# Evaluating the Effect of Oxygen Concentrations on Antibiotic Sensitivity, Growth, and Biofilm Formation of Human Pathogens

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**ABSTRACT:** Standard antimicrobial susceptibility tests are performed in vitro under normal room oxygen conditions to predict the in vivo effectiveness of antimicrobial therapy. The aim of this study was to conduct a comprehensive analysis of the effect of different oxygen levels on the antibiotic susceptibility of two strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. It was found that anoxic conditions caused reduced sensitivity of bacteria to aminoglycoside antibiotics in four of six bacteria used in the study. In addition, oxygen limitation decreased the susceptibility of *P. aeruginosa* strains and *K. pneumoniae* strains to piperacillin/tazobactam and azithromycin, respectively. In contrast, five of six bacteria became more susceptible to tetracycline antibiotics under oxygen-limiting conditions. Our data highlight the importance of considering the potential in vivo oxygen levels within the infection site when setting susceptibility breakpoints for evaluating the therapeutic potential of a drug and its effect on antibiotic sensitivity of the pathogen.

**KEYWORDS:** oxygen concentration, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, antibiotic sensitivity, minimum inhibitory concentration

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## Introduction

Since their introduction in the early 1940s, antibiotics have saved millions of lives and are considered a marvel of modern medicine. However, in the past few years, there has been an alarming increase in antibiotic resistance.[1] The most common microorganisms developing resistance and responsible for two-thirds of healthcare-associated infections have been reported to belong to the "ESKAPE" group of pathogens, which include the gram-negative bacteria *Acinetobacter baumannii*, *Enterobacter* species, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* and the gram-positive bacteria *Enterococcus faecium* and *Staphylococcus aureus*.[2,3]

To determine efficacy of an antibiotic or possible drug resistance in a specific pathogen, it is required to isolate and examine the antibiotic susceptibility of the pathogen in a clinical microbiology laboratory. Antimicrobial susceptibility tests are performed in vitro to predict the in vivo effectiveness of antimicrobial therapy and help guide the choice and dosage of the antibiotic. The most commonly used testing methods include broth dilution, disk diffusion, and gradient diffusion methods, along with the use of automated instrument systems.[4] The key parameter used as a measure of antibiotic sensitivity is minimum inhibitory concentration (MIC). It is calculated as the lowest concentration of the antibiotic required to inhibit the visible growth of a microorganism after overnight incubation.[5,6] One of the methods for MIC determination on agar medium is the use of E-test antibiotic strips.[7] The strips have a predefined antibiotic concentration gradient and the MIC values are determined based on where the zone of inhibition intersects the strip. The E-test has been found to be fairly accurate and comparable with the other conventional susceptibility tests.[8,9]

The standard antibiotic susceptibility tests (ASTs) are routinely performed on bacteria grown planktonically or on agar plates under *normal* ambient room oxygen conditions. However, oxygen levels could be low under clinically relevant environments such as in burn wounds, lungs of cystic fibrosis patients, intra-abdominal abscess, the oral cavity, and others, contributing to increased pathogen persistence.[10–12] Furthermore, reduced oxygen levels might facilitate biofilm formation for pathogens such as *Pseudomonas* and *Staphylococcus*, contributing toward their increasing tolerance to traditionally recommended antibiotics.[13,14] On the other hand, hyperbaric oxygen therapy ($HBO_2$) and oxygen therapy[15,16] could increase the exposure of a pathogen to high levels of oxygen, which again might alter the antibiotic susceptibility from that measured under ambient oxygen levels.[17,18]

The purpose of this study was to conduct a comprehensive analysis of the effect of oxygen on antibiotic susceptibility of three key human pathogens. In this study, a

system was developed, which allowed experiments to be conducted in enriched oxygen environments. Together with other oxygen-limiting systems, the MICs of 14 antibiotics, representing 7 different classes, were determined using both laboratory strains and clinical isolates of *S. aureus, K. pneumoniae*, and *P. aeruginosa*. Our data show that oxygen levels can greatly alter the antibiotic sensitivity of the pathogen and should be taken into consideration when setting up susceptibility breakpoints and evaluating the therapeutic potential of a drug.

## Materials and Methods

**Bacterial strains and culture conditions.** The following bacteria were used in the study: *S. aureus* FPR3757, a multidrug-resistant strain USA300,[19] *S. aureus* SH1000,[20] *K. pneumoniae* AZ1169,[21] *K. pneumoniae* ATCC 33495, *P. aeruginosa* UCBPP-PA14,[22] and *P. aeruginosa* PaA, a clinical isolate from keratitis patients.[23] The bacteria represent both relatively fresh clinical isolates and commercially obtained laboratory strains. Bacteria were grown routinely in Luria Broth (LB) media at 37°C.

**Antibiotic sensitivity test.** Fourteen antibiotics were chosen to represent different classes and modes of action, which included the following: β-lactam antibiotics (meropenem, doripenem, ampicillin, and piperacillin/tazobactam), glycopeptide (vancomycin), tetracyclines (tetracycline and tigecycline), aminoglycosides (gentamicin, amikacin, tobramycin, and kanamycin), macrolides (azithromycin), fluoroquinolones (ciprofloxacin), and rifampicin. Although not all tested antibiotics are clinically relevant for each of the bacteria evaluated, to maintain consistency, all of the 14 selected antibiotics were tested on all the bacteria. Antibiotic sensitivity assays were performed using E-test antibiotic strips (bioMerieux), according to the manufacturer's protocol, with some modifications. In brief, a single colony of each bacterial strain was inoculated overnight in LB. Thereafter, the cultures were washed and diluted in phosphate-buffered saline (PBS) to bring the concentrations to an optical density ($OD_{600}$) of 0.1, corresponding to $1 \times 10^8$, $4 \times 10^8$, and $6 \times 10^7$ colony forming units/mL (CFU/mL) for *S. aureus*, *P. aeruginosa*, and *K. pneumoniae*, respectively. A total of 100 µL of *S. aureus* or *K. pneumoniae* cells were placed onto a 20 mm Petri plate containing tryptic soy agar (1.5% agar; TSA). *P. aeruginosa* cells were placed onto plates containing LB supplemented with 1.5% agar and 1% $KNO_3$. The medium used in the present study was selected as it allowed the growth of bacteria in all oxygen conditions. Sterile glass beads were used to spread the inoculums on the plates and produce an evenly distributed lawn. Once the agar surface was completely dry, E-test antibiotic strips were placed on top of the microbial lawn with sterile forceps. Plates were placed in the appropriate oxygen environment and incubated at 37°C for 24 hours. Experiments were conducted three times in triplicate. MIC values were determined according to manufacturer's guidelines (E-test Antimicrobial Susceptibility Testing, 2012), which specified values at the point of complete inhibition of all growth. Antibiotic concentrations on the strips used for meropenem, doripenem, ciprofloxacin, and rifampicin were 0.003–32 µg/mL. The remaining antibiotics that were tested had antibiotic gradients 0.016–256 µg/mL.

