Iranian Journal of Fisheries Sciences 19(5) 2258-2273 2020
DOI: 10.22092/ijfs.2020.122373

Determination of the optimal conditions of cloning Aerolysin gene from the common carp pathogen *Aeromonas hydrophila* in *Escherichia coli* BL21

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Received: May 2019 Accepted: July 2019

Abstract

Aeromonas hydrophila is a gram-negative bacterium which associated with gastrointestinal diseases and septicaemia. This pathogenic bacterium has several virulence factors ranging from pili to the excreted protein which called (Aerolysin) with minor and major effects, respectively. Additionally, *Aeromonas hydrophila* is a widely distributed bacterium that commonly causes ulcers in cyprinid fish such as carps and secondary diseases in humans as well. In the present study, characteristics and haemolytic activities of the recombinant Aerolysin protein and optimal conditions for cloning are determined using the synthesized cloning/expression Aerolysin gene, assembled into the *Escherichia coli* BL21 (DE3) through pGEX-6P1 vector, using SDS-PAGE and western blotting techniques. The results declared that, the Aerolysin gene (1482 bp) was cloned by transforming the recombinant pGEX-6P1 vector into *Escherichia coli* BL21 (DE3) as a prokaryotic expression host. The SDS-PAGE results indicated that the estimated protein size was 54 KDa. Recombinant Aerolysin protein synthesis at both selected temperatures, 25°C and 37°C, indicated that 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was the optimum concentration for induction. However, the recombinant protein was unable to synthesize in the absence of IPTG inducer. Western blot analysis indicated the efficient sensitivity and specificity of the recombinant Aerolysin protein. In conclusion, the recombinant protein showed potential advantages for immunoassay approaches in order to decrease the economic losses caused by disease in the aquaculture industry.

Keywords: *Aeromonas hydrophila*, *Escherichia coli* common Carp, Fish disease, Haemolytic activity, Recombinant protein, Aerolysin

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[DOR: 20.1001.1.15622916.2020.19.5.34.4]

Introduction

The genus *Aeromonas* belongs to the gram-negative group of bacteria, characterized by its rod shape and facultative anaerobic and pathogenic properties. Usually, many aquaculture fishes are considerably infected by motile *Aeromonas*, and the term "Motile *Aeromonas septicaemia*" is used to describe motile aeromonas infections of warm water fish, including common carp (*Cyprinus carpio*) (Allen *et al.*, 2010) and (Orozova *et al*., 2018). Also *Aeromonas hydrophila* is among the highly pathogenic bacterium which isolated by previous studies from the fresh water crayfish, *Pacifastacus leniusculus* (Jiravanichpaisal *et al*., 2009). Even in humans, *Aeromonas hydrophila* plays an important role in two major diseases associated with the *Aeromonas* genus such as the common gastroenteritis and wound infections, with or without bacteraemia. Gastroenteritis typically occurs as an indirect infection that occurs after ingestion of carp contaminated by *Aeromonas hydrophila*, but wound infections occur by direct contact with water contaminated by *Aeromonas hydrophila* with open skin injuries (Minnaganti *et al*., 2000). *Aeromonas hydrophila* has many virulence factors, including protease, Aerolysin, and enterotoxin toxin, which cause disease in humans and fish (Zhu *et al*., 2007).

The whole nucleotide sequence of the Aerolysin gene is located on a 1.8-kb ApaI-EcoRI fragment and consists of 1,479 bp that contains an ATG initiation codon and a TAA termination codon (Khan *et al*., 1998). Aerolysin toxin is considered the most common virulence factor due to its involvement in diarrhoeal diseases, deep wound infections and haemorrhagic septicaemia (Igbinosa *et al*., 2017). This toxin is first synthesized by *Aeromonas hydrophila* as an inactive form called pro-aerolysin. Then, the N-terminal signal sequence and 45 amino acids from the C terminus are removed for the final release of the functional folded protein, Aerolysin. A previous study confirmed the presence of the Aerolysin gene using PCR, but they did not determine the characteristics of the Aerolysin protein (Ørmen and Østensvik, 2001).

The present study aimed to investigate an adequate amount of recombinant Aerolysin protein using recombinant cloning to determine the characteristics and haemolytic activities of the Aerolysin toxin. A better characterisation of these effects in farmed fish species will lead to much improved bespoken immunoassay-diagnostic techniques for disease identification in the early phases prior to the onset of severe infection resulting in significant mortality and economic losses.

