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Biochemical and immunological characterization of haemolysin produced by *Pseudomonas aeruginosa* PAO1 isolated from burn wounds

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Abstract:

Background: Infection of burn wounds by multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) is a leading cause of morbidity and mortality and remains one of the most challenging concerns for the burns unit. The aim of this study is purify and characterize the haemolysin produced by multidrug resistant *P. aeruginosa* PAO1 isolated from burn wounds.
Methods: Isolation and identification of *P. aeruginosa* from burns was done by standard bacteriological methods. *P. aeruginosa* PAO1 was identified by PCR amplification and sequencing of the 16S rRNA gene. The haemolysin of *P. aeruginosa* PAO1 was purified by 70% ammonium sulphate precipitation followed by gel filtration on Sephadex G-100, and separation by SDS-Poly Acrylamide Gel Electrophoresis. *In vivo* toxicity of the purified haemolysin was determined by intraperitoneal injection of Swiss albino mice, and *in vitro* toxin-antitoxin neutralization test was performed as previously described.
Results: The pure haemolysin had a molecular weight of 37 kDa, with maximum activity at 25°C for 30 minutes and stable within pH range of 4-9 (maximum activity at pH 7). The haemolysin was activated by Ca^{2+}, Fe^{3+} and Cu^{2+}. Intraperitoneal injection of mice with 0.5ml of haemolysin (128 HU/ml) caused 100% mortality while 0.5 and 0.1 ml of haemolytic titer (64 HU/ml) of the heated haemolysin (toxoid) caused 50% and 0% mortality respectively. *In vitro* toxin-antitoxin neutralization test revealed that anti-haemolysin antitoxin was present in the serum of the mice that were previously vaccinated with heated toxin.
Conclusion: This study concluded that haemolysin can be a potential vaccine component for prevention of haemolysis caused by multidrug resistant *P. aeruginosa* in burn patients.

Keywords: haemolysin, *Pseudomonas aeruginosa*, multidrug resistant organism

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Caractérisation biochimique et immunologique de l'hémolysine produite par *Pseudomonas aeruginosa* PAO1 isolée de brûlures

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Abstrait:

Contexte: L'infection des plaies par brûlures par *Pseudomonas aeruginosa* (*P. aeruginosa*) multirésistante (MDR) est l'une des principales causes de morbidité et de mortalité et demeure l'une des préoccupations les

plus difficiles pour l'unité des brûlures. Le but de cette étude est de purifier et de caractériser l'hémolysine produite par *P. aeruginosa* PAO1 multirésistante isolée de brûlures.
Méthodes: L'isolement et l'identification de *P. aeruginosa* des brûlures ont été effectués par des méthodes bactériologiques standard. *P. aeruginosa* PAO1 a été identifié par amplification par PCR et séquençage du gène d'ARNr 16S. L'hémolysine de *P. aeruginosa* PAO1 a été purifiée par une précipitation au sulfate d'ammonium à 70% suivie d'une filtration sur gel sur Sephadex G-100 et d'une séparation sur électrophorèse par gel SDS-Poly Acrylamide. La toxicité *in vivo* de l'hémolysine purifiée a été déterminée par injection intrapéritonéale de souris albinos suisses et un test de neutralisation *in vitro* toxine-antitoxine a été effectué comme décrit précédemment.
Résultats: L'hémolysine pure avait un poids moléculaire de 37 kDa, avec une activité maximale à 25°C pendant 30 minutes et stable dans une plage de pH de 4 à 9 (activité maximale à pH 7). L'hémolysine a été activée par Ca^{2+}, Fe^{3+} et Cu^{2+}. L'injection intrapéritonéale de souris avec 0,5 ml d'hémolysine (128 HU/ml) a causé une mortalité de 100% tandis que 0,5 et 0,1 ml de titre hémolytique (64 HU/ml) de l'hémolysine chauffée (anatoxine) ont causé respectivement 50% et 0% de mortalité. Un test de neutralisation *in vitro* toxine-antitoxine a révélé que l'antitoxine anti-hémolysine était présente dans le sérum des souris préalablement vaccinées avec de la toxine chauffée.
Conclusion: Cette étude a conclu que l'hémolysine peut être un composant vaccinal potentiel pour la prévention de l'hémolyse causée par *P. aeruginosa* multirésistante chez les patients brûlés.

