Cloning and Expression of Alpha Hemolysin Toxin Gene of \textit{Staphylococcus aureus} Against Human Cancer Tissue

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Abstract

Recombinant technology has crucial impact in therapy development. In microbial environment, Pathogenic bacteria such as \textit{Staphylococcus aureus} produces alpha hemolysin protein. This protein is used as anticancer protein. In our study, alpha hemolysin toxin (\textit{hia}) gene was isolated from \textit{S. aureus} isolate. The isolate of \textit{S. aureus} was isolated from blood samples of patients at Microbiology laboratory of Children Mansoura University Hospital. The purified PCR of \textit{hia} gene of \textit{S. aureus} strain was subjected to sequencing and cloning. Recombinant alpha hemolysin toxin cloning was detected by PCR, and \textit{α}-hemolysin protein was purified by Sphadex. The purified \textit{α}-protein was used as anticancer against HepG-2 cells (human Hepatocellular carcinoma), HCT-116 cells (human colon carcinoma), MCF-7 cells (human breast cancer cell line) and A-549 cells (human Lung cancer cell line).

Keywords: \textit{Staphylococcus aureus}; Alpha hemolysin toxin; Sequencing; Cloning; Anticancer tissue.

Abbreviations: \textit{hia}: Alpha hemolysin toxin; PCR: Polymerase Chain Reaction; HepG-2 cells: human Hepatocellular carcinoma; HCT-116 cells: human colon carcinoma; MCF-7 cells: human breast cancer cell-line; A-549 cells: human Lung cancer cell line.

Introduction

In therapeutic system, \textit{Staphylococcus aureus} play crucial role in pathogenic world (Reddy et al., 2014). \textit{S. aureus} is facultative human pathogenic that causes a wide range of infections from skin and soft tissue to invasive such as endocarditis, osteomyelitis, and pneumonia (Bartlett and Hulten, 2010; Aman and Adhikari, 2014; Borrello et al., 2007).

Pathogenic \textit{S. aureus} have a wide range of virulence factors as superantigens (enterotoxins, toxic shock syndrome toxin and exfoliative toxins), cytoxins (alpha-hemolysin, beta-hemolysin, gamma-hemolysin, delta-hemolysin, Panton-Valentine leukocidin), phagocytosis inhibitors (polysaccharide capsule, protein A), and immune evasion molecules (chemotaxis inhibitory protein, staphylokinase, aureolysin) (Bartlett and Hulten, 2010; Aman and Adhikari, 2014; Borrello et al., 2007).

Alpha hemolysin toxin of \textit{S. aureus (hia)} is monomer water-soluble 34 KDa exotoxin protein of 293 amino acids and chromosomally gene. Alpha-toxin is a pore-forming hemolytic toxin that brings about membrane damage to many types of mammalian cells. Alpha hemolysin toxin can be increased the productivity by cloning (Leng et al., 2011; Tavares et al., 2014; Gurnev and Netorovich, 2014; Kong et al., 2016). Alpha hemolysin toxin plays a role in inducing apoptosis in tumor cells, so it can be used as anticancer (Wang et al., 2011).

In this connection, the main objective of this paper is to isolate and identify the most effective isolate of \textit{S. aureus} for producing \textit{α}-hemolysin toxins and more deeply to evaluate the potential efficacy of this toxins against cancer of human cells tissues.

Materials and Methods

Collection and isolation of \textit{S. aureus} isolate

\textit{S. aureus} isolate used in this study was isolated from blood samples of patients at Children Mansoura University Hospital (CMUH). \textit{S. aureus} isolate was identified according to morphological and biochemical identification.

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according to methods of Sogaard et al. (2007) and Rubin et al. (2010).

Isolation of Alpha Hemolysin Toxin of S. aureus

Primer

*hia* primer sequences was selected as Tavares (2014):

RT_Foward: 5'-TAATGAATCCTGTGCTAATGCC-3',
RT_reverse: 5'-CACCTGTTTTTACTGTAGTTATTGCTTCC-3'.