Materials and methods

Aeromonas hydrophila isolated from infected fishes were collected from the Agriculture College Fish Farm located in Grda-rasha 8 kilometres away from Erbil province Iraq, from October 2016

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to August 2017. Bacterial specimens were taken from the skin of the infected fishes using sterile swabs, and transferred to the laboratory at Salahaddin University, Science College Biology Department in the capital city of Erbil for future processing. The LB broths were incubated at 37°C for 24-48 hours after detecting positive turbidity. The samples were inoculated on LB agar and blood agar plates at 37°C for 24-48 hours, and the plates were then examined for colony morphology parameters (Colour, size and margins pattern).

The polymerase chain reaction (PCR) and primers

Bacterial DNA was extracted after incubation at 37°C in LB broth by using Jena Bioscience DNA Extraction Kit (Germany) according to the manufacturer's instructions with some modification. Primers were designed using DNA sequences for PCR to obtain an adequate amount of Aerolysin gene for recombinant plasmid construction.

The sequences of forward and reverse primers were as following:

Aero-F: 5'CGCGGATCCGGCTTGTCATTGATCATATCC 3' and
Aero-R: 5'CCGCTCGAGTTATTGATTGGCAGCTGGC 3', respectively.

The Aerolysin gene of *Aeromonas hydrophila* was amplified by PCR. The 50 µl reaction mixture consisted of 25 µl of master mix, 1 µl of each primer, 20 µl of ddH_2O and 3 µl (30 ng) of bacterial genomic DNA. Amplification conditions included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, annealing at 65°C for 30 sec, and 72°C for 1 min with a final extension at 72°C for 4 min (Chacon *et al*., 2003).

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using 1-2% agarose gels to analyse DNA according to its molecular weight mobility. Each PCR product and recombinant plasmid sample (6 µl) was mixed with 2 µl of loading dye and 4 µl of SYBR® Safe™ DNA Gel Stain (Life Technologies, UK) per 100 mL of agarose and run using 1× TAE buffer at 45 V for 15 min and then 90 V for 35-45 min. The size of DNA fragments in the tested samples was evaluated using the GeneRuler 1 kb DNA ladder (Quick-Load®,UK). The bands were visualized using a UV lamp at 365 nm, and images were then captured by a Canon D100 (Canon Co., Japan).

Construction of the pGEX-6P1-Aerolysin plasmid

Construction of recombinant plasmids was performed by restriction enzymes and ligation. PCR products and plasmid pGEX-6P1 vectors were treated with two restriction enzymes including BamHI and XhoI and the Ligation Kit was used according to the manufacturer's instructions (Fig. 1; Tables 1and 2).

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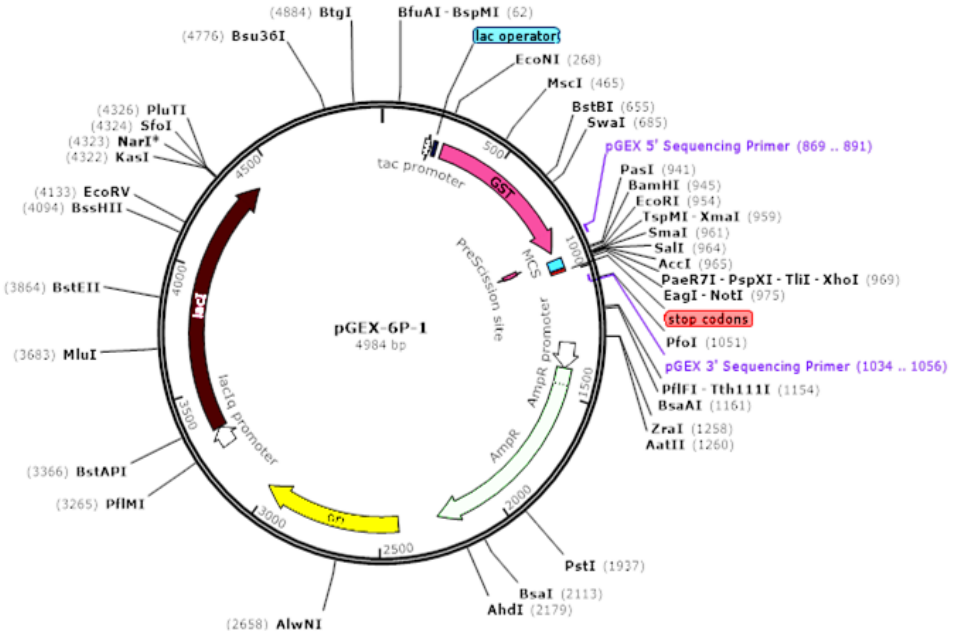

Figure 1: Map of the plasmid vector pGEX-6P1; the map was created with the SnapGene program. (www.snapgene.com)

Table 1: Utilized restriction enzymes.