Mots-clés: hémolysine, *Pseudomonas aeruginosa*, organisme multirésistant

Introduction:

Infection in burn patients is a leading cause of morbidity and mortality and remains one of the most challenging concerns for the burns unit (1). Multidrug-resistant (MDR) *Pseudomonas aeruginosa* (*P. aeruginosa*) is an emerging cause of mortality and morbidity in burn patients, which is estimated to cause 4-60% of nosocomial infections in different parts of the world (2).

Numerous *P. aeruginosa* virulence factors contribute to the pathogenesis of wound infections in patients with burns. Pili and flagella are essential for the organism ability to persist in burns wound and cause disseminated infections (3). *P. aeruginosa* elaborate many toxic products and enzymes such as catalase, lipase, lecithinase, elastase, proteases and haemolysins, which enable the organism to invade and destroy the host tissues (4-6). In particular, haemolysin contributes to virulence of *P. aeruginosa*, and decreased haemolytic activity has been associated with decrease virulence (7).

The aim of current study is to purify and characterize the haemolysin produced by MDR *P. aeruginosa* PAO1 isolated from burn patients as a preliminary step to producing toxoid from it.

Materials and method:

Study setting

This study was conducted in the Medical Microbiology and Immunology Department, Faculty of Medicine, and Botany Department Faculty of Science, Zagazig University, Egypt. Approval for performing the study was obtained from Institutional Review Board of Faculty of Medicine, Zagazig University. Consent was obtained from each patient enrolled in the study. All experiments were carried out in compliance with the relevant laws and guidelines.

Collection of specimens

Forty five swab specimens were collected using sterile cotton swabs from patients with burn wound infections in Central Hehia Hospital, Hehia city, El-Sharkia, Egypt during the period July to September 2013. All samples were transported to the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, within two hours of collection.

Isolation and identification of *Pseudomonas aeruginosa*

The swabs were cultured on nutrient, MacConkey and blood agar plates, and incubated aerobically at 37°C for 24 hours. Colonies were identified as *P. aeruginosa* on culture plates using standard bacteriological methods (8). Antibiotic susceptibility of *P. aeruginosa* isolates was determined by the CLSI disk diffusion method (9). The identity of the MDR *P. aeruginosa* was confirmed by PCR amplification and sequencing of the 16S rRNA gene at the Sequence Unit, Sigma Company, Giza, Egypt as *P. aeruginosa* PAO1 (NR 074828 1).

Determination of haemolysin level

The haemolysin level of *P. aeruginosa* PAO1 (NR 074828 1) was determined by the modified disk diffusion method (10). The overnight broth culture of the isolates was diluted in sterile saline to 0.5 McFarland standards. Sterile filter paper disk was immersed in each bacterial suspension to absorb 0.01ml. The disks were placed on the surface of blood agar medium and incubated overnight at 37°C. Diameters of haemolysis (H) and growth (G) zones were measured and the H/G ratio was determined.

Haemolysin purification

P. aeruginosa PAO1 (NR 074828 1) was grown in nutrient broth medium at optimum conditions for maximum haemolysin produc-

tion. The medium was optimized in presence of glucose as carbon source and yeast extract as nitrogen source at pH 7.0 for 2 days. The culture supernatant was mixed with 70% ammonium sulphate concentration, with constant stirring at 4°C overnight. The precipitate was then separated by centrifugation at 4000 rpm for 20 minutes and re-suspended in small amount of phosphate buffer (pH 7.0). The re-suspended pellets were applied to a Sephdex G-100 column pre-equilibrated with the same buffer. The active fractions were collected, pooled and concentrated by dialysis against the same buffer. For each fraction, protein content and haemolytic activity were determined by methods previously described (11,12).

Haemolytic titer

The titer of *P. aeruginosa* PAO1 haemolysin was determined using two fold dilution method (13). 0.5ml of haemolysin of was diluted by two fold serial dilutions in 0.5ml phosphate buffered saline (pH 7.4). 0.5ml of 1% suspension of red cells was added to tubes containing the dilutions of the haemolysin. The final readings were made visually after 2hours of incubation at 37°C in a water bath. The reciprocal of the highest dilution showing complete haemolysis was taken as the number of haemolytic units per ml (HU/ml) present in the preparations

Determination of molecular weight

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the purity and molecular weight of the protein, as described by Laemmli (14) using 12% separating acrylamide gel.

Amino acid analysis

Amino acid composition of the purified protein was determined by applying the GLC hydrolytic technique (15) with a Beckman amino acid analyzer system (Sykam-S 7130 Amino Acids, Reagent Orgaizer).

Biochemical properties of purified haemolysin

Thermal stability of the haemolysin was studied after pre-incubation at various temperature (25, 37, 45, 55 and75°C) using 0.01M phosphate buffer (pH 7.0) for different periods of time (30, 60, 90 and 120 minutes). A 1% RBCs suspension was added to the haemolysin (0.5 ml/0.5 ml v/v). The thermal inactivation rate (Kr min) was described by the first-order kinetic model (16)**;** Ln (A_t/A_0) k_tT, where A_o and A_t are the specific activity at zero and 't' time respectively. The $T_{1/2}$ (time at which the haemolysin loses 50% of its activity) was calculated from the linear equation for each temperature. The temperature at which the haemolysin loses 50% of its activity (Tm) was calculated from the linear equation of different pre-incubation temperature at 60 minutes.

The stability of the haemolysin was examined after pre-incubation for 30 minutes at different pH values (4, 5, 6, 7, 8 and 9). Acetate (0.2M), phosphate (0.2M) and Tris HCl (0.2M) buffers were used to create pH range 4-5, 6-7 and 8-9 respectively. After adding 1% RBCs suspension and incubating at 37°C for 2 hours, the haemolytic activity was determined for each pH

To assay the metal ions effect, the purified haemolysin was pre-incubated in 1mM EDTA at 37°C for 10 minutes and then dialyzed against 0.01M phosphate buffer (pH 7.0). The haemolysin was incubated with each metal ion separately for 30 minutes before adding 1% RBCs suspension. Metal ions in form of FeSO4, $CuSO_4$, $MgSO_4$, $ZnSO_4$, $CaCO_3$, Iodine (5mM) as well as EDTA (1 and 5mM) were used. The haemolytic activity was determined after incubation at 37°C for 2 hours.

Mice toxicity of fresh and heated haemolysin

The purified haemolysin was heated for 15minutes in a boiling water bath to prepare the toxoid (17). The haemolytic titer of the fresh (toxin) and heated (toxoid) haemolysin was determined as described previously (13). Six groups of four male Swiss albino mice (6-8 weeks old) were injected intra-peritoneally with doses of 0.5, 0.3 and 0.1ml of the purified toxin (groups A, B and C) and toxoid (groups D, E and F). The toxicity reflected by the mortality rate in each group was measured using the following equation (18);

$$\text{Mortality rate} = \frac{\text{Number of animals that died}}{\text{Total number of animals per group}}$$

***In vitro* toxin-anti-toxin neutralization test**

Toxin neutralization test was performed to detect the presence of serum antibodies that can neutralize the haemolytic properties of the haemolysin. Blood sample was obtained from five Swiss albino mice previously immunized by repeatedly injecting them with 0.1ml toxoid after which their sera were separated. Fifty, 45, 40, 35 and 30µL of serum from the immunized mice were added to 0, 5, 10, 15 and 20µL of haemolysin respectively. 25µL of 1% RBC suspension was added to each tube and incubated at 37°C for 6 hours. All tubes were centrifuged for 2 minutes and the supernatants were examined for haemolysis. The last tube with no haemolysis was the endpoint of the test (19), which contains the least concentration of the antibody that neutralizes the haemolysin.

Results:

Purification of haemolysin

As shown in Table 1, the haemolytic activity was 32, 64 and 128 HU/ml for the crude broth culture filtrate, after precipitation with 70% ammonium sulphate and following gel filtration chromatography on Sephadex G-100 respectively. Compared to the crude culture broth filtrate, the haemolysin was more purified with purification factor of 29.7 and 10.7 fold using chromatography and 70% ammonium sulphate precipitation resp-ectively. The haemolysin was purified as an extracellular enzyme from the liquid cultures of *P. aeruginosa* PAO1 growing in broth medium (pH7). The purification profile of tested haemolysin using sephadex-G100 showed a sharp peak containing the active haemolysin in fractions 9 to 11 with specific activity 196.9 Umg^{-1} proteins compared to 6.6 Umg^{-1} of the crude protein (Table 1).

Molecular weight of purified haemolysin

Fig 1 shows the SDS-PAGE of the purified haemolysin of *P. aeruginosa* with one major band of approximately 37 KDa.

Amino acids of purified haemolysin

As shown in Table 2, the haemolysin was composed of 40.2% neutral amino acids (glycine, valine, leucine, isoleucine and alanine), 20.7% acidic amino acids (glutamic acid and aspartic acid), 19.7% basic amino acids (lysine and arginine), and 60.9% oxy amino acids (serine, threonine and proline). The haemolysin has high concentration of glycine (26.7%) among other detected amino acids.

Table 1: Purification of the haemolysin from *Pseudomonas aeruginosa* PAO1 using different methods

Purification step	Haemolytic activity (HU/ml)	Protein (mg/ml)	Specific activity (HU/mg protein)	Purification factor (folds)	% Recovery
Culture broth (crude)	32	4.83	6.62	1.0	100
70% ammonium sulphate	64	1.12	70.90	10.7	20.9
Sephadex G-100	128	0.65	196.92	29.7	15.6

HU = haemolytic units

Table 2: Amino acids composition of *P. aeruginosa* PAO1 haemolysin

Amino acid name	Concentration (mg/100ml)	Ratio (%)
Aspartic	2.32	5.76
Threonine	1.21	3.00
Serine	1.31	3.25
Glutamic	6.00	14.92
Proline	5.42	13.47
Glycine	10.74	26.7
Alanine	1.48	3.68
Valine	1.30	3.23
Isoleucine	0.94	2.33
Leucine	1.72	4.27
Histidine	2.54	6.31
Lysine	2.81	6.98
Arginine	2.42	6.01

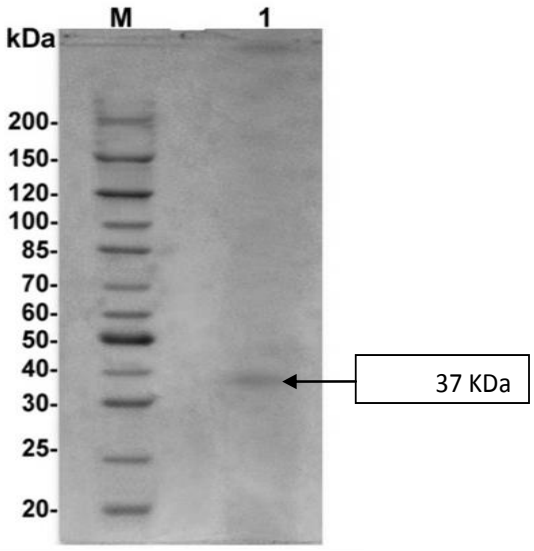

Fig.1: SDS-PAGE profile of purified haemolysin of *Pseudomonas aeruginosa* PAO1 shows that molecular weight of the purified enzyme was 37 KDa (Lane 1) when compared to the protein markers (Lane M)

Table 3: Biochemical characters of *Pseudomonas aeruginosa* haemolysin

Temperature (°C)	**Time (minute)**	**Hemolytic activity (HU/ml)**	**Relative activity %**
25	30	127.4	100
	60	120.9	94.8
	90	115.6	90.7
	120	112.8	88.5
37	30	107.4	84.3
	60	104.9	82.3
	90	95.4	74.8
	120	93.9	73.7
45	30	87.3	68.5
	60	85.8	67.3
	90	84.7	66.4
	120	83.0	65.1
55	30	75.9	59.6
	60	68.1	53.4
	90	64.0	50.2
	120	63.1	49.5
70	30	55.3	43.4
	60	53.7	42.1
	90	52.8	41.4
	120	48.9	38.4

Temperature (°C)	**$T_{½}$ (min)***	**Kr min^{-1}****	**Tm*** (°C)**
25	196.96	0.162	61
37	230.78	0.145	
45	129.41	0.305	
55	109.79	0.379	
70	85.06	0.499	

