Current Microbiology (2020) 77:479–490
https://doi.org/10.1007/s00284-019-01858-7

Oxidative Stress Influences *Pseudomonas aeruginosa* Susceptibility to Antibiotics and Reduces Its Pathogenesis in Host

Fatma A. Mohamed[1] · Ghada H. Shaker[1] · Momen M. Askoura[1]

Received: 9 November 2019 / Accepted: 24 December 2019 / Published online: 7 January 2020
© Springer Science+Business Media, LLC, part of Springer Nature 2020

Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes serious infections in humans, notably cystic fibrosis. *P. aeruginosa* faces various stresses such as oxidative stress either in the environment or within the host during infection. In the present study, the influence of oxidative stress on both *Pseudomonas* antibiotic susceptibility and host pathogenesis was characterized. Prior exposure to H_2O_2 significantly altered *P. aeruginosa* susceptibility to tested antibiotics; colistin, ciprofloxacin, tobramycin, and ceftazidime. The minimum inhibitory concentrations (MICs) of tested antibiotics either increased or decreased following H_2O_2 exposure. Importantly, RT-qPCR revealed that expression of quorum sensing genes, that regulate virulence factors production in *P. aeruginosa*, was significantly higher in unstressed relative to H_2O_2-stressed cells. The impact of *P. aeruginosa* exposure to oxidative stress by H_2O_2 on bacterial pathogenesis was investigated using in vivo mice infection model. Interestingly, exposure to oxidative stress markedly reduces *P. aeruginosa* pathogenesis in mice. Unstressed *P. aeruginosa* was able to kill more mice as compared to H_2O_2-stressed bacteria. In addition, body weight of mice infected with unstressed *P. aeruginosa* was lower than that of mice inoculated with stressed bacteria. Isolated organs (spleen, liver, and kidney) from mice infected with unstressed bacteria exhibited increased weight as well as bacterial load in comparison with mice infected with stressed bacteria. In summary, current data highlight the impact of oxidative stress on *P. aeruginosa* antibiotic susceptibility as well as host pathogenesis. These findings could be helpful in treatment of infections caused by this important pathogen.

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen. It is a ubiquitous bacterium that is found and isolated from various environments including plants, animals, soil, and humans [1]. *P. aeruginosa* accounts for 10–15% of nosocomial infections worldwide [2] and is considered the third most common organism associated with hospital-acquired infections such as urinary catheter-associated infections, ventilator-associated pneumonia, blood, burn, and wound infections. In addition, *P. aeruginosa* is the causative agent of a wide variety of life-threatening infections, particularly in immunocompromised patients [3, 4]. *P. aeruginosa* acquired its reputation due to having an arsenal of virulence factors that are under regulation by quorum sensing (QS) genes. These virulence factors include hemolysins, pyoverdin, pyocyanin, rhamnolipids, and proteases that largely contribute to the bacterium pathogenesis [5, 6].

The ability of *P. aeruginosa* to tolerate various unfavorable conditions in addition to its increased resistance to antimicrobial agents that highly contribute to the organism's success to be one of the most important opportunistic pathogens [7]. The increased bacterial resistance to commonly used antimicrobial agents encourage the search for alternative techniques to fight infections caused by *P. aeruginosa* such as phage therapy and quorum sensing inhibitors [8].

Environmental stress is generally defined as an external factor that adversely affects the physiological state of bacteria [9]. Bacteria are exposed to various stressful conditions, either in natural environments or within the host during infection such as osmotic pressure, extremes of temperature, pH, and starvation [10]. Moreover, many food-borne pathogens have to survive various stresses during food

✉ Momen M. Askoura
momenaskora@yahoo.com

Fatma A. Mohamed
fatmaelfayed@yahoo.com

Ghada H. Shaker
ghada_shaker61@yahoo.com

[1] Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

processing such as pH and high salt concentration [11]. In addition to previously mentioned stresses, the exposure to growth-inhibitory compounds such as disinfectants and antibiotics represents another type of stresses that bacteria have to survive [9].

Bacterial exposure to stress could lead to reduction in growth rate, inhibition of metabolic functions or even death, in more severe circumstances, at individual cell or population levels [9]. Stresses adversely affect bacterial physiology through inducing many metabolic changes in bacterial cells in order to respond to such stressful conditions [12]. For instance, bacteria can reduce the damages caused by many stresses through both phenotypic and genotypic adaptations [12].

Aerobically growing bacteria including *P. aeruginosa* are routinely exposed to oxidative stress in the form of Reactive Oxygen Species (ROSs) such as peroxide and superoxide. These ROSs are unavoidable by-products of aerobic respiration and capable of causing severe damage to various cellular macromolecules [13]. During host infection, phagocytic cells generate ROSs, such as superoxide, that are involved in antibacterial activity in order to limit bacterial infection [14]. In addition, being a nosocomial pathogen, *P. aeruginosa* is at high risk of exposure to various disinfectants and detergents that create oxidative stress and consequently a harmful environment for bacterial survival [15]. Therefore, many bacteria possess defense mechanisms that enable their survival under such stressful conditions. These mechanisms include DNA-repair systems, scavenging substrates in addition to antioxidant enzymes such as superoxide dismutase and catalase [14].

Importantly, adaptation to a specific stress could affect bacterial response to other stresses as well as bacterial susceptibility to antimicrobial agents in a process termed "cross-protection" [9]. A link between exposure to various stresses such as oxidative stress and bacterial susceptibility to antimicrobial agents has been previously reported [15]. For instance, exposure to oxidative stress influence the expression of many bacterial multidrug efflux systems which contribute to ameliorating adverse effects associated with stress exposure [16]. Similarly, induction of antioxidant mechanisms within bacterial cells in response to exposure to antimicrobial agents has been previously demonstrated. Many antimicrobials exhibit their bacterial inhibitory activity through the generation of ROSs which could account for the observation that bacterial response to oxidative stress largely affects its susceptibility to various antimicrobials [16].

The current study aimed to characterize the influence of oxidative stress on the susceptibility of *P. aeruginosa* to antibiotics. Moreover, the contribution of oxidative stress to *P. aeruginosa* pathogenesis was also investigated using murine infection model. Treatment of *P. aeruginosa* infections is highly difficult due to the increased bacterial resistance to commonly used antimicrobial agents. Therefore, the findings of this study could expand our familiarity with *P. aeruginosa* and consequently help us in controling infections caused by this opportunistic pathogen.

Materials and Methods

Bacterial Strains

Pseudomonas aeruginosa PAO1 and six clinical isolates of *P. aeruginosa* collected from infected patients attending the University Hospital and El Ahrar General Hospital, Zagazig, Egypt according to standard methods of sample collection. *P. aeruginosa* clinical isolates were recovered from patients suffering from wound and eye infections (1DF & 3OP), respiratory tract infection (2SP & 6SP), and urinary tract infection (4U & 5U) and were used in this study. Bacterial isolates were identified morphologically and biochemically according to [17]. Stock cultures of bacterial isolates were stored at − 80 °C in glycerol.

Killing Curve of P. aeruginosa to H_2O_2

Pseudomonas aeruginosa PAO1 was grown overnight in Müller–Hinton (MH) broth at 37 °C. Cells were harvested by centrifugation (6000 rpm for 10 min), washed twice in sterile phosphate-buffered saline (PBS) and exposed to H_2O_2 (3.5 and 5 mM) in MH broth. The percent bacterial survival was determined immediately and at 5, 10, 15, 20, 25, and 30 min after H_2O_2 exposure. Samples were taken at specified times, numbers of viable cells were determined by serial dilution in PBS (pH 7.4), plating on MH agar plates, and incubation for 24–48 h at 37 °C under aerobic conditions. Bacterial survival following exposure to H_2O_2 was expressed as the percent survival as a function of the duration of exposure to H_2O_2 (survival at time zero represents 100%). The results are expressed as the means ± standard errors from three independent biological experiments.