Name of restriction enzyme	Supplier	Volumes
BamHI	Fermentas	concentration 10 U μl^{-1}
XhoI	Fermentas	concentration 10 U μl^{-1}
10× TangoTM buffer	Fermentas	This buffer is comprised of 33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, and 0.1 mg ml^{-1} BSA

BamHI Resrtiction enzyme, * XhoI Resrtiction enzyme, *10× Tango^{TM} buffer used with restriction enzymes for remaining pH (pH 7.9).

Table 2: Utilized DNA ligation components.

Components	Brands	Volumes
T4 DNA ligase	Fermentas	concentration 5 U μl^{-1}
10× DNA ligation buffer	Fermentas	Composed of 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, and 5 mM ATP (pH 7.4)
Nuclease-free water	Fermentas	

T4 DNA ligase enzyme, *10× DNA ligation buffer used with restriction enzymes for remaining pH at (pH 7.4)., * Nuclease-free water from Fermentas.

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Plasmid Vector pGEX-6P1 included the following components and characteristics

Each digestion reaction was set up in a total volume of 20 µl, which consisted of 5 µl of PCR product or plasmid DNA, 1 µl of BamHI, 1 µl of XhoI, 2 µl of Tango buffer, and ddH2O up to 20 µl, and then incubated at 37°C for 16 hours (Tillett and Neilan, 1999). Then, the enzymes were inactivated at 65ºC for 10 min, and the mixture was used for ligation (Fig. 2).

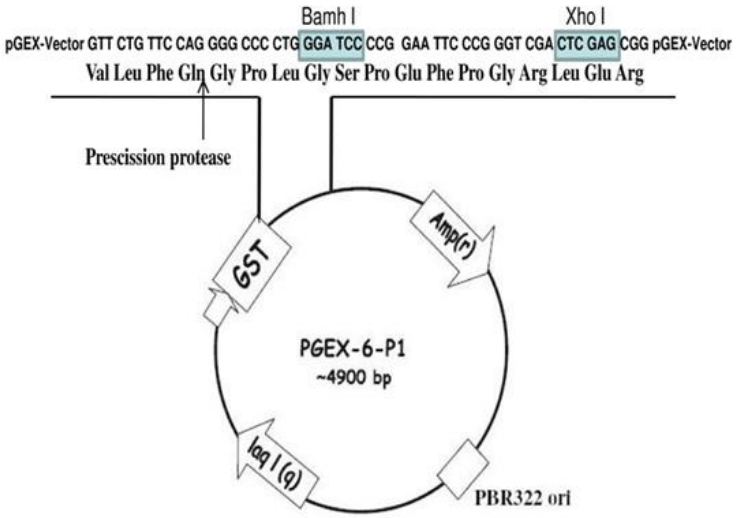

Figure 2: Map of the plasmid vector pGEX-6P1 showing the restriction sites by RE.

The ligation of the insert and vector was carried out in the smallest volume possible, typically 10 µl containing 2 µl of vector, 1 µl of insert (at a 6:1 insert to vector molar ratio), 1 µl of T4 DNA ligase, 2 µl of 10× reaction buffer, and 4 µl of ddH_2O. The reaction was allowed to incubate at room temperature (approximately 21°C) for 4 hours and then transferred into competent bacterial cells (Aslanidis and De Jong, 1990).

Transformation of recombinant pGEX-6P1-Aerolysin into Escherichia coli DH5α

Recombinant pGEX-6P1-Aerolysin was transformed into competent *Escherichia coli* DH5α using a heat-shock approach. Two hundred microliters of *Escherichia coli* DH5α was thawed on ice for 10 min, and 3 µl of the ligation reaction was added to the cells, which was mixed gently by stirring using a pipette tip. The mixture was incubated on ice for 30 min, and the cells were heat-shocked in a 45°C water bath for 90 seconds. The cells were then immediately transferred onto ice for 10 min. Then, 500 µl of LB broth was added, and the cell suspension was incubated at 37°C for 1 hour while shaking at 250 rpm. Finally, 100 µl of the cell suspension was plated onto LB agar supplemented with 100 µg $µl^{-1}$

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ampicillins and incubated at 37°C overnight (Froger and Hall, 2007).

Screening of successful pGEX-6P1-Aerolysin recombinant clones

The constructed plasmid containing the target Aerolysin gene was sequenced in order to confirm the pGEX-6P1-Aerolysin recombinant clones, and double restriction digestions were carried out to confirm the presence of the Aerolysin gene in the new prokaryotic host *Escherichia coli* DH5α that is summarized in Table 2.