Bacterial Suspension Preparation

*S. aureus* isolate used in this study was grown in 5 ml of trypton soya bean (TSB) broth at 37 °C for 24 hours then the pellets were collected by centrifugation at 12000 rpm for 5 minutes according the method of Liang et al. (2011). The cell pellets were stocked at 4°C until used.

RNA Extraction by Kit Method

RNA isolation from bacterial cells was carried out using the protocol of RNAasy kit (Intronbio- technology, Korea).

Preparation of Complementary Deoxyribonucleic acid (cDNA)

The cDNA reaction was performed using reverse transcriptase enzyme. The reaction was carried out in a 20 µl reaction mixture containing: 3 µl of RNA extraction, 2.5 µl of buffer, 2.5 µl of dNTPs (dATP, dCTP, dGTP and dTTP), 5 µl of the primer (reverse primer of specific *hia* gene), 0.2 µl of reverse transcriptase enzyme, 6.8 µl of PCR water (nuclease – free). The tubes containing reaction mixture were mixed, and transferred to the PCR apparatus and the program was entered as the following: The reaction was initiated by first step at 42°C for 1 hour, followed by second step at 72°C for 10 minutes. cDNA products were stored at -20°C until used.

25 µl of reaction mixture PCR reaction was made with 2.5 µl of stored cDNA, 5 µl of buffer, 4 µl of M MgCl₂, 3 µl of dNTPs (2.5 mM of each ), 3 µl of the primer (1.5 µl of forward + 1.5µl of reverse), 0.4 µl of Taq DNA polymerase (Promega, U.S.A.), 7.1 µl of PCR water (nuclease-free). The tubes containing reaction mixture were vortexed, and transferred to the PCR-apparatus. The thermal program was run as follows: The initial denaturing step at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute and finally the reaction was terminated with an extension step at 72°C for 5 minutes. At the final, the program was stopped. Amplified products were electrophoresed in 2% agarose with ethidium bromide, photographed and analyzed (Tavares, 2014; Ouyang et al., 2016).

PCR product Purification

PCR product of α-toxin gene was purified by using DNA gel extraction kit (Biobasic Inc, Canada). Purified DNA was stored at -20°C until used in gene sequencing and gene cloning expression.

Sequence Analysis of Alpha Hemolysin Toxin of *S. aureus*

The alpha hemolysin toxin specific PCR products of the strain *S. aureus* was sequenced using automated DNA sequence by Macrogen (Macrogen com, Korea). The obtained DNA analysis using comparative tool such as Blast DNA (www.ncbi.nlm.nih.gov) (Rajendhran and Gunasekaran, 2010).

Cloning of *S. aureus* Alpha Hemolysin Toxin

The purified PCR product of *S. aureus* and expression vector (pH6HTN His6HaloTagT7) (Promega, U.S.A.) were treated with the same suitable restriction enzyme (EcoRI) (Promega, U.S.A.). Restriction enzyme was chosen by using the sequence of purified PCR of *S. aureus* in online site (www.restrictionmapping.com). The restricted purified PCR product was ligated with the restricted vector by using T4 DNA ligase enzyme (Promega, U.S.A.). Cloned vector was transmitted into competent *E. coli* DH5α cells. Transformed cells were identified by the colour of colonies using antibiotic (ampicillin) containing medium with presence of X-gal and IPTG (isopropyl B-D-1-thiogalactopyranoside). The above methods have been described by Inoue et al. (1990), Reddy et al. (2014) and Pulicherla et al. (2013).

Detection of Gene Cloning by using PCR

Plasmid DNA Isolation

White colony was inoculated in 5 ml of Luria Bertani (LB) medium supplemented with Ampicillin (50mg/ ml) and incubated at 37 °C for 24 hours in shaking condition (Pulicherla et al., 2013). The bacterial cells were harvested by using centrifugation at 12000 rpm for 10 minutes. Plasmid was extracted by using extraction plasmid kit (Biobasic Inc, made in Canada). The plasmid DNA was eluted by adding 30 µl of elution buffer and the plasmid stored at -20°C until used.

