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Resistance, Persistence and Tolerance Significance of MDR Nosocomial Bacterial Infections in ICUs in Relation to MBC/MIC

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NOSOCOMIAL infections are a major challenge, as one third of yearly admissions estimated as deaths. In intensive care units (ICUs), Gram-negative pathogens are responsible for the majority of these deadly infections. Multidrug resistance (MDR) in the seorganisms has been attributed to production of modified enzymes responsible of elevated MICs and MBC/MICs putting them in the transient stages for persistence and tolerance. Bacterial cells those can survive high doses of antibiotics comprise a subpopulation of persistent but not resistant cells.Bacterial isolates (326) were collected from nosocomial infections via microbiology laboratories of police hospitals in Cairo. Frequency-wise, it was found that *Escherichia* is the most frequent (155), *Pseudomonas* (96), *Staphylococcus* (34), *Klebsiella* (24), *Proteus* (16) while *Citrobacter* is the least frequent (1). *Pseudomonas*, *Klebsiella*, *Proteus* and *Citrobacter* showed the highest MDRs, with the highest MICs in a descending order. Results clearly indicated the high risk of the two *Pseudomonas aeruginosa* from sputum and urine persisting (inhibited but not killed) against MICs 3 tested antibiotics; CRO, AMC and FEB. It is highly recommended here to run tests for MBC/MICs to choose the antibiotic with least MBC/MIC ratio; less than four, in order to reduce the transient persistent and/ or tolerant stages.

Keywords: ICUs, MDR, MBC/MIC, Nosocomial Bacterial Infections, Persistence and tolerance, *Pseudomonas*.

Introduction

Nosocomial infections are a major challenge to patient`s safety and thought to be the sixth cause of death amongst admissions worldwide. More annoyable that two thirds of these infections are still out of control. The significant role in the occurrence and re-emergence of such infections relies on the diverse population of pathogens (Diab et al., 2008; Muhammad et al., 2013; Diab et al., 2018). Ventilator-associated pneumonia, urinary tract infections bloodstream are predominate, (Hidron et al., 2006–2007; Diab & Al-Turk, 2013). Tolerant, acclimatized, adapted and persistent bacterial pathogens could reach the sick patients through sources like central air conditioning and laundries (Osaro et al., 2008). The antibiotic resistance aggravate the extent of bacterial load (Davane et al., 2014; Asghar & Faidah, 2009). The poor infection control quality of the hospital environment gives persistent bacteria easy access and spread which badly-affect, not only patients admitted to rooms in which the prior occupants tested positive for a pathogen, but also patients in other facilities, increasing the health care costs resulting from treatment failures, and longer hospital stays (Roy et al., 2014; Muhammad et al., 2013; WHO, 2012; Nunes et al., 2005).

It is significantly-important here to know that bacterial tolerance to antibiotics stemmed from the failure of these antibiotics to inhibit and/or kill such pathogenic bacteria. While resistance is clearly defined and accurately measured as MIC, tolerance is poorly characterized and lack precise quantitative method of measurement, except the

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minimum duration of killing (MDK). Tolerome studies; which are the genes associated with tolerance are still at its very beginning (Fridman & Balaban, 2016). Bacterial persister cells represent a subpopulation of cells that can survive intensive antibiotic treatment without being resistance (Balaban et al., 2019). Acclimation, persistance and adaptation which defined as the second phase of coping stresses, are intermediate steps to tolerance (Biagianti-Risbourg et al., 2013).

Unfortunately, many multidrug-resistant bacteria emerged from persistence subpopulations, including *Pseudomonas aeruginosa, Staphylococcus aureus*, *Acinetobacter baumannii*, and extended-spectrum *β*-lactamase (ESBL)–producing or carbapenemase-producing, are increasingly reported worldwide (Dortet et al., 2014; Robilotti & Deresinski, 2014; Pitout et al., 2015). Emergence and re-emergence of difficult-to-treat nosocomial infections in patients is function in the increased antibiotic MBC/MICs ratio (Lee et al.,, 1998; Wise et al.,, 1998).