Expression, solubility and purification of recombinant pGEX-6P1-Aerolysin

The expression of recombinant Aerolysin recombinant in *Escherichia coli* BL21 (DE3) was performed, as *Escherichia coli* BL21 (DE3) competent cells contain the T7 lysozyme gene in their genomic DNA. T7 lysozyme increases the tolerance of *Escherichia coli* cells to protein toxicity. In addition, the bacteria lack both the Ion protease and the outer membrane protease ompT, which degrade the expressed proteins in *Escherichia coli.* Strain BL21 (DE3) is also the most widely used host for target gene expression (Sadeghi *et al*., 2011). Single colonies of competent cells with transformed recombinant plasmid were grown in 10 ml LB medium containing 100 µg μl^{-1} of ampicillin overnight at 37°C. Then, this culture was diluted at a ratio of 1:100 into 100 ml of fresh LB medium and incubated at 150 rpm at 37°C. When OD450 reached 0.5, IPTG was added to the medium to final concentrations of 0.5 mM, 1 mM, and 2 mM. These media were further incubated at 25ºC or 37°C for 3 hours. The cell pellet was harvested by centrifugation at 5000 rpm for 10 min at 4°C, and the best conditions and solubility for pGEX-6P1-Aerolysin expression were detected by SDS-polyacrylamide gel electrophoresis (Peti and Page, 2007).

Solubility determination of the GST (glutathione S-transferase) fusion protein by SDS-PAGE was performed in suspended whole cells in 50 mL of 1×binding buffer with a pH of approximately 8, which contained 0.5 M NaCl and 20 mM Tris-HCl, that was disrupted by sonication on ice. The lysate was separated by centrifugation into soluble fractions (supernatant) or insoluble fractions (pellet) and run separately for solubility screening of the recombinant protein using SDS-PAGE.

Affinity chromatography resin

All steps of purification by affinity chromatography using an immobilized glutathione Sepharose column was carried out at 4°C to prevent proteolysis. The 2.5 × 8 cm column size was prepared by washing with double-deionized, distilled water. Pellets of *Escherichia coli* cells were re-suspended in 15 ml of cold lysis buffer after sonication on ice, and the lysate was then centrifuged at 48,000×g for 20 min at 4°C. The pellets were re-suspended in 15 mL PBS buffer using a

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double homogenizer to obtain recombinant protein for loading on the equilibrated glutathione Sepharose column. The protocol included a flow rate of 0.1 ml min^{-1}, followed by a washing step with 10 bed volumes of PBS/EDTA at a flow rate of 1.5 ml min^{-1}, elution of proteins with a GST tag, and storage at 4°C. The protein should be ~90% pure at this stage (Wu *et al*., 2017).

Size exclusion chromatography (gel filtration)

Using an Äkta FPLC system (Pharmacia) final purification of Aerolysin was accomplished by gel filtration on Superdex-75 (HighLoad 16/60 gel filtration column) 120 ml column pre-equilibrated with 50mM Tris-HCl, pH 8.0, at a flow rate of 0.5 ml min^{-1}. Care was taken to not let the pressure exceed 0.5 MPa. Individual and pooled fractions were analysed using SDS-PAGE.

Evaluation of biological haemolytic activity

The agar diffusion haemolysis test (ADHT) is an effective way to evaluate the biological haemolytic activity of the target cloned Aerolysin gene after the affinity chromatography resin technique of recombinant protein purification. Blood agar plates contained 3% sheep red blood cells, 1.5% agar, 0.8% NaCl, 0.02% KCl and 25 mM Tris (pH 7.4) were sterilized at 1.034×10^5 Pa for 15 min. The solution was mixed and poured into a petri dish at a temperature of approximately 35°C after solidification. A hole was created in the centre of the plate with a sterilized 1 µl micropipette tip. Then, 50 µl of soluble recombinant protein in 1×elution buffer and 50 µl of denatured recombinant protein in 1×elution buffer containing 6 M urea were added to the holes. Fifty microliters of 1×elution buffer and 1× elution buffer with 6 M urea were used as negative controls. Finally, the haemolysis on each dish was visualized and scanned after incubating at 37°C for 24 hours.

Western blot analysis

Antibodies against recombinant Aerolysin were collected from the sera of sheep after 40 days of injections with 300 µg of recombinant Aerolysin. Serum was collected and stored as aliquots at -20°C. Polyclonal antibodies against the WCs was prepared by using injected formalin-killed WCs and the aliquots stored at -20°C (Chervyakova *et al.*, 2016).

