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RESEARCH ARTICLE

Exposure to stress minimizes the zone of antimicrobial action: a phenotypic demonstration with six *Acinetobacter baumannii* strains

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ABSTRACT

Aim: To phenotypically study the role of domestic environmental stress in the emergence of antimicrobial resistance in *Acinetobacter baumannii*. **Materials and Methods:** Six strains of *A. baumannii* were initially subjected to AST and then were exposed to various stresses (temperature, pH and random combinations). Stressed cells were subcultured and then subjected for AST. The ZOIs before and after exposure to stress were compared. Statistical analysis was done using Student t-test at $p < 0.10$. **Results:** Exposure to stresses and combination of stresses resulted in substantial reduction in the ZOIs. Stress hardening was associated with further reduction in ZOIs. **Conclusion:** Exposure to domestic environmental stress imparted a significant and substantial reduction in the susceptibility of *A. baumannii* strains to antibiotics.

Keywords: *Acinetobacter baumannii*; Environmental stress; Antimicrobial resistance; Bacterial stress response; Stress-induced resistance.

INTRODUCTION

The advent of many potent antibiotics in the late 1960s rendered great hope in the effective management of infectious diseases while a few projected the utility of antibiotics to an extent of eradication [1, 2]. The upsurge in the global incidence of drug-resistant infections holds out a significant intimidation in the management of infectious diseases and to the attributed mortality and morbidity corresponding to all the levels of health care. Numerous initiatives have been directed by global health agencies to tackle this issue. Progressing prospectively across the timeline, we are no far from stumbling upon the 'end of antibiotic era' since the development of AMR has been entitled to an inevitable phenomenon [3-5]. ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus auerus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.) derive special attention of any infection preventionist (IP) due to their ability to persist refractory to antibiotics and the associated financial and morbidity burden [6].

Acinetobacter baumannii is an aerobic, Gram-negative, non-fermenting cocco-bacillus discovered in 1911 by a Dutch microbiologist, Martinus Willem Beigerinck. Early Acinetobacter outbreaks were reported in terminally ill patients and were effectively managed with sulfonamides and beta-lactams [2, 7, 8]. The isolation of multi-drug resistant (MDR) strains of *Acinetobacter baumannii* was reported in the early 1990s. Around the same time, the introduction of imipenem aided the effective treatment of MDR *Acinetobacter baumannii* infections. Unfortunately, resistance to imipenem vs reported in the same year due to the elaboration of OXA beta-lactamases making them extensively drug resistant (XDR) [9]. *Acinetobacter baumannii* have accumulated multiple drug resistance mechanisms which enable them to tolerate potent antimicrobials like carbapenems [10-12]. Surveillance carried out in 2010 revealed the incidence of isolation of MDR *A. baumannii* to be 74% and is currently the second most commonly isolated nosocomial pathogen [13, 14]. Currently, colistin is considered to be the drug of choice for the treatment of MDR and XDR *A. baumannii* infections [15]. However, the isolation of Pan-drug resistant (PDR) *A. baumannii* strains resistant to colistin has also been reported across the globe [16-18]. Owing to these challenges in effective management, it has been documented that isolation of *A. baumannii* from a hospitalized patient presents an approximate mortality of 30 % and is also associated with an escalation in morbidity, health-care costs and length of hospital stay [19-21].

In spite of the recent advancements in infection control and sterilization modalities, *A. baumannii* persist to be a liability in an intensive-care setup [12]. Environmental and human colonization, prolonged persistence on the surface of inanimate objects, the ability for spontaneous resistance development and biofilm formation are the exceptional properties of this organism [22-24]. Persistence of *A. baumannii* in the environment exposes them to multiple adverse factors that endanger their survival. These adverse factors elicit a strong protective stress response that enhances the probability of bacterial persistence. This stress response comprises of a definitive sequence of sub-cellular events resulting in altered gene expression and overall cellular physiology [25]. These protective stress responses are known to influence the action of antibiotics since antimicrobials are also considered to be growth-threatening factors [26-29]. Efforts to precisely decipher the interface of interaction between the action of domestic environmental stress factors, bacterial stress response and mechanisms of tolerating antimicrobial agents will be productive in understanding the molecular epidemiology of drug resistance development. Meanwhile, few scientists are also working on innovative alternative methods [30-32]. To our knowledge, the relation between AMR and stress due to food preservation has been studied in food-related pathogens such as *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes*. Previous works were directed to understand the effect of sublethal bacteriostatic stresses on antibiotic susceptibility [33-36] and few have worked on the relation between environmental factors and virulence [37]. But no work has been done to establish the influence of environmental conditions in harnessing AMR. *A. baumannii* has been chosen to be extensively studied owing to its long bacteria-environment interaction duration as a nosocomial pathogen which in turn predisposes it to adaptive responses in the form of higher frequencies of resistance-promoting genotypic and phenotypic alterations. As a preliminary step, in this study we phenotypically demonstrate the influence of exposure to domestic environmental stress on the antimicrobial susceptibility of *A. baumannii* by comparing the zones of inhibition (ZOI) before and after exposure to stress. Moreover, in this study, the model of demonstration was designed in such a way to possess a close resemblance to the actual scenario of contracting a health-care associated infection.

MATERIALS AND METHODS

The clinical isolates and the control strains were subjected to antimicrobial susceptibility testing by Kirby Bauer's disc diffusion method for nine commonly used drugs and their zones of inhibition were noted. Later they were exposed to various sub-lethal stresses, sub-cultured and again were subjected to antibiotic challenge. Owing to the non-availability of any evidence suggesting a standardized procedure to study the influence of stress on antimicrobial resistance, a novel scientifically coherent procedure was formulated and adopted throughout the study.

Ethical conduct

Ethical clearance for conducting the study was obtained from the Institutional Ethical Committee (IEC), Shridevi Institute of Medical Sciences and Research Hospital, Tumkur, Karnataka, India.

Bacterial strains

Five isolates (n=5) of *A. baumannii* grown from an array of samples isolated from the patients admitted to our hospital and other tertiary care centers in our locality were used in this study. The phenotypical and biochemical confirmation of the isolates was undertaken as per the recommendations of CLSI 2015 [38]. At first, the isolates were cultured in BHI broth (Himedia labs, Mumbai, India). Following this, the antibiotic susceptibility testing (AST) of the isolates was carried out for a few selected antibiotic agents that are commonly used for the treatment of *A. baumannii* infections by Kirby- Bauer disc diffusion method and were interpreted with reference to CLSI 2015 [38] guidelines. Standard strain *Acinetobacter baumannii* ATCC BAA 747 was included in the study for standardization and for comparison of variations in antibiotic susceptibility with other clinical isolates. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* 27592 were used as quality controls for interpretation of AST.

Antibiotic susceptibility testing

AST was carried out using Kirby- Bauer disc diffusion method. The inoculum was prepared by adjusting a 2 to 4-hour BHI broth to match the 0.5 McFarland turbidity standard, using saline. The dried surface of Muller- Hinton agar was inoculated by streaking a sterile swab dipped in the saline suspension over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left ajar for 3 to 5 minutes, to allow for any excess surface moisture to be absorbed before applying the antibiotic impregnated disks. After 16 to 24 hours of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of an inverted perti plate. Susceptibility of the isolates for cefuroxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), amikacin (30 µg), norfloxacin (10 µg), imipenem (10 µg), meropenem (30 µg), ampicillin+sulbactam (10 µg + 10 µg), and piperacillin+tazobactam (1000 µg + 10 µg) were evaluated, recorded and interpreted according to CLSI 2015 [38] guidelines.

