JJBS

Volume 14, Number 4,December 2021
ISSN 1995-6673
Pages 813 – 817

Jordan Journal of Biological Sciences

Evaluation of Quorum-Sensing, Antibiotics Resistance, and Biofilm Formation in Pathogenic Bacteria from the Hospital Environments

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Received: October 8, 2020; Revised: December 19, 2020; Accepted: January 7, 2021

Abstract

Background: Multidrug-resistant bacteria (MDR) often contaminate hospital environment and cause serious illnesses. Quorum Sensing (QS) regulates a variety of downstream cellular processes, including antibiotics resistance mechanisms and biofilm formation, and causes harm to the host. This study investigates antibacterial susceptibility and biofilm formation of pathogenic bacteria in hospital environment.

Methods: Hundred bacterial isolates were collected from various environments in the Medical City hospital. The antimicrobial susceptibility technique was evaluated through disk diffusion method. Next, biofilms formation was detected by the microliter plate assay. Finally, PCR was used to analyze the frequency of QS system genes.

Results: Current findings showed that the predominant isolates were *Acinetobacter baumannii* (34%), *Escherichia coli* (30%), *Pseudomonas aeruginosa* (19%), and *Klebsiella pneumonia* (17%). In general, significant resistance was found related to trimethoprim (88%), Augmentin (88%), and cefotaxime (72%). Among all isolates, 62% of sensitivity was related to ciprofloxacin. Biofilm had been formed by 39% of isolates. PCR results showed that the frequency of *lasI* and *rhlI* gene was 70% and 61%, respectively.

Conclusion: Current findings revealed that the hospital environment is a potential reservoir of MDR gram-negative pathogenic bacteria. Thus, we suggest that the health policymakers in Iraq must critically apply the guidelines and recommendations for monitoring the environments in the health sector.

Keywords: Antibiotics Footprint, *Acinetobacter baumannii*, Antibiotics Resistance, Quorum-Sensing, PCR.

1. Introduction

Nosocomial infections, also known as hospital-acquired infections, are serious global health concerns, mainly occurring during hospitalization and causing increased morbidity and mortality (Labi *et al.,* 2019). A hospital environment is undoubtedly a great source of potentially pathogenic bacteria (Bouzada *et al.,* 2010). It can be contaminated with bacterial pathogens, mainly Gram-negative (G-ve) rods such as *Acinetobacter*, *Escherichia coli*, *Pseudomonas spp*, *Klebsiella sp*, *Shigella spp*, *Salmonella spp* and *Proteus spp*, and Gram-positive (G+ve) cocci such as *Staphylococcus aureus*, *Enterococcus* and *Streptococcus*. Environmental surfaces serve as a reservoir for pathogenic bacteria (Otter *et al.,* 2013). The development of nosocomial infection depends on a multifaceted relationship between the rate of contamination of the hospital environment, characteristics of the pathogen, and a susceptible host (Worku *et al.,* 2018). Biofilm bacteria can share nutrients and are shielded from harmful environmental factors such as desiccation, antibiotics, and the immune system of a host body (Nirwati *et al.,* 2019). In the hospital environment, biofilm-forming bacteria can associate with the ability to survive on surfaces, resist antibiotics, and face host defenses. Therefore, it contributes to cause chronic infections (Ali *et al.,* 2019).

Quorum sensing are used by pathogenic bacteria to regulate gene expression. QS bacteria produce and release signals called autoinducers molecules (Häussler, 2010). Target genes regulate virulence factors, biofilm formation, and broad behaviors including swarming, swimming, twitching motility, and conjugation (Rutherford and Bassler, 2012). The most common QS system in G-ve bacteria involves the production of N- acylated homoserine lactones (AHLs) or autoinducer (Netotea *et al.,* 2009). QS signaling will trigger biofilm formation, resulting in antimicrobial resistance of the pathogens, thereby increasing the therapeutic complexity of bacterial diseases (Jiang *et al.,* 2019). In this regard, the main aim of

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the current study is to identify the seriousness of a hospital environment as a potential reservoir of multidrug-resistant bacteria capable of infecting patients.

2. Materials and Methods

2.1. Bacterial isolates collection and identification

A total of one hundred bacterial isolates were collected from the surfaces, laundries, health care workers, and medical equipment in Medical City hospital in Baghdad, Iraq. Sterile swabs were used for the collected samples. These isolates were identified by routine biochemical tests and the Vitek 2 system.

