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Gentamicin enhances *toxA* expression in *Pseudomonas aeruginosa* isolated form cow mastitis

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Abstract | The present study was undertaken in order to investigate the role of gentamicin in the gene expression of *toxA* in *Pseudomonas aeruginosa* isolated from cow mastitis. A total of ten *P. aeruginosa* strains originally isolated from cows infected with mastitis. Agar dilution methodology was performed to determine the minimal inhibitory concentration of gentamicin, all of which developed resistance toward gentamicin. The findings presented here demonstrated that all these strains harboured *toxA* depending on PCR-based assay. Nonetheless, RT-PCR technique revealed a wide variation in expression of *toxA*. Moreover, the cultivation of *P. aeruginosa* in the presence of gentamicin, significantly ($P< 0.05$), induced the expression of *toxA,* in addition to the possibility of enhancing the virulence of this bacterium. In conclusion, using gentamicin to treat infections caused by *P. aeruginosa* may participate in more severe outcomes.

Keywords | Gentamicin, Mastitis, *Pseudomonas aeruginosa*, *toxA*

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INTRODUCTION

Pseudomonas aeruginosa is an adaptable bacterial pathogen related with a wide range of infections in humans and animals. Due to the innate capacity of resistance to antimicrobial agents, this bacterium is greatly difficult to treat. What's more, such resistance is being progressively a problematic issue because of increasingly development of resistance to agents regarded as powerful therapeutic options (Kerr and Snelling, 2009).

This pathogen has a propensity to initiate sudden clinical, or perhaps, subclinical cases of mastitis outbreaks in a number of cows within short period of time. A group of signs and symptoms accompany the acute cases of bovine mastitis with noticeable udder swelling; fever, in addition to abnormal watery as well as clotty milk, which contains flakes or blood. Moreover, severe signs might be seen as well, including toxemia, however, high mortality rate occurred regardless of aggressive treatment. Yet, those being survived will be unfit for productive use (Park et al., 2014). Locally, in Iraq, twenty-two samples comprising eight milk and 14 udder wounds were positive for *P. aeruginosa* out of 140 samples (Saleh et al., 2016). Furthermore, Naser and Ismaeel, (2016) alongside with Neamah, (2017) separately reported that *P. aeruginosa* represented 44% of cow mastitis.

Exotoxin A is the most important virulence factor elaborated by most of the *P. aeruginosa* strains. It plays a crucial role in the *P. aeruginosa* pathogenesis. Markedly, this toxin has been recognized as very toxic for mammalian cells (Dong et al., 2015). It has the ability to inhibit protein synthesis of the bacterial cell via the ADP-ribosylation of cellular elongation factor 2. Eventually, this toxin action leads to restricted damage in tissue and therefore bacterial invasion (Michalska and Wolf, 2015). The *toxA* gene is a chromosome characteristic genetic sequence of *P. aerugi-*

nosa responsible for regulating exotoxin A synthesis, has been extensively used in molecular detection of *P. aeruginosa* (Dong et al., 2015).

Subinhibitory concentrations of antibiotics are known to provoke extensive transcriptional changes in bacteria (Ohlsen et al., 1998), to our knowledge, this is the first report that investigating the influence of gentamicin at sub minimal inhibitory (sub MIC) on gene expression of *toxA*. Therefore, the main goal of this study is investigating the effect of gentamicin at sub MIC on the gene expression of *toxA* of *P. aeruginosa* isolated form cow mastitis.

MATERIALS AND METHODS

A total of ten *P. aeruginosa* isolates were obtained from microbiology laboratory, College of veterinary medicine, University of Baghdad. Originally, these isolates were isolated from milk samples obtained from mastitis-affected cows. Nevertheless, re-identification was accomplished using the conventional biochemical tests (Gram stain, lactose fermentation, catalase, oxidase, IMViC, motility, and hemolysis pattern) in addition to VITEK 2 compact and API 20NE (bioMe´rieux, France) identification systems.

Determination of Minimum Inhibitory Concentration of Gentamicin

Different concentrations of gentamicin (2 – q1024 µg/ml) were used to estimate the MIC following agar dilution method and the results were interpreted in accordance to the guidelines of Clinical Laboratory Standards Institute (2016), *P. aeruginosa* ATCC 27853was used as a quality control strain.

Molecular Detection of *ToxA*

In order to amplify a fragment of *toxA* (352 bp), the DNA was amplified in a thermal cycler (Bio rad, USA) using the primers (FP 5´-GGTAACCAGCTCAGCCACAT-3´, RP 5´-TGATG TCCAGGTCATGCTTC3´) (Banerjee et al., 2017). Amplicon was visualized in 1.5% agarose gel electrophoresis.