Phenotypical detection of enzyme production

All the isolates were screened for the production of enzymes such as ESBL, AmpC, and carbapenemase using disc diffusion method and were interpreted according to the manufacturer's instructions (Himedialabs, Mumbai, India). β-lactamase production was detected by comparing the ZOIs of drug and drug-inhibitor combination discs. Both the discs were applied aseptically to MHA plates inoculated with the *A. baumannii* in lawn pattern. The discs were placed at least 20 mm apart. The plates were incubated for 18 to 24 hours following which the ZOIs around drug and drug-inhibitor combination were measured and compared for interpretation. The results were interpreted as producer or non-producer.

Extended spectrum β- lactamase (ESBL) [39]

For ESBL detection, ceftazidime (30 µg) and ceftazidime-clavulanic acid (30 µg + 10 µg) combination discs were used. Any strain of *A. baumannii* which shows a difference in the zone diameter of ≥ 2 mm between the two discs was interpreted as ESBL producer.

AmpC cephalosporinase

Though not recommended by CLSI, AmpC production has been known to produce tolerance to extended spectrum cephalosporins and beta-lactam/beta-lactamase inhibitor combinations (40). *A. baumannii* was detected by comparing the ZOIs of cefoxitin (30 µg) and cefoxitin-cloxacillin (30 µg + 200 µg) combination. Any strain of *A. baumannii* which shows a difference in the zone diameter of ≥ 4 mm between the two discs was interpreted as AmpC producer.

Carbapenemase [41]

Carbapenemase production was detected by using two discs - imipenem (10 µg) and imipenem-EDTA (10 µg + 750 µg) which were placed not less than 20 mm apart. Any strain exhibiting a difference in ZOI ≥ 7 mm between the two discs was said to be an MBL producer.

Exposure to sublethal stress

Each of the five isolates and the control strain (*A. baumannii* ATCC BAA 747) were inoculated in BHI broth and were incubated for 24 hours under optimal conditions (37°C, pH 7.4). The overnight cultures were exposed to a variety of sub-lethal domestic environmental stresses such as sub-optimal temperature, super-optimal temperature, acidic pH, alkaline pH and random combinations of all for a specified duration of time.

Control

The temperature of 37°C and pH of 7.4 were considered to be the control conditions. All the obtained results were interpreted with reference to the ZOIs obtained by incubation at standard optimal conditions for 18 to 24 hours.

Temperature

All the strains were exposed to a range of temperature (5°C to 45°C) inclusive of sub-optimal and super-optimal temperatures.

Sub-optimal temperature
The overnight incubated broth of each bacterial strain was exposed to sub-optimal temperatures of 5°C, 20°C and 30°C for a duration of 24 hours.

Super-optimal temperature
BHI broth of each strain incubated for 24 hours was subjected to heat stress by exposing them to 40°C and 45°C for 2 to 4 hours in temperature controlled water bath.

pH

24-hour old inoculated BHI broths were acidified or alkalinized using acid or alkali to yield a range of pH from 3.0 to 10.0 and were incubated for 2 to 4 hours duration.

Acidic pH
Overnight incubated BHI tubes were acidified with diluted sulphuric acid (H_2SO_4) to attain final acidic pH of 3.0, 5.0 and 6.0. The tubes with supplemented acid were incubated at 37°C for 2 to 4 hours.

Alkaline pH

Alkalization was carried out by the addition of diluted potassium hydroxide (KOH) to render a final alkaline pH of 9.0 and 10.0 and the supplemented broth suspensions were incubated at 37°C for 2 to 4 hours.

Combination

All the strains were treated with random combinations of pH and temperature such as 5.0, 20°C; 5.0, 40°C; 9.0, 20°C; and 9.0, 40°C respectively. For this, 24-hour old broth cultures of each strain were adjusted to the specified pH by addition of acid/alkali and then stressed at a particular temperature for 2 to 4 hours. Moreover, a few additional test conditions were included. These additional conditions included exposure of cells which survived pH 3.0 to pH 1.0; pH 10.0 to pH 12.0; pH 3.0 to pH 12.0 and pH 10.0 to pH 1.0. The cells that survived the sub-lethal pH were cultured in BHI broth. 24-hour old broth cultures were then acidified or alkalinized to attain the specified pH and were incubated at 37°C for 2 to 4 hours.

Isolation of stressed bacterial cells

All the strains cultured in BHI broth were subjected to the action of various sub-lethal stresses for specified duration of time. Following exposure to stress, each strain was plated on an MHA plate which was further incubated for 18 to 24 hours at 37°C. From the colonies that appeared on the MHA plate, one or two were inoculated into another tube containing BHI broth and incubated for 2 to 4 hours. Growth in the broth was adjusted to 0.5 McFarland turbidity units by the addition of sterile physiological saline and then was subjected to antibiotic susceptibility testing as per the above-described procedure.

Statistical analysis

The ZOIs for various antibiotic agents at different test conditions were recorded on a spreadsheet. The mean and standard deviation of Zones of inhibition (mm) of all strains to a specific antibiotic were estimated. The differences in ZOIs between test conditions and standard condition for each antibiotic were compared and analyzed using MS Excel spreadsheet application. The significance of variation was assessed by one-tailed studentt-test. Since a novel, non-standardized methodology was adopted and due to the small sample size, a value of $p < 0.10$ was considered significant. At diverse test conditions, variation in ZOI was observed only with amikacin, norfloxacin, piperacillin-tazobactam, imipenem, and meropenem. Hence, only the susceptibility for these five (n=5) antibiotics were further analyzed for statistical significance.

RESULTS

Antimicrobial susceptibility of unstressed *A. baumannii*

This study involved five (n=5) clinical isolates and one standard ATCC BAA 747 strain of *A. baumannii*. The data of five (n=5) clinical isolates has been furnished in Table 1. Initial AST was carried out by Kirby-Bauer disc diffusion method and the susceptibility pattern is shown in Table 2. All the five clinical isolates were resistant to a majority of antibiotics used in the study except for variable susceptibility to norfloxacin and amikacin. The diameter of the zone of inhibition for each drug was compared with the standard ATCC strain. Zone of inhibition (ZOI) was deemed to be the parameter for comparison and analysis. The diameter of zones around amikacin, norfloxacin, piperacillin-tazobactam, imipenem, and meropenem for the clinical isolates were considerable and comparable. Hence, the clinical isolates, though exhibited resistance to most of the antibiotics, were included in the study. ATCC BAA 747 strain was susceptible to amikacin, norfloxacin, imipenem and meropenem; intermediately susceptible to piperacillin-tazobactam; and resistant to

cefuroxime, ceftazidime, cefepime, and ampicillin-sulbactam. ATCC BAA 747 was used as a substitute for susceptible strain.