MIC is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic (prevents the visible growth of bacteria) is used to evaluate the antimicrobial efficacy. These evaluations can be quite useful during the (R&D) phase, as the concentration of drug required is several thousands of times less than the concentration found in the finished dosage form. A commonly used cocktail of bacteria is known as the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species). The ESKAPE pathogens are considered the leading cause of nosocomial infections and are known to be resistant to antimicrobial products (Boucher et al., 2009). The FDA Tentative Final Monograph (TFM- section 333.470, page 31444) also provides guidance on organisms to use to determine the efficacy of such products. MBC is the lowest concentration of an antibacterial agent required to kill a bacterium over a fixed time. It can be determined from the broth dilution of MIC tests by sub-culturing onto agar plates that do not contain the test agent. MBC is, an obligatory, complementary to the MIC; which makes MIC alone invalid fact to relay on. Antibacterial agents are regarded as bactericidal if the MBC/MIC is not more than four. MBC testing is a must, good and relatively inexpensive tool to simultaneously evaluate multiple antimicrobial agents for potency. So the MBC/MIC ratio is not only more accurate and reliable, but also empowered research to avoid or at least reduce the intermediate steps of acclimation, persistence, tolerance and adaptation resulting in MDR strains.

Materials and Methods

Sampling sites

Three police hospitals namely; (Aguza Police Hospital, Nasser City Police Hospital and New Cairo Police Academy Hospital), were chosen as the most popular and famous police hospitals at Cairo, Egypt. They were chosen apart from each other representing relatively different environments of the city. Not less important is the date of installation and the variable life style of the three districts where they are. They receive and admit only members of the "Ministry of Interior Affairs".

Sampling period

Six months; from March 2016 to August 2017. During these months, hospitals, in general, receive and admit higher numbers of patients than the rest of the year.

Sampled cases

The concept of choosing cases in this study based upon ICUs patients acquired infections during they stay period. This means they should be went under investigation and have been admitted for reasons other than the infection. These infections occur up to 48hrs- 3 days after hospital admission. Accordingly, 326 patients (222 males and 104 females) their age ranged between 18 years and 57 years were targeted directly from the microbiology laboratory results. Bacterial cultures were collected from different sources including; urine, sputum, blood culture and wound, throat, eye, bed sore swabs.

All isolates (326) were identified to the species level following morphological (staining and microscopic investigation) and biochemical (Microbact GNB 12E ThermoFisher Scientific) tests required.

The Clinical and Laboratory Standards Institute (CLSI) protocols for MIC and MBC determination, were adopted. A common methodology utilized for MIC is CLSI M07-A9, Methods for Dilution Antimicrobial Susceptibility

Tests for Bacteria That Grow Aerobically. For MBC determination, CLSI M26-A, Methods for Determining Bactericidal Activity of Antimicrobial Agents, was followed.

Results

The 326 bacterial isolates collected from the study sites were confirmed to belong to species from 6 different genera. Site-wise Nasser City Hospital showed intensive lab-investigations reflected on the highest number of samples (175), Aguza came later (96) and New Cairo last (55). Frequency-wise, *Escherichia* was the most frequent (155), followed by *Pseudomonas* (96), then came *Staphylococcus* (34), *Klebsiella* (24), *Proteus* (16) and *Citrobacter* (1) (Table 1, Fig. 1). Source-wise the majority of identified isolates were from urine (181) followed by wound (45), sputum (33), throat (24), blood (23) and finally bed-sore (20), (Table 2). More deeply, urine harbored all of the 6 identified bacterial strains, followed by wound and bed-sore had 5, blood and sputum 3 and finally came throat only 2 strains. Spread-wise, *Escherichia coli* and *Staphylococcus aureus* were detected in all 6 sources of study, while; *Pseudomonas aeruginosa*, *Klebsiella sp*., *Proteus sp*. and *Citrobacter freundii* were detected and one of the studied sources, respectively (Table 2, Fig. 2).