Preparation of Unstressed and H_2O_2-Stressed Bacteria

Unstressed and H_2O_2-stressed bacteria were prepared according to McMahon et al. [9] with some modifications. *P. aeruginosa* was cultured in MH broth and incubated at 37 °C for 14 h with shaking at 250 rpm. Cells were harvested by centrifugation at 6000 rpm for 10 min and washed twice in sterile PBS. Washed bacterial cells were resuspended in PBS to form standard inocula with an optical density at 600 nm (OD_{600}) of 0.1 which corresponds to 10^8 CFU/mL. H_2O_2-stressed bacteria were prepared as follows: a total

volume of 0.5 mL of bacterial culture prepared as mentioned above for unstressed bacteria was added to 50 mL of MH broth containing 3.5 mM H_2O_2 and incubated at 37 °C for 30 min. Next, bacterial cells were harvested by centrifugation at 6000 rpm for 10 min and washed twice in sterile PBS. Washed cells were resuspended in PBS of to achieve a bacterial count of approximately 10^5–10^6 CFU/mL.

Determination of Minimum Inhibitory Concentration (MIC) by Dilution Method

The minimum inhibitory concentrations (MICs) of tobramycin, colistin, ceftazidime, and ciprofloxacin were determined for both unstressed and H_2O_2-stressed bacteria as described before by Jo¨rgensen and Ferraro [18] using microtiter plate. Antibiotics were dissolved in MH broth following the manufacturer's recommendations. Stock antibiotic solutions were stored at − 20 °C and used within 5 days to determine MICs for both standardized stressed and unstressed control bacterial cells as follows: serial twofold dilutions of each antibiotic were prepared in MH broth in sterile 96-well microdilution plates. Fixed amount of bacterial suspension was added to each well containing antibiotic. Both negative control wells containing broth but no bacteria as well as positive control wells containing broth and bacteria were included in the experiment. MIC plates were incubated in a humidified chamber at 37 °C for 24 h and results were taken. MIC for selected antibiotics was defined as the lowest concentration of antibiotic that is needed to inhibit the growth of stressed or unstressed bacteria after 24 h. MICs were determined in triplicate and the presence of significant differences ($P < 0.05$) between unstressed and stressed *P. aeruginosa* was examined by Mann–Whitney U analysis using the GraphPad Prism 5 software package.

Total RNA Extraction for qRT-PCR

H_2O_2-stressed and unstressed *P. aeruginosa* PAO1 were prepared exactly as described above. RNA of H_2O_2-stressed and unstressed *P. aeruginosa* PAO1 was extracted using GeneJET RNA Purification Kit (Thermoscientific, USA) following the manufacturer instructions. Briefly, bacterial cells of both H_2O_2-stressed and unstressed bacteria were collected by centrifugation (6000 rpm for 10 min, 4 °C). Bacterial pellets were resuspended in 100 µL of Tris–EDTA buffer containing lysozyme and incubated for 5 min at 25 °C. Next, lysis buffer supplemented with β-mercaptoethanol was added and mixed thoroughly. The obtained RNA was further purified using a Qiagen RNeasy minikit (Qiagen, Valencia, CA). Following purification, 100 µL of nuclease-free water was added to elute RNA which is stored at − 70 °C until use.

Quantitative RT-PCR

The expression levels of quorum sensing genes *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsR*, and *pqsA* in both unstressed and H_2O_2-stressed *P. aeruginosa* PAO1 were analyzed by qRT-PCR. The expression level of each gene was normalized to *ropD*, a gene for which the expression levels remained unchanged in both unstressed and H_2O_2-stressed bacteria. Primers used in this study are listed in Table 1 [19]. The analysis was conducted following the protocol described in SensiFAST™ SYBR® Hi-ROX One-Step Kit (Bioline, UK) using StepOne Real-Time PCR system (Applied Biosystem, USA). Specific PCR amplification was confirmed by both agarose gel electrophoresis and a melting curve analysis of products according to the manufacturer recommendation. The relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

In Vivo Mice Infection

The influence of oxidative stress on the pathogenesis of *P. aeruginosa* PAO1 was characterized using mice infection models. The experiment was performed as described before [20–22] with modifications, considering animal welfare guidelines of Medical Research Center, Zagazig University. Three-week-old healthy albino mice (*Mus musculus*) were used in this study. Briefly, unstressed and H_2O_2-stressed bacteria were prepared as mentioned before. Bacterial pellets were resuspended in PBS (pH 7.4) to the desired bacterial density (2.5×10^7 CFU/mL) that was confirmed by plate colony counting. One hundred µL of suspended bacterial cells of both unstressed and H_2O_2-stressed cells

Table 1 Primers used in qRT-PCR [19]

Gene name	Primer sequence	Amplicon size (bp)
ropD (F)	CGAACTGCTTGCCGACTT	131
ropD (R)	GCGAGAGCCTCAAGGATAC	
lasI (F)	CGCACATCTGGGAACTCA	176
lasI (R)	CGGCACGGATCATCATCT	
lasR (F)	CTGTGGATGCTCAAGGACTAC	133
lasR (R)	AACTGGTCTTGCCGATGG	
rhlI (F)	GTAGCGGGTTTGCGGATG	101
rhlI (R)	CGGCATCAGGTCTTCATCG	
rhlR (F)	GCCAGCGTCTTGTTCGG	160
rhlR (R)	CGGTCTGCCTGAGCCATC	
pqsA (F)	GACCGGCTGTATTCGATTC	74
pqsA (R)	GCTGAACCAGGGAAAGAAC	
pqsR (F)	CTGATCTGCCGGTAATTGG	142
pqsR (R)	ATCGACGAGGAACTGAAGA	

F forward, *R* reverse

were aseptically injected into the abdominal cavity of each mouse using 1 mL-insulin syringe, G28½ needle (AMECO, Egypt). Mice were divided and allocated randomly into four groups (6 mice each). Mice in the first and second groups were injected with H_2O_2-stressed and unstressed bacteria, respectively. As negative controls, a cohort of 6 mice was injected with 100 µL of sterile PBS, and another 6 mice were left uninfected. Infected and control mice were maintained at room temperature under aerobic conditions with feeding. Mice survival in each group was monitored every 24 h for 4 days, plotted using Kaplan–Meier method and significance was calculated using Log-rank test, GraphPad Prism 5 at $P < 0.05$**.** In addition, mice body weights were determined for each group just before bacterial challenge and every 24 h over the experiment period. The body weight gain for mice in each group was calculated and expressed as the mean percent of body weight ± standard errors considering body weight just before bacterial challenge as 100%. Bacterial burden from mouse tissues was determined as follows: two groups; 6 mice each were injected with H_2O_2-stressed and unstressed bacteria. Control mice were included exactly as mentioned above. Control and bacteria-infected mice were anesthetized by inhalation of ether at 24 h postinfection. Mice were sacrificed and spleen, liver, and kidney samples were obtained aseptically and weighed. In order to calculate bacterial burden in each organ, isolated organs were homogenized by hand in a sterile glass plate containing 5 mL of PBS buffer. Serial tenfold dilutions were made for homogenized tissue samples in PBS (pH 7.4) and 0.1 mL aliquots were plated on cetrimide agar plates. The plates were incubated at 37 °C for 24 h under aerobic conditions to determine the bacterial loads for each organ and expressed as (CFU/g). The bacterial load in each organ was calculated and expressed as means ± standard errors. The statistical analysis was examined by Mann–Whitney U analysis using the GraphPad Prism 5 software package with a *P* value of < 0.05 was considered significant.