Results

For expression of the Aerolysin gene, the whole coding region of the Aerolysin gene (1482 bp) was isolated and amplified from native bacterial cells (*Aeromonas hydrophila*) by a conventional PCR system including designed oligonucleotide primers for this gene. Forward and reverse primers were designed with restriction site overhangs at the 5’ and 3’ ends (Fig. 3).

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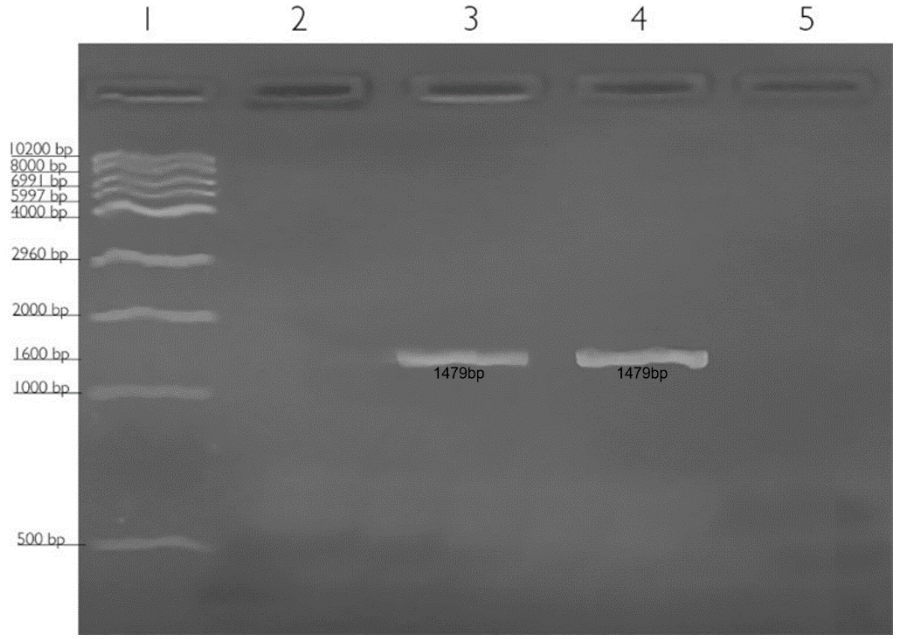

Figure 3: PCR products from cloning of aerolysin gene analysed using agarose gel electrophoresis (2%) under UV light. Lane 1: Ladder; Lane 2: Negative control; Lane 3: Positive control; Lane 4: Positive sample; Lane 5: Negative sample.

Amplicon segments inserted into the multiple cloning sites (MCS) of the pGEX-6P1 vector generated the expression plasmid pGEX-6P1-Aerolysin, which was subjected to double restriction digestion (BamHI and XhoI), to confirm the presence of the aArolysin gene. Agarose gel electrophoresis detected the size of the uncut extracted pGEX-6P1-Aerolysin plasmid, which was approximately 6400 bp, and confirmed the presence of the Aerolysin gene in the constructed plasmid after sequencing and double digestion (Fig. 4). The resulting sequences were aligned with sequences of the Aerolysin gene using Geneious 5.0.3 software. The results of the alignment showed that the inserted sequences were 100% identical to the synthesized gene, and no mutation or variation in the gene nucleotides occurred during the cloning process.

The expression of truncated recombinant Aerolysin protein in *Escherichia coli* was examined using SDS-PAGE analysis. The results suggested that the recombinant protein was only expressed in competent bacteria with the pGEX-6P1-Aerolysin vector, and determined that the size of the expressed protein was approximately 54 kDa. The SDS-PAGE analysis also determined the best expression conditions regarding temperatures and IPTG concentrations; the results suggested the optimal conditions for expression of pGEX-6P1-Aerolysin in *Escherichia coli* were 37°C at IPTG concentrations of 0.5 mM and 1 mM (Figs. 5 and 6).