TABLE 1. Distribution of the six identified genera represented by 326 isolates collected from the microbiological laboratories (belonging only to ICUs) of the three studied police hospitals namely; Naser City Hospital, Aguza Hospital and New Cairo Hospital.

Site of isolation / Isolates	Nasser City	Aguza	New Cairo	Total No.
E. Coli	87	42	26	155
Klebsiella oxytoca	12	7	5	24
Proteus mirabilis	9	6	1	16
P. aeruginosa	18	9	7	34
Staph.aureus	49	31	16	96
Citrobacter freundii	0	1	0	1
Total	175	96	55	326

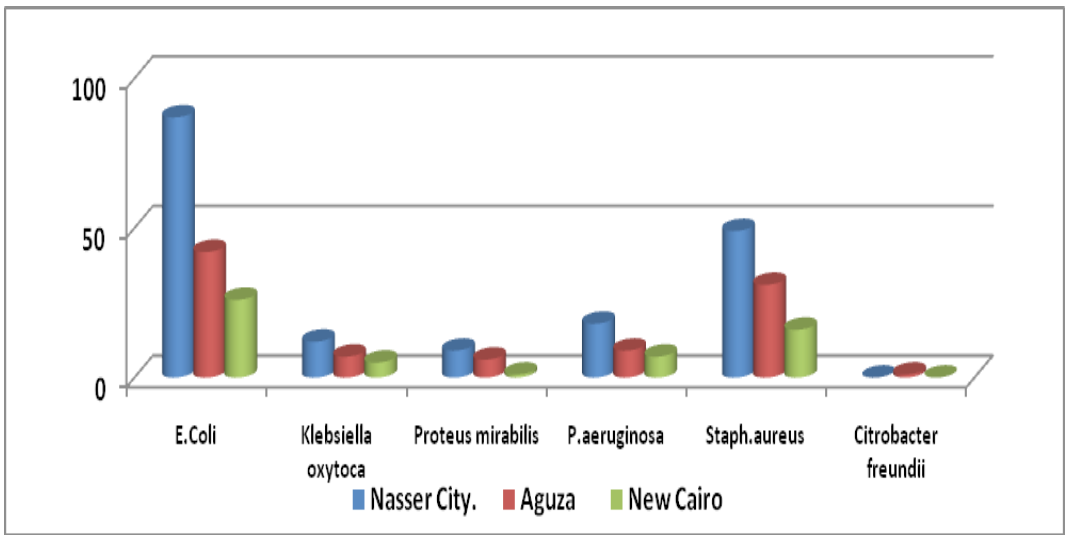

Fig. 1. Distribution of the six identified genera represented by 326 isolates collected from the microbiological laboratories (belonging only to ICUs) of the three studied police hospitals namely; Naser City Hospital, Aguza Hospital and New Cairo Hospital.

TABLE 2. Frequency of each of the six genera (N. 326) in the six studied sources of sampling namely; urine, sputum, blood, wound throat and bed-sore.

Source of sampling / Isolates	Urine	Sputum	Blood	Wound	Throate	Bed-Sore Swab	Total
E. Coli	86	16	11	27	4	11	155
Klebsiella oxytoca	17	0	0	5	0	2	24
Proteus mirabilis	9	0	0	5	0	2	16
P. aeruginosa	21	6	2	2	0	3	34
Stoph. aureus	47	11	10	6	20	2	96
Citrobacter freundii	1	0	0	0	0	0	1
Total	181	33	23	45	24	20	326

