In Vitro Anti-Biofilm Activity of Curcumin Nanoparticles in Acinetobacter baumannii: A Culture-Based and Molecular Approach

Atefeh Hosseini 1, Taher Nejadsattari 1 and Mohsen Zargar 2, *

1Department of Microbiology, Sciences and Research Branch, Islamic Azad University, Tehran, Iran
2Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran

*Corresponding author: Department of Microbiology, Qom Branch, Islamic Azad University, Postal Code: 37161-92797, Qom, Iran. Tel: +98-9121539288, Email: zmohsen2002@yahoo.com

Received 2018 August 12; Revised 2019 July 21; Accepted 2019 August 04.

Abstract

Background: Acinetobacter baumannii is one of the most important opportunistic and biofilms-forming microorganisms. Its biofilm structure provides an effective barrier against the antimicrobial penetration and increases the drug resistance of Acinetobacter baumannii by 1000 × compared with plankton cells. However, nanoparticles have recently reported as suitable tools to prevent biofilm formation.

Objectives: The aim of this study was to investigate the effect of curcumin nanoparticles on biofilm gene expression in Acinetobacter baumannii strains in vitro.

Methods: Seventy clinical isolates of Acinetobacter baumannii were collected from different specimens and all specimens were diagnosed biochemically. Antibiotic resistance pattern was detected by disc diffusion method. Isolates of high biofilm producers were selected to investigate the effect of curcumin nanoparticles. The antibiofilm activity of curcumin nanoparticles was studied using Real-time PCR technique in the molecular level.

Results: This bacterium showed high resistance to most antibiotics, except for polymyxin B. Twenty-five strains had high biofilm formation capacity. MIC for curcumin was 128 µg/mL in all 25 strains. The expression results showed that CsuE gene was downregulated to 0.31 fold in curcumin-treated samples compared to untreated samples.

Conclusions: The study findings suggest that curcumin nanoparticles can be helpful as a candidate for inhibiting the formation of Acinetobacter biofilms.

Keywords: Acinetobacter baumannii, Curcumin Nanoparticle, Real-Time PCR, Biofilm

1. Background

Acinetobacter is an obligate Gram-negative and aerobic coccobacillus, which has been among the most important bacterial infections in the last three decades (1). The most common problem with the treatment of infections caused by this bacterium is the resistance to most antibiotics (2). Antibiotic resistance capacity can be transmitted among bacteria, leading to an increasing resistance day by day (3). Acinetobacter baumannii is mainly responsible for the most antibiotic-resistant cases in this genus in which the multiresistance has led to many problems in the treatment of hospital infections both in the costs of treatment and in the recovery of patients (4-6). A few studies have been done to investigate the virulence factors of this bacterium; however, the most important ones that contribute to the pathogenesis of A. baumannii include the ability of biofilm formation, the presence of polysaccharide in the cell wall, phospholipases, iron absorption capability, external membrane vesicles, OmpA outer membrane proteins, and penicillin-binding protein (7, 8).

It has been established that A. baumannii can appear on artificial surfaces for a long period; thereby, allowing it to persist in the hospital environment due to its ability to form biofilms (9). Biofilms provide bacteria with three main advantages, including trapping the essential elements and nutrients for bacterial usage, tolerate hard conditions as well as the protection against the host’s immune system, and the opportunity to transfer antibiotic resistance genes (10). Furthermore, the availability of fewer nutrients in deeper space within the biofilm and consequently a slower metabolism can prevent the bacteria from taking up an antibiotic and antimicrobial efficacy (11, 12). The process of biofilm formation in many bacteria is mediated by flagella; however, for A. baumannii, pili seem to be involved in this process under the regulation...
of CsuA/BABCDE operon (13). It has been shown the inactivation of CsuE results in the prevention of pili production and the formation of biofilms. This indicates that the CsuA/BABCDE system plays a prominent role in the initial stage of biofilm formation (14).

Owing to the lack of new synthetic antimicrobials to treat MDR Gram-negative infections, attention should be increasingly focused on natural compounds either as stand-alone or adjunctive therapies. Curcumin is a polyphenol mixture extracted from the yellow root of turmeric, which has several pharmacological effects including anti-inflammatory, anti-oxidant, