

USING REAL-TIME POLYMERASE CHAIN REACTION TO STUDY THE EFFECT OF SALICYLIC ACID AND SODIUM CHLORIDE ON THE PYOCYANIN PRODUCTION OF *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa* is a bacterium that seizes the opportunity to cause serious infections in both man and animals. Pyocyanin is a strong virulence factor produced by *P. aeruginosa*. The present study was performed to explain the effect of salicylic acid (aspirin) and sodium chloride (NaCl) on *P. aeruginosa* and Pyocyanin using concentrations of 1.5%, 3% and 6% for aspirin and 3%, 6% and 9% for NaCl. The effectiveness of aspirin and NaCl was detected by real time PCR (qPCR). The *P. aeruginosa* isolate was from broilers suffering from septicemia in Zagazig city, Egypt. The study revealed that 6% and 9% concentration of NaCl had a bactericidal effect on the bacterium but 3% concentration of NaCl has an inhibitory effect on both the growth and the expression of Pyocyanin (by 0.5-fold lesser than the untreated sample). Also, data revealed that 1.5%, 3% and 6% concentrations of aspirin inhibited the growth and reduced the expression of pyocyanin with slight various degrees (by 0.7955, 0.4234, 0.1948-fold lesser than untreated sample, relatively). The results of this study could be useful in pharmacological intervention and clinical therapy as aspirin and NaCl could be combined with antimicrobial agents to treat *Pseudomonas* infections.

Key words: *Pseudomonas aeruginosa*; pyocyanin; sodium chloride; salicylic acid

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a broad, Gram-negative bacterium that seizes the opportunity to infect the immune-compromised hosts including people infected by HIV, patients exposed to chemotherapy and patients suffering from severe burns (1).

The infection with *P. aeruginosa* gives rise to mortality in chickens and clinical

symptoms including respiratory signs and septicemia (2). Proteases (elastase, protease, and alkaline protease), toxins (exoenzyme S and exotoxin A), hemolysins and phenazines are virulence factors in *P. aeruginosa*. They have been found to participate in *P. aeruginosa* pathogenicity in man and animals. Pyocyanin is a phenazine compound (5-N-methyl-1-hydroxy phenazine) considered as both a signal molecule and an important

virulence factor. In cystic fibrosis lungs in vitro, pyocyanin interferes with the ciliary function of the respiratory system and can change the host immune response. Also, pyocyanin is considered as the last signal in the quorum-sensing (QS) system, and organizes many genes in *P. aeruginosa* (3).

Some compounds can inhibit the quorum-sensing system such as salicylic acid (aspirin) (4). Aspirin can inhibit not only biofilm forming of *P. aeruginosa*, but also it can decrease the pyocyanin production (5). It has been reported that salicylic acid cause changes in the membrane of *P. aeruginosa*. Salicylic acid is regarded as an antimicrobial agent in the treatment of *Pseudomonas* keratitis (6).

Many environmental stimuli such as osmolarity, pH, NaCl and the diversity of ions have a distinct influence on *P. aeruginosa* (7, 8). The swarming motility of the bacterium is affected by NaCl. Bacterial growth is inhibited by 6% NaCl solution. Bactericidal action occurs at 10% NaCl solution (8).

16S rDNA-based PCR assays provide fast, plain and dependable identification of *P. aeruginosa* and its discrimination from other *Pseudomonas* species which are closely related in phylogenesis (9). Real-Time PCR (qPCR) was applied to study the fluorescence level in a particular selected gene. It is a highly sensitive technique that has remarkable potential for the high-volume analysis of gene expression in both research and routine medical diagnostics. This technique provides real-time quantitation of an initial template of DNA or RNA (10).

The present study was designed to illustrate the effect of NaCl and aspirin on an Egyptian *P. aeruginosa* strain isolated from septicemic broilers.

Material and methods

The origin of the strain

P. aeruginosa was isolated from broilers suffering from septicemia in Zagazig City, Egypt since 2017. *P. aeruginosa* was identified after examining for phenotypic and genotypic characteristics in the laboratory of

microbiology department in Zagazig University. The isolate was maintained on Tryptone Soya Agar (Oxoid, CM0131, UK) (TSA) slopes at 5 °C and sub-cultured every month. *P. aeruginosa* was transferred on TSA slopes to Kafrelsheikh University at the laboratory of bacteriology.

Determination of the effect of Sodium Chloride on P. aeruginosa

P. aeruginosa subculture occurred in King A broth (Himedia M1544) supplemented with 30, 60, and 90g/l of NaCl (Bio Jet) to obtain 3%, 6%, and 9% concentrations respectively. Cultures were incubated in a shaker incubator (180 rpm at 30°C) for 48h. The growth was determined visually and compared with the untreated sample of *P. aeruginosa* (3).

Determination of the effect of aspirin

P. aeruginosa subculture was occurred in nutrient broth overnight (PO) at 37 °C. Aspirin (100 mg, BAYER) was used. 2-fold serial dilutions in nutrient broth were prepared resulting in 100, 50, 25, 12.5, 6, 3, 1.5 mg/ml concentrations. Seven tubes each one contains ml from nutrient broth. In the first tube, one tablet from aspirin (aspirin protect 100mg) was added. The concentration became 100 mg /ml in the first tube. 0.5 ml from the first tube was taken to second tube giving concentration 50 mg /ml in the second tube. Repeat the same in other tubes giving the concentrations 25, 12.5, 6, 3, 1.5 mg/ml). Then discard 0.5 ml from the last tube. The last three tubes No. 5(D),6(E) and 7(F) contained 6,3,1.5 % Aspirin respectively. Diluted aspirin (D, E, F) was inoculated with 100 Micron PO containing 6 _ 10⁵ CFU/ml and incubated at 37 °C for 24 h. Minimum inhibitory concentration (MIC) (the lowest concentration of antibacterial agent that inhibit the apparent growth of the bacteria) was measured. PO was treated with sub-inhibitory concentrations (sub-MIC) of aspirin (1/4 MIC). The number of viable emerged bacterial cells was estimated and collated to the number of untreated PO using the plating

method (12). The growth of both PO treated with 1/4 MIC of aspirin and untreated PO was monitored. Inoculation of an overnight culture of PO into nutrient broth tubes treated with aspirin (1/4 MIC) and control ones without aspirin occurred. Incubation at 37 °C for 24 h. Every hour, Optical Density 600 nm for treated and untreated samples was measured (4).

Molecular identification

The extraction of DNA was done by QI-Aamp DNA Mini kit instructions (Qiagen, Germany, GmbH, Catalog no. 51304) with some changes. Incubation of 200 µl of the bacterial suspension occurred at 56°C for 10 min after addition of 10 µl of proteinase K and 200 µl of lysis buffer. Then, 200 µl of ethanol 100% (Applichem) were appended to the lysate. The sample was washed and centrifuged according to the manufacturer's index. Then, elution of the nucleic acid occurred with 100 µl of elution buffer presented in the kit. The reaction contained 25 µl of the following PCR master mix. Emerald Amp GT PCR master mix (2x premix) was 12.5 µl, PCR grade water was 4.5 µl. Forward primer (20 pmol) was 1 µl. Reverse primer (20 pmol) was 1 µl. Template DNA was 6 µl. The amplification of target gene phenazine (phzM) of *Pseudomonas* genus was performed using primer pair of phzM; 5'-ATGGAGAGCGGGATCGACAG- '3 and 5'- ATGCGGGTTTCCATCGGCAG- '3 amplifying 875 bp (13). The used primers were supplied from Metabion (Germany). Amplification cycles (n=35) were performed under these conditions; primary denaturation for 5 min at 94°C, secondary denaturation for 30 sec at 94°C, 1 min of annealing at 54°C and 1 min of extension at 72°C. Electrophoresis of amplified products in 1.5% agarose gel (ABgene) was done. The sizes of the amplified product were determined by a gene ruler 100 bp DNA Ladder (catalog No. SM0243) (Fermentas, Thermo Scientific, Germany). Photographing was done by Alpha imager (Innotech, Biomedica). A computerized analysis was performed.