Results

The capacity of *P. aeruginosa* to survive different concentrations of H_2O_2 was characterized. Bacteria were exposed to various H_2O_2 concentrations (3.5 and 5 mM) followed by determining the number of viable cells at different time points over 30 min of exposure. As shown in Fig. 1, *P.*

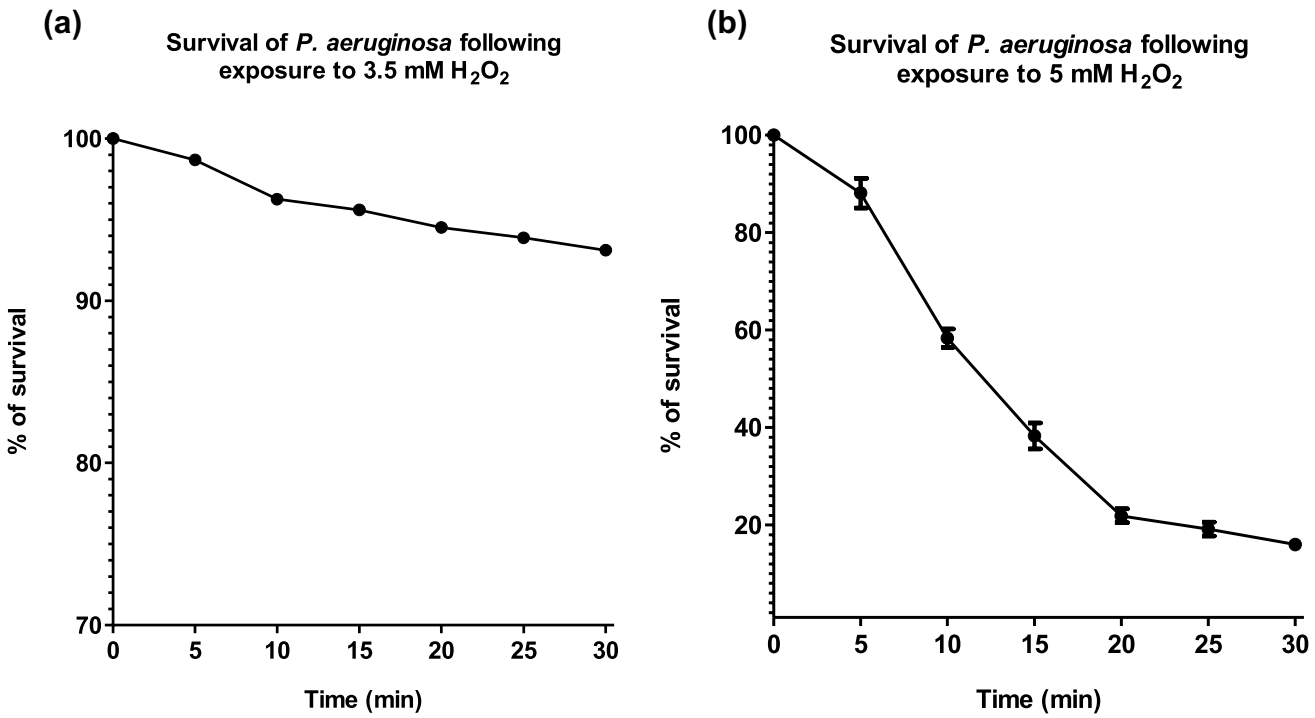

Fig. 1 **a** *Pseudomonas aeruginosa* was capable of surviving 3.5 mM H_2O_2 without a significant decrease in viability. **b** There was a marked decrease in *Pseudomonas aeruginosa* viability following exposure to 5 mM H_2O_2. *Pseudomonas aeruginosa* was exposed to 3.5 mM or 5 mM H_2O_2 and the percent bacterial survival was determined immediately, and at 5, 10, 15, 20, 25, and 30 min after H_2O_2 exposure. Samples were taken at specified times, numbers of viable cells were determined by serial dilution in PBS (pH 7.4), plating on MH agar plates, and incubation for 24–48 h at 37 °C under aerobic conditions. Bacterial survival was expressed as the percent survival as a function of the duration of exposure to H_2O_2 (survival at time zero represents 100%). The results are expressed as the means ± standard errors from three independent biological experiments. Error bars are present but too small when are not seen

aeruginosa was capable of surviving 3.5 mM H_2O_2 over 30 min without a significant decrease in viability (Fig. 1a). On the other hand, the number of viable bacteria markedly decreased upon exposure to higher concentration (5 mM) of H_2O_2 (Fig. 1b). Therefore, the oxidative stress experiments were conducted using 3.5 mM H_2O_2 which exhibits the required oxidative stressful environment without drastically affecting bacterial viability.

Next, the minimum inhibitory concentrations (MICs) of tested antibiotics; colistin, ciprofloxacin, tobramycin, and ceftazidime were determined against *P. aeruginosa* PAO1 and 6 *Pseudomonas* isolates recovered from different clinical sources. As shown in Table 2, there was a significant difference ($P < 0.05$) in MICs for tested antibiotics between unstressed (control) and stressed-bacteria. All *Pseudomonas* clinical isolates, on contrast to *P. aeruginosa* PAO1, showed at least twofold decrease in MIC For ceftazidime following H_2O_2 exposure as compared to unstressed bacteria. All tested bacteria (*P. aeruginosa* PAO1 and clinical isolates) showed two to > fourfold decrease in MIC for ciprofloxacin following H_2O_2 exposure. For colistin, all oxidative stressed bacteria, except two clinical isolates (4U and 5U), showed two to > fourfold increase in MIC as compared to unstressed cells. Finally, two clinical isolates; 1DF and 2 SP exhibited twofold increase in MIC for tobramycin, while other clinical isolates as well as PAO1 showed at least twofold decrease in MIC following H_2O_2 exposure.

Furthermore, the contribution of H_2O_2 stress to bacterial virulence was investigated. The expression levels of QS genes; *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsR,* and *pqsA*, that regulate production of *P. aeruginosa* virulence factors, were determined in both unstressed and H_2O_2-stressed *P. aeruginosa* using qRT-PCR. As shown in Fig. 2, the transcript levels of QS genes were significantly higher in unstressed *P. aeruginosa* relative to H_2O_2-stressed bacteria. These results clearly indicate that exposure of *P. aeruginosa* to H_2O_2 negatively affects the expression of QS genes and consequently would decrease the production of virulence genes in *P. aeruginosa*.

In addition, the contribution of oxidative stress to *P. aeruginosa* pathogenesis was further evaluated using mice infection models. As shown in Fig. 3, no death was recorded for the control groups, either uninoculated or PBS-injected mice. Unstressed *P. aeruginosa* PAO1 killed significantly more mice than H_2O_2-stressed bacteria ($P < 0.01$).

Moreover, as compared to mice infected with stressed bacteria, the mice inoculated with unstressed *Pseudomonas* exhibited a significant decrease in body weight over the infection course (Fig. 4).

Furthermore, organs weights were determined for both mice infected with H_2O_2-stressed and unstressed bacteria. Spleen,liver, and kidney samples obtained from mice infected with unstressed bacteria showed a significant increase in weight relative to those obtained from both control mice as well as mice injected with H_2O_2-stressed bacteria (Fig. 5).

Finally, bacterial load in each organ was determined for control and bacteria-inoculated mice. The numbers of unstressed bacteria were significantly higher in examined mice tissues as compared to H_2O_2-stressed bacteria (Fig. 6).