2.2. Antibiotic Susceptibility test

The pattern of antibiotic susceptibility was done by the Kirby-Bauer method and interpreted according to the Clinical Laboratory Standard Institute guidelines (CLSI, 2020). Eight antibiotic discs were used in this study, which are Amikacin (AK, 30µg), Augmentin (AMG, 20 µg), Cefotaxime (CTX, 30µg), Cefepime (CPM, 30µg), Ceftriaxone (CTR, 30µg), Ciprofloxacin (CIP, 5µg), Trimethoprim (TMP, 5µg) and Piperacillin (PI, 30µg). These discs are provided from Bioanalyse in Turkey. The duplicated antibiotic was made and quality controled using *P. aeruginosa* ATCC 27853 (incubated at 37°C for 18-24hr.). Bacterial isolates were resistant to at least three different antimicrobials classes considered as MDR.

2.3. Biofilm formation assay

Microliter Plate Assay was performed for biofilm formation according to the method described by Babapour *et al*. (2016). First, 200 µl of bacterial suspension overnight culture (equivalent to 0.5 McFarland standard) was used to inoculate wells of a flat-bottom 96-well polystyrene microtiter plate (Coastar, USA), containing 180 µl of Brain Heart Infusion broth (Himedia, India) with 2% sucrose. After incubation at 37C^{o} for 24 hours, unattached cells were gently rinsed three times with phosphate buffer saline (pH: 7.2). Then, cells were dried at room temperature for 15 min. Later, adherent bacteria were fixed with 200 µl of 99% methanol per well. The bacteria attached to the surface were stained with 200 µl of crystal violate (0.1%), rewashed, and extracted with 200 µl ethanol (95%). A total of 200 µl of the mixed solution was analysis using a spectrophotometer at optical density (OD_{630}) 630 nm (OPTIMA/Japan). Each assay was performed in triplicate, and the mean OD_{630} value of tested wells was applied to biofilm-forming ability. Uninoculated medium was considered as negative control. Finally, adherence capabilities of the isolates were divided into four categories, and above the mean optical density of the negative control was considered as the cut-off optical density (ODc). Based on the ODs in brain heart infusion broth, the isolates with OD360 < ODc360 were defined as biofilm non-formers, isolates with (ODc < OD < 2×ODc) were defined as biofilm formers of weak level, isolates with (2×ODc < OD < 4×OD) were defined as moderate level, and isolates with (4×OD < OD) were defined as strong level.

2.4. DNA extraction and gene amplification

DNA was extracted according to the manufacturer's instructions of the DNA extraction kit (WizPrep™ gDNA Mini Kit, South Korea). Polymerase Chain Reaction was performed for amplification of *lasR*, *rhlR*, *lasI* and *rhlI* quorum sensing genes (See Table 1). Each PCR mix (25µl) was composed from 12.5µl of Go Taq® Green master mix, template DNA 5µl, forward & reverse primers (1µl for each), and 5.5µl of deionized nuclease–free water (Promega, USA).

PCR amplification conditions were as follows (all primers): initial denaturation at 95°C/5min followed by 36 cycles of 95ºC/30sec, 59°C/1min and 72°C/1min, and a final extension at 72°C/10min (Cotar *et al.*, 2010). The products were analyzed by 1% gel agarose (Promega, USA), containing 0.5 µg/mL of ethidium bromide and visualized under UV light.

Table 1: Primers oligonucleotide sequence and molecular size of PCR products

Gene	Oligonucleotide	Product size bp	Reference
lasR	F: 5′-TGCCGATTTTCTGGGAACC-3′	401	(Cotar *et al*., 2010)
	R: 5′-CCGCCGAATATTTCCCATATG-3′		
lasI	F: 5′-TCGACGAGATGGAAATCGATG-3′	402	
	R: 5′-GCTCGATGCCGATCTTCAG-3′		
rhlI	F: 5′-CGAATTGCTCTCTGAATCGCT-3′	182	
	R: 5′-GGCTCATGGCGACGATGTA-3′		
rhlR	F: 5′-TCGATTACTACGCCTATGGCG-3′	208	
	R: 5′-TTCCAGAGCATCCGGCTCT-3′		

3. Results

The study aimed to identify the seriousness of hospitals environment as a potential reservoir of multidrug-resistant bacteria and inform policy to monitoring the hospital environment. Thus, the current finding showed that pathogenic bacteria heavily contaminate the surfaces of the hospitals. A hundred of bacterial Gram-negative isolates were identified. Among all isolates, *A. baumannii* were 34%, *E. coli* were 30%, *P. aeruginosa* were 19%, and *K. pneumonia* were 17%. The results of the antibiotics susceptibility test showed that most isolates were highly resistant against most antibiotics. The highest resistance was recorded for trimethoprim (88%), Augmentin (88%), and cefotaxime (72%). At the same time, ciprofloxacin (38%) was recorded as the most effective antibiotic against isolates. All isolates showed resistance to the rest of antibiotics ranging between (44%) and (66%), as shown in Figure 1.