RNA extraction for qPCR was done following RNeasy Mini Kit instructions (Qiagen, Catalog no. 74104) with some modifications. To protect RNA from degradation, a double volume (1 ml) of the RNA protect Bacteria Reagent (Qiagen, Germany, GmbH Catalog no. 76506), was added to one volume (0.5 ml) of the broth of *P. aeruginosa* culture, then the mix was incubated at room temperature for 5min. Then add 200 µl of Tris EDTA buffer (TE buffer) (Thermo Fisher, Catalog no. 12090-015) containing 1 mg/ml Lysozyme (Biochemica, Applichem, Catalog no. A3711) and 700 µl of lysis buffer. Then, 500 µl of 96% ethanol (Applichem) were added to the lysate. Washing and centrifugation of the sample were performed according to the manufacturer's index. Then, elution of RNA by 50 µl RNase free water (Quantitect SYBR green PCR kit) (catalog. No .204141). The reaction contained 25 µl of the R master mix according to Quantitect SYBR green PCR kit. QuantiTect SYBR Green PCR master mix (2x premix) was 12.5 µl. RevertAid Reverse Transcriptase (Thermo Fisher, Catalog number: EP0441) was 0.25 µl. Forward primer (20 pmol) was 0.5 µl. Reverse primer (20 pmol) was 0.5 µl. Template RNA was 3 µl. RNase free water was 8.5 µl. The target genes: phenazine (*phzM*) of *Pseudomonas* genus was performed using primer pair of *phzM* (mentioned previously), a primer pair of 16S rRNA gene;5'- GGGGGATCTTCG-GACCTCA- '3 and 5'- TCCTTAGAG-TGCCACCCG- '3 (14). The following cycling condition protocol was used: 30 min for reversed transcription at 50 °C, 5 min for primary denaturation at 94 °C, amplification cycles (n=40) were performed under the following conditions; 15 min of secondary denaturation at 94°C, annealing (Optics on) at 52 °C for 45 min for 16S rRNA but at 54 °C for 45 min for *phzM*, 30 sec for extension at 72 °C, dissociation curve (one cycle) conditions; 1 min of secondary denaturation at 94 °C, 1 min of annealing at 52 °C for 16S rRNA but at 54 °C for 1 min for *phzM*, 1 min of final denaturation at 94 °C. The curves of amplification and cycle threshold (Ct) values

were evaluated by the Stratagene MX3005P qPCR software. To assign the difference in the gene expression on the RNA of the samples, a comparison between the Ct of each sample and that of the control sample occurred according to the " $2^{-\Delta\Delta C_t}$ " equation (15).

Results

The effect of NaCl

Growth appeared at 3% concentration NaCl but no growth occurred at 6, 9%. Growth turbidity at 3% concentration of NaCl was less than that of untreated sample.

The effect of Aspirin

Initially, the minimum inhibitory concentration of aspirin against *P. aeruginosa* PO was revealed as 24 mg/ml. The sub-inhibitory concentrations of aspirin (1/4, 1/8 and 1/16 MIC) corresponding to 6, 3 and 1.5 mg/ml concentrations of aspirin respectively were applied to evaluate the inhibitory influence on both quorum sensing system and the virulence factors of *P. aeruginosa* PO. The treated *P. aeruginosa* PO with sub-MIC of aspirin was as similar as the untreated PO in its viability. Example for that, the bacterial number was 154 ± 104 CFU/ml at 1/4 MIC in treated PO but the bacterial number of the untreated PO was 160 ± 105 CFU/ml. Also, both treated and untreated PO attained a stationary phase after incubation for 9 h at 37 °C, signifying no impact on the growth. But the bluish metallic sheen of *P. aeruginosa* (11) was changed to some extent.