Culture Method

Tryptone soy broth (TSB; HiMedia, India) with or without subinhibitory concentrations of gentamicin were inoculated with each isolate at concentration compatible to MacFarland (1-1.5 × 10^8 CFU/ml) at 37°C for 24 hr. thereafter, they were sent for RT-PCR study.

Real-Time Quantitative Polymerase Chain Reaction

Extraction of RNA was performed using protocol of TRIzol™ Reagent (Rio et al., 2010). cDNA was created following the manufacturer's instructions of the RT–PCR kit supplied byInvitrogen.

The gene (*toxA*) expression in *P. aeruginosa* culture was accomplished via RT-q PCR technique by Magnetic induction cycler (Bimolecular system, Australia) using primers listed in Table 1. Nonetheless, the reaction mixture is demonstrated in Table 2. Control samples were created by using 10.5 µl nuclease-free water and no cDNA template. However, *16SrRNA* was employed to normalized the gene expression of *toxA*.

Table 1: qPCR Primers used throughout this study (Goldsworthy 2008)

Gene Primer	Sequence (5´ - 3´)
16srDNA	F- ACCTGGACTGATACTGACACTGA
	R- GTGGACTACCAGGG-TATCTAATCCT
toxA	F- GGAGCGCAACTATCCCACT
	R- TGGTAGCCGACGAACACATA

Table 2: RT-qPCR reactants

Reactant	Volume (µl)
iQ SYBR Green supermix	12.5
forward primer	1
reverse primer	1
nuclease-free water	9.5
cDNA template	1

Reaction conditions for *16SrRNA* and *toxA* genes amplification were achieved as described by Goldsworthy (11). For every qPCR a plate read-out was taken after each cycle. Moreover, a melting curve using 1°C increments was also performed following the 40 cycles in order to determine that the correct gene was amplified.

The relative expression ratios were calculated by using the cycle threshold (C_t) of the *16SrRNA* as the calibrator (*n*-fold expression = $2^{-\Delta Ct}$, where ΔC_t represents the difference between the C_t of the *toxA* and the C_t of the *16SrRNA*).

Statistical Analysis

Data were presented as the mean of three replicates ± standard error of the mean. The statistical significance of *toxA* gene expression differences between *P. aeruginosa* isolates was determined based on repeated analysis of variance (ANOVA) using the statistical program IBMSPSS version 25. *P* values of <0.05 were regarded as significant (Paulson, 2008).

RESULTS

The identification was confirmed for all isolates included

in the present work depending on the results summarized in Table 3. In regard to MIC, all ten isolates were resistant to gentamicin (MIC ≥ 16 µg/ml) in accordance to CLSI breakpoints (Table 4).

Table 3: Results of identification of study isolates.

Id	Test	Result
1	Gram stain	Negative
2	MacConkey agar	Pale colonies
4	Oxidase	Negative
5	Catalase	Positive
6	Indole Test	Negative
7	Methyl Red Test	Negative
8	Voges-Proskauer	Positive
9	Citrate Utilization	Positive
10	Motility Test	Positive
11	β Hemolysis	Positive

Table 4: MIC of gentamicin of *P. aeruginosa* isolates included in present work

Isolate code	MIC µg/ml
P1	32
P2	64
P3	64
P4	128
P5	32
P6	32
P7	128
P8	32
P9	64
P10	64

The current findings revealed that all isolates of *P. aeruginosa* harboured *toxA* (Figure 1). Moreover, these ten isolates developed very low expression levels of mean C_t value of 28.0 ± 1.02.

Pseudomonas aeruginosa 16SrRNA displayed, similarly, moderate levels of expression, giving mean C_t values of 22.93 ± 0.67.

When *P. aeruginosa* isolates grown in the presence of gentamicin; *toxA* expression mean has increased significantly (P< 0.05) over their spouses before the addition of gentamicin. Fold change ranged from 5.11 – 323.36 and it varied significantly (P < 0.05) among the isolates as it is depicted in Figure 2.

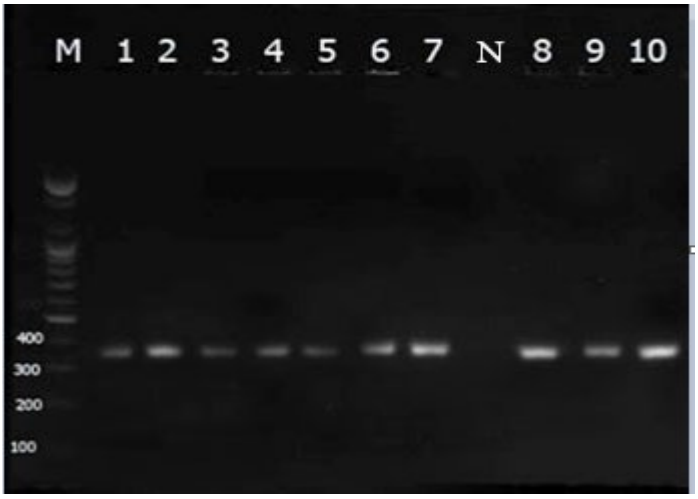

Figure 1: Visualization of *toxA* gene (352 pb) by 1.5% agarose gel analysis. The shown bands are representative of PCR products amplified from *P. aeruginosa* (lanes 1 -10), negative control (lane N), lane M represents 100 bp DNA ladder.