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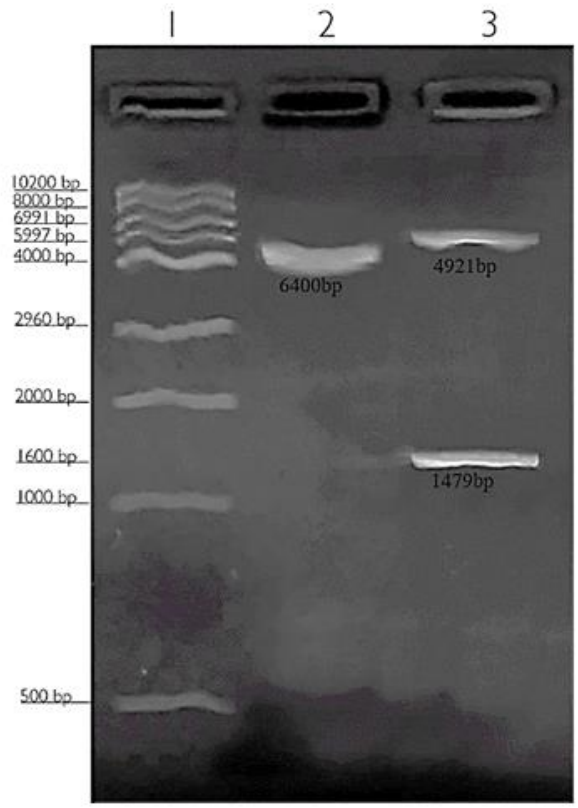

Figure 4: The pGEX-6P1-Aerolysin digestion using agarose gel electrophoresis (1%) under UV light. Lane 1: Ladder, Lane 2: Recombinant plasmid (pGEX-6P1-aerolysin); Lane 3: pGEX-6P1-aerolysin double-digested with BamHI and XhoI.

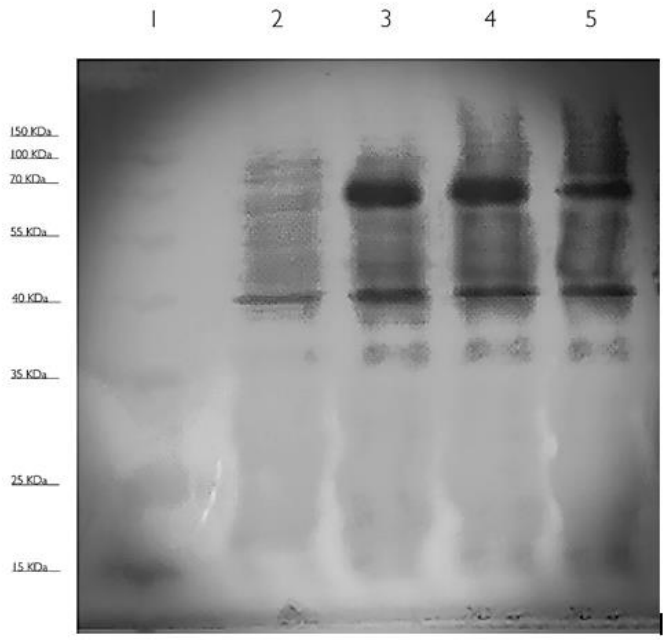

Figure 5: The SDS–PAGE of the expression of the GST-Aerolysin fusion protein at 25°C after 3 hrs of induction by different IPTG concentration. Lane 1: Marker; Lane 2: 0 mM IPTG; Lane 3: 0.5 mM IPTG; Lane 4: 1 mM IPTG; Lane 5: 2 mM IPTG.

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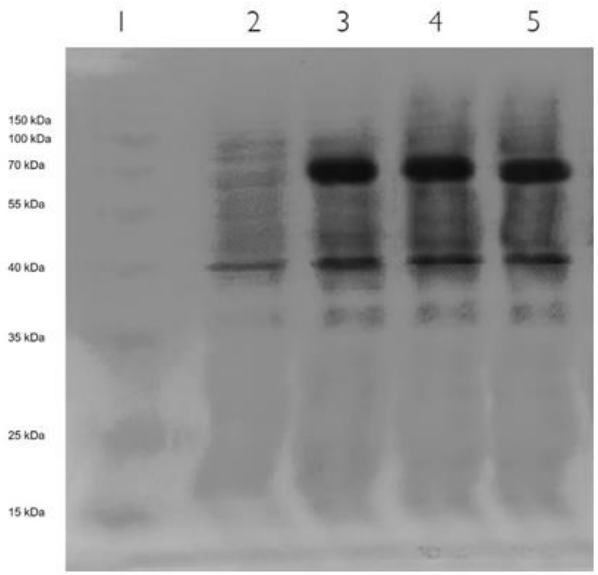

Figure 6: The SDS–PAGE of the expression of the GST-Aerolysin fusion protein at 37°C after 3 hrs of induction by different IPTG concentration. Lane 1: Marker; Lane 2: 0 mM IPTG; Lane 3: 0.5 mM IPTG; Lane 4: 1 mM IPTG; Lane 5: 2 mM IPTG.