Turbidity degree was inversely proportional with the concentration of Aspirin. Treated *Pseudomonas* with 1/4 MIC showed the least turbidity. The turbidity of treated *Pseudomonas* with

1/16 MIC was lesser than the untreated sample.

Polymerase Chain Reaction

PCR reaction was done to diagnose *P. aeruginosa* using 16S rDNA. The results showed that one band 875 bp (product size) as a result of agarose gel compare with DNA marker (100 bp) (figure 1).

Quantitative real-time PCR

The fluorescence light emitted within qPCR is directly proportional to the formed DNA and can be assumed as an amplification plot. As a result of Ct values (table 1), the data revealed that 3% concentration of NaCl reduced the expression of *PhzM* (by 0.5-fold less than the untreated sample). The reduction percentage of *PhzM* was 50%. But for Aspirin, 1.5%, 3% and 6% concentrations caused decrease in the expression of pyocyanin with various degrees (by 0.7955, 0.4234, 0.1948 -fold less than untreated sample relatively), i.e., the reduction in expression of *PhzM* with 80.52 %, 57.66% and 20.45% in sub MIC (1/4 (6mg/ml), 1/8 (3mg/ml) and 1/16 (1.5mg/ml)) of Aspirin respectively (chart1). The experiment had been repeated three times. The amplification curve of 16SrDNA (figure 2) but that of *PhzM* (figure 3) were showed.

Table 1: The Ct values of 16SrDNA and *PhzM* for each sample with the fold change of them were analyzed. The Control sample was the untreated isolated *P. aeruginosa*

Sample No.	Sample ID	<i>16S rDNA</i>			<i>phzM</i>	
		CT	CT	Fold change	CT	Fold change
B	Control	19.78	22.86	-	-	-
C	NACL 3%	20.84	24.92	0.5000	22.86	0.5000
D	Aspirin 6%	20.39	25.83	0.1948	22.86	0.1948
E	Aspirin 3%	19.21	23.53	0.4234	22.86	0.4234
F	Aspirin 1.5%	20.54	23.95	0.7955	22.86	0.7955

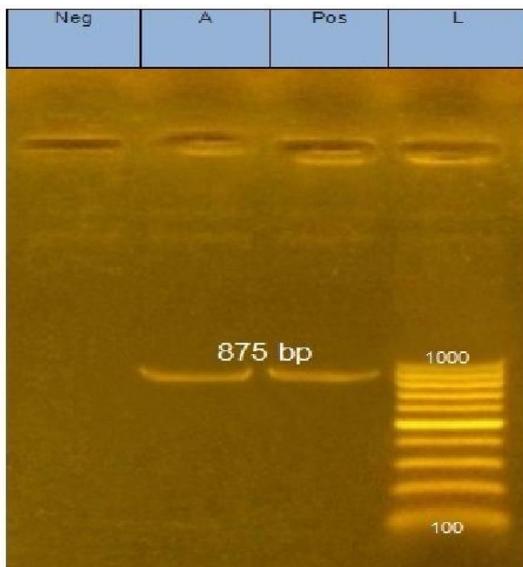


Figure 1: agarose gel electrophoresis of pyocyanin gene amplification of *Pseudomonas aeruginosa* strain. L: 100bp ladder. Lane (A): positive sample. Pos: positive control and neg: negative control. Positive and /or negative controls were represented by field sample that was previously confirmed to be positive or negative by PCR for the related gene in the Reference laboratory of Animal health research institute, Zagazig University. Amplicon size: 875 bp