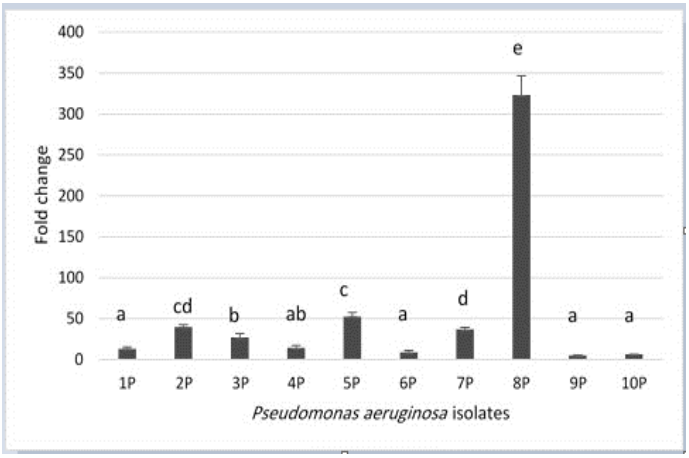

Figure 2: Fold change of *toxA* gene expression levels in *P. aeruginosa* isolates from bovine mastitis. P= 4.56 × 10^{-21}, $LSD_{0.05}$= 13.008. Columns with similar letter have insignificant differences. Error bars represent standard error of mean.

DISCUSSION

Observational data investigated the correlation between virulence and antibiotic resistance has established a simultaneous rising of both of them (Schroeder et al., 2017). However, the current findings are in agreement with a study done by Neamah, (2017) as he stated the *toxA* was detected in all *P. aeruginosa* strains that isolated from cow mastitis. While, Raziq, (2017) has detected *toxA* gene in 84% of the isolates.In compatible with our results, Banerjee et al. (2017) in a study done in South Bengal reported that,nearly, 5.4% of bovine subclinical mastitis were due to *toxA* containing *P. aeruginosa*.

Markedly, the link between toxin production and antibi-

otic resistance has been analyzed by many authors. Ohlsen et al. (1998) demonstrated that some methicillin-resistant *S. aureus* isolates produced up to 30-fold more alpha-toxin in the presence of 10 mg of methicillin per ml than in its absence. The authors explained such an interaction may induce signal transduction mechanisms, resulting in activation of the *hla* promoter. However, it cannot be explained by increased levels of the regulatory molecule RNA III. Moreover, Tofik (2011) confirmed that toxin production by *S. aureus* in the presence of oxacillin was significantly up-regulated. Another hypothesis was adopted by Kimmitt et al. (2000), SOS-inducing antimicrobial agents, particularly the quinolones, trimethoprim, and furazolidone, were shown to induce toxin gene expression.

The regulatory mechanisms govern the interconnection between virulence determinants and genes responsible for the antibiotic resistance is highly complicated. Occasionally these mechanisms are believed of as distinct events. Yet, form the genetic point of view, such regulatory mechanisms are interweaved and connected. Frequently, the expression of antibiotic resistance genes can be influenced by the regulatory mechanisms of virulence determinants and vice versa. Unluckily, the host can affect this gene regulation directly or indirectly via many forms. Of these, stress response, environmental sensing, post-transcriptional modifications, ribo-regulation, multi-networks of regulation, and formation of biofilm govern by quorum sensing (Schroeder et al., 2017).

CONCLUSION

The findings demonstrated in the present work confirmed that growing *P. aeruginosa* in the presence of gentamicin induced *toxA* expression and might enhance the virulence of this pathogen; upon that, including gentamicin in the treatment regimen of infections caused by *P. aeruginosa* might severely worsen the outcomes.

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CONFLICT OF INTERESTS

No conflict of interest.

AUTHORS CONTRIBUTION

Atheer Abdul Razzaq, Ansam Khalid Mahmood, and Nuhad Mohammed Hammed: Sample collection, and manuscript preparation.

Kifah A.Jasim and Zaid Saifuldeen Abdulqader: Measurements, Acquisition and Analysis of data.

Harith Jabbar Fahad Al-Mathkhury: Study conception and design, Interpretation of data and Drafting of manuscript.

All authors read and approved the final manuscript.

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