Effect of pH on haemolysin activity		**Effect of ions on haemolysin activity**	
pH values	Hemolytic activity (HU/ml)	Metal ion (mM)	Hemolytic activity (HU/ml)
4	112.8 ± 0.1	Fe^{3+} (5 mM)	126.1 ± 0.2
5	114.5 ± 0.4	Cu^{2+} (5 mm)	120.7 ± 0.7
6	116.8 ± 0.1	Ca^{2+} (5 mm)	135.1 ± 0.2
7	126.7 ± 0.2	Mg^{2+} (5 mM)	82.5 ± 0.6
8	115.6 ± 0.4	Zn^{2+} (5 mM)	74.0 ± 0.3
9	100.3 ± 0.7	Iodine (5 mM)	59.9 ± 1.0
		EDTA (5 mM)	61.3 ± 0.3
		EDTA (1 mM)	104.8 ± 0.8

*Half-life times; **Thermal inactivation rate; *** Half-life temperature

Table 4: *In vivo* toxicity of the fresh and heated haemolysin in mice

Injected material	Hemolytic titre (HU/ml)	Dose (ml)	Mortality of mice after days									
			1	%	2	%	3	%	4	%	5	%
Fresh (Toxin)	128	0.5	0/4	0	1/4	25	2/4	50	3/4	75	4/4	100
		0.3	0/4	0	0/4	0	0/4	0	0/4	0	¾	75
		0.1	0/4	0	0/4	0	0/4	0	0/4	0	2/4	50
Heated (Toxoid)	64	0.5	0/4	0	0/4	0	0/4	0	0/4	0	2/4	50
		0.3	0/4	0	0/4	0	0/4	0	0/4	0	¼	25
		0.1	0/4	0	0/4	0	0/4	0	0/4	0	0/4	0

HU = haemolytic units

Biochemical properties of purified haemolysin

As shown in Table 3, maximum activity of the haemolysin was at 25°C for 30 minutes and decline of activity with increased exposure time. Also increasing temperature decreased enzymatic activity. With regards to the thermal kinetic parameters of the haemolysin, the half-life ($T_{1/2}$) was longest (230.78 min) at 37°C and half-life temperature (Tm) was estimated to be 61°C. The haemolysin was approximately stable within pH range of 4-9 and maximum activity was at pH 7.0. The activity of the haemolysin was increased by the divalent ions Ca^{2+} followed by $Fe2^{+}$, Cu^{2+} and 1mM EDTA in that order. The haemolysin was inhibited in the presence iodine followed by 5mM EDTA, Mg^{2+} and Zn^{2} in that order.

Mice toxicity of fresh and heated haemolysin

As shown in Table 4, intraperitoneal (IP) injection of 0.5, 0.3 and 0.1 ml of fresh toxin (128 HU/ml) caused mortality rate of 100%, 75%, and 50% respectively in the injected mice. The haemolysin lost its toxicity after heating for 15 minutes in boiling water. The unprotected mice survived intraperitoneal (IP) injection of 0.1 ml heated haemolysin. The haemolysin partially lost its toxicity at a dose of 0.5 and 0.3 ml, causing mortality rate of 50% and 25% respectively in the mice. With respect to the *invitro* toxin-antitoxin neutralization test shown in Table 5, three samples (1, 2, 3) showed no haemolysis with different endpoints while the last 2 samples (4, 5) showed haemolysis.

Table 5: *In vitro* toxin-anti-toxin neutralization test

Sample	Haemolysis	Endpoint
1	-ve	35 µl
2	-ve	40 µl
3	-ve	35 µl
4	+ve	--
5	+ve	--

Discussion:

Infection by multidrug resistant *P. aeruginosa* is a big challenge in patients with burn wounds with tendency to development of septicaemia and high risk of death. The organism is naturally resistant to a significant number of antimicrobials, and infections caused by it is therefore associated with high mortality rate because treatment is difficult due to reduced availability of effective antimicrobials (20). Research into novel therapies aside conventional antimicrobials is recommended to combat these multi-drug and sometimes pan-drug resistant organisms (21).

The anti-virulence approach is one of the new strategies to disarm *P. aeruginosa* infective arsenals by inhibiting the expression and activity of its virulence factors. This has the tendency to reduce its invasiveness and avoid emergence of resistance since proliferation is not affected (22). Haemolysin is one of the virulence factors of many organisms. *P. aeruginosa* produces haemolysin to sense and sequester iron from its environment, which also helps in survival of the pathogen by inhibiting host factors (23). Alpha-haemolysin of uropathogenic *Escherichia coli* can induce apoptosis of target host cells including neutrophils, T-lymphocytes and renal cells, which promotes exfoliation of epithelial cells of the bladder, contributing to nephropathogenicity (24).