**Oxygen growth conditions.** Experiments were conducted in five oxygen conditions. For *anoxic* growth environment (0% $O_2$), plates were placed in a CoyLab anaerobic chamber (Coy Laboratory Products) using anoxic gas mix of 10% $H_2$, 10% $CO_2$, and 80% $N_2$. *Hypoxic* conditions with low oxygen levels (7%–9% $O_2$, 5%–8% $CO_2$) were obtained by placing the plates in a sealed Mitsubishi™ AnaeroPack™ 2.5 L Rectangular Jar system containing an AnaeroPack™-MicroAero Gas Generator pack (Mitsubishi Gas Chemical America Inc). For *normoxic* room oxygen environment (20.8% $O_2$), plates were placed in a standard benchtop incubator (VWR). A benchtop $CO_2$ incubator was used to obtain enriched $CO_2$ environment of 5.5%, while maintaining ambient room oxygen levels (20.8%). Finally, for hyperoxic oxygen environment with elevated $O_2$ levels (95%–99% $O_2$), a modification of our gasbag system was used (Fig. 1).[24]


**Figure 1.** Closed container system with valve used to maintain hyperoxic gas environment. Plates were placed in a 1 L airtight container. Air was removed using a vacuum tube connected to a standard laboratory vacuum gas tap. Pure oxygen was inserted via a PVC tube connected to a compressed gas cylinder and the container was incubated at 37°C.

A 1 L polypropylene airtight container with a sealing O-ring was used (Fisherbrand™ Infecon™ 3000 Infectious Substance Shipper Kit). Two Luer Stopcock valves were placed on 3 mL syringes, which were inserted into the lid and secured using clear silicone sealant. One valve was connected via a clear polyvinyl chloride (PVC) tube to a standard laboratory vacuum gas tap. The second valve was connected to a gas regulator attached to a compressed gas cylinder containing 99.99% pure medical grade oxygen (Gts-Welco). To conduct the experiment, plates were inserted into the jar and the jar was sealed. Air was removed using the vacuum tube. Thereafter, the vacuum tube was closed and pure oxygen was inserted (final Psi reading of ~8). The oxygen was removed once more via vacuum and reintroduced. This gas-flushing procedure was performed five times in order to remove all ambient air. The jars were placed at 37°C and incubated for 24 hours. No change in gas pressure was measured, conforming that the jar was airtight. Oxygen levels were confirmed by using a Traceable® Portable Dissolved Oxygen Meter (Fisher Scientific).

**Growth curve and biofilm formation.** To measure microbial growth in each oxygen condition, each bacterial strain was inoculated overnight in LB. Thereafter, the cells were washed and diluted in PBS to bring the concentrations to an $OD_{600}$ of 0.1. The cells were diluted 1:10 in fresh TSB (for *S. aureus* and *K. pneumoniae*) or LB/$KNO_3$ media (for *P. aeruginosa*). The cultures were placed in 96-well micro titer wells (120 µL per well). Each plate was placed at a different oxygen condition, one plate for each time point in a separate jar. At each time point (0, 10, 20, 28, and 46 hours), the plates were removed and the growth was measured at 600 nm ($OD_{600}$) using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). For biofilm assays, the plates were prepared as described above. After 24 hours of incubation, plates were removed, washed in order to detach loosely attached cells, and stained with 0.1% crystal violet (CV). CV was solubilized using acetic acid 50% (v/v), and relative biofilm biomass was assayed by measuring the optical density of the CV solution at 600 nm ($OD_{600}$).[25] The experiments were conducted twice in quadruplicate.

**Statistical analysis.** GraphPad Prism 6 (GraphPad Software, Inc.) was used to perform one-way analysis of variance followed by Tukey's multiple comparison test. Results were considered significant at $P$-value $<0.05$.

## Results

**Antibiotic sensitivity tests.** To measure the effect of different oxygen levels on antibiotic efficacy, MIC tests were conducted using E-test strips. As anticipated, the MIC values varied greatly among the different bacteria and conditions. Furthermore, large differences in susceptibility were measured ranging from 2-fold to a greater than 30-fold for different oxygen environments when compared to the MIC values under normal room ambient air incubation.

Compared to room $O_2$ levels, anoxic conditions altered *S. aureus* strain FPR 3757 susceptibility in 80% of the tested antibiotics. Increased MIC values were measured for meropenem (11-fold), doripenem (20-fold), ampicillin (6-fold), piperacillin/tazobactam (5-fold), kanamycin (7-fold), gentamicin (10-fold), amikacin (12-fold), and tobramycin (23-fold). In comparison, 2–3-fold decreases in MIC values for tetracycline and tigecycline were observed. On the other hand, hyperoxic incubation did not produce any notable change in the MIC values. Hypoxic environment and elevated $CO_2$ conditions increased the sensitivity of bacteria to piperacillin/tazobactam 2–3-fold. In contrast, hypoxia decreased the efficacy of amikacin and tobramycin, increasing the MICs 3- and 6-fold, respectively (Table 1).

*S. aureus* strain SH1000 demonstrated similar reductions in susceptibility to aminoglycosides under anoxic conditions indicated by increases in MIC values by 12-, 18-, and 26-fold for gentamicin, amikacin, and tobramycin, respectively. Hypoxic incubation as well as incubation under high $O_2$ environment decreased the efficacy of gentamicin and azithromycin by increasing the MICs 3- to 5-fold. Additionally, there was a 2-fold reduction in MIC values for piperacillin/tazobactam under hypoxic incubation and for meropenem under high $CO_2$ conditions, as compared to values under normal ambient air (Table 2).

Investigating the sensitivity of *K. pneumoniae* strain AZ1169 to the various antibiotics, we found that 50% of the antibiotics had altered efficacy under oxygen-limiting conditions. Of the 14 antibiotics tested, anoxic incubation made the pathogen more susceptible to tetracycline and ciprofloxacin by 2–3-fold. A 3-fold increase in MIC values was measured for gentamicin, kanamycin, and azithromycin, whereas, a 10-fold increase in MIC values was measured for tobramycin. A hypoxic environment shifted the MIC value up by 3-fold for tobramycin and over 15-fold for azithromycin moving the value above the maximum concentration imbedded on the strip. On the other hand, a hyperoxic environment decreased the MIC value for ampicillin by almost 2-fold, in contrast to a 3-fold increase for azithromycin (Table 3).

*K. pneumoniae* ATCC strain 33495 showed a similar decline in sensitivity to five of the antibiotics under anoxic incubation, with approximately 3-fold increases in MIC values for gentamicin, amikacin, and kanamycin, and 5- and 8-fold increases for azithromycin and tobramycin, respectively. In contrast, susceptibility toward tigecycline increased 2-fold under anoxic condition. Hypoxic incubation increased the MIC values for gentamicin, kanamycin, and ciprofloxacin by around 2.5-fold, but caused a notable 30-fold increase in MIC value for azithromycin, suggestive of a drastic decrease in efficacy under oxygen-limiting conditions. Incubation under elevated $CO_2$ environment increased the MIC values for kanamycin and azithromycin by 2- and 13.5-folds, respectively. In addition, hyperoxic environment increased the MIC values of tobramycin, kanamycin, azithromycin, and ciprofloxacin by 2-, 2.5-, 33-, and 8.5-folds, respectively (Table 4).

**Table 1.** Antibiotic sensitivity of *S. aureus* FPR 3757 under various oxygen conditions.

| NO. | ANTIBIOTIC | NORMOXIA | ANOXIA | HYPOXIA | ELEVATED $CO_2$ | HYPEROXIA |
|---|---|---|---|---|---|---|
| 1 | Meropenem | 0.25 ± 0.00 | **3 ± 0.00** | 0.09 ± 0.00 | 0.09 ± 0.00 | 0.42 ± 0.07 |
| 2 | Doripenem | 0.13 ± 0.00 | **2.75 ± 0.96** | 0.11 ± 0.12 | **0.04 ± 0.01** | 0.15 ± 0.04 |
| 3 | Vancomycin | 9.33 ± 2.31 | 6 ± 0.00 | 6.67 ± 2.31 | 6.67 ± 2.31 | 5.33 ± 1.15 |
| 4 | Ampicillin | 0.5 ± 0.00 | **3.67 ± 0.58** | 0.23 ± 0.03 | **0.15 ± 0.04** | 0.83 ± 0.58 |
| 5 | Piperacillin/tazobactam | 4 ± 0.00 | **24 ± 0.00** | **1.33 ± 0.29** | **1 ± 0.00** | 1.5 ± 0.00 |
| 6 | Tetracycline | 21.33 ± 4.62 | **6 ± 0.00** | 10.67 ± 2.31 | 10.67 ± 2.31 | 10.67 ± 2.31 |
| 7 | Tigecycline | 0.75 ± 0.00 | **0.19 ± 0.00** | 0.29 ± 0.08 | 0.46 ± 0.07 | 0.42 ± 0.29 |
| 8 | Gentamicin | 0.63 ± 0.14 | **6.67 ± 1.15** | 1.83 ± 0.29 | 1.33 ± 0.29 | 1.56 ± 0.59 |
| 9 | Amikacin | 4 ± 0.00 | **53.33 ± 9.24** | **16 ± 6.93** | 5.33 ± 1.15 | 6.67 ± 1.15 |
| 10 | Tobramycin | 0.38 ± 0.00 | **9.33 ± 2.31** | **2.67 ± 1.15** | 1 ± 0.00 | 1 ± 0.00 |
| 11 | Kanamycin* | 4 ± 0.00 | **32 ± 0.00** | 6 ± 0.00 | 6 ± 0.00 | 6 ± 0.00 |
| 12 | Azithromycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 13 | Ciprofloxacin | >32 ± 0.00 | 18.67 ± 4.62 | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 |
| 14 | Rifampicin | 0.01 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |

**Notes:** Data represent MIC (μg/mL) values. Each experiment was conducted three times, with each value representing the mean and standard deviation. Values in bold represent 2-fold or higher differences in the values compared to that measured under normal room oxygen conditions. Bold gray boxes represent 5-fold or higher differences in the values compared to that measured under normal room oxygen conditions. The MIC values with ">" symbol are above the maximum concentration on the E-test strips. *Data represent values from only two experiments, as the antibiotic became unavailable from the manufacturer.

Antibiotic sensitivity testing on *P. aeruginosa* strain Pa14 showed the highest sensitivity to tetracycline under anoxic condition, which was 5-fold more than that measured under normal ambient oxygen conditions. However, oxygen limitation increased MIC values of amikacin by approximately 2-fold, but remarkably shifted the MIC for piperacillin/tazobactam combination to values above the maximum concentration on the E-test strip. $CO_2$-enriched environment led to a 3-fold decrease in MIC values for ampicillin. Finally, hyperoxic environment decreased the sensitivity of the bacterium to around one-third of the antibiotics, with 3- and 4-fold increases in MIC values measured for amikacin and ciprofloxacin, respectively (Table 5).