Discussion

Pseudomonas aeruginosa is challenged by many stresses and similar to other human pathogens, it has to overcome these unfavorable conditions [2]. While, there was more focus on foodborne pathogens, the impact of various stresses on *P. aeruginosa* has not been adequately demonstrated [9]. In addition, the effect of oxidative stress on bacteria has been characterized only using standard bacterial strains and did not include clinical isolates of bacteria. Investigating the impact of oxidative stress on clinical isolates, as performed in the current study, is important in order to investigate the contribution of environment on bacterial response to oxidative stress.

Currently, the antibiotic susceptibility of six clinical isolates of *P. aeruginosa* recovered from different sources in addition to the standard strain PAO1 was characterized before and after exposure to H_2O_2 stress. Primarily, it was important to optimize the experimental conditions in order

Table 2 MICs of *P. aeruginosa* before and after exposure to H_2O_2 stress

Antibiotic	MIC (µg/mL) Unstressed bacteria							MIC (µg/mL) Stressed bacteria[a]						
	PAO1	1DF	2 SP	3OP	4 U	5 U	6 SP	PAO1	1DF	2SP	3O	4U	5U	6SP
Colistin	1	1	0.5	1	4	4	4	+	+	+++	+	−	−−−	+++
Ciprofloxacin	0.5	1	0.125	0.25	128	512	512	−−−	−−	−	−−−	−−	−−	−−
Tobramycin	2	0.125	0.5	1	1024	4096	4096	−	+	+	−−	−	−	−
Ceftazidime	0.5	1	4	1	1024	4096	4096	+	−	−−−	−	−−	−	−

[a](+) 1.5- to 2-fold increase in MIC ($P < 0.05$); (++) 2.1- to 4-fold increase in M IC ($P < 0.05$); (+++) greater than fourfold increase in MIC ($P < 0.05$); (−) 1.5- to 2-fold decrease in MIC ($P < 0.05$); (−−) 2.1- to 4-fold decrease in MIC ($P < 0.05$); (−−−) greater than fourfold decrease in MIC ($P < 0.05$)

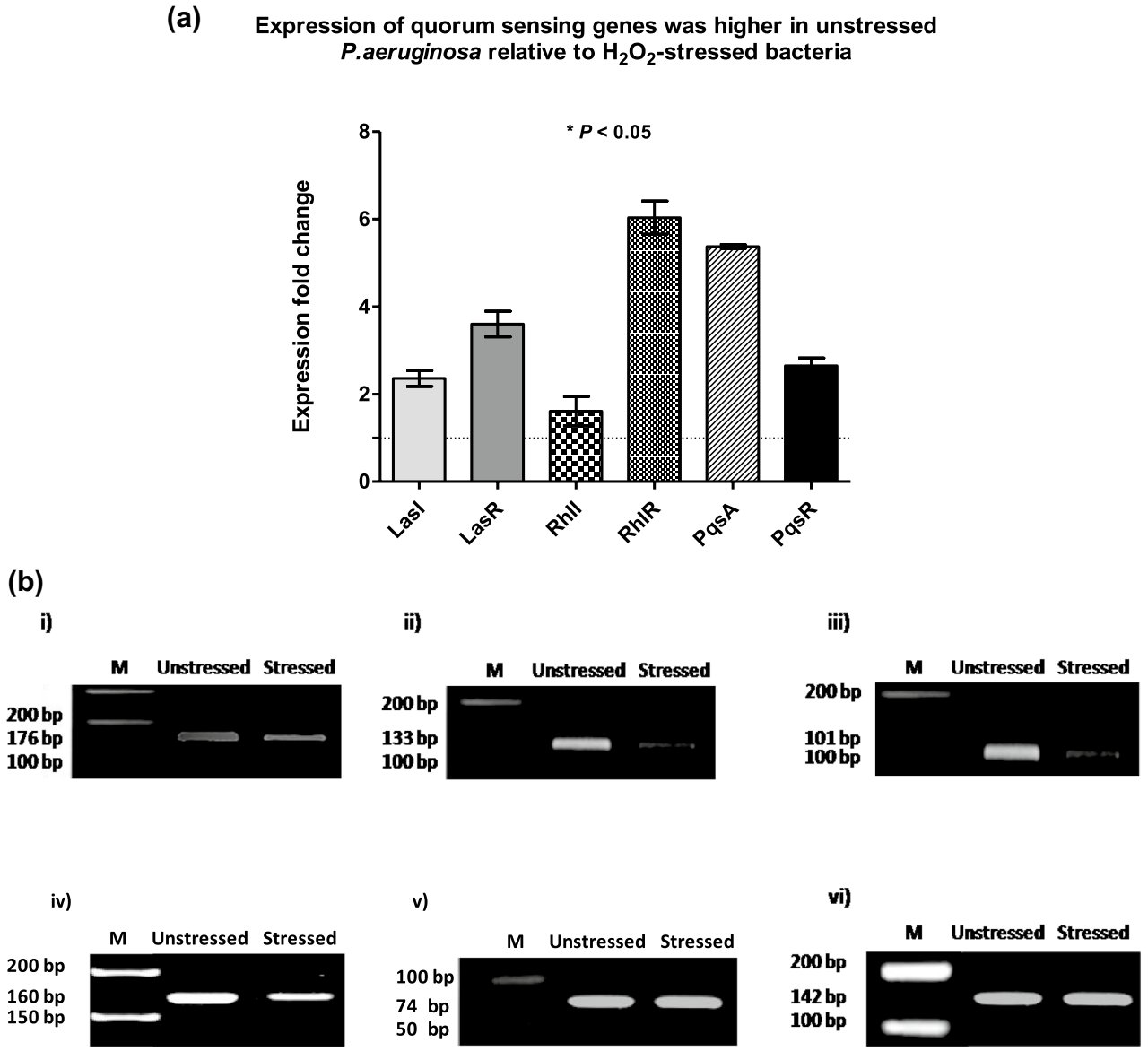

Fig. 2 **a** RT- qPCR revealed increased expression of quorum sensing genes in unstressed *P. aeruginosa* relative to H_2O_2-stressed bacteria. The expression levels of *lasI*, *lasR*, *rhlI*, *rhlR*, *pqsA*, and *pqsR* in both unstressed and H_2O_2-stressed *P. aeruginosa* PAO1 were analyzed by qRT-PCR. The expression level of each gene was normalized to *ropD*. Expression fold change in gene expression in unstressed *P. aeruginosa* was calculated by the $2^{-\Delta\Delta CT}$ method compared to H_2O_2-stressed *P. aeruginosa*. The data shown are the means ± standard errors from three biological experiments with three technical replicates each. * $P < 0.05$ was considered significant using Student's *t* test. **b** Agarose gel electrophoresis of; (i) *lasI* amplicon (176 bp), (ii) *lasR* amplicon (133 bp), (iii) *rhlI* amplicon (101 bp), (iv) *rhlR* amplicon (160 bp), (v) *pqsA* amplicon, (74 bp), and (vi) *pqsR* amplicon (142 bp)

to achieve the required stressful environment without drastically affecting bacterial survival. Characterization of the impact of oxidative stress on bacterial cells was conducted herein using 3.5 mM H_2O_2 that offers the required stressful environment without affecting bacterial survival. On the other hand, there was a higher mortality in bacteria following exposure to 5 mM H_2O_2.