Haemolytic activity of recombinant Aerolysin

The expression protein was purified by affinity chromatography (Fig. 7), and haemolytic activity was tested after purification of the cloned Aerolysin protein in its active form (fully folded structure). The soluble recombinant Aerolysin protein showed weak haemolytic activity by producing a haemolytic zone around the hole in the agar, while the negative control indicated no haemolytic zone area.

Western blot analysis

The antigenicity of recombinant Aerolysin from *Escherichia coli* BL21 was demonstrated via western blotting using laboratory sheep antisera against whole proteins isolated from *Escherichia coli*. The results showed the clear presence of Aerolysin protein, as shown in Fig. 8.

Discussion

Although, DNA cloning has been reported for many *Aeromonas hydrophila* genes in *Escherichia coli*, few studies of active Aerolysin protein production in *Escherichia coli* have been reported (Dong *et al*., 2017).

Production of any recombinant protein in a heterologous host cell or organism is a complex multi-step process and is often a problematic issue. Protein expression levels can be low, or the purified protein may be insoluble or unstable. To generate large amounts of recombinant proteins, optimal conditions must be determined, which requires selection of an appropriate expression vector and expression host (Hannig and Makrides, 1998).

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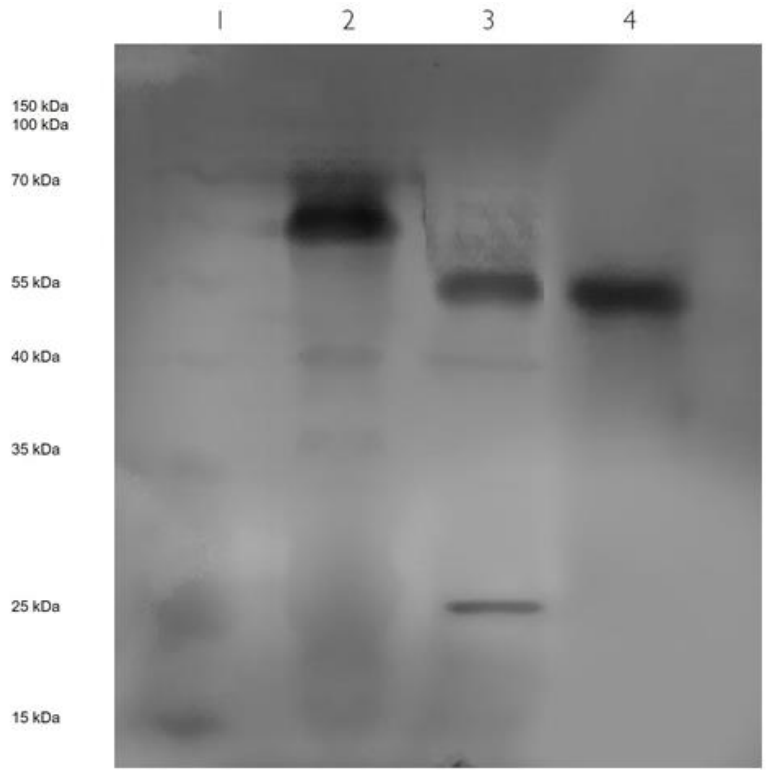

Figure 7: GST-Aerolysin Purification and digestion with PreScission. Lane 1: Marker, Lane 2: GST-Aerolysin eluted from a glutathione-Sepharose, Lane 3: Cleaved GST-Aerolysin, Lane 4: Aerolysin purified by gel filtration on a Superdex 75 column.

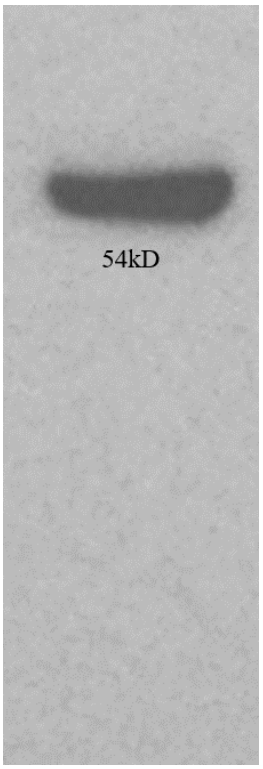

Figure 8: SDS-PAGE western blotting showing the presence of the recombinant aerolysin protein.

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Recombinant protein expression is largely host cell-dependent, and several expression conditions such as selected vector, expression host, composition of the growth medium, incubation temperature and incubation time are also involved. Moreover, the nature and source of the protein encoding sequence also play an important role in the level of product expression (Beckwith *et al*., 2018).