PCR for Isolated Plasmid

PCR reaction was same as described above for PCR of *S. aureus*.

Recombinant Alpha Hemolysin Toxin Extraction and Purification

The sample preparation and the protein purification were done by the methods of Reddy et al. (2014). Expression of *hia* gene and separation of *hia* protein were carried out by using SDS-PAGE. White colonies were cultured in 200 ml of LB broth medium with inducer IPTG and ampicillin for 24 hours. The culture was centrifuged at 10000 rpm for 30 minutes in cooling centrifuge. The harvested pellets were suspended with 25 ml of gel permission buffer and sonicated on ice for 5 minutes, and then centrifuged at 10000 rpm for 20 minutes. The supernatants were collected in a new sterilized flukene tube and transferred to sphadex column for protein purification (El-Gayar, 2015). The protein...
fractionated was taken 1 ml per 1 minute. The obtained α-toxin protein were determined by using method of Bradford (1976). The purified protein SDS-PAGE is shown in (Fig. 4C) which was performed the methods of Harlow and lane (1988). The purified α-protein was dried by lyphlization.

Application of Recombinant A-Hemolysin Protein In Different Human Cancer Tissues

Mammalian cell lines
HepG-2 cells (human Hepatocellular carcinoma), HCT-116 (colon carcinoma), MCF-7 cells (human breast cancer cell line) and A-549 cells (human Lung cancer cell line) were obtained from VACSERA Tissue Culture Unit.

Cell lines Propagation
The cell lines (HepG-2, HCT-116, MCF-7, A-549) propagation were described by Mosmann (1983).

Cytotoxicity of Alpha-Hemolysin Protein Using Viability Assay
For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1x10⁴ cells per well in 100µl of growth medium. Fresh medium containing different concentrations of recombinant alpha hemolysin protein was added after 24 h of seeding. Serial two-fold dilutions of the tested α-protein were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA). The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO₂ for a period of 48 h. Three wells were used for each concentration of α-protein. Control cells were incubated without test sample and with or without DMSO. After incubation of the cells for 37°C, various concentrations of α-hemolysin protein were added, and the incubation was continued for 24 h and viable cells yield was determined by a colorimetric method which the cells were stained by the crystal violet stain (Mosmann, 1983; Gomha et al., 2015).

The relation between surviving cells and α-toxin concentration is plotted to get the survival curve of each tumor cell line after treatment with α-toxin. The 50% inhibitory concentration (IC₅₀), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) (Mosmann, 1983; Gomha et al., 2015).

Results and Discussion
Alpha hemolysin toxin of S. aureus is one of the most important virulence factors. hia gene of S. aureus used in this study was detected and isolated by using PCR. Isolated α-toxin gene was subjected to sequencing and cloning. Alpha hemolysin protein was generated from recombinant hia gene in competent E. coli cells. The purified protein was treated human cancer cell lines.

Isolation and Identification of S. aureus Isolate
The isolate produced yellow colonies when grown on blood agar medium as shown in Fig. (1-A). This isolate was Gram-positive cocci shaped, coagulase positive. According, this strain was preliminary identified to be S. aureus. Alpha hemolysin expression was detected on blood agar medium, which the color turned to brownish color as showed in Fig. (1B). These results showed agreement with those published by Reddy et al. (2014); Stulik et al. (2014).

Fig. 1: A) Yellow colonies of S. aureus on blood agar. B) S. aureus was produced alpha hemolysin toxin on blood agar medium which turned to brownish color.

Analysis of Sequence of Alpha Hemolysin Toxin of S. aureus
α-toxin gene (hia) and purified hia gene were exactly 680 bp as showed in Fig.2. Purified hia gene was sequenced as Fig. 3. The Purified PCR product was sequenced by Microgene (in Korea) then the sequences were submitted to NCBI, and Blast sequence analysis showed that α-toxin has been 99% according with sequences published in Gene Bank. This result is agreement with this published by Fei et al. (2011).