Table 1. Clinical sites of isolation of *Acinetobacter baumannii.*

S No	Strain No	Age	Gender	Clinical Diagnosis	Sample	Source
1	ET400	60 yrs	M	VAP (Cardiac arrest)	Bronchial wash	ICU
2	ET401	65 yrs	F	VAP (COPD)	Tracheal aspirate	ICU
3	B2023	17 days	M	Neonatal sepsis	Blood	NICU
4	CT57	59 yrs	M	Catheter-associated UTI	Urine	ICU
5	CT58	68 yrs	F	Catheter-associated UTI	Urine	ICU

Table 2. Antibiogram of unstressed *Acinetobacter baumannii* isolates.

S. No.	Antibiotic	ET400	ET401	B2023	CT57	CT58	ATCC BAA 747
1.	Cefuroxime	R	R	R	R	R	R
2.	Ceftazidime	R	R	R	R	R	R
3.	Cefepime	R	R	R	R	R	R
4.	Amikacin	R	R	R	S	S	S
5.	Norfloxacin	I	R	I	S	S	S
6.	Amp-Sulb	R	R	R	R	R	R
7.	Pip-Tazo	R	R	R	R	R	I
8.	Imipenem	R	R	R	R	R	S
9.	Meropenem	R	R	R	R	R	S

Enzyme production

All the five (n=5) clinical isolates were screened for beta-lactamase (ESBL, AmpC cephalosporinase, and Carbapenemase) production. Among the five strains, only blood isolate (B2023) was found to be an AmpC cephalosporinase producer (Table 3).

Table 3. β-lactamase production among clinical isolates.

S. No.	Strain	ESBL	AmpC	Carbapenemase
1.	**ET400**	-	-	-
2.	**ET401**	-	-	-
3.	**B2023**	-	+	-
4.	**CT57**	-	-	-
5.	**CT58**	-	-	-

Influence of temperature stress on antibiotic susceptibility

Clinical isolates (n=5) and ATCC BAA 747 strain were exposed to a range of sub-optimal (5°C, 20°C, and 30°C) and super-optimal (40°C and 45°C) temperatures. Though the zones of inhibition mildly reduced

following exposure to sub-optimal temperatures of 20°C and 30°C, a significant reduction in ZOIs was observed on exposure to a super-optimal temperature of 45°C. A marked decrease in ZOI of ≥ 9mm for norfloxacin was observed when strains B2023 and CT57 were exposed to 45° C temperature. Effects of exposure to sub-optimal and super-optimal temperatures on the susceptibility for amikacin, norfloxacin, piperacillin-tazobactam, imipenem, and meropenem were relatively significant. At 45°C, a majority of the bacterial strains exhibited resistance to all the antibiotics. UTI isolates (CT57 and CT58) were initially sensitive to amikacin and norfloxacin but exposure to 40°C and 45°C rendered them resistant. ATCC BAA 747 strain also showed a similar pattern of variation at different temperatures. Exposure to 5°C for 24 hours resulted in a mild reduction in the mean ZOI for a majority of the antibiotics but this variation in susceptibility was statistically insignificant ($p > 0.10$). Eventually, a significant reduction ($p < 0.10$) in the mean ZOI was recorded at 45°C for all antibiotics. The influence of other test temperatures on mean ZOI was statistically insignificant ($p > 0.10$) except for norfloxacin which exhibited significant variation in susceptibility at 20°C ($p=0.0693$) and at 40°C ($p=0.02908$). As a general trend, the mean ZOI decreased at sub-optimal and super-optimal temperatures. However, a significant reduction was observed only at 45°C (Figure 1, Table 4).

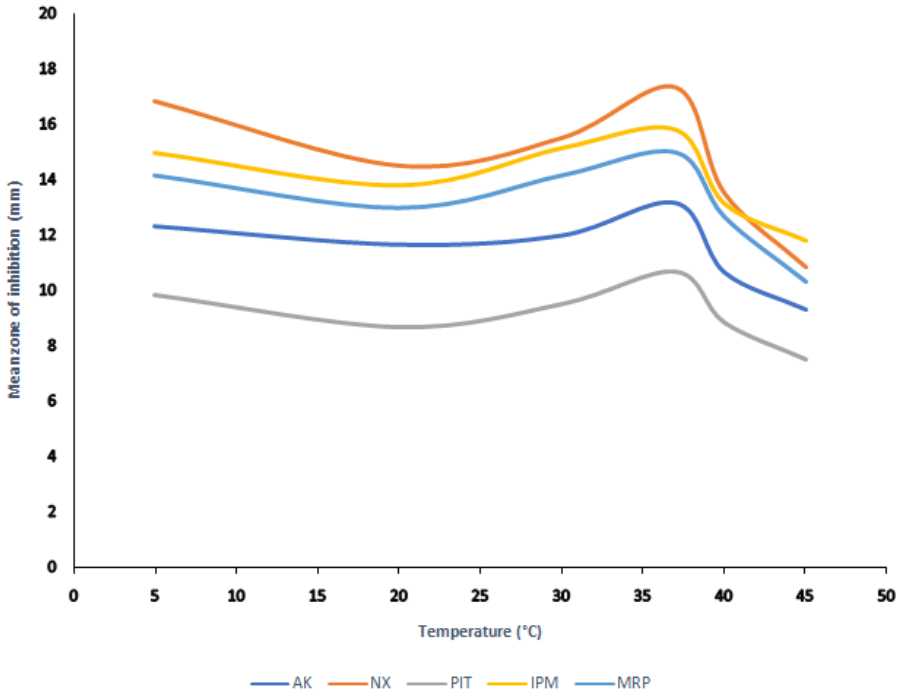


Figure 1. Influence of temperature on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is Temperature (°C) at pH 7.4 and along y-axis is the mean zone of inhibition (mm).

Influence of pH stress on antibiotic susceptibility

Overnight broth cultures of each *A. baumannii* strain was exposed to a collection of acidic (3.0, 5.0 and 6.0) and alkaline (9.0 and 10.0) pH for 2 hours and then were subjected to antibiotic challenge. On exposure to increasing pH from 3.0 to 7.4, there was a consistent increase in ZOI following which a steady fall in ZOI was recorded (Figure 2, Table 5) till an alkaline pH of 10.0 was attained. Least ZOI values were obtained at the extremes of acidic (3.0) and alkaline (10.0) pH. ATCC BAA 747 strain also followed the same trend of variation. The degree of reduction in ZOI was equivalent with all the bacterial strains. Exposure to acidic pH conferred greater resistance to the majority of the antibiotics when compared to alkaline pH (Table 5). A

significant fall in ZOI of ≥ 10 mm for norfloxacin was observed at pH 3.0 in UTI isolates (CT57 and CT58). ATCC strain did not survive an acidic pH of 3.0. At extremes of acidity and alkalinity, the ATCC strain showed a marked reduction in ZOI of ≥ 10 mm for amikacin and imipenem. A gross reduction in mean ZOI ≥ 4 mm was recorded for norfloxacin and carbapenems at extremes of acidic and alkaline pH. The reduction in mean ZOI for amikacin, piperacillin-tazobactam, and imipenem at pH 6.0 and 9.0 was statistically not significant. However, the mean ZOI for norfloxacin showed significant reduction at all levels of tested acidity and alkalinity.