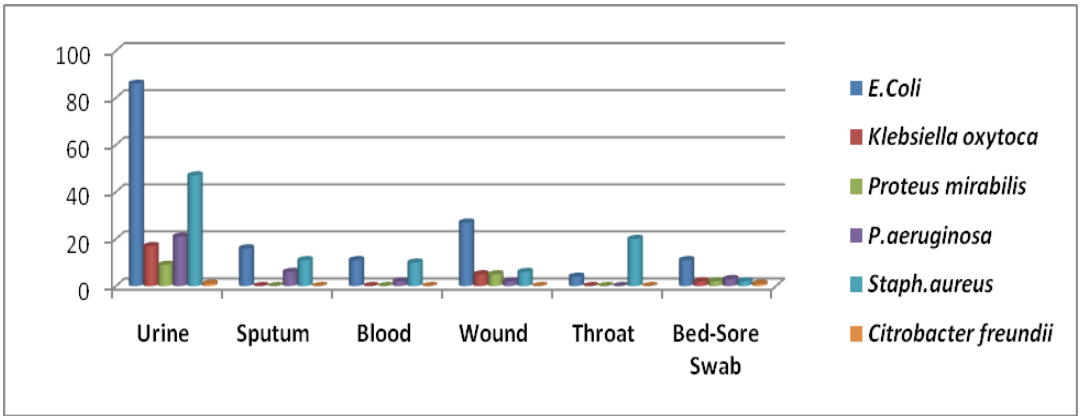

Fig. 2. Frequency of each of the six genera (N. 326) in the six studied sources of sampling namely; urine, sputum, blood, wound throat and bed-sore.

Only one representative isolate of each of the identified 6 Genera underwent further identification using Microbact Biochemical Kits and 16s rRNA gene sequencing gave accurate specific names for the *Klebseilla* sp isolates to be *Klebsiella oxytoca* and the *Proteus* sp. isolates to be *Proteus mirabilis*, in addition to *E. coli*, *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Staphylococcus aureus*. Species-wise, 3 *Pseudomonas aeruginosa*; strains were picked up from different sites and different sources; Aguza Hospital/urine sample, Naser City Hospital/ sputum sample and New Cairo Hospital/blood sample, in addition to *Klebseilla oxytoca* from Naser City Hospital/bed sore sample, *Proteus mirabilis* from New Cairo Hospital/wound sample and *Citrobacter freundii* from Aguza Hospital/ urine sample. *Pseudomonas aeruginosa* isolates were chosen as they have been detected in all the studied hospitals and sources, where *Klebseilla oxytoca*, *Proteus mirabilis* and *Citrobacter freundii* were, relatively, uncommon on the specific level. So, we enriched the strains choice to cover a wider range of suspected nosocomial infectious bacteria at ICUs, both in the studied sites and the studied sources. Antibiotics (25) were chosen as to cover, not only the most commonly- used for treatment in the three studied hospitals, but also different antibiotic families and generations. Results showed MDR capabilities of all the six studied isolates. *Pseudomonas aeruginosa* resisted (16), *Klebsiella oxytoca* (9), *Staphylococcus aureus* (9), *Escherichia coli* (8), *Citrobacter freundii* (8) and *Proteus mirabilis* (3), (Table 3). A narrower range of antibiotics was adopted for detailed studies. For 12 antibiotics namely; Gentamicin (CN 30µg), Meropenem (MEM 30µg), Ciprofloxacin (CIP 30µg), Cefotaxime (CTX 30µg), Amikacin

(AK 30µg), Ampicillin/Sulbactam (SAM 30µg), Levofloxacin (LEV 30µg), Slbactam/ Cefoperazone (SCF 30µg), Amoxycillin/ Clavulanic acid (AMC 30µg), Imepinem (IMP 30µg), Cefepime (FEP 30µg) and Ceftriaxone (CRO 30µg), the resistance profile of the six isolates was examined and found as presented in (Table 4). The antibiotic-profiles for the six isolates considerably varied from each other, not only in the number of antibiotics they resist, but also in the type of antibiotics they resist. The *Pseudomonas aeruginosa* (3 isolates), although identified as one species, seemed to be different strains as they showed different antibiotic-resistance profiles. They shared resistance to six out of 12 tested antibiotics ; CN, CIP, CRO, AMC and FEP. Urine *Pseudomonas aeruginosa* isolate resist 9, while blood and sputum ones resist 7 out of 12 tested antibiotics. *Proteus mirabilis* resist 10 antibiotics followed by *Klebsiella oxytoca* (9) and *Citrobacter freundii* (7). All the 6 isolates shared resistance to CRO, AMC and FEP.

TABLE 3. Antibiotic-resistance profiles of six randomly-chosen representatives of the six preliminary-identified species against 26 most commonly-used antibiotics belong to different families and generations.

No.	Antibiotics 30mg / Isolates	*E.coli*	*K. oxytoca*	*Protues mirabilis*	*P.aeruginse*	*Citrobacter freundii*	*Staph.aurus*
1	Amox/Clov (AMC 30mg)	+	+	+	-	+	-
2	Amp/Sulba (SAM 30mg)	+	-	-	-	+	-
3	Ampicillin (AMP 30mg)	-	-	-	-	-	+
4	Cefoperazone (SCF 30mg)	-	+	+	-	+	+
5	Cefazolin	-	-	-	+	-	-
6	Cefepnme (FEP 30mg)	+	+	+	-	-	-
7	Ceftriaxone (CRO 30mg)	-	-	-	-	-	-
8	Ceplalothin	-	-	-	-	-	-
9	Chioromphein	+	+	+	-	+	+
10	Ciprofloxacin (CIP 30mg)	+	+	-	+	+	+
11	Clindamycin (C 30mg)	+	+	+	-	-	+
12	Erythromycin (E 30mg)	-	-	+	-	+	-
13	Gentomicin (CN 30mg)	-	+	-	+	+	-
14	Imipenem (IMP 30mg)	+	-	+	-	+	+
15	Levofloxacin (LEV 30mg)	+	+	+	+	-	+
16	Linezhid	-	-	-	-	+	-
17	Ofloxacin (OFX 30mg)	-	+	+	-	-	+
18	Meropenem (MEM 30mg)	-	+	-	-	+	+
19	Oxacillin (OX 30mg)	+	-	-	-	-	-
20	Penicillin (P 30mg)	-	-	+	-	-	-
21	Rifampicin (RD 30mg)	-	-	+	-	+	+
22	Tetracycline (TET 30mg)	-	+	-	-	-	+
23	trimeth/Sulfa	+	+	+	+	+	-
24	Vancomycin (V 30mg)	-	-	-	-	+	+
25	cefotaxim	-	+	-	-	-	+
26	cefoxitin	+	+	+	-	-	-

Ceftriaxone (CRO), Amoxycillin/Clavulanic acid (AMC) and Cefepime (FEP) went under further antibiotic assay study because all the six isolates resist them at 30 µg/disc, (Table 4). Serial dilutions; 20, 30, 40, 50, 60, 70 and 90 µg/ml, on Muller Hinton broth and Mcfarland standard bacterial inocula, were used to determine the MIC (s) of the six isolates. *Proteus mirabilis* and *Citrobacter freundii* isolates were completely inhibited at 30 µg/ml, where the 3 *Pseudomonas aerginosa* and *Klebseilla oxytoca* isolates showed complete inhibition at 40 µg/ml (Table 5). Streaks on Muller Hinton agar containing the 30, 40, 50, 60, 70 and 90 µg/ml of the same antibiotics using inocula from the completely inhibited broth of each isolate showed MBC (s) of 30 µg/ml for both *Proteus mirabilis* and *Citrobacter freundii*, while the 3 *Pseudomonas aerginosa* and *Klebseilla oxytoca* isolates showed MBC (s) of 50 µg/ml (Table 5). Cells in yellow in Table (5) have MBC/MICs more than 4, meaning that their MICs and recommended concentrations in the final products (commercial antibiotics), inhibit but not killing the pathogens. These pathogens are not resistant, they are persistent and/or tolerant.