*P. aeruginosa* strain PaA demonstrated higher susceptibility to one-third of the test antibiotics under oxygen-limiting conditions. MIC values for tetracycline and tigecycline in

**Table 2.** Antibiotic sensitivity of *S. aureus* SH1000 under varying oxygen conditions.

| NO. | ANTIBIOTIC | NORMOXIA | ANOXIA | HYPOXIA | ELEVATED $CO_2$ | HYPEROXIA |
|---|---|---|---|---|---|---|
| 1 | Meropenem | 0.13 ± 0.00 | 0.09 ± 0.00 | 0.05 ± 0.01 | **0.04 ± 0.01** | 0.1 ± 0.02 |
| 2 | Doripenem | 0.04 ± 0.01 | 0.03 ± 0.00 | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.04 ± 0.01 |
| 3 | Vancomycin | 10.67 ± 2.31 | 8 ± 0.00 | 8 ± 0.00 | 8 ± 0.00 | 8 ± 0.00 |
| 4 | Ampicillin | 0.11 ± 0.02 | 0.13 ± 0.00 | 0.08 ± 0.02 | 0.05 ± 0.01 | 0.11 ± 0.02 |
| 5 | Piperacillin/tazobactam | 0.46 ± 0.07 | 0.38 ± 0.00 | **0.13 ± 0.00** | 0.23 ± 0.03 | 0.75 ± 0.00 |
| 6 | Tetracycline | 0.63 ± 0.14 | 0.58 ± 0.29 | 0.5 ± 0.00 | 0.46 ± 0.07 | 0.38 ± 0.00 |
| 7 | Tigecycline | 0.38 ± 0.00 | 0.32 ±0.11 | 0.42 ± 0.14 | 0.27 ± 0.08 | 0.23 ± 0.03 |
| 8 | Gentamicin | 0.25 ± 0.00 | **3.33 ± 0.58** | **1 ± 0.00** | 0.58 ± 0.14 | **1 ± 0.00** |
| 9 | Amikacin | 3 ± 0.00 | **58.67 ± 33.3** | 3.33 ± 0.58 | 2.67 ± 0.58 | 4.67 ± 1.15 |
| 10 | Tobramycin | 0.29 ± 0.08 | **8 ± 0.00** | 0.67 ± 0.29 | 0.5 ± 0.00 | 0.75 ± 0.00 |
| 11 | Kanamycin* | – | – | – | – | – |
| 12 | Azithromycin | 2 ± 0.00 | 2 ± 0.00 | **13.33 ± 2.31** | 4.67 ± 1.15 | **6.67 ± 2.31** |
| 13 | Ciprofloxacin | 0.5 ± 0.00 | 0.21 ± 0.03 | 0.5 ± 0.00 | 0.46 ± 0.07 | 0.58 ± 0.14 |
| 14 | Rifampicin | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |

**Notes:** Data represent MIC (μg/mL) values. Each experiment was conducted three times, with each value representing the mean and standard deviation. Values in bold represent 2-fold or higher differences in the values compared to that measured under normal room oxygen conditions. Bold gray boxes represent 5-fold or higher differences in the values compared to that measured under normal room oxygen conditions. The MIC values with ">" symbol are above the maximum concentration on the E-test strips. *Antibiotic was not available from the manufacturer.

**Table 3.** Antibiotic sensitivity of *K. pneumoniae* AZ1169 under varying oxygen conditions.

| NO. | ANTIBIOTIC | NORMOXIA | ANOXIA | HYPOXIA | ELEVATED $CO_2$ | HYPEROXIA |
|---|---|---|---|---|---|---|
| 1 | Meropenem | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| 2 | Doripenem | 0.03 ± 0.01 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.01 ± 0.00 |
| 3 | Vancomycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 4 | Ampicillin | 160 ± 55.43 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | **56 ± 11.13** |
| 5 | Piperacillin/tazobactam | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 6 | Tetracycline | 12 ± 0.00 | **4 ± 0.00** | 12 ± 0.00 | 12 ± 0.00 | 8 ± 0.00 |
| 7 | Tigecycline | 4 ± 0.00 | 3 ± 0.00 | 6 ± 0.00 | 6 ± 0.00 | 6 ± 0.00 |
| 8 | Gentamicin | 0.75 ± 0.00 | **3.33 ± 0.58** | 3 ± 0.00 | 1.83 ± 0.29 | 1.83 ± 0.29 |
| 9 | Amikacin | 3 ± 0.00 | 8 ± 3.46 | 5 ± 0.00 | 4 ± 1.73 | 4.67 ± 1.15 |
| 10 | Tobramycin | 0.75 ± 0.00 | **8 ± 3.46** | 3 ± 0.00 | 1.83 ± 0.29 | 1.5 ± 0.00 |
| 11 | Kanamycin | 2 ± 0.00 | **8 ± 0.00** | 4.67 ± 1.15 | 3.33 ± 0.58 | 4 ± 0.00 |
| 12 | Azithromycin | 16 ± 0.00 | **64 ± 0.00** | **>256 ± 0.00** | **64 ± 0.00** | **64 ± 0.00** |
| 13 | Ciprofloxacin | >32 ± 0.00 | **6.67 ± 1.15** | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 |
| 14 | Rifampicin | >32 ± 0.00 | 26.67 ± 4.62 | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 |

**Notes:** Data represent MIC (μg/mL) values. Each experiment was conducted three times, with each value representing the mean and standard deviation. Values in bold represent 2-fold or higher differences in the values compared to that measured under normal room oxygen conditions. Bold gray boxes represent 5-fold or higher differences in the values compared to that measured under normal room oxygen conditions. The MIC values with ">" symbol are above the maximum concentration on the E-test strips.

normoxic conditions were found to be over the maximum concentration on the strip. However, anoxic, hypoxic, and hyperoxic environments reduced the values by at least 8- to 12-, 7-, and 4- to 8-folds, respectively. Anoxic environment decreased the MIC values for meropenem and rifampicin by more than 2-fold, in contrast to an increase of 2-fold for amikacin and notably more for piperacillin/tazobactam, suggestive of a considerable decrease in bacterial susceptibility as compared to that under normal ambient air incubation. Elevated $CO_2$ potentiated the activity of carbapenems and tigecycline by decreasing the MICs 3-fold (Table 6).