Influence of combination of test conditions on antibiotic susceptibility

Bacterial cultures were subjected to stress in the form of a few combinations of pH and temperature; 5.0, 20°; 9.0, 20°C; 5.0, 40°C; and 9.0, 40°C respectively. The combinations of acidic pH with sub-optimal temperature and alkaline pH with super-optimal temperature induced a higher degree of resistance than other combinations (Table 6). It is noticeable that the combination of temperature and pH stresses caused a greater reduction in the ZOIs than the reduction caused due to the action of individual stresses themselves. For example, the ZOI of CT57 for amikacin at 20°C was 18 mm and at pH 5.0 was 14 mm while the zone markedly decreased to 12 mm when subjected to the combination of 5.0 pH and 20°C temperature. ATCC BAA 747 that was susceptible to carbapenems following exposure to all individual stresses, developed resistance when subjected to a combination of cold and acidic stress. But the ATCC strain did not withstand the combination of super-optimal temperature and acidity. A significant reduction ($p < 0.10$) in mean ZOI was observed at all combinations of temperature and pH (Table 6 and Figure 3). Hence, subjection to a random combination of stresses consistently enhanced resistance to all the antibiotic agents.

Table 4. Effect of temperature on susceptibility to various antibiotics.

Temperature (°C)		**37**	**5**	**20**	**30**	**40**	**45**
AK	Mean	13.17	12.33	11.67	12	10.67	9.33
	SD	7.91	6.98	6.25	6.66	5.2	3.88
	CI	±6.33	±5.58	±5.00	±5.33	±4.16	±3.10
	p- Value		0.39037	0.29069	0.34252	0.14579	0.03002
NX	Mean	17.33	16.83	14.5	15.5	13.5	10.83
	SD	5.09	5.42	3.94	4.04	3.83	3.97
	CI	±4.07	±4.34	±3.15	±3.23	±3.06	±3.18
	p- Value		0.41563	0.0693	0.1587	0.02908	0.00512
PIT	Mean	10.67	9.83	8.67	9.5	8.83	7.5
	SD	4.93	5.46	4.27	5.05	4.58	3.67
	CI	±3.94	±4.37	±3.42	±4.04	±3.66	±2.94
	p- Value		0.3613	0.15143	0.29744	0.18548	0.04412
IPM	Mean	15.83	15	13.83	15.17	13.17	11.83
	SD	8.18	8.44	7.05	7.52	7.17	6.55
	CI	±6.54	±6.75	±5.64	±6.02	±5.74	±5.24
	p- Value		0.40958	0.25951	0.41875	0.20222	0.09776
MRP	Mean	15	14.17	13	14.17	12.67	10.33
	SD	5.18	5.08	4.94	5.31	4.23	3.83
	CI	±4.14	±4.06	±3.95	±4.25	±3.38	±3.06
	p- Value		0.3521	0.18342	0.35817	0.11713	0.01532

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

Table 5. Effect of pH on susceptibility to various antibiotics.

pH		7.4	3	5	6	9	10
AK	Mean	13.17	7.5	9.67	12	11.17	10.5
	SD	7.91	5.21	4.03	6.6	5.71	4.93
	CI	±6.33	±4.17	±3.22	±5.28	±4.14	±3.94
	p-Value		0.0222	0.0433	0.3412	0.2146	0.0222
NX	Mean	17.3	7.83	11.83	14.5	12.67	11.5
	SD	5.09	4.36	2.71	3.89	3.5	3.89
	CI	±4.07	±3.49	±2.17	±3.11	±2.80	±3.11
	p-Value		0.0015	0.0021	0.0673	0.0112	0.0072
PIT	Mean	10.7	5	8.17	9.17	8.17	7.67
	SD	4.93	2.45	3.49	4.07	4.36	4.08
	CI	±3.94	±1.96	±2.79	±3.26	±3.49	±3.26
	p-Value		0.0012	0.0695	0.2036	0.1091	0.0657
IPM	Mean	15.8	7.83	11.5	13.67	12.67	11.5
	SD	8.18	4.26	3.45	6.98	6.41	6.12
	CI	±6.54	±3.72	±2.76	±5.58	±5.13	±4.90
	p-Value		0.0029	0.0138	0.2409	0.1403	0.0719
MRP	Mean	15	8.17	12.17	12.83	12.33	11.33
	SD	5.18	4.49	3.66	4.45	4.41	4.72
	CI	±4.14	±3.92	±2.93	±3.56	±3.53	±3.78
	p-Value		0.0068	0.0581	0.1431	0.0994	0.0577

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

Table 6. Effect of random combinations of temperature and pH on the susceptibility to various antibiotics.

Conditions (Temp, pH)		37, 7.4	20, 5.0	40, 5.0	20, 9.0	40, 9.0
AK	Mean	13.17	8.83	10	9.5	9.67
	SD	7.91	3.13	4.43	3.99	4.13
	CI	±6.33	±2.50	±3.54	±3.19	±3.30
	p- Value		0.00964	0.06991	0.03693	0.04619
NX	Mean	17.33	11	12.17	10.67	10.83
	SD	5.09	1.67	2.32	2.58	2.04
	CI	±4.07	±1.34	±1.86	±2.06	±1.63
	p- Value		0.00012	0.0014	0.00073	0.00028
PIT	Mean	10.67	7.17	7.83	7.33	7.33
	SD	4.93	2.86	4.49	3.27	3.27
	CI	±3.94	±2.29	±3.59	±2.62	±2.62
	p- Value		0.015	0.09124	0.02716	0.02716
IPM	Mean	15.83	10.5	11.33	11.67	11
	SD	8.18	1.76	5.24	5.82	5.18
	CI	±6.54	±1.41	±4.19	±4.66	±4.14
	p- Value		0.00035	0.04478	0.07005	0.03553
MRP	Mean	15	10.83	11.5	12	10.83
	SD	5.18	3.31	4.46	4.29	3.76
	CI	±4.14	±2.65	±3.57	±3.43	±3.01
	p- Value		0.0137	0.05633	0.07368	0.0211

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

Table 7. Effect of stress hardening on susceptibility to various antibiotics.

Conditions (Pre-stressed pH, exposed pH)		37, 7.4	3.0, 1.0	10.0, 12.0	3.0, 12.0	10.0, 1.0
AK	Mean	13.17	6	4.67	7.67	7
	SD	7.91	5.37	3.93	5.47	6.03
	CI	±6.33	±4.30	±3.14	±4.38	±4.82
	p- Value		0.01107	0.0016	0.02838	0.02708
NX	Mean	17.33	6	7	7.67	6.67
	SD	5.09	4.9	5.66	4.27	5.61
	CI	±4.07	±3.92	±4.53	±3.42	±4.49
	p- Value		0.00119	0.00328	0.00132	0.00278
PIT	Mean	10.67	4	5.33	5	5
	SD	4.93	3.1	5.16	2.45	4.52
	CI	±3.94	±2.48	±4.13	±1.96	±3.62
	p- Value		0.00163	0.02622	0.00119	0.01381
IPM	Mean	15.83	5.83	6.83	7.67	8.33
	SD	8.18	4.67	7.31	4.5	7.66
	CI	±6.54	±3.74	±5.85	±3.60	±6.13
	p- Value		0.00167	0.01477	0.00338	0.01205
MRP	Mean	15	5.83	6.5	7.67	8.17
	SD	5.18	4.62	6.63	4.13	7.49
	CI	±4.14	±3.70	±5.30	±3.30	±5.99
	p- Value		0.00232	0.0128	0.00369	0.03792

Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem, CI - 95% confidence interval; Note: $p < 0.10$ is considered significant. Significant - indicated by blue color, not significant - indicated by red color.