Prior exposure of *P. aeruginosa* to oxidative stress by H_2O_2 significantly affected bacterial sensitivity to tested antibiotics when compared with unstressed bacteria. The difference between clinical and *P. aeruginosa* PAO1 standard strain in their susceptibility to some antibiotics such as ceftazidime following H_2O_2 could be attributed to the influence of environment on clinical isolates. As mentioned before, clinical isolates were recovered from patients suffering from various infections. Clinical isolates have already faced many challenges in their environment either within or outside the host such as pH fluctuations, osmotic, or even

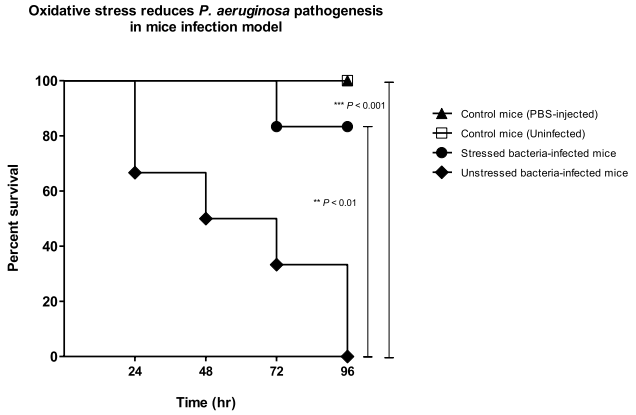

Fig. 3 Prior exposure to oxidative stress (3.5 mM H_2O_2/30 min) significantly reduced *P. aeruginosa* pathogenesis in mice. Mice ($n=6$ mice/ group) were inoculated intraperitoneally with 100 µL of suspended bacterial cells (2.5×10^7 CFU/mL) of H_2O_2-stressed and unstressed *P. aeruginosa*. As negative controls, 6 mice were injected with 100 µL of sterile PBS, and another 6 mice were left uninfected. Mice survival in each group was monitored every 24 h for 4 days and plotted using Kaplan–Meier survival curve. Unstressed *P. aeruginosa* killed more mice than H_2O_2-stressed bacteria (**$P<0.01$; log-rank test). No death was recorded for mice in negative controls; either uninfected or PBS-injected mice.

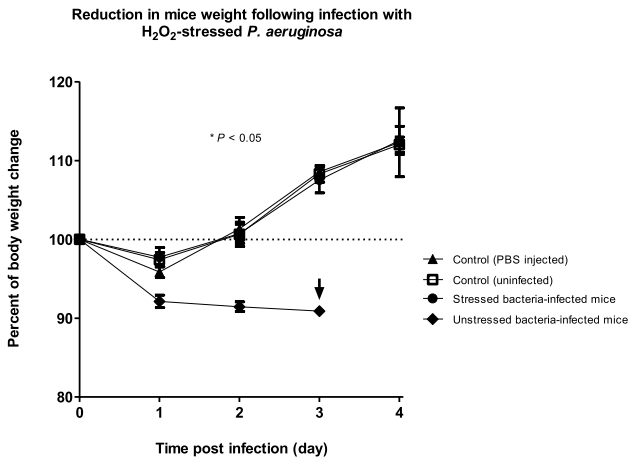

Fig. 4 Mice infected with unstressed *Pseudomonas aeruginosa* exhibited a significant decrease in body weight as compared to mice infected with H_2O_2 stressed-bacteria. Mice ($n=6$ mice/group) were inoculated intraperitoneally with 100 µL of suspended bacterial cells (2.5×10^7 CFU/mL) of H_2O_2-stressed and unstressed *Pseudomonas aeruginosa*. Two mice groups; one contained 6 mice injected with 100 µL of sterile PBS, and the other one contained 6 mice left uninfected, were included as negative controls. Body weights were determined for mice in each group just before bacterial challenge and every day over the experiment period. The body weight gain for mice in each group was calculated and expressed as the mean percent of body weight ± standard errors considering body weight just before bacterial challenge as 100%. There was a significant reduction (*$P<0.05$; Mann–Whitney *U* analysis) in body weight of mice inoculated with unstressed *Pseudomonas aeruginosa* as compared to mice infected with H_2O_2-stressed bacteria decrease over the infection course. The arrow indicates that all mice infected with unstressed *Pseudomonas aeruginosa* died after this time point.

oxidative stress [10]. All these stresses could have affected bacterial physiology and structure more as compared with standard strain which was just exposed to experimental H_2O_2 stress [16]. However, this variation between *P. aeruginosa* clinical isolates and PAO1 was not seen in bacterial response to ciprofloxacin. Similar to ciprofloxacin, stressed bacteria (PAO1 and most of clinical isolates) showed similar changes in MIC, either increase or decrease in MIC, for both colistin and tobramycin in comparison with unstressed cells.

Change in *Pseudomonas* susceptibility to antibiotics following exposure to oxidative stress observed herein is in agreement with that has been shown before. Exposure to stressful conditions was found to noticeably affect bacterial susceptibility to antibiotics [9]. Moreover, there is a link between *Pseudomonas* response to oxidative stress and both antibiotic susceptibility and host pathogenesis [23, 24]. For instance, constitutive expression of the redox-active regulator OspR (oxidative stress response regulator) alters β-lactam resistance in *P. aeruginosa*. In addition, OspR was found to regulate additional genes involved in quorum sensing and consequently could affect bacterial survival and pathogenesis [25].

Exposure to stress could direct bacteria to develop highly regulated adaptive mechanisms that in addition to protecting bacteria against such stress, these mechanisms induce changes in bacterial cells that largely affect their antimicrobial susceptibility [16]. In addition, stress could alter cell physiology or promote physiological changes that may interfere with bacterial response to various antimicrobials [16]. Bacteria usually respond to environmental stresses by developing either phenotypic or genotypic adaptations [26]. Phenotypic alterations include expression of shock proteins that protect bacteria against subsequent challenge with the same stress in a process known as stress hardening [27]. Similarly, bacterial resistance to antibiotics can be induced following bacterial exposure to certain stress in a phenomenon known as cross-protection [9, 28–31]. On the other hand, genetic plasticity which means increased rates of mutagenesis as well as intra- and inter-cellular genes transfer, is considered an example for genotypic alterations that take place in bacteria upon stress exposure [31, 32]. Genotypic alterations enhance population diversity, increasing the possibility that some cells become more capable of surviving harsh conditions. Environmental stresses such as oxidative stress, pH extremes, starvation in addition to heat and osmotic shock were shown to induce mutation in bacterial cells [32].These adaptive mutations in bacterial cells are associated with changes in bacterial response to antibiotics either by increase or decrease in bacterial sensitivity [33].

Other studies further demonstrated that stress impact on bacterial response to antibiotics could be either directly or indirectly. The direct influence could occur through many mechanisms. For example, it could result from recruitment