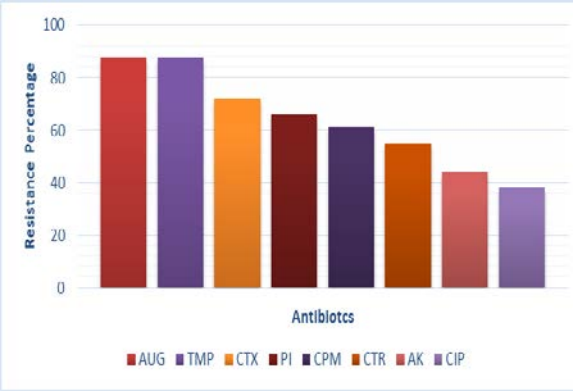

Figure 1: Percentage of Antibiotic susceptibility test of bacterial isolates.

The current obtained results showed that 39% of the environmental isolates were biofilm producers. The results recorded that four isolates with a percentage of 11.76% of *A. baumannii* isolates were strong biofilm producers. At the same time, most isolates were producers with weak biofilm with percentage of 66.66%. In general, *K. pneumoniae* was the high biofilm producers with percentage of 52.94%, followed by *E. coli* (43.33%), *A. baumannii* (35.29%), and *P. aeruginosa* (26.32%) (See Table 2).

Table 2: Adhesion patterns of isolates

Isolates (Number)	Adhesion patterns			Total (%)
	Strong	Moderate	Weak	
A. baumannii (n=34)	4	Non former	8	12 (35.29%)
E. coli (n=30)	Non former	5	8	13 (43.33%)
P. aeruginosa (n=19)	Non former	Non former	5	5 (26.32%)
K. pneumonia (n=17)	Non former	4	5	9 (52.94%)
Total (%)	4 (10.26%)	9 (23.08%)	26 (66.66%)	39 (39%)

PCR analysis revealed that 70% of isolates carried the *lasI* gene, 61% of isolates carried the *rhlI* gene, 57% of isolates had the *lasR* gene, while 4% isolates carried the *rhIR* gene (See Figure 2 and Table 3). *A. baumannii* was the most bacterial isolate harboring quorum sensing genes, *lasI*, *lasR*, and *rhlI* genes found in 25 (73.52%) isolates, while 4 (11.76%) of isolates carried *rhIR* gene. The *lasI*, *lasR*, and *rhlI* genes were found in all isolates of *P. aeruginosa* (100%) and 13 (76.43%) isolates of *K. pneumonia*. Finally, 13 (43.33%) *E. coli* isolates contained *lasI* gene, and 4 (13.33%) isolates had *rhlI* genes.

Table 3: Number and percentage of QS genes presence in isolates

Genes / Isolates	*lasI*	*lasR*	*rhlI*	*rhIR*
baumannii (n=34)	25 (73.52%)	25 (73.52%)	25 (73.52%)	4 (11.76%)
P.aeruginosa (n=19)	19 (100%)	19 (100%)	19 (100%)	Negative
K. pneumoniae (n=17)	13 (76.43%)	13 (76.43%)	13 (76.43%)	Negative
E. coli (n=30)	13 (43.33%)	Negative	4 (13.33%)	Negative
Total	70 (70%)	57 (57%)	61 (61%)	4 (4%)

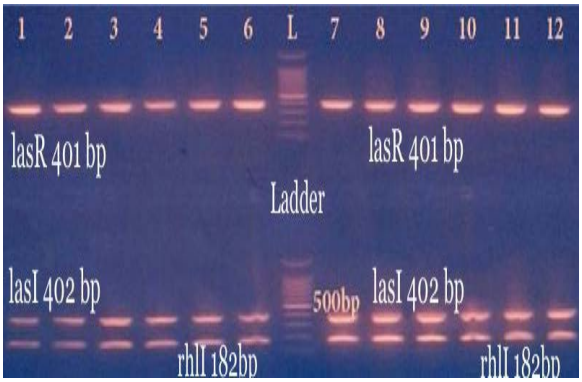

Figure 2: Agarose Gel Electrophoresis (1% agarose, 5-10 V/cm for 50 min) of *lasR*, *lasI*, and *rhlI* genes. Lane L 100 bp DNA Ladder, Lanes 1-12 Represent of Isolates Bands.