Protein tagged with GST enables purification of recombinant proteins using affinity chromatography, which is widely used in molecular approaches for determining the characteristics of unknown proteins (Mahajan *et al*., 2012). GST consists of 211 amino acids (26 KDa), in which the DNA sequence is often integrated into expression PGEX-6P1 vector for production of recombinant proteins. The result of expression in vector is a GST-tagged fusion protein, in which the functional GST protein is fused to the N terminus of the recombinant protein. The induction of GST yields a greater amount of recombinant protein due to rapid folding of GST into a stable and highly soluble protein upon translation, and GST-tagged fusion proteins can be purified or detected based on the ability of GST to bind its substrate, glutathione (GSH) (Nühse *et al*., 2003).

According to this study, *Escherichia coli* BL21 (DE3) was used as the expression host for the production of Aerolysin, and the pGEX-6P1 vector system has several advantages for the expression of the Aerolysin gene. Consistent with previous studies, pGEX-6P1 is one of the most powerful systems developed for the expression of recombinant proteins in *Escherichia coli*. Strain BL21 (DE3) is also the most widely used host for target gene expression (Sørensen and Mortensen, 2005).

Constructed vectors are transformed into *Escherichia coli* BL21 (DE3)-competent cells, which contain the T7 lysozyme gene in their genomic DNA. T7 lysozyme increases the tolerance of *Escherichia coli* cells to protein toxicity. In addition, the bacteria lack both the Ion protease and the outer membrane protease ompT, which degrade expressed protein (Studier, 2018).

In comparison with another study, optimization condition demonstrated some similarity, as temperature and IPTG concentration both showed a positive effect on the expression of the protein (Guan and Dixon, 1991).

The concentration of IPTG dramatically influences the induction of lac repressor-regulated promoters. Regarding some proteins, it is important to slowly induce transcription of the expression plasmid with lower IPTG concentrations, while for some others, production of high amounts of protein is desired (Sadeghi *et al*., 2011). In this study, the effect of different concentrations of IPTG on Aerolysin gene expression was examined, and 1 mM was selected as the optimum concentration at both 25°C and 37°C.

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In agreement with previous studies, the maximum amount of Aerolysin recombinant protein production was obtained by incubation at 37°C, and another study confirmed that 37°C is the best temperature for heterologous protein expression in *Escherichia coli* BL21 (Kim *et al*., 2003). The active form of a recombinant protein is required for various applications, such as diagnosis of PNH, and for progress of vaccine applications (Kaur *et al*., 2018). The recombinant Aerolysin protein showed weak haemolytic activity by producing a tiny haemolytic zone that may be due to the optimized incubation at the temperature of 37°C. However, other studies indicated 25°C as the best incubation temperature for evaluation of the haemolytic activity of recombinant Aerolysin protein. This preliminary method successfully generated a viable protein cloned and expressed in a microbial vector (*E. coli*) and implicated in the pathology of an important farmed fish species such as carp. The technique can serve as a basis for the molecular based identification of the pathogen and shows considerable potential for more advanced applications when refined further. Optimisation will require more attention to the haemolytic and functional characteristics of the gene expression in *E. coli* with regard to post-translational modification of the protein in this organism. In this respect, in may be interesting to compare the proteome of Aerolysin from *Aeromonas hydrophila* obtained from infected carp, and the protein generated by the recombinant technique in *E. coli*. Gene expression may not translate equivocally into counterparts with exact metabolic and physiological effects in the host organisms. Further work could be undertaken with various challenge tests in carp and other fish species to examine the efficacy of the recombinant Aerolysin via the diet, aqueous exposure or by IP (Intra-Peritoneal) injection routes to elucidate the efficacy of virulence and toxicity under simulated farm conditions.

In conclusion, the Aerolysin gene was successfully isolated from *Aeromonas hydrophila* and cloned into the expression host *Escherichia coli* BL21. The active form of recombinant Aerolysin with the removed GST tag was expressed in an adequate amount. The recombinant Aerolysin protein demonstrated weak haemolytic activity, and western blotting was used to determine the antigenicity of the recombinant Aerolysin protein. This technique provides a novel platform for continued research and development for practical dissemination to the aquaculture industry, and with implications for disease prevention and control as well as reducing our dependency on antibiotics through superior management.

Acknowledgements

The authors are grateful to Dr. Kareem Kharabayee (PhD, Veterinary) Salahaddin University- College of Science- Biology Department for assisting in the revision of this manuscript. The authors are also

[DOR: 20.1001.1.15622916.2020.19.5.34.4]

thankful to Dr. Abbas Salihi (PhD, Physiologist) Salahaddin University-College of Science- Biology department, for providing timely suggestions and valuable guidance.

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