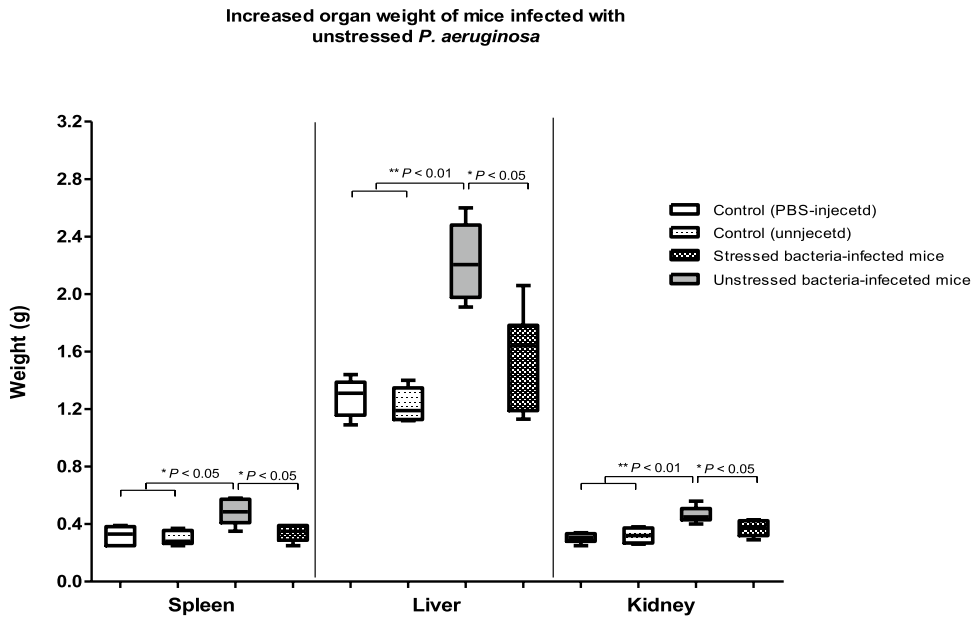

Fig. 5 Mice infected with unstressed bacteria showed increase in organs weights as compared with mice infected with H_2O_2-stressed bacteria. Mice (n = 6 mice/group) were inoculated intraperitoneally with 100 µL of suspended bacterial cells (2.5×10^7 CFU/mL) of H_2O_2-stressed and unstressed *Pseudomonas aeruginosa*. As negative controls, 6 mice were injected with 100 µL of sterile PBS, and another 6 mice were left uninfected. Bacteria-infected and control mice were anesthetized by inhalation of ether 24 h postinfection. Spleen, liver, and kidney samples were obtained aseptically and weighed. There was a significant increase (*$P < 0.05$; Mann–Whitney *U* analysis) in weight of organs obtained from mice infected with unstressed bacteria relative to those obtained from both control mice as well as mice injected with H_2O_2-stressed bacteria. Organs weights were calculated and expressed as the mean ± standard errors

of resistance determinants such as antimicrobial efflux upon exposure to stress condition [34, 35]. Moreover, changes in antimicrobial targets [34, 36, 37] and induced formation of resistant biofilms [38, 39] following stress exposure could play a role in bacterial susceptibility to antibiotics. For instance, enhanced biofilm formation was observed in *P. aeruginosa* following exposure to oxidative hypochlorite through the activation of c-di-GMP synthesis [40]. Finally, alterations to the membrane structure and/or function [41–43] under stress conditions were shown to affect bacterial sensitivity to antibiotics. Since antimicrobial agents act on growing cells [44], the stress-induced growth cessation or dormancy [45–47], could indirectly affect bacterial susceptibility to antimicrobials. Furthermore, it has been shown that antimicrobial agents can induce bacterial resistance mechanisms since these compounds themselves are growth-inhibiting stressors that trigger bacteria to develop protective responses [48].

Current data clearly demonstrate that prior exposure of *P. aeruginosa* to oxidative stress adversely affects expression of virulence genes. Expression of QS genes in H_2O_2-stressed *P. aeruginosa* was significantly reduced as compared to unstressed bacteria. In addition, exposure of *P. aeruginosa* to oxidative stress largely affects its pathogenesis in mice infection models. As previously shown, the QS machinery systems are connected in a hierarchical manner and play an important role in the pathogenesis of *P. aeruginosa*. For instance, lasI/R (regulate the production of elastase, protease, and exotoxin A), rhlI/R (regulate the production of elastase, pyocyanin, rhamnolipids, protease, and biofilm formation), and pqsA/R (regulate the production of pyocyanin) [6, 49]. The las system is present at the top of the signaling hierarchy and considered as the chief regulator, once it is stimulated it activates both rhl and pqs systems [50]. Reduced expression of the las system results in inhibition of the subsequent cascade including the rhl and pqs systems, and in turn las, rhl, and pqs-dependent virulence factors production would be depressed [50, 51]. As mentioned before, bacterial exposure to stress conditions could result in severe damage to cell components [10, 52–56].

Bacteria exhibit a significant decline in ATP production following H_2O_2 exposure [30]. It was shown that bacteria have H_2O_2-sensitive sites in ATP production machinery or that H_2O_2 disrupts bacterial energy-production pathways [57]. In addition to interfering with bacterial membrane permeability and ATP production, oxidants were found to be capable of causing substantial harms to bacterial proteins. For instance, exposure to oxidants leads to extensive protein unfolding and aggregation as these oxidants rapidly oxidize a variety of bacterial amino acid side chains [53, 58, 59]. In addition, excessive ROSs generated within bacteria following exposure to various oxidants could result in oxidizing

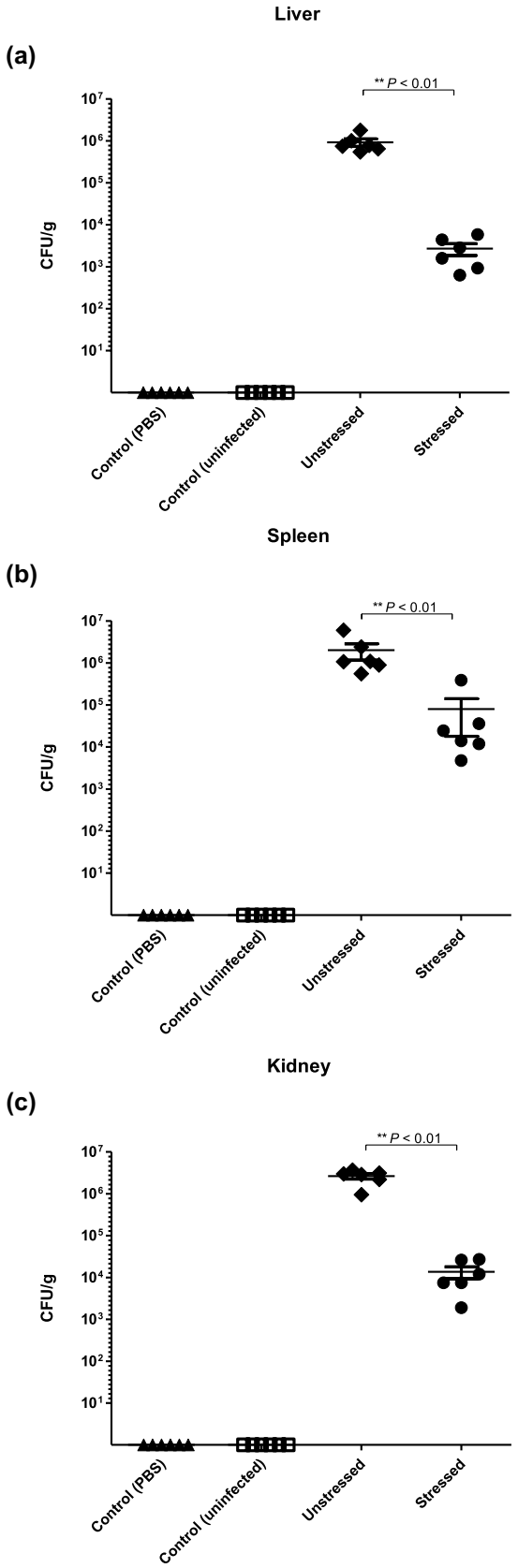

Fig. 6 Organs isolated from mice infected with unstressed *Pseudomonas aeruginosa* showed increased bacterial load as compared to mice injected with H_2O_2-stressed bacteria. Two groups (6 mice each) were injected with H_2O_2-stressed and unstressed bacteria. Control mice were included exactly as mentioned before. Infected and control mice were anesthetized by inhalation of ether at the designated time point (24 h postinfection) and mice were sacrificed. Liver (**a**), spleen (**b**), and kidney (**c**) were obtained, homogenized, serially diluted in PBS, and aliquots were plated on cetrimide agar plates. The plates were incubated at 37 °C for 24 h under aerobic conditions to determine the bacterial loads for each organ and expressed as (CFU/g). The bacterial load in each organ was calculated and expressed as means ± standard errors. Bacterial load of unstressed *Pseudomonas aeruginosa* in mice organs was significantly higher than H_2O_2-stressed bacteria (*$P < 0.05$; Mann–Whitney *U* test)

of membrane fatty acids, lipid peroxidation [60], and DNA damage [61, 62]. Finally, bacterial uptake of glucose and amino acids was found to be inhibited by exposure to various oxidizing agents. Oxidants most likely target and damage membrane-bound transport proteins that are responsible for uptaking of these valuable nutrients [63]. Therefore, we suggest that upon exposure to oxidative stress, *P. aeruginosa* directs its machinery towards fixing and repairing the damage that takes place in cellular structures at the expense of enhancing bacterial virulence capability. Moreover, bacterial attenuation upon stress exposure could facilitate bacterial clearance by the host immune especially when cells are phagocytosed and encounter further phagocyte-produced oxidants.

In contrast to current findings, a correlation between oxidative stress response and increased *P. aeruginosa* host virulence has been previously reported [64, 65]. These studies indicated that exposure to certain levels of oxidative stress could render *P. aeruginosa* more resistant to killing by immune cells through enhancing bacterial defense mechanisms [25, 66]. In fact, the difference between present outcomes and those previous studies is not surprising. This difference could be attributed to many reasons such as variations in experimental conditions including bacterial growth phase, level, and exposure time to oxidative stress employed in each study. In support of this explanation and in light of the hierarchical oxidative stress model, it has been shown that low level of oxidative stress could just enhance bacterial defense machinery, while at increased levels, the excessive production of ROSs drastically damage bacterial cells [67].

In conclusion, the current study highlights the influence of exposure to oxidative stress on both *P. aeruginosa* antibiotic susceptibility and host pathogenesis. *P. aeruginosa* sensitivity to commonly used antibiotics was found to be significantly affected upon exposure to oxidative stress. In addition, a difference in antibiotic susceptibility following exposure to oxidative stress between *P. aeruginosa* clinical isolates and the standard strain has been demonstrated herein. This finding sheds light on the importance of

environment in reshaping bacterial response to antimicrobial agents. More attention should be paid to patients suffering from *Pseudomonas* infections both during treatment by antibiotics and bacterial control using disinfectants and/or antiseptics. To our knowledge, this is the first study to illustrate that *P. aeruginosa* pathogenesis is significantly reduced upon exposure to oxidative stress using mice as infection model. The current results are valuable and could broaden our understanding of virulence mechanisms of *P. aeruginosa* and hence help us control and treat infections caused by this important pathogen.

Authors' contributions MMA and GHS conceived and designed the experiments; FAM performed the experiments; MMA designed the tables & figures, interpreted the results, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in animal study were in accordance with the ethical standards of The Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC) with approval number ZU-IACUC3/F/138/2019. All animals were handled according to guidelines from the Animal Ethics Board (Zagazig University). All participants provided written informed consent prior to enrolment in the study.

References

1. Filiatrault MJ, Picardo KF, Ngai H, Passador L, Iglewski BH (2006) Identification of *Pseudomonas aeruginosa* genes involved in virulence and anaerobic growth. Infect Immunity 74(7):4237–4245
2. Strateva T, Yordanov D (2009) *Pseudomonas aeruginosa*: a phenomenon of bacterial resistance. J Med Microbiol 58(9):1133–1148
3. Moreau-Marquis S, Bomberger JM, Anderson GG, SwiateckaUrban A, Ye S, O'Toole GA, Stanton BA (2008) The ΔF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. Am J Physiol-Lung Cell Mol Physiol 295(1):L25–L37
4. Stover C, Pham X, Erwin A, Mizoguchi S, Warrener P, Hickey M, Brinkman F, Hufnagle W, Kowalik D, Lagrou M (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406(6799):959
5. Zhou JW, Luo HZ, Jiang H, Jian TK, Chen ZQ, Jia AQ (2018) Hordenine: a novel quorum sensing inhibitor and antibiofilm agent against *Pseudomonas aeruginosa*. J Agric Food Chem 66(7):1620–1628. https://doi.org/10.1021/acs.jafc.7b05035
6. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ (2012) The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 76(1):46–65
7. Lister PD, Wolter DJ, Hanson ND (2009) Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 22(4):582–610. https://doi.org/10.1128/cmr.00040-09
8. Gill EE, Franco OL, Hancock RE (2015) Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens. Chem Biol Drug Des 85(1):56–78
9. McMahon MA, Xu J, Moore JE, Blair IS, McDowell DA (2007) Environmental stress and antibiotic resistance in food-related pathogens. Appl Environ Microbiol 73(1):211–217. https://doi.org/10.1128/AEM.00578-06
10. AlNabulsi AA, Osaili TM, Elabedeen NAZ, Jaradat ZW, Shaker RR, Kheirallah KA, Tarazi YH, Holley RA (2011) Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of Cronobacter sakazakii. Int J Food Microbiol 146(2):137–143
11. Yousef AE, Courtney PD (2003) Basics of stress adaptation and implications in new generation food. In: Yousef AE, Juneja VK (eds) Microbial stress adaptation and food safety. CRC Press, Boca Raton, Florida, pp 1–30
12. Bower CK, Daeschel MA (1999) Resistance responses of microorganisms in food environments. Int J Food Microbiol 50(1–2):33–44
13. Storz G, Imlayt JA (1999) Oxidative stress. Curr Opin Microbiol 2(2):188–194
14. Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D (2013) The stringent response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen peroxide and antibiotic tolerance. J Bacteriol 195(9):2011–2020. https://doi.org/10.1128/jb.02061-12
15. Moradali MF, Ghods S, Rehm BHA (2017) *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol. https://doi.org/10.3389/fcimb.2017.00039
16. Poole K (2012) Bacterial stress responses as determinants of antimicrobial resistance. J Antimicrob Chemother 67(9):2069–2089. https://doi.org/10.1093/jac/dks196
17. Koneman E PG, Schreckenberger P, Woods G, Winnw W, Allen S, Janda W (2006) Koneman'scolor atlas and textbook of diagnostic microbiology, 6th ed. Lippincott Williams and willkins, Philadelphia, Baltimore, New York, London
18. Jorgensen JH, Ferraro MJ (2009) Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis 49(11):1749–1755. https://doi.org/10.1086/647952
19. Saleh MM, Abbas HA, Askoura MM (2019) Repositioning secnidazole as a novel virulence factors attenuating agent in *Pseudomonas aeruginosa*. Microb Pathog 127:31–38. https://doi.org/10.1016/j.micpath.2018.11.042
20. Lee S, Hinz A, Bauerle E, Angermeyer A, Juhaszova K, Kaneko Y, Singh PK, Manoil C (2009) Targeting a bacterial stress response to enhance antibiotic action. Proc Natl Acad Sci USA 106(34):14570–14575. https://doi.org/10.1073/pnas.0903619106
21. Kobayashi F (1971) Experimental infection with *Pseudomonas aeruginosa* in mice. II. The fate of highly and low virulent strains in the peritoneal cavity and organs of mice. Jpn J Microbiol 15(4):301–307
22. Weng Y, Chen F, Liu Y, Zhao Q, Chen R, Pan X, Liu C, Cheng Z, Jin S, Jin Y, Wu W (2016) *Pseudomonas aeruginosa* enolase influences bacterial tolerance to oxidative stresses and virulence. Front Microbiol 7:1999. https://doi.org/10.3389/fmicb.2016.01999
23. Poole K (2012) Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol 20(5):227–234
24. Small DA, Chang W, Toghrol F, Bentley WE (2007) Comparative global transcription analysis of sodium hypochlorite,