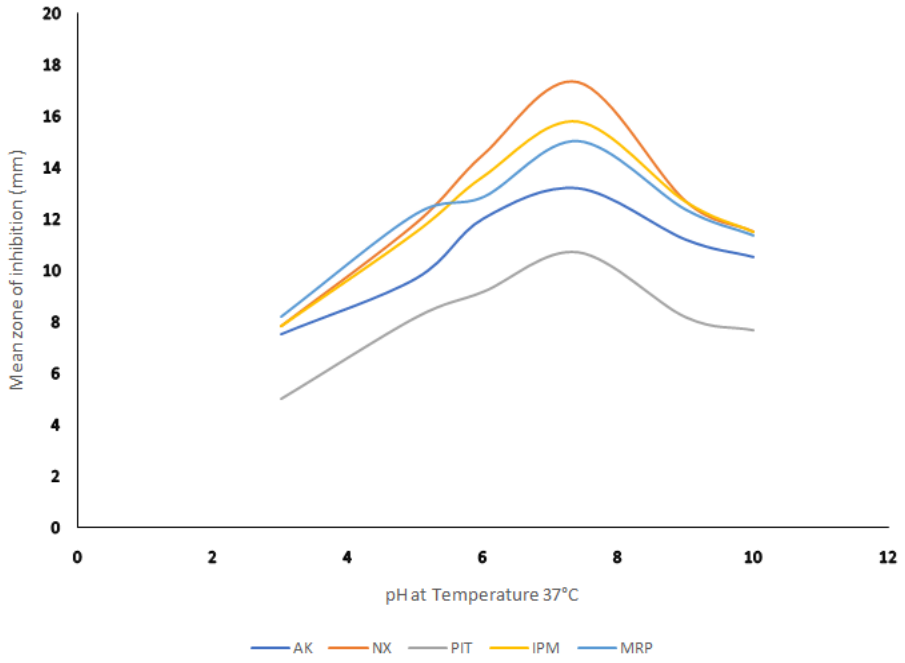


Figure 2. Influence of pH on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+ Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is pH at 37°C and along y-axis is the mean zone of inhibition (mm).

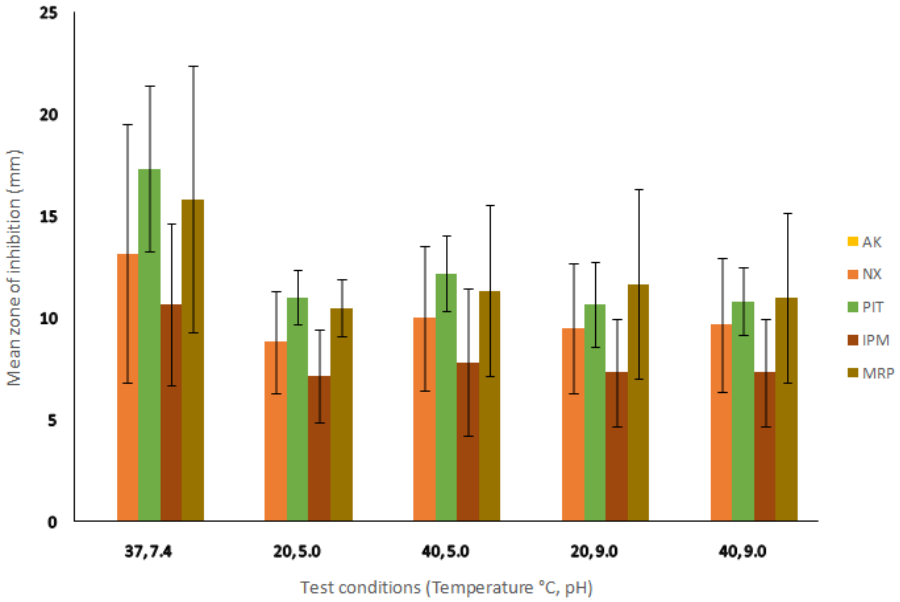


Figure 3. Influence of combined action of stresses on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is the test condition (temperature °C, pH) and along y-axis is the mean zone of inhibition (mm).

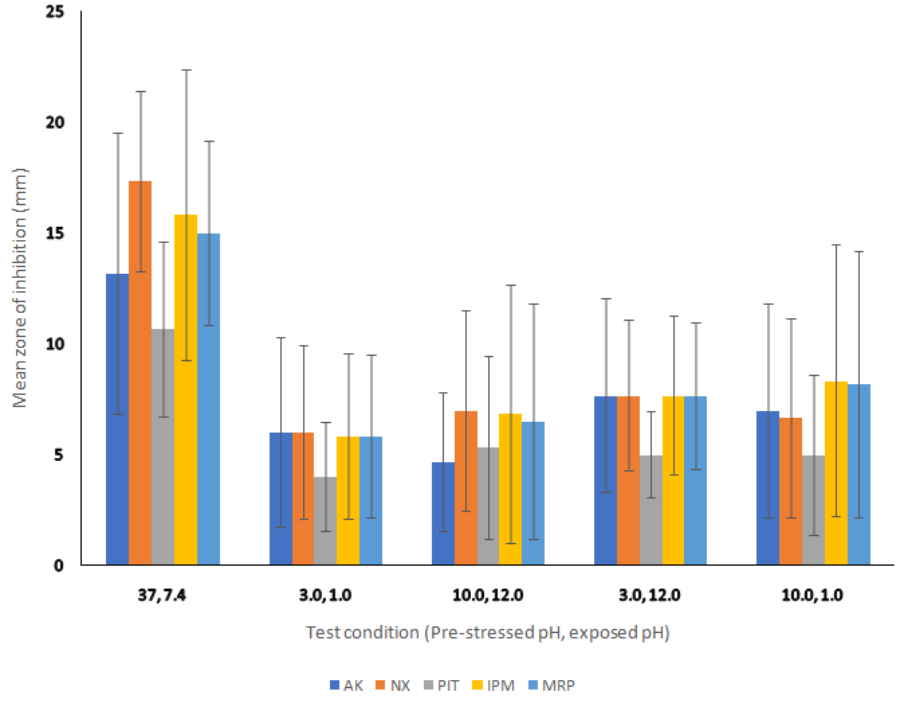


Figure 4. Effect of stress hardening on antimicrobial susceptibility.
Abbreviations: AK - Amikacin, NX - Norfloxacin, PIT - Piperacillin+Tazobactam, IPM - Imipenem, MRP - Meropenem; Note: Plotted against x-axis is pH (sub-lethal pH, lethal pH) and along y-axis is the mean zone of inhibition (mm).

Few other combinations like exposure of pH-stressed cells to another stratified degree of acidity or alkalinity were also included in the study. The bacterial cells stressed at pH 3.0 and 10.0 were then exposed to pH 1.0 and 12.0 following which they were plated and then subjected for AST. It was observed that acid-stressed (pH 3.0) cells of all strains except for that of ET401 survived a higher acidic pH of 1.0 while all strains

survived alkaline pH 12.0. VAP isolates (ET400 and ET401) stressed at pH 10.0 did not survive pH 1.0. Likewise, UTI isolates stressed at pH 10.0 did not survive a higher alkaline pH 12.0. Notably, the post-stressed cells of strain B2023 tolerated all the designated higher levels of acidity and alkalinity. As a general rule, subjecting the post-stressed cells to a different degree of acidity or alkalinity resulted in a further curtailment of antibiotic susceptibility ($p < 0.10$) (Table 7 and Figure 4).