MBC/MICs more than 4 give these isolates the term "Persistent or Tolerant", where less than 4 described as "Resistant". Results clearly indicated the high risk of the two *Pseudomonas aeruginosa* from Naser City H/sputum and Aguza H/urine persisting (inhibited but not killed) the 3 tested antibiotics. Relatively less risk emerged from *Pseudomonas aeruginosa* from New Cairo H/blood and *Citrobacter freundii* from Aguza H/urine and least for the *Klebseilla oxytoca* from New Cairo H/bed-sore and *Proteus mirabilis* from Naser City H/wound.

TABLE 4. Profiles of antibiotic-resistance of the most suspected nosocomial bacterial species isolated from different sites and sources of study.

Bacterial species/ Site/source / Antibiotic	*Pseudomonas aeruginosa* (New Cairo H./blood)	*Pseudomonas aeruginosa* (Naser City H./ sputum)	*Pseudomonas aeruginosa* (Aguza H./ urine)	*Klebsiellaoxytoca* (Naser City H./ bed- sore)	*Proteus mirabilis* (Naser City H./ wound)	*Citrobacter freundii* (Aguza H./ urine)
MEM30µg	-	-	+	+	-	-
CN 30µg	-	-	-	+	+	+
LEV 30µg	-	+	-	-	-	+
SAM30µg	+	-	+	-	-	-
CIP 30µg	-	-	-	-	-	+
CRO30µg	-	-	-	-	-	-
AK 30µg	+	+	-	+	-	+
CTX 30µg	-	-	-	-	+	-
SCF 30µg	-	+	-	-	-	-
AMC30µg	-	-	-	-	-	-
FEP 30µg	-	-	-	-	-	-
IMP 30µg	-	-	+	-	-	+

- No. of species (6) and No. of antibiotics (12) namely; Gentamicin (CN 30µg), Meropenem (MEM 30µg), Ciprofloxacin (CIP 30µg), Cefotaxime (CTX 30µg), Amikacin (AK 30µg), Ampicillin/Sulbactam (SAM 30µg), Levofloxacin (LEV 30µg), Slbactam/ Cefoperazone (SCF 30µg), Amoxycillin/Clavulanic acid (AMC 30µg), Imepinem (IMP 30µg), Cefepime (FEP 30µg) and Ceftriaxone (CRO 30µg).
- (-) Resistant showed no clear zone and (+) sensitive with clear zone.

TABLE 5. Results of the MBC (s) and MIC (s) µg/Ml for the six studied isolates; 3 *Pseudomonas aeruginosa, Klebseillaoxytoca, Proteus mirabilis* and *Citrobacter freundii* to the 3 investigated antibiotics; Ceftriaxone (CRO), Amoxycillin/Clavulanic acid (AMC) and Cefepime (FEP).

Bacterial sp. / Antibiotics	*Pseudomonas aeruginosa* (New Cairo H./ blood) MBC/MIC µg/Ml	*Pseudomonas aeruginosa* (Naser City H./ sputum) MBC/MIC µg/Ml	*Pseudomonas aeruginosa* (Aguza H./urine) MBC/MIC µg/Ml	*Klebsiella oxytoca* (New Cairo H./ bed- sore) MBC/MIC µg/Ml	*Proteus mirabilis* (Naser City H./wound) MBC/MIC µg/Ml	*Citrobacter freundii* (Aguza H./ urine) MBC/MIC µg/Ml
CRO 30mg	150/70	450/60	450/60	50/40	130/40	330/90
AMC 30mg	150/30	250/30	350/30	270/30	300/50	300/40
FEP 30mg	250/40	350/60	550/70	90/30	70/30	320/50

Cells in yellow have MBC/MICs more than 4, meaning that their MICs and recommended concentrations in the final products (commercial antibiotics), inhibit but not killing the pathogens. These pathogens are not resistant, they are persistent and/or tolerant.