Fig. 2: A) Amplified PCR of hia gene of S. aureus from blood, with the amplicon size of exactly 680 bp. B) Purified α-toxin of S. aureus strain. Amplified PCR of hia gene was ran in 2% of agarose gel and then bands were eluted by the gel elution kit. M: 1000 bp DNA marker (100, 200, 300, 400, 500, 700, 1000, 1600, 2000, 5000).
CCTGATCGGAGAGTGCTACAAAAGTGGTTTAGCCTGGGCTTCGAGCTTAAAGGTAACAGTT
GCAACACTCGATAGTAAGTACAGCTCAATATCTGATGATTATATCAAGGAAATTCGAGAT
ACACAAAGATATATGAGTACTTTACTTTATGAGATTCAACGGTAAATGTTACTGTTGATGAT
ACAGGAAAATCCGCGCGCTTATTGTTGCAAAATGTTTCCGATTGGTGCTGCAACACTGAAATAT
GTTCACACCTGATTTCAAAACAATTTTAGAGAGCCCAACTGATAAAAAGTAGGCTGGAA
AGTAGATTTAACAAATATGTGAATCAAATATGGGACCAATAITGATAGATTCTTTGGAA
CCCCGCGATAGGAACACTTTTCTCTTAGAATTAGGACTTGAAAGCAGCAGTA
TAACCTCCTTTGATCTTATAACAAAGCAATCTTCTATTTACTCTTAGCTTCACCAGACTT
CGCTACAGTTATTACTATGGGATAAGAAGCATTCCAACACAAACAACAAATATGATAT
ATACGAACAGTTGCGGTATGATTACCAATTCGATGTTGACCTCAACAAATTGGAAGGTA
CCAAATTCAAGGATTAGGACAGCTTCTTGCAGAAAGATATAAACATCTCGAGTGGAA

Fig. 3: Sequence of alpha hemolysin toxin of *S. aureus* form PCR product. Amplified gene was isolated from agarose gel and sequenced to confirm the present of *hia* gene of *S. aureus*.

![Blue colonies](image1.png)
![White colonies](image2.png)

**Fig. 4:** Expression of recombinant *hia* protein from recombinant *E. coli*. A) The recombinant *E.coli* colonies on L. B. medium. The white colonies were detected to the gene insertion while the blue colonies were not inserted the gene. The white colonies were selected. B): PCR-detection of alpha hemolysin toxin after cloning. PCR of purified plasmid after cloning. M: 1000 bp DNA marker. C): Purified alpha hemolysin toxin (1-6 purified fractional protein) after cloning in *E. coli*. M: Marker protein (94, 66.2, 45, 33, 26, 20).

**Cloning of Alpha Hemolysin Toxin Gene Into Ph6hnalotagt7 Expression Vector**

The PCR product of α-toxin of *S. aureus* was separated on 2% agarose gel and purified PCR using gel extraction kit (thermo). The purified PCR and the expression vector were cut with the same restriction enzyme (EcoR1) then ligated with ligase enzyme (promega). The recombinant vector was transformed into *E.coli* DH5α and white colonies were selected (Fig. 4-A). Plasmid DNA min prep form the white colonies were performed using plasmid isolation kit. The purified plasmid was used in PCR test for detection of α-toxin gene (Fig.4-B). The recombinant bacteria were induced in L. B. broth medium using IPTG and Ampicillin and the production of alpha hemolysin toxin was evaluated. The recombinant alpha hemolysin toxin was purified by using sphadex column and the obtained α-toxin were determined by using Bradford method. The activity of all
obtain fraction were screened and separated by using SDS-PAGE as showed in the Fig. (4-C). α-toxin protein was expressed at 34 KDa and this result is agreement with those published by Bantel et al. (2001) and Swofford et al. (2014).