Cultures of *P. aeruginosa* considered to be of proven virulence have been reported to have higher titers of extracellular haemolysin than cultures of less virulence ones. Intra-corneal injection of purified haemolysin produced extensive corneal opacification with extensive leukocytic infiltration of the tissue (25), and it was suggested that *P. aeruginosa* haemolysin played a role in the pathogenesis by effecting lysis of host cells and/or subcellular organelles, leading to the release of enzymes destructive to corneal tissue (25). Therefore targeting the haemolysin of *P. aeruginosa* can be a new non-antibiotic approach in the therapy of MDR strains of this organism in burn patients.

In this study, we purified, and chemically and immunologically characterized haemolysin of MDR *P. aeruginosa* PAO1 as a preliminary step to producing a vaccine for combating the effect of this virulence factor in burn patients. The haemolysin was more purified following chromatography with a factor of 29.7 fold. The purification profile using sephadex-G100 shows a sharp peak containing the active haemolysin in fractions 9 to 11 with specific activity 196.9 U mg^{-1} proteins compared to 6.6 U mg^{-1} of the crude protein. Our result agrees with another study on *P. florescens* (26) which reported that high performance liquid chromatography (HPLC) gives 32.6 fold increases in haemolysin purification. HPLC increased the purity of haemolysin of *Bacillus mycoides* by 40.8 folds with the assumption that filtration step is the key to the homogeneity observed. HPLC increases the purity of haemolysin because it polymerizes with other impurities (27).

Our study showed that using SDS-PAGE, purified haemolysin of *P. aeruginosa* had one major band with molecular weight of approximately 37 kDa. Amino acid analysis of the haemolysin also showed that it is a hetero polymer. One study estimated the molecular weight of haemolysin produced by *P. aeruginosa* to be 78 kDa using both SDS-PAGE and Sephacryal S-200 column chromatography while another one estimated it to be 76 kDa with high-performance size exclusion chromatography (28). The molecular weight of haemolysins of other organisms reported includes 12 kDa for *Actinobacillus actinomycetemcomitans* (29) and 45 kDa for *Porphyromonas gingivalis* (30). Similarly, extracellular protein showing haemolysin with SDS-PAGE profile of approximately 50 kDa was characterized from a pathogenic *Aeromonas hydrophila* strain An4 isolated from marine catfish (31).

The amino acid composition of the haemolysin makes it a good immunogen (33), which can induce antibody formation. We investigated whether this haemolysin can be converted to toxoid by heating it for 15 minutes in boiling water to prepare the toxoid (17)**.** The lack of haemolysis in some tubes in the *in vitro* toxin-antitoxin neutralization test can be attributed to presence of antitoxin in the serum obtained from a previously immunized (injected with heated

toxin) mice. This serum is believed to contain antitoxin which neutralized the haemolysin. The samples that showed haemolysis may be due to the fact that the titer of the antitoxin was too low to neutralize the toxin (haemolysin). Other toxins of *P. aeruginosa* have been found to be immunogenic and mounted protective immune response in mice. Passive immunization of mice with antitoxin A gamma globulin protected mice against the lethality of intraperitoneal infection with *P. aeruginosa* (34). Pavlovskis et al., (35) and Snell et al., (36) observed that passive immunization of mice with specific antitoxin increased the survival of burned mice infected with toxigenic strains (but not with non-toxigenic strains) compared to control mice. Combining antibiotic therapy (gentamicin) with passive antitoxin therapy significantly improved the survival of the burned infected mice compared to infected mice receiving only antitoxin (37).

Conclusion:

This study concluded that haemolysin of *P. aeruginosa* PAO1 is a protein of 37kDa with 27.7% glycine content. The crude haemolysin has a haemolytic activity of 32 HU/ml and 128 HU/ml after purification with gel filtration on Sephadex G-100, with maximum activity at 25^{o}C for 30 minutes at pH7 with half life temperature ($T_{1/2}$) of 61^{o}C. The haemolysin activity is enhanced by Ca^{2+} and Fe^{3+} and inhibited by iodine and 5mM EDTA. The haemolysin lost its activity by heating for 15 minutes in boiling water and induces neutralizing antibodies in vaccinated mice. It is recommended that haemolysin of *P. aeruginosa* PAO1 be investigated further as a potential component of vaccine for prevention of haemolysis caused by multi-drug resistant strains of *P. aeruginosa* in burn patients.

