highly effective more than 80% of pure protein can be obtained in one chromatographic step as stated by Kimple et al (2013). His-tag was also used by Nickerson et al. (2008) study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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