**Application of Recombinant A- Hemolysin Protein in Different Human Cancer Tissues**

Purified recombinant alpha hemolysin obtained during this study was dried by lyophilization. Cytotoxicity of recombinant alpha hemolysin protein was tested against four cell lines such as HepG-2, HCT-116, MCF-7, A-549. In this study showed that different concentrations of alpha hemolysin toxin was effected on the cell viability of four cell lines (HepG-2, HCT-116, MCF-7, A-549) and determined IC50 under these experimental conditions. From Fig. 5 and Fig. 7A, inhibitory activity of α-toxin against Hepatocellular carcinoma cells was detected with IC50 = 210 µg/ml. From Fig. 6 and Fig. 7B, inhibitory activity of α-toxin against colon carcinoma cells was detected with IC50 = 244 µg/ml. From Fig. 7C and Fig. 7D, weak inhibitory activity of alpha toxin against Breast carcinoma cells and Lung carcinoma cells were detected with IC50 = >500 µg/ml. Our results confirmed that alpha hemolysin toxin is used as anticancer. These results are agreement with those published by Bantel et al. (2001); Swofford et al. (2014). In this connection, Vandenesch et al. (2012) has reported that α-hemolysin is the most characterized virulence factor of *S. aureus*. Upon binding to the cell surface, α-hemolysin monomers assemble into a homo heptamer, forming a preapore. The preapore subsequently transitions to a mature β-barrel transmembrane pore. This pore allows the transport of molecules smaller than 2kD such as K+ and Ca2+ ions, leading to necrotic death of the target cell.

![Fig. 5](image-url)

*Fig. 5: Microscopic examination for the effect of α-hemolysin on hepatocellular cancer cells (HepG-2). The cells were incubated with (A) 0, (B) 62.5, (C) 250 or (D) 500 µg/ml of α-hemolysin at 37°C for 48 hours. After that, the cell viability was determined by using 1% crystal violet stain.*

![Fig. 6](image-url)

*Fig. 6: Microscopic examination for the effect of α-hemolysin on colon cancer cells (HCT-116). The cells were incubated with (A) 0, (B) 62.5 or (C) 500 µg/ml of α-hemolysin at 37°C for 48 hours. After that, the cell viability was determined by using 1% crystal violet stain.*
In this study, that high concentration of α-toxin (500 µl/ml) was effected on the cell viability of four cells line (HepG-2, HCT-116, MCF-7, A-549) as showed in Table (1). The cell viability of cell lines were represented 31.69% in HepG-2, 36.27% in HCT-116, 78.95% in MCF-7, 67.28% in A-549. From our results, Alpha hemolysin protein had high inhibitory activity against HepG-2 and HCT-116, and had weak inhibitory activity against MCF-7 and A-549. In this connection, Haslinger et al. (2003); Essmann et al. (2003) had reported that S. aureus α-toxin is a pore-forming toxin that can caused apoptosis of tumor cells at higher concentrations. In this study, it was found that the cell viability of MCF-7 was represented 78.95% per 48 hours. Dissimilar results were reported by Swofford et al. (2014) where the cell viability represented 85% in less 1 h of in human breast cancer (MCF-7). In this study, the cell viability of A-549 was represented 67.28%. This result of lung cancer cell line is agreement with Johansson et al. (2008).

Table 1: Crystal violet staining percentage of viability of human cancer cells.

| Cell line                              | Cell viability (%) |
|----------------------------------------|--------------------|
| Human hepatocellular cancer cell line  | 31.69              |
| (HepG-2)                               |                    |
| Human colon cancer cell line (HCT-116) | 36.27              |
| Human breast cancer cell line (MCF-7)  | 78.95              |
| Human lung cancer cell line (A-549)    | 67.28              |

**Conclusions**

Alpha hemolysin protein was isolated from pathogenic S. aureus that was isolated from blood samples of patients (children). This recombinant hia gene was produced alpha hemolysin protein by using competent E. coli as a host. The recombinant α-toxin protein was used as anticancer agent. The production of recombinant α-toxin was very simple process, low cost, high efficiency, non-toxic, high yield and no side effect.

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