Conflict of interest:

No conflicts of interest is declared

Authors' contributions:

AAA and WEH were responsible for concept and design of the study. All authors contributed to data collection. AME and EHAH undertook acquisition and interpretation of data and drafting of the manuscript. AAA undertook critical review of the manuscript. All authors agreed to the final draft.

References:

1. Cochran, A., Morris, S.E., Edelman, L.S., et al. Systemic Candida infection in burn patients. Surg Infect (Larchmt). 2002; 3 (4): 367-374.
2. Biswal, I., Arora, B. S., Kasana, D., et al. Incidence of multidrug resistant *Pseudomonas aeruginosa* isolated from burn patients and environment of teaching institution. J Clin Diagn Res. 2014; 8 (5): DC 26-29. doi: 10.7860/JCDR/2014/7483.4383.
3. Gillespie, S. H., and Bamford, K. B. Medical Microbiology and Infection at a Glance. 4th edition, Wiley-Blackwell; 2012.
4. Kolmos, H. J., Thuesen, B., Nielsen, S., et al. Outbreak of infection in a burns unit due to *Pseudomonas aeruginosa* originating from contaminated tubing used for irrigation of patients. J Hosp Infect. 1993; 24: 11–21.
5. Bownik, A., and Siwicki, A. Effects of staphylo coccal haemolysins on the immune system of vertebrates. Centr Eur J Immunol. 2008; 33 (2): 87-90.
6. Schaber, J. A., Carty, N. L., MacDonald, N.A., et al. Analysis of quorum sensing-deficient isolates of *Pseudomonas aeruginosa*. J Med Microbiol. 2004; 53: 841-853.
7. Attila, C., Ueda, A., Cirillo, S. L., et al. *Pseudo monas aeruginosa* PAO1 virulence factors and poplar tree response in the rhizosphere. Microb Biotechnol. 2007; 1(1): 17–29.
8. Cheesbrough, M. District Laboratory Practice in Tropical Countries Part-2. Cambridge University Press, Cambridge, New York, Melbourne, Madrid, Cape Town, Singapore, São Paulo.2006.
9. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk susceptibility tests; 26th approved standards. Document M1002-S. Wayne, PA: CLSI; 2016.
10. Beecher, D. J., and Wong, A. C. Identification of haemolysin BL-producing *Bacillus cereus* isolated by a discontinuous hemolytic pattern in blood agar. Environ Microbiol. 1994; 60 (5): 1646-1651.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., et al. Protein measurements with the Folin phenol reagent. J Biol Chem., 1951; 193: 265-275.
12. Berk, R. S. Production and characterization of *Pseudomonas aeruginosa* hemolysin. J Bacteriol. 1962; 88 (3):559-565.
13. Liu, P. V. Survey of haemolysin production among species of Pseudomonads. J Bacteriol. 1957; 74:718-727.
14. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacterio phage T4. Nature (London). 1970; 227: 680-685.
15. Tous, G. I., Fausnaugh, J. L., Akinyosoye, O., et al. Amino acid analysis on polyvinylidene difluoride membranes. Anal Biochem. 1989; 179: 50-55.
16. Whitaker, J. R. Principles of enzymology for food sciences. In Enzyme inhibitors, New York: Marcel Dekker. 1972; 255-282.
17. Khalil, M. A. Studies on the control of some haemolytic bacteria. Ph.D. Thesis, Botany Department, Faculty of Science, Tanta University. 2009.
18. Abraham, E. Intranasal immunization with bacterial polysaccharide containing liposomes enhances antigen-specific pulmonary secretory antibody response. Vaccine. 1992; 10 (7): 461-468.
19. Kotrla, T. Fall 2014 tentative schedule'. MLAB 1335 Immunology/Serology 2014. Available at <http://www.austincc.edu/mlt/ser/ser_schedule.html/> Accessed at October 14, 2014.
20. Bhatt, P., Rathi, K. R., Hazra, S., et al. Pre valence of multidrug resistant *Pseudomonas aeruginosa* infection in burn patients at a tertiary care centre. IJB. 2015; 23 (1): 56-59.
21. Kaur, I. Novel Strategies to Combat Anti-microbial Resistance. J Infect Dis Ther. 2016; 4: 292.
22. Galdino, A. C. M., de Oliveira, M. P., Ramalho, T. C., et al. Anti-Virulence Strategy against the