DISCUSSION

In the past two decades, *A. baumannii* has emerged out as a successful nosocomial pathogen exhibiting salient challenging features such as prolonged environmental persistence, spontaneous emergence of drug resistance and biofilm formation [20]. Especially, the spread of MDR *A. baumannii* worldwide poses a serious threat to the management of *A. baumannii* infections in health care setup. In the present study, five (n=5) clinical isolates of *A. baumannii* and one (n=1) standard ATCC BAA 747 strain were included. According to the recent definition [42], three isolates (ET400, ET401, and B2023) were extensively drug resistant (XDR) while the other two strains (CT57 and CT58) were multidrug resistant (MDR). Since all the clinical isolates were drug resistant, ATCC BAA 747 strain was used as a substitute to susceptible strain for interpretation of the results. The purpose of this study was to understand the influence of environmental stress-bacterial cell interaction on its susceptibility to antibiotic agents. Production of beta-lactamase enzymes is one of the chief mechanisms that confer a high degree of resistance to beta-lactam antibiotics. Of the collection of beta-lactamases, the roles of extended-spectrum β-lactamses, Metalloβ-lactamases (such as carbapenemases) and AmpC cephalosporinases are significant with special reference to *A. baumannii* [39-43]. We made an attempt to screen all the clinical isolates for β-lactamase production phenotype. Only one strain (B2023) was found to produce AmpC cephalosporinase while other strains were non-enzyme producers in spite of exhibiting resistance to all the β-lactam antibiotic agents. Hence, it can be inferred that the resistance exhibited by clinical isolates of *A. baumannii* is probably due to other mechanisms such as alterations in the expression of porin channels [43, 44] and induction of efflux pump expression [43]. A total of six (n=6) strains of *A. baumannii* were subjected to various forms of environmental stresses (sub-optimal and super-optimal temperature; acidic and alkaline pH; and few combinations of both) in vitro. The stationary phase of bacterial growth provides an advantage of enhancing the chances of environmental persistence [45]. As an effort to understand the survival strategies of bacteria, in our study, all the strains were exposed to sub-lethal domestic environmental stresses during their stationary phase of growth. Then, the post-stressed bacterial cells were subjected to antibiotic challenge.

All (n=6) *A. baumannii* strains were exposed to super-optimal and sub-optimal temperatures. On exposure of the bacterial cells to super-optimal temperatures of 40°C and 45°C, a consistent increase in the degree of resistance was observed. Remarkably at 45°C, a majority of the bacterial strains were resistant to all the test antibiotics. Similar observations were recorded by Faezi Ghasemi [34] where exposure of *Listeria monocytogenes* PTCC1297 strain to a high temperature of 45°C for 2 hours exhibited a marked 2 to 4 fold increase in the MIC of all the antibiotics tested. In contrary to this, McMahon et al. [33] reported that increased temperature stress enhances the susceptibility of food related pathogens like *S. aureus, E. coli* and *S. typhimurium* to antibiotics. Similar observations were reported by Bahram [46] that exposure of *Stenotrophomonsmaltophilia* to temperature stresses increased its susceptibility to aminoglycosides. It should be noticed that McMahon et al. and Bahram exposed the bacteria to environmental stress during lag phase or the phase of adaptation which leads to inhibition of bacterial proliferation and thus reduction in MIC. As discussed earlier, stationary phase of bacterial growth is considered to be the appropriate juncture to understand the instincts of bacterial persistence. Recent evidences suggest that exposure to heat stress induces the expression and synthesis of stress proteins especially sigma factors (SigB, SigX, and RpoE) which are regulated by σB, σX and σE respectively [45, 47-50]. These sigma factors have been shown to induce the expression of *mexCD-OprJ* multidrug efflux operon which is responsible for the reduction in susceptibility to a majority of the antibiotics [45]. Exposure to a sub-optimal temperature of 5°C did not significantly alter the

susceptibility of bacterial strains except for a mild reduction in the mean ZOI for all the antibiotics ($p > 0.10$). But a moderate reduction in susceptibility was noticed at 20°C and 30°C. According to McMahon et al. [33], exposure to cold stress did not alter the susceptibility of *E. coli* and *S. aureus* to a majority of antibiotics with a mild degree of increase in resistance to a few antibiotics while *S. typhimurium* showed an increased susceptibility. Al-Nabulsi [35] reported a two to four-fold increase in resistance (MIC) of *L. monocytogenes* following exposure to a low temperature of 10°C for 24 hours. In *E. coli* exposure to cold stress induced the expression of *RcsCDB* gene which in turn participates in protection against β-lactam agents and capsule synthesis [45, 51]. Holler [52] also demonstrated that 4°C had least stress effect while intermediate temperatures (10°C and 20°C) cause significant injury to the bacterial cell membrane of *Campylobacter coli* SP10 leading to adaptive changes in morphology and metabolism. However, the mechanism of enhanced susceptibility at low temperature has not yet been understood.

Antibiotic susceptibility of all the strains of *A. baumannii* reduced consistently at both acidic and alkaline pH. Susceptibility was proportional to pH as the pH was raised from 3.0 to 7.4. When the pH was further raised from 7.4 to 10.0, a fall in susceptibility was noted. In other words, both acidity and alkalinity reduced the susceptibility of *A. baumannii* strains to antibiotics. Exposure to acidity conferred a higher degree of resistance to antibiotics than alkalinity. However, at the extremes of acidic (3.0) and alkaline pH (10.0), all the *A. baumannii* strains were resistant to all the antibiotics. Al-Nabulsi [53] also reported that exposure to acidic and alkaline environment increased the resistance of *Cronobacter sakazakii* for antibiotics such as ampicillin, amoxicillin, kanamycin, neomycin, etc. McMahon reports a marked rise in MIC for *S.aureus, S. typhimurium* and *E.coli* following subjection to acid stress (pH 5.0). Another study by Hernando [54] demonstrated a fall in antibiotic susceptibility of *L. monocytogenes* consequential to citric acid exposure. Similar findings were recorded by Al-Nabulsi [35] on exposing three strains of *L. monocytogenes* to acidic stress. Controversial to the findings of our study, Faezi-Ghasemi [34] reported an increase in susceptibility of *L. monocytogenes* to beta-lactam agents, aminoglycosides, and rifampicin on exposure to acidic pH of 5.0. Faezi-Ghasemi exposed the bacteria to acidic pH in the log phase of bacterial growth during which active proliferation occurs and the bacteria are more vulnerable to any form of stress [55] while in this study bacteria were subjected to any form of stress during their stationary phase. Acidic and alkaline environmental pH enhance the expression of sigma factor, SigB [56, 57] and CpxRA TCS [58, 59] which in turn modulate the preferential synthesis of outer membrane proteins (OmpF and OmpC) [45, 60] and alter the cell membrane fluidity by modifying the membrane lipid composition [45, 57]. These changes in the membrane composition and permeability possess a direct influence on the influx and efflux of antibiotics across the cell membrane and thus on the antibiotic susceptibility of the bacterial cell itself.