Discussion

The study was keen and carefully deal with only nosocomial bacterial infections by looking only at infections that appear on ICUs patients and were not the cause of their admission to the studied hospitals. The number of collected samples; 175, 96 and 55 from Naser City, Aguza and New Cairo Hospitals respectively, reflected, may be, the obvious differences in the life style, category of Police officers allowed to visit the hospitals and average income. New Cairo is the most recent installed and equipped one. *E. coli* and *Staph. aureus* strains were isolated from all the investigated sites; urine, sputum, blood, wound, throat and bedsore. This high incidence was expected and have been repeatedly documented by many researchers (Rubin et al., 1999; Karchmer et al., 2002; Wertheim et al., 2005; Winn, 2006; Mohamudha et al., 2012). The absence of *Klebsiella oxytoca* and *Proteous mirabilis* from sputum, blood and throat may be due to immunological aspects in blood and the alkalinity of the throat and sputum in addition to the many studies suggesting these to organisms as emerging nosocomial pathogens (Singh et al., 2016; Foris & Snowden, 2018). *Pseudomonas aeruginosa* appeared in all sampling sources except throat alarming, may be, insufficient infection control actions, although its regular appearance all over the world` hospitals was recorded (Stehling et al., 2008; Akpaka et al., 2009; Morales et al., 2012; Davane et al., 2014). The *Citrobacter freundii* isolated only from urine and was assigned as serious new comer for the pyelonephritis etiologic members. Detection of *E. coli, Staph. aureus* and *P. aeruginosa* in blood samples (septicemic cases) indicated late stages of bacterial systemic invasion through high virulence capabilities of these pathogens (Micek et al., 2005; Patel et al., 2017; Davis & Stoppler, 2019; Li et al., 2019). Surveyed samples revealed the occurrence and spreading of eight MDR (3–16 out of 26 antibiotics) species belong to six genera were not connected with neither the hospital nor the sample source. Four of these MDR isolates are representatives of 1/2 (SKP) of the EKAPSE cocktail bacterial pathogens recommended by the FDA to determine the efficacy of antimicrobial products. The remarkable notice was that non of the 26 screened antibiotics showed +ve activity against all the six studied isolates, but only two antibiotics; Ciprofloxacin (30mg) and Trimethprime/sulphate, gave +ve activity towards five of them (Boucher et al., 2009). Ceftriaxone (30mg) and Ceplalothin (30mg) were –ve acted on all the six isolates those showed real resistance as the MBC/MICs were less than four for four of them. Amoxicillin/clav (30mg) which actively inhibited the growth of five of the tested isolates, gave MBC/MICs less than four indicating transient action; persistence and tolerance, explaining the nosocomial behavior of them (Balaban et al., 2019). *Pseudomonas aeruginosa*, for example isolated from all the three hospitals and almost all the six sources with the same antibiotic-resistance profile. But, the MBC/MICs were different and no previous studies investigated this so far. MBC/MIC values of these three *Pseudomonas aeruginosa* strains were higher than four, which mean they are in the persistence and/or tolerance stationary

phase, rather than resistance. Make it worse, the concept of MIC definition that only 50% or 90% of the bacterial cell are inhibited when the estimated MICs are 50 or 90, respectively, leaving behind 50% and 10% of cell uninhibited causing nosocomial infections. *Pseudomonas aeruginosa* is a major CRISPR Cas model system, which make phage therapy application preferable instead of antibiotics. Knock-out and gene silencing applications are also promising in the future, but till this, it is very important to re-evaluate the validity of most antibiotics on the MBC/MIC bases.

Conclusion and Recommendation

Antibiotic persistence is not only an interesting example of non-genetic single-cell heterogeneity, it may also have a role in the failure of antibiotic treatments. Tolerance and persistence are similar phenomena of increased survival in the presence of antibiotic. The misunderstanding of resistance, persistence and tolerance in MDR pathogens based on only MICs is thought to be a main cause of spreading of such pathogens as nosocomial. This is very important specially when performing culture and sensitivity tests in microbiology labs.

It is highly recommended here to run tests for MBC/MICs precisely to choose the antibiotic with least MBC/MIC ratio; less than four, in order to reduce the nosocomial hazardous ramification of such pathogen during the transient persistent and/ or tolerant phases.

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