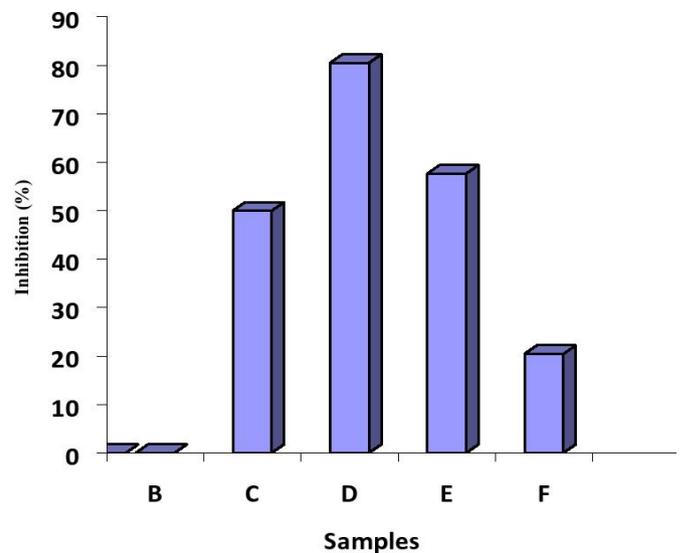


Chart 1: The inhibition percentage of *PhzM* expression in each sample. B: control (untreated sample). C: a sample containing NaCl 3%. D: a sample containing Aspirin 6%. E: a sample containing Aspirin 3%. F: a sample containing Aspirin 1.5%

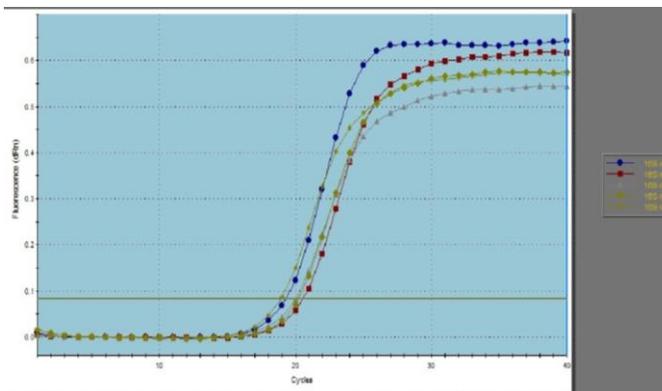


Figure 2: Amplification curve of 16SrDNA. X-axis: amplification cycles. Y-axis: distinctive fluorescence. The axis that was parallel to X axis was a threshold axis. Each point of each sample of the curve which crossed the threshold axis was the Ct value

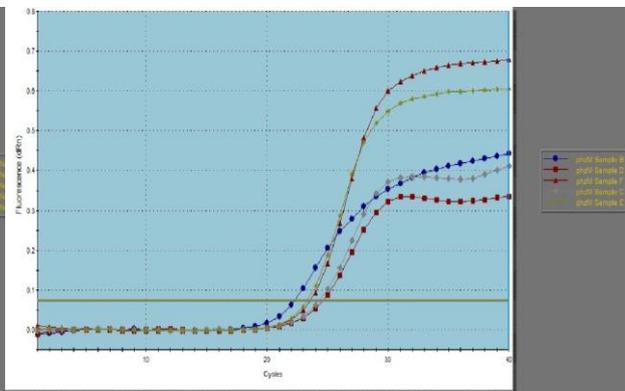


Figure 3: Amplification curve of *PhzM*. X-axis: amplification cycles. Y-axis: distinctive fluorescence. The axis that was parallel to X-axis is threshold axis. Each point of each sample of the curve which crossed the threshold axis was Ct value

Discussion

In the present study, the influence of NaCl and Salicylic acid on the *P. aeruginosa* growth and expression of pyocyanin virulence factor of it was investigated. Results demonstrated

that NaCl and Aspirin had a significant impact on the *P. aeruginosa* growth and pyocyanin production. This study revealed that 6% and 9% concentration of NaCl had a bactericidal effect on the bacterium but 3% concentration of NaCl has an inhibitory effect on the growth

and also on the expression of Pyocyanin (by 0.5-fold lesser than the untreated sample). The reduction percentage of *PhzM* was 50%. But for Aspirin, 1.5%, 3% and 6% concentrations caused inhibition of growth and decrease in the expression of pyocyanin with slight various degrees (by 0.7955, 0.4234, 0.1948 -fold lesser than untreated sample relatively), i.e., the reduction in expression of *PhzM* with 80.52%, 57.66% and 20.45% in sub MIC (1/4 (6mg/ml), 1/8 (3mg/ml) and 1/16 (1.5mg/ml) of Aspirin respectively.