Multidrug-Resistant Bacterial Pathogen *Pseudomonas aeruginosa*: Pseudolysin (Elastase B) as a Potential Druggable Target. Curr Protein Pept Sci. 2019; 20 (5): 471-487

23. Gupta, P., Gupta, R. K., and Harjai, K. Multiple virulence factors regulated by quorum sensing may help in establishment and colonization of urinary tract by *Pseudomonas aeruginosa* during experimental urinary tract infection, Indian J Med Microbiol. 2016; 31(1): 29-33.
24. Parvez, S. A., and Rahman, D. Virulence Factors of Uropathogenic *E. coli.* In Behzadi P. (ed). Microbiology of Urinary Tract Infections-Microbial Agents and Predisposing Factors, 2018. DOI: 10.5772/intechopen.79557
25. Johnson, M. K., and Allen, J. H. The role of hemolysin in corneal infections with Pseudo-*monas aeruginosa.* Invest Ophthalmol Vis Sci. 1979; 17 (5): 480-483.
26. Crevel, C., Sally U., Carne, A., et al. Purification and properties of Zinc Metallophospholipase C from *Pseudomonas fluorescens*. Eur J Biochem. 1994; 224 (3): 845-852.
27. Wang, C. G., Chen, M. K., and Chen, T. Improved purification and some properties of a novel phospholipase C from *Bacillus mycoides* strains 970. Afr J Microbiol Res. 2010; 4 (7): 523-526.
28. Berka, R. M., and Vasil, M. L. Phospholipase C (heat-labile hemolysin) of *Pseudomonas aeruginosa*: purification and preliminary characterization. J Bacteriol. 2002; 152 (1): 239-245.
29. Kimizuka, R., Miura, T., and Okuda, K. Characterization of *Actinobacillus actinomycetemco mitans* hemolysin. Microbiol Immunol. 1996; 40: 717–723.
30. Deshpande, R.G., and Khan, M.B. Purification and characterization of hemolysin from *Porphy romonas gingivalis* A7436. FEMS Microbiol Lett. 1999; 176:387–394.
31. Pandey, A, Milind, N. M., and Dubey, S. K. Hemolysin, Protease, and EPS Producing Pathogenic *Aeromonas hydrophila* Strain An4 Shows Antibacterial Activity against Marine Bacterial Fish Pathogens. J Marine Biol. 2010; Article ID 563205 http://dx.doi.org/10.1155/2010/563205
32. Russo, T. A., Davidson, B. A., Genagon, S. A., et al. *E. coli* virulence factor haemolysin induces neutrophil apoptosis and necrosis/lysis in vitro and necrosis/lysis and lung injury in a rat pneumonia model. Am J Physiol Lung Cell Mol Physiol. 2005; 289 (2): L207-L216
33. Doan, T., Melvold, R., Viselli, S., et al. Lippincott Illustrated Reviews: Immunology. 2nd edition. Harvey RA editor. Wolters Kluwer, Lippincott Williams &Wilkins.
34. Liu, P. V., and Hsieh, H. Exotoxins of *Pseudomonas aeruginosa* III. Characteristics of antitoxin A. J Infect Dis 1973; 128: 520-526.
35. Pavlovskis, O.R., Pollack, M., Callahan, L.T., et al. Passive protection by antitoxin in experimental *Pseudomonas aeruginosa* burn infections. Infect Immun. 1977; 18: 596-602
36. Snell, K., Holder, I. A., Leppla, S. A., et al. Role of exotoxin and protease as possible virulence factors in experimental infections with Pseudo-*monas aeruginosa*. Infect Immun. 1978; 19: 839-845
37. Woods, D. E., and Iglewski, B. H. Toxins of *Pseudomonas aeruginosa*: New Perspectives. Rev Infect Dis. 1983;5 Suppl 4:S715-22.