**Microbial growth.** To determine if there is a correlation between sensitivity of the pathogens to various antibiotics under different oxygen conditions and the growth capability of

**Table 4.** Antibiotic sensitivity of *K. pneumoniae* ATCC 33495 under varying oxygen conditions.

| NO. | ANTIBIOTIC | NORMOXIA | ANOXIA | HYPOXIA | ELEVATED $CO_2$ | HYPEROXIA |
|---|---|---|---|---|---|---|
| 1 | Meropenem | 0.03 ± 0.01 | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| 2 | Doripenem | 0.05 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.01 | 0.04 ± 0.02 | 0.03 ± 0.00 |
| 3 | Vancomycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 4 | Ampicillin | 3.5 ± 0.58 | 3.67 ± 0.58 | 6.75 ± 4.11 | 5.33 ± 1.15 | 5.33 ± 2.31 |
| 5 | Piperacillin/Tazobactam | 4 ± 0.00 | 3.67 ± 0.58 | 6 ± 0.00 | 6.67 ± 2.31 | 8 ± 0.00 |
| 6 | Tetracycline | 42.67 ± 9.24 | 21.33 ± 4.62 | 32 ± 0.00 | 106.67 ± 18.48 | 96 ± 0.00 |
| 7 | Tigecycline | 0.75 ± 0.00 | **0.25 ± 0.00** | 1 ± 0.00 | 1 ± 0.00 | 1.5 ± 0.00 |
| 8 | Gentamicin | 0.75 ± 0.00 | **3 ± 0.00** | **2.67 ± 0.58** | 1.67 ± 0.29 | 1.83 ± 0.29 |
| 9 | Amikacin | 2 ± 0.00 | **9.33 ± 2.31** | 4 ± 0.00 | 3.67 ± 0.58 | 4 ± 0.00 |
| 10 | Tobramycin | 0.67 ± 0.14 | **6 ± 0.00** | **3 ± 0.00** | **2.33 ± 0.58** | **2 ± 0.87** |
| 11 | Kanamycin | 1.27 ± 1.48 | **5.33 ± 2.31** | **4.67 ± 1.15** | **4 ± 1.73** | **4.67 ± 1.15** |
| 12 | Azithromycin | 3.67 ± 0.58 | **21.33 ± 4.62** | **117.33 ± 18.48** | **53.33 ± 18.48** | **128 ± 0.00** |
| 13 | Ciprofloxacin | 0.02 ± 0.00 | 0.04 ± 0.01 | **0.07 ± 0.02** | 0.04 ± 0.01 | **0.19 ± 0.00** |
| 14 | Rifampicin | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 |

**Notes:** Data represent MIC (μg/mL) values. Each experiment was conducted three times, with each value representing the mean and standard deviation. Values in bold represent 2-fold or higher differences in the values compared to that measured under normal room oxygen conditions. Bold gray boxes represent 5-fold or higher differences in the values compared to that measured under normal room oxygen conditions. The MIC values with ">" symbol are above the maximum concentration on the E-test strips.

**Table 5.** Antibiotic sensitivity of *P. aeruginosa* Pa14 under varying oxygen conditions.

| NO. | ANTIBIOTIC | NORMOXIA | ANOXIA | HYPOXIA | ELEVATED $CO_2$ | HYPEROXIA |
|---|---|---|---|---|---|---|
| 1 | Meropenem | 0.13 ± 0.00 | 0.11 ± 0.02 | 0.13 ± 0.00 | 0.11 ± 0.02 | 0.19 ± 0.00 |
| 2 | Doripenem | 0.1 ± 0.04 | 0.13 ± 0.00 | 0.19 ± 0.00 | 0.13 ± 0.00 | 0.17 ± 0.04 |
| 3 | Vancomycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 4 | Ampicillin* | 24 | 24 | 32 | **8** | 64 |
| 5 | Piperacillin/tazobactam | 2 ± 0.00 | **>256 ± 0.00** | 1.5 ± 0.00 | 1.17 ± 0.29 | 2.67 ± 0.58 |
| 6 | Tetracycline | 85.33 ± 18.48 | **13.33 ± 2.31** | 48 ± 0.00 | 37.33 ± 9.24 | 42.67 ± 9.24 |
| 7 | Tigecycline | 24 ± 0.00 | 18.67 ± 4.62 | 12 ± 0.00 | 29.33 ± 4.62 | 24 ± 0.00 |
| 8 | Gentamicin | 2.67 ± 0.58 | 3.33 ± 0.58 | 3.33 ± 0.00 | 2.67 ± 0.58 | 5.33 ± 1.15 |
| 9 | Amikacin | 8 ± 0.00 | **26.67 ± 4.62** | 13.33 ± 0.00 | 9.33 ± 2.31 | **32 ± 0.00** |
| 10 | Tobramycin | 1.5 ± 0.00 | **6.67 ± 2.31** | 4 ± 0.00 | 2.67 ± 0.58 | 4.67 ± 1.15 |
| 11 | Kanamycin** | – | – | – | – | – |
| 12 | Azithromycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 13 | Ciprofloxacin | 0.13 ± 0.00 | 0.13 ± 0.00 | 0.29 ± 0.08 | 0.34 ± 0.08 | **0.75 ± 0.00** |
| 14 | Rifampicin | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 |

**Notes:** Data represent MIC (μg/mL) values. Each experiment was conducted three times, with each value representing the mean and standard deviation. Values in bold represent 2-fold or higher differences in the values compared to that measured under normal room oxygen conditions. Bold gray boxes represent 5-fold or higher differences in the values compared to that measured under normal room oxygen conditions. The MIC values with ">" symbol are above the maximum concentration on the E-test strips. *Data represent values from only one experiment as the manufacturer discontinued the antibiotic. **Antibiotic was not available from the manufacturer.

different bacteria, optical density of the bacterial cultures was measured after 0, 10, 20, 28, and 46 hours of incubation under different oxygen environments. For *S. aureus,* both FPR 3757 and SH1000 displayed the highest growth under hyperoxic environment, followed by ambient environment, least being under the oxygen-limiting conditions. For both the strains of *K. pneumoniae*, anoxic environment was the least favorable, with all other conditions being similar in facilitating growth as compared to growth under normal room air. Growth curves for both the strains of *P. aeruginosa* indicate least growth under anoxic conditions, but Pa14 strain had the highest optical density under hyperoxia, in contrast to a hypoxic environment favoring the growth of PaA (Fig. 2). In conclusion, differences in growth of the bacteria under varied environments were