peracetic acid, and hydrogen peroxide on *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 76(5):1093–1105. https://doi.org/10.1007/s00253-007-1072-z
25. Lan L, Murray TS, Kazmierczak BI, He C (2010) *Pseudomonas aeruginosa* OspR is an oxidative stress sensing regulator that affects pigment production, antibiotic resistance and dissemination during infection. Mol Microbiol 75(1):76–91
26. Hengge-Aronis R (2000) The general stress response in Escherichia coli. Bacterial stress responses
27. Rowan NJ (1999) Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. Trends Food Sci Technol 10(8):261–270
28. Katzif S, Danavall D, Bowers S, Balthazar JT, Shafer WM (2003) The major cold shock gene, cspA, is involved in the susceptibility of *Staphylococcus aureus* to an antimicrobial peptide of human cathepsin G. Infect Immun 71(8):4304–4312
29. Alekshun MN, Levy SB (1997) Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. Antimicrob Agents Chemother 41(10):2067
30. Hannum DM, Barrette WC Jr, Hurst JK (1995) Subunit sites of oxidative inactivation of *Escherichia coli* F1-ATPase by HOCl. Biochem Biophys Res Commun 212(3):868–874
31. Velkov VV (1999) How environmental factors regulate mutagenesis and gene transfer in microorganisms. J Biosci 24(4):529–559
32. Jolivet-Gougeon A, David-Jobert S, Tamanai-Shacoori Z, Ménard C, Cormier M (2000) Osmotic stress-induced genetic rearrangements in *Escherichia coli* H10407 detected by randomly amplified polymorphic DNA analysis. Appl Environ Microbiol 66(12):5484–5487
33. Foster PL (2000) Adaptive mutation: implications for evolution. BioEssays 22(12):1067–1074
34. Poole K (2008) Bacterial multidrug efflux pumps serve other functions. Microbe 3(4):179
35. Lipus D, Vikram A, Gulliver D, Bibby K (2019) Upregulation of peroxide scavenging enzymes and multidrug efflux proteins highlight an active sodium hypochlorite response in *Pseudomonas fluorescens* biofilms. Biofouling 35(3):329–339. https://doi.org/10.1080/08927014.2019.1605357
36. Ishiguro EE, Ramey WD (1980) Inhibition of in vitro peptidoglycan biosynthesis in *Escherichia coli* by guanosine 5′-diphosphate 3′-diphosphate. Can J Microbiol 26(12):1514–1518
37. Wu J, Long Q, Xie J (2010) (p) ppGpp and drug resistance. J Cell Physiol 224(2):300–304
38. Landini P (2009) Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. Res Microbiol 160(4):259–266
39. Tan X, Qin N, Wu C, Sheng J, Yang R, Zheng B, Ma Z, Liu L, Peng X, Jia A (2015) Transcriptome analysis of the biofilm formed by methicillin-susceptible Staphylococcus aureus. Sci Rep 5:11997. https://doi.org/10.1038/srep11997
40. Strempel N, Nusser M, Neidig A, Brenner-Weiss G, Overhage J (2017) The oxidative stress agent hypochlorite stimulates c-di-GMP synthesis and biofilm formation in *Pseudomonas aeruginosa*. Front Microbiol 8:2311. https://doi.org/10.3389/fmicb.2017.02311
41. Gunn JS (2001) Bacterial modification of LPS and resistance to antimicrobial peptides. J Endotoxin Res 7(1):57–62
42. Kato A, Groisman EA (2008) The PhoQ/PhoP regulatory network of Salmonella enterica. In: Bacterial signal transduction: networks and drug targets. Springer, Berlin, pp 7–21
43. Meehl M, Herbert S, Götz F, Cheung A (2007) Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 51(8):2679–2689. https://doi.org/10.1128/AAC.00209-07
44. Eng RH, Padberg FT, Smith SM, Tan EN, Cherubin CE (1991) Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Chemother 35(9):1824–1828. https://doi.org/10.1128/aac.35.9.1824
45. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 4(4):295–305. https://doi.org/10.1038/nrmicro1384
46. Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372. https://doi.org/10.1146/annurev.micro.112408.134306
47. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN (2004) SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. Science (New York, NY) 305(5690):1629–1631. https://doi.org/10.1126/science.1101630
48. Morita Y, Sobel ML, Poole K (2006) Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: involvement of the antibiotic-inducible PA5471 gene product. J Bacteriol 188(5):1847–1855. https://doi.org/10.1128/JB.188.5.1847-1855.2006
49. El-Mowafy SA, El Galil KHA, El-Messery SM, Shaaban MI (2014) Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in *Pseudomonas aeruginosa*. Microb Pathog 74:25–32
50. Kiratisin P, Tucker KD, Passador L (2002) LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer. J Bacteriol 184(17):4912–4919. https://doi.org/10.1128/jb.184.17.4912-4919.2002
51. El-Mowafy SA, Shaaban MI, Abd El Galil KH (2014) Sodium ascorbate as a quorum sensing inhibitor of *Pseudomonas aeruginosa*. J Appl Microbiol 117(5):1388–1399. https://doi.org/10.1111/jam.12631
52. Albrich JM, Gilbaugh JH 3rd, Callahan KB, Hurst JK (1986) Effects of the putative neutrophil-generated toxin, hypochlorous acid, on membrane permeability and transport systems of Escherichia coli. J Clin Investig 78(1):177–184. https://doi.org/10.1172/jci112548
53. Dahl J-U, Gray MJ, Jakob U (2015) Protein quality control under oxidative stress conditions. J Mol Biol 427(7):1549–1563
54. Halliwell B (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The lancet 344(8924):721–724
55. Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem 77:755–776
56. Imlay JA (2013) The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol 11(7):443–454. https://doi.org/10.1038/nrmicro3032
57. Bonvillain RW, Painter RG, Ledet EM, Wang G (2011) Comparisons of resistance of CF and non-CF pathogens to hydrogen peroxide and hypochlorous acid oxidants in vitro. BMC Microbiol 11(1):112
58. Winter J, Ilbert M, Graf P, Özcelik D, Jakob U (2008) Bleach activates a redox-regulated chaperone by oxidative protein unfolding. Cell 135(4):691–701
59. Winterbourn CC (2008) Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol 4(5):278
60. Mead J (1976) In Free radicals in, biology edn. Academic Press, New York, WA Pryor
61. Demple B, Linn S (1982) 5, 6-Saturated thymine lesions in DNA: production by ultraviolet light or hydrogen peroxide. Nucleic Acids Res 10(12):3781–3789
62. Levin DE, Hollstein M, Christman MF, Schwiers EA, Ames BN (1982) A new Salmonella tester strain (TA102) with A X T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci USA 79(23):7445–7449. https://doi.org/10.1073/pnas.79.23.7445

63. Chandler JD, Day BJ (2015) Biochemical mechanisms and therapeutic potential of pseudohalide thiocyanate in human health. Free Radic Res 49(6):695–710
64. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D (2004) *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. Infect Immun 72(7):4275–4278
65. Lau GW, Hassett DJ, Ran H, Kong F (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol Med 10(12):599–606
66. Rodriguez-Rojas A, Mena A, Martin S, Borrell N, Oliver A, Blazquez J (2009) Inactivation of the hmgA gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology (Reading, England) 155(Pt 4):1050–1057. https://doi.org/10.1099/mic.0.024745-0
67. Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, Sioutas C, Yeh JI, Wiesner MR, Nel AE (2006) Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. Nano Lett 6(8):1794–1807

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.