Combined action of temperature and pH stress resulted in the development of a higher degree of resistance to antibiotics. Hot and cold temperatures; and acidic and alkaline pH initiate a sequence of specific or non-specific pathways that bring about alterations in the genotype and phenotype of a stressed bacterial cell that enable its survival. Most of the environmental stresses like osmotic pressure, acidity, alkalinity and cell wall-active antibiotic agents pose a direct impact on the bacterial cell envelope [45]. When a collective spectrum of envelope stresses acts simultaneously, the probability of induction of cross-resistance to other stresses is maximum [33, 35, 45]. Especially, in an intensive care setup, a myriad of stresses operate simultaneously on the bacteria. This enhances the chances of bacterial persistence in the environment with a significant reduction in antibiotic susceptibility. Al-Mahin [61] constructed a genetically modified strain of *Lactococcus lactis* NZ9000 that expressed an *E. coli dnaK* stress protein on exposure to various stresses. They reported that the genetically modified strain of *L. lacti* remained resilient to all the stresses. This higher degree of tolerance was attributed to the product of *dnaK* expression, hsp70, a heat shock protein. Hence, it can be inferred that induction of heat shock proteins or molecular chaperones non-specifically stimulate multiple pathways which enable a high degree of tolerance to various stresses. Other stress proteins such as PhoPQ, ParRS, BraRS and AlgU are responsible for induction of broad-spectrum cross-resistance to various cell-wall damaging factors, antibiotics, detergents and unfavorable temperature [45, 62-67]. In this study, we also tried to evaluate the relation between stress-hardening and antibiotic susceptibility. Our preliminary workup to determine the sub-

lethal levels of pH revealed that an acidic pH 1.0 and an alkaline pH 12.0 to be lethal to all the bacterial strains employed in the study. However, following exposure to sub-lethal levels of acidity and alkalinity, a majority of the strains survived lethal surges of acidity and alkalinity. Enhanced tolerance to higher levels of acidity and alkalinity were associated with a substantial reduction in the susceptibility of *A. baumannii* to various antibiotic agents. This proves that the genotypic and proteomic adaptive changes in the bacterial cell that are responsible for stress hardening also initiate mechanistic consequences that impart a significant measure of cross resistance to other stresses.

The control *Acinetobacter baumannii* ATCC BAA 747 strain followed a trend of variations similar to the other clinical strains following exposure to different test conditions which is supported by Al-Nabulsi. He reported that *Listeria monocytogenes* ATCC 7644 showed a pattern of variation similar to other two isolates from food products [35]. However, in our study, the tolerance of ATCC strain to various test conditions was much reduced. Clinical isolates remained viable after following exposure to a wide range of stresses compared to the ATCC strain. This is because the clinical isolates were multi-drug resistant and resistance to antibiotics, in turn, confers a certain degree of tolerance to environmental stress [33, 68]. From this it can be understood, that both carbapenem susceptible *Acinetobacter baumannii* (CSAB) and carbapenem resistant *Acinetobacter baumannii* (CRAB) project similar distinctions in antibiotic susceptibility following the exposure to various stresses.

McMahon et al. [33] reported that increase in MIC following exposure to stress was not associated with any change in the diameter of the zone of inhibition (ZOI). They accredited the increase in MIC to the occurrence of hyper-resistant mutants. But in this study, an obvious and significant reduction in the zones of inhibition (ZOIs) was noted following the exposure to stresses.

It has been understood that exposure to stress results in the emergence of a phenotypically heterogeneous population of bacterial cells that possess a higher degree of adaptability or resilience. Directed mutation or stress adaptation is a consequence of hypermutability, amplification of resistance genes, stress-induced inter- and intra-bacterial genetic transfer and recombination, and defects in DNA repair [33, 68-73]. These amendments in the bacterial genotype that enable environmental persistence of the bacteria also possess a direct influence on the bacterial susceptibility to antibiotic agents [72, 73]. In the current study, bacterial strains were subcultured following exposure to stress to ensure that the acquired resistant phenotype is not a transient phenomenon. The scientific reliability and acceptability of this work were ensured by exposing the *A. baumannii* strains to stresses in their stationary phase of growth and by calibrating the methodology to closely mimic the realistic picture of acquiring a nosocomial infection. For further evaluation of this hypothesis, demonstration of stress gene expression, stress protein elaboration, and expression of genetic determinants of antibiotic resistance by employing nucleic acid amplification techniques and advanced proteomic studies would be suggested. A major drawback of this study is the limited sample size. Hence, it would be recommended to include a considerable number of bacterial strains exhibiting heterologous patterns of antibiotic susceptibility. Structuring, designing and developing novel chemotherapeutic agents that will inhibit the action of stress proteins or the adaptive changes secondary to the production of stress proteins would serve a great deal in mitigating the emergence and spread of antimicrobial resistance.

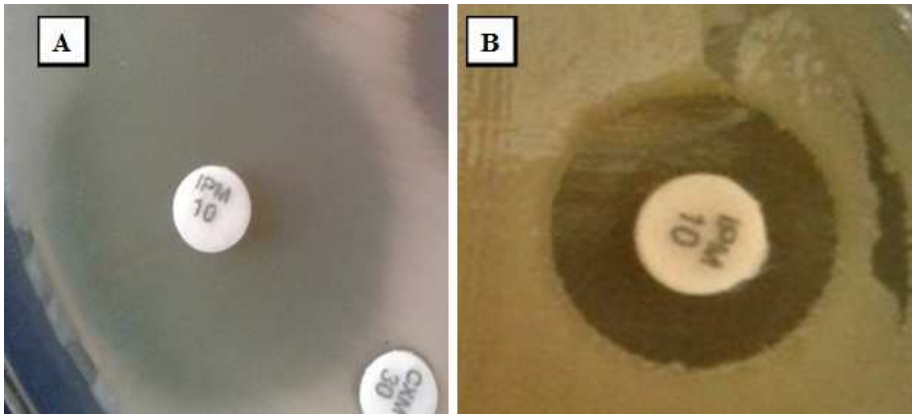
CONCLUSIONS

Acinetobacter baumannii has emerged to be a notorious superbug. This study was determined to elucidate the effect of interaction between domestic environmental stress and bacterial existence on bacterial susceptibility to antibiotics. There was a steady reduction in the antibiotic susceptibility of *A. baumannii* with exposure to various stresses. Post-stressed cells of carbapenem resistant *A. baumannii* (CRAB) strains (all five clinical isolates) and those of carbapenem sensitive *A. baumannii* (CSAB) strain (ATCC BAA 747) did not show any significant difference in susceptibility to antibiotics. Therefore, an intense and prolonged interaction between bacterial cell and environmental stress fosters the development of antimicrobial resistance. Thus, the

symbiotic interaction between environmental stresses and bacterial stress responses in the emergence of antimicrobial resistance is established.