**Table 6.** Antibiotic sensitivity of *P. aeruginosa* PaA under varying oxygen conditions.

| NO. | ANTIBIOTIC | NORMOXIA | ANOXIA | HYPOXIA | ELEVATED $CO_2$ | HYPEROXIA |
|---|---|---|---|---|---|---|
| 1 | Meropenem | 0.29 ± 0.08 | **0.08 ± 0.02** | **0.06 ± 0.01** | **0.06 ± 0.00** | 0.15 ± 0.04 |
| 2 | Doripenem | 0.32 ± 0.11 | 0.17 ± 0.07 | 0.17 ± 0.04 | **0.08 ± 0.02** | 0.25 ± 0.00 |
| 3 | Vancomycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 4 | Ampicillin* | – | – | – | – | – |
| 5 | Piperacillin/tazobactam | 1.83 ± 0.29 | **>256 ± 0.00** | 2 ± 0.00 | 1.5 ± 0.00 | 1.5 ± 0.00 |
| 6 | Tetracycline | >256 ± 0.00 | **18.67 ± 4.62** | **32 ± 0.00** | >256 ± 0.00 | **58.67 ± 9.24** |
| 7 | Tigecycline | >256 ± 0.00 | **26.67 ± 4.62** | **32 ± 0.00** | **58.67 ± 9.24** | **29.33 ± 4.62** |
| 8 | Gentamicin | 5.33 ± 1.15 | 14.67 ± 2.31 | 10.67 ± 2.31 | 7.33 ± 1.15 | 12 ± 4.00 |
| 9 | Amikacin | 26.67 ± 4.62 | **85.33 ± 36.95** | 42.67 ± 9.24 | 26.67 ± 4.62 | 37.33 ± 9.24 |
| 10 | Tobramycin | 3.33 ± 0.58 | 8 ± 0.00 | 6.67 ± 1.15 | 4.75 ± 1.50 | 4 ± 0.00 |
| 11 | Kanamycin* | – | – | – | – | – |
| 12 | Azithromycin | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 | >256 ± 0.00 |
| 13 | Ciprofloxacin | 0.19 ± 0.00 | 0.15 ± 0.04 | 0.38 ± 0.00 | 0.23 ± 0.03 | 0.46 ± 0.07 |
| 14 | Rifampicin | >32 ± 0.00 | **13.33 ± 2.31** | >32 ± 0.00 | >32 ± 0.00 | >32 ± 0.00 |

**Notes:** Data represent MIC (μg/mL) values. Each experiment was conducted three times, with each value representing the mean and standard deviation. Values in bold represent 2-fold or higher differences in the values compared to that measured under normal room oxygen conditions. Bold gray boxes represent 5-fold or higher differences in the values compared to that measured under normal room oxygen conditions. The MIC values with ">" symbol are above the maximum concentration on the E-test strips. *Antibiotic was not available from the manufacturer.

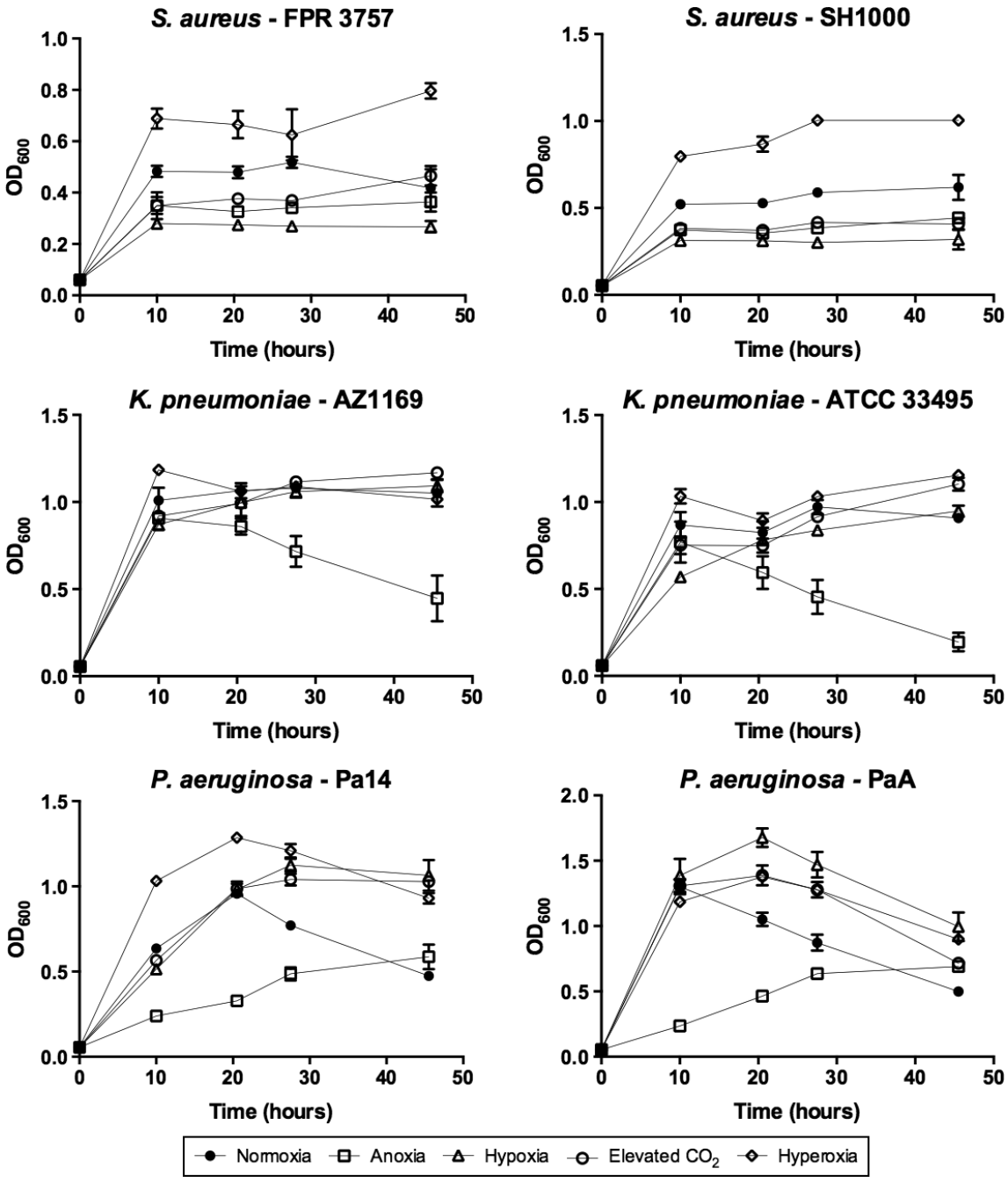


**Figure 2.** Effect of ambient oxygen levels on microbial growth. Ninety-six-well plates were inoculated with *S. aureus* FPR 3757, *S. aureus* SH1000, *K. pneumoniae* AZ1169, *K. pneumoniae* ATCC 33495, *P. aeruginosa* Pa14, and *P. aeruginosa* PaA. Plates were placed under varying oxygen conditions (five plates for each condition containing all six bacteria). At each time point (0, 10, 20, 28, and 46 hours), one plate was removed from its designated incubation chamber and growth was monitored by the change in culture turbidity measured as optical density at 600 nm ($OD_{600}$). Each value represents the mean of 8 wells. Error bars are shown as one standard deviation.

observed, with anoxic conditions being the least favorable for all the pathogens tested.

**Biofilm formation.** As biofilm formation greatly influences antibiotic tolerance,[26] we measured the ability of each bacterium to form biofilm under conditions similar to those under which the antibiotic sensitivity tests were performed. To this end, biofilm formation was measured after 24 hours of incubation under the different oxygen conditions. For one-third of the bacteria tested, a significant ($P < 0.0001$) positive correlation between oxygen levels and biofilm formation was observed, with the highest biofilm biomass seen for *S. aureus* SH1000 and *P. aeruginosa* Pa14 in 100% oxygen environments, as compared to that under ambient air environment. In contrast, *P. aeruginosa* strain PaA showed a decrease in biofilm formation under low as well as elevated oxygen environments (Fig. 3).

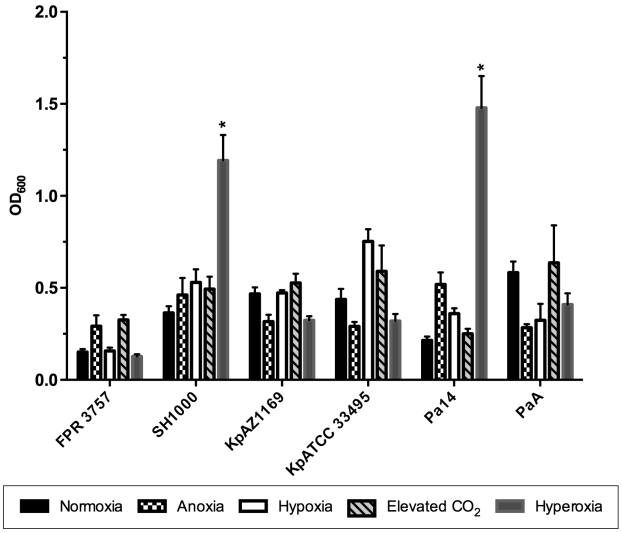


**Figure 3.** Effect of ambient oxygen levels on biofilm formation. Ninety-six-well plates were inoculated with *S. aureus* FPR 3757 (FPR 3757), *S. aureus* SH1000 (SH1000), *K. pneumoniae* AZ1169 (AZ1169), *K. pneumoniae* ATCC 33495 (ATCC 33495), *P. aeruginosa* Pa14 (Pa14), and *P. aeruginosa* PaA (PaA). Plates were placed under varying oxygen conditions. Data represent the amount of CV staining following 24 hours of incubation and measured at 600 nm ($OD_{600}$). Each value represents the mean of 8 wells. Error bars are shown as one standard deviation. Asterisks indicate significant differences ($P < 0.0001$) as compared to values under normal ambient air.

## Discussion

In recent years, nonprudent use of antibiotics has contributed to the surge of multidrug-resistant infections.[27] In order to make accurate therapeutic decisions, it might be important to determine antibiotic susceptibility under clinically relevant environmental conditions. Standard ASTs are done under normal room oxygen conditions, despite the fact that different sites of infection in the body have different oxygen and carbon dioxide concentrations.[28] The purpose of this study was to conduct a comprehensive analysis of the effect of oxygen on susceptibility of key human pathogens to a range of antibiotics.

Aminoglycosides are the most commonly used broad-spectrum antibiotics that inhibit bacterial protein synthesis by an energy-dependent mechanism for transport into the bacterial cells.[29] We observed that anoxic conditions caused the most notable reduction of the susceptibilities of *S. aureus* and *K. pneumoniae* strains to aminoglycoside antibiotics (Tables 1–4). This observation was in accordance with previous studies, which indicate that the bacterial uptake of these antibiotics is oxygen dependent, and thereby an anoxic environment markedly curtails their efficacy.[14,30]

In addition, we observed that incubation under anoxic environment led to substantial reduction in sensitivity of *S. aureus* strain FPR3757 to the β-lactam group of antibiotics (Table 1). β-Lactam antibiotics are known to inhibit bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs) on the cell membrane.[31] Oxygen deprivation may alter the expression of PBPs or decrease the affinity of the drugs for the PBP enzymes, leading to decreased sensitivity. However, this correlation is not well documented. Furthermore, *K. pneumoniae* strains demonstrated reduced sensitivity to the macrolide antibiotic, azithromycin, under anoxic conditions (Tables 3 and 4). Macrolide antibiotics are bacterial protein synthesis inhibitors. Reduction in efficacy of these antimicrobial agents suggests a modification of cell metabolic signaling pathways under oxygen-deficient environment; however, the mechanism is not clear.[32]

Piperacillin/tazobactam is a β-lactam/β-lactamase inhibitor antibiotic combination that inhibits bacterial cell wall synthesis. In this study, we found that both the *P. aeruginosa* strains show an increase in resistance to piperacillin/tazobactam under anoxic conditions (Tables 5 and 6). Possible reason for resistance could be altered membrane permeability to the drug under oxygen-deprived conditions.[33] In addition, an overexpression of multidrug efflux pump protein might lead to development of resistance.[34] Our data were not consistent with a previous study, which found no change in MIC values for piperacillin/tazobactam under anoxic conditions.[35] The different results in our study compared to the published work could be attributed to different bacterial strains used in the two studies. Piperacillin/tazobactam E-tests manufactured between December 2012 and October 2015 were recalled by manufacturer for issues in the results provided. However, the purpose of this study was to determine the impact of oxygen levels on antimicrobial susceptibility and not to obtain actual MIC values to be used for clinical applications.

In contrast to observed reduction in aminoglycoside, β-lactam, and macrolide antibiotic efficacies under anoxic environment, tetracycline and tigecycline antibiotics were found to be more effective against majority of the pathogens under limited oxygen conditions. There is a lack of documented studies investigating the effect of oxygen on tetracycline antibiotics. To our knowledge, it has not been previously shown that tetracycline antibiotics might be more effective under oxygen-limiting conditions. We found that the MIC values for *P. aeruginosa* strains decreased by greater extent compared to those for the other pathogens. *P. aeruginosa* strain PaA was resistant to tetracycline antibiotics at normal air incubation, but became susceptible under altered oxygen concentrations (Table 6). Mechanisms attributed to tetracycline resistance include its energy-dependent efflux, ribosomal protection, and oxygen-modulated chemical alteration of the antibiotic.[36,37] Hence, under depleted oxygen environment, there might be downregulation of these pathways, leading to increased susceptibility. In addition, oxygen limitation might favor loss of antibiotic resistance genes due to elevated metabolic burden.[38] However, we found an increase in susceptibility of *P. aeruginosa* PaA to tetracyclines under hyperoxic conditions as well, the mechanism for which is not clear. These

findings emphasize the importance of relevant oxygen levels for AST. In vitro sensitivity testing under normoxia might suggest resistance of the pathogen to tetracycline antibiotics, but in fact, the existent clinical environment might be oxygen deprived and the antibiotic may actually be effective.

In comparison to oxygen limitation, testing susceptibility of the pathogens to antimicrobials at high $CO_2$ and $O_2$ conditions did not show any consistent trend for most of the pathogens tested. The only noteworthy finding was the decreased susceptibility of both the *K. pneumoniae* strains to azithromycin under high $CO_2$ and high $O_2$ concentrations. Similar reductions in sensitivity were found under limited oxygen environments (Tables 3 and 4). These findings suggest that normoxic environment is best suited for azithromycin efficacy. Target site modification or efflux of the drug in response to altered oxygen environments may be some of the possible factors responsible for reduction in efficacy of the drug.[39–41]

In order to correlate antibiotic efficacy to growth patterns of the pathogens in each oxygen environment, growth curve assays were performed. As anticipated, there were differences in growth of the bacteria under limited or enriched $O_2$ environments in comparison to that under normal room oxygen levels. Hyperoxia facilitated growth of 50% of the pathogens tested, whereas anoxic and hypoxic environments were least favorable for all the pathogens (Fig. 2). However, when comparing the antibiotic MICs to the growth of these bacteria under the different environments, there does not seem to be a significant correlation between the environment that is most favorable for the growth of a bacterium and its susceptibility toward the antibiotics under those conditions. For example, hyperoxic conditions favored growth of both the strains of *S. aureus*. However, SH1000 strain showed a decreased sensitivity to gentamicin and azithromycin, whereas FPR3757 did not show any change in the MIC values under elevated oxygen conditions, as compared to those under normal incubation.

It is widely believed that biofilm formation protects bacteria from antibiotic challenges and increases their tolerance to antimicrobials, contributing to the chronic nature of infections.[42,43] Biofilm formation assay in the current study demonstrated that one-third of the pathogens tested differ in their ability to form biofilms under varied oxygen environments. We found that *S. aureus* SH1000 and *P. aeruginosa* Pa14 showed a higher biofilm biomass buildup under hyperoxic environment (Fig. 3). This increase can be linked to a higher growth of these pathogens under elevated oxygen environment, although no such association was found with respect to antibiotic sensitivity. Hence, the influence of different oxygen environments on biofilm formation by the pathogens does not necessarily translate into differences in their susceptibilities to antibiotics. This underlines the fact that the differences observed in MICs under various oxygen environments are not reflective of mere differences in growth rates and/or biofilm production. However, there might be additional in vivo differences in sensitivities depending on the best growth conditions available for a pathogen and ambient environment at the site of infection.

Hyperoxia has been proposed to increase the antimicrobial efficacy of some of the antibiotics as well as to help restore normal oxygen tension in ischemic tissues.[18] Although exposure to elevated oxygen alone might be bacteriostatic, a combination therapy with antimicrobials is recommended.[17] Thus, it is important to determine the changes in bacterial susceptibility to antimicrobials in the presence of elevated oxygen levels. For this study, we have modified and developed a low-cost system that allows experiments to be conducted in a variety of ambient gas environments (Fig. 1). Since the container is sealed, it could be used with gases that are considered unsafe for use in a standard laboratory environment, such as elevated oxygen levels. The ability to insert Petri dishes and standard 6–96-well plates allows experiments to be conducted using standard laboratory protocols. Finally, the small size of the unit allows compatibility with *standard* laboratory equipment such as incubators and shakers.

In this study, MICs were measured using E-test as the simplest estimate of antibacterial effect. Being a gradient diffusion test, E-test strips make it possible to determine MIC values between the conventional two-fold dilution values suggested by breakpoints.[44] It is important to note that the *AST-like* protocols used in this study were for research purposes only, and the MIC values obtained might not reflect actual drug concentration for therapeutic use. It is essential that the clinical applications be based on the most current breakpoints from international organizations such as CLSI and EUCAST.[45,46]

## Conclusion

In this study, we have conducted a thorough analysis of the effects of different oxygen environments on susceptibility of medically relevant bacteria to an extensive range of antibiotics. We found that oxygen limitation decreases the sensitivity of the pathogens to most of the antibiotics. We also observed that enriched oxygen environment might favor growth and biofilm formation of some pathogens, but not necessarily reflect onto a significant difference in antimicrobial efficacy. We conclude that use of clinically relevant oxygen environments should be a parameter in antimicrobial susceptibility testing and the breakpoints should be set accordingly. This would help physicians make better therapeutic decisions by predicting more accurately the susceptibility of the pathogens in vivo, thereby leading to improved clinical outcome.

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## Author Contributions

Conceived and designed the experiments: DEK. Analyzed the data: SG, NL, and DEK. Wrote the first draft of the manuscript: SG and DEK. Contributed to the writing of the manuscript: SG, NL, and DEK. Agreed with manuscript results and conclusions: SG, NL, and DEK. Jointly developed the structure and arguments for the paper: SG and DEK. Made critical revisions and approved the final version: SG and DEK. All the authors reviewed and approved the final manuscript.

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