4. Discussion

Many studies showed that hospitals' environment (surfaces, clothes, air, water, food, waste, and medical devices) harbor bacteria such as *Staphylococcus*, *Enterococcus*, *A. baumannii*, *E. coli*, *P. aeruginosa*, and *K. pneumonia*. Bacterial isolates in the hospital setting are characterized as MDR, not only for the irrational use of antibiotics but also for the presence of antibiotics residues in fluid effluents (Dougnon *et al.*, 2020). Hospitals' environments are characterized by heavy bacterial density (Ory *et al.*, 2016). Antibiotic resistant bacteria pose a significant threat to public health in the hospitals' environment (Osińska *et al.*, 2017).

Our findings are consistent with several previous studies that showed various components of the hospitals' environment could accommodate many pathogenic bacteria. According to Kim and co-workers, the areas around patients are generally contaminated by bacteria. The bacterial contaminantion on surfaces was supported by the formation of biofilms and prolonged survival in the environment (Kim *et al.*, 1981,Talon, 1999, Bertrou *et al.*, 2000).

Current findings revealed local isolates carrying one or more QS genes. The Iraqi study conducted by Sallman *et al*. (2018) reported 82.53% isolates carrying *lasI/lasR* and *rhlI/rhlR* genes. Senturk *et al*. (2012) displayed that 77.7%, 88.8%, 66.6%, and 77.7% of isolates were positive for *rhlR*, *rhlI* , *lasR*, and *lasI*, respectively. QS involves generation, release and detection of extracellular signal molecules called auto-inducers (AI). It regulates behaviors requiring cells to synchronize in order to achieve successful results (Paluch *et al.,* 2020). The QS system facilitates the bacterial population to grow and proliferate in an environment with effective intercellular communication (Subhadra *et al.,* 2016). QS-controlled processes include antibiotic resistance, biofilm formation and virulence (Paluch *et al.,* 2020).

Antibiotic-resistant *A. baumannii* has been represented as one of the most problematic hospitals acquired bacteria. *A. baumannii* can colonize in the hospital setting, and constitutes a significant problem in intensive care units (Espinal *et al.,* 2012). *A. baumannii* was isolated from 11% (7/64) of air samples. Hospitals and healthcare settings are regarded as reservoirs of *Pseudomonas spp* isolates, which are a worldwide health concern due to the increasing development of MDR isolate (Alhusseini *et al.,* 2019). Several therapeutic challenges exist with MDR *P. aeruginosa* due to the limit of effective treatment strategies (Aloush *et al.,* 2006). The presence of pathogenic bacteria in the hospitals' environment poses a significant risk to health. *K. pneumonia* is recognized as an urgent threat to human health because of the emergence of MDR isolates associated with hospital outbreaks and hyper-virulent strains associated with severe community-acquired infections (Holt *et al.,* 2015). Recorded hospital settings showed the highest percentage of 23% of extended-spectrum β-lactamase producing *K. pneumonia* (Chaudhry *et al.,* 2019). Biofilm becomes a significant problem in health care (Dewasthale *et al.,* 2018). Bacteria in a biofilm are a protective mechanism to survive in harsh conditions. These bacteria become more resistant to antibiotics; therefore, this biofilm structure represents an important virulence factor (Espinal *et al.,* 2012).

Antibiotics resistance in biofilms is complex and results from contributions of intrinsic, acquired, and adaptive mechanisms. Most notably, biofilm specific features such as the differential expression of multiple gene networks, extracellular matrix, and the metabolic heterogeneity of subpopulations within a biofilm colony are significant contributors to antibiotic resistance (Taylor *et al.,* 2014). *P. aeruginosa* and *K. pneumoniae* exhibited strong biofilm-forming ability on hospital clinical laboratory surfaces. *Klebsiella spp*. was found to persist on dry inanimate surfaces between 2 Hr. to 30 months, while the persistence of *P. aeruginosa* was 6 Hr. to 16 months (Chen *et al.,* 2020).

5. Conclusion

The extracted findings from this study reported the prevalence of gram-negative pathogenic isolates in the hospitals' environment. So, appropriate measures could help reduce pollutants in the hospitals' environment and reduce related serious illnesses. As a result, the current findings recommend the routine screening and disinfection of the hospitals' environment to prevent contamination.

6. Author's contributions

Laith B. Alhusseini, Dunya J. Ridha, Zahraa A. Khadam: Conceptualization, Design of methodology. Mohammed F. Al Marjani: Supervision, Validation. Laith B. Alhusseini, Zahraa A. Khadam: Writing-Reviewing and Editing. Fadhl A. S. Al Gasha, Shayma M. A. Al Baker, Awas H. Al Rahal: Writing and Reviewing the manuscript.

7. Ethical approval

The authors do not see any ethical issues that may arise after the publication of this manuscript.

Acknowledgements

We thank the clinical staff of the Medical City hospital in Baghdad. We are grateful for the useful comments and suggestions from anonymous referees.

Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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