IMAGES

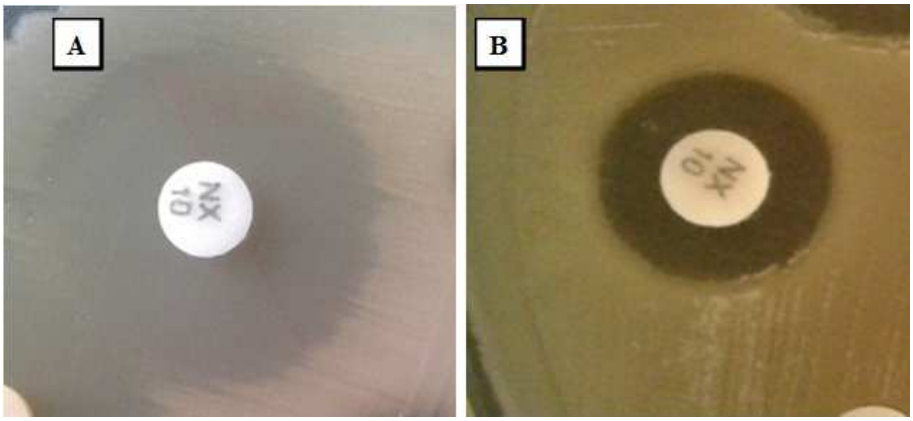
Image 1. Depiction of variations in the susceptibility of ATCC BAA 747 strain to imipenem following exposure to various stresses.



A. ZOI formed around imipenem disc in ATCC BAA 747 before exposure to stress.
B. ZOI formed around imipenem disc in ATCC BAA 747 after exposure to a pH of 5.0 at 20°C.

The ZOI of imipenem before exposure to stress was 32 mm. A least ZOI of 12 mm was obtained when ATCC BAA 747 was exposed to pH of 5.0 at 20°C.

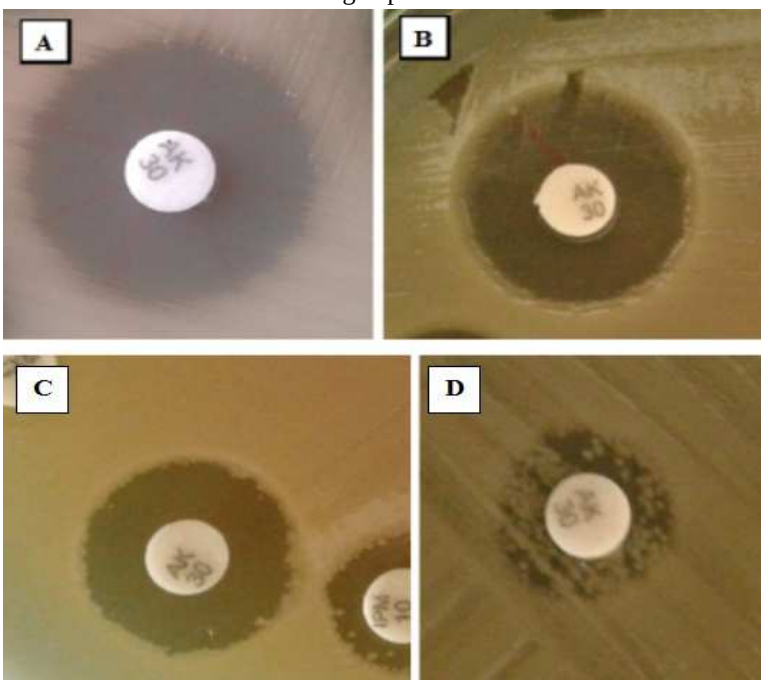
Image 2. Depiction of variations in the susceptibility of ATCC BAA 747 strain to norfloxacin following exposure to various stresses.



A. ZOI formed around norfloxacin disc in ATCC BAA 747 before exposure to stress.
B. ZOI formed around norfloxacin disc in ATCC BAA 747 after exposure to a pH of 9 at 40°C.

Before exposure to stress a ZOI of 24 mm was recorded. It is noted that the ZOI was significantly reduced to 12 mm after exposure to a pH of 9 at 40°C.

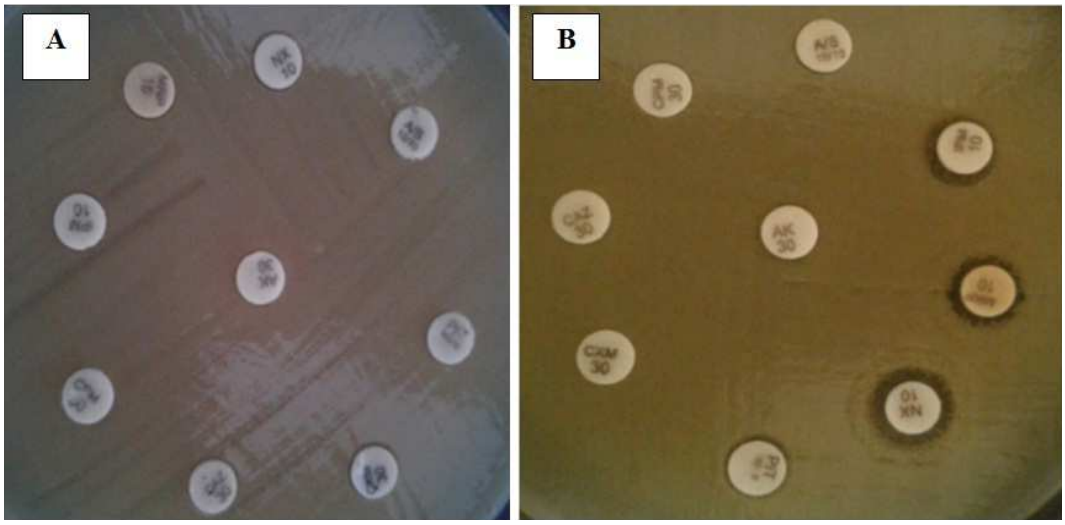
Image 3. Depiction of variations in the susceptibility of ATCC BAA 747 strain to amikacin following exposure to various stresses.



A. ZOI formed around amikacin disc in ATCC BAA 747 before exposure to stress.
B. ZOI formed around amikacin disc in ATCC BAA 747 after exposure to 20°C.
C. ZOI formed around amikacin disc in ATCC BAA 747 after exposure to pH 10.0.
D. ZOI formed around amikacin disc in ATCC BAA 747 after exposure to pH 9.0 at 40°C.

The ZOI for amikacin recorded before exposure to stress was 22 mm. It was reduced to 18 mm following the exposure to a temperature of 20°C. A further reduction in ZOI to 15 mm was observed when exposed to a pH of 10.0. A least ZOI of 12 mm was recorded after exposure to pH 9.0 at 40°C. Also note multiple hyper-resistant clones of *A. baumannii* growing up to to the margins of the disc (3D). It was initially thought to be a contaminant but was later confirmed to be *A. baumannii*.

Image 4. Absence of Zone of inhibition (ZOI).



A. Acid stressed ET401 after exposure to a pH 12.0.
B. B2023 following exposure to 45°C.

A significant fall in susceptibility to antimicrobial agents was observed when acid stresses ET401 was exposed to a lethal alkaline pH of 12.0 and when B2023 was heat stressed at a super-optimal temperature of 45°C. In these two test conditions, the strains exhibited absolute resistance to all the tested antibiotics. When acid stressed ET401 was further exposed to a pH 12.0, the cells did not only survive this lethal level of alkalinity but also were absolutely resistant to antibiotic action. After exposure to 45°C, a very negligible ZOI was noted. However, the bacterial cells were able to tolerate all the antibiotics just producing a very minimal ZOI for a few antibiotics.

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AUTHORS' CONTRIBUTION

AE conceptualized the study. AE and GSVK analyzed, interpreted data and wrote the manuscript. AE and TSK carried out bench work, generated data. The final manuscript was read and approved by all authors.

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