Previous studies revealed that *Pseudomonas* influenced by a different concentration of salinity. Pyocyanin production increased proportionally with salinity concentration. The high amount of pyocyanin (29.57 µg/ml) was obtained with 20g/L of NaCl (2% concentration). The result also indicated that salinity above 20g/L have totally affected pyocyanin production but did not affect the bacterium growth which was completely inhibited at 60 g/L of NaCl (6% concentration). The production of pyocyanin was salinity-dependent when *P. aeruginosa* had grown in King A medium supplemented with 2.5 to 20g/l of NaCl. The highest amount of pyocyanin was obtained at 20g/l (2% NaCl) and the growth of *P. aeruginosa* was completely inhibited at 50 g/l of NaCl (5 % NaCl) (3); However, another study showed that maximum of productivity was found in a medium salinity ranging from 5-10g/l. but at concentration 5 g /l or less, there was not significantly inhibition of the growth and the pyocyanin production. (16, 17).

Other studies revealed that the normal growth of *P. aeruginosa* appeared on a medium which contained 0.25 up to 1.25% concentration of NaCl in comparison to control medium. Above 1.75% NaCl concentration in the medium, it can survive up to 2.75% NaCl but the survival number of *Pseudomonas* gradually reduced. The inhibition impact was observable at a solution containing 4% concentration of NaCl. A solution containing 6% concentration of NaCl could inhibit the growth of *Pseudomonas* after incubation for 24 h. The bactericidal effect on *Pseudomonas* occurred by 8% and 10% of NaCl (18, 8).

For patients suffering from cystic fibrosis in the lung, aerosols containing hypertonic saline (7% NaCl) are useful in the treatment due to abolish both motility and growth of *P. aeruginosa* (19).

Salicylic acid can inhibit production of pyocyanin (4,6). Distinct reduction in the virulence factors of *P. aeruginosa* treated with 6 mg/ml (1/4 MIC) and 3 mg/ml (1/8 MIC) concentrations of aspirin. Pyocyanin reduction was 78% due to treating with aspirin (1/4 MIC) (4). Growth in the sub-inhibitory concentrations of salicylic acid resulted in a significant reduction in bacterial cell number, cell density and in quorum-sensing signaling molecules (20). Salicylic acid plays a role in down-regulating some of the virulence factors (especially pyocyanin) in *P. aeruginosa* and attenuating the virulence of the bacterium on *Caenorhabditis elegans* and *Arabidopsis thaliana*. A 50% reduction of Pyocyanin production was resolved after treatment with 0.1 mM salicylic acid but an 80% reduction of pyocyanin production with no visible influence on the bacterial growth was determined after treatment with 1.0 mM SA (20, 21).

Another study revealed that *P. aeruginosa* 1604 was still able to produce a considerable amount (0.44 µgml⁻¹) of pyocyanin after a 72 hours lag period in the presence of 0.1 % aspirin. At 0.3 % concentration, the inhibitory effect was evident for 120 hours. For long-term inhibitions, a higher amount of applied salicylic compounds (0.3 and 0.4 %) are necessary (5). Salicylic acid can reduce bacterial numbers and its virulence during keratitis so it is effective as an antimicrobial substance to treat *Pseudomonas* keratitis (6).

Conclusion

In this study, *P. aeruginosa* isolate was from broilers suffering from septicemia in Zagazig city, Egypt. By using PCR and qPCR, the data revealed that Aspirin and Sodium chloride inhibit the *P. aeruginosa* growth and decrease pyocyanin production. This new therapeutic approach can be used in combination with antimicrobial agents to treat *P. aeruginosa* cases.

Conflict of interest

None of the authors disclose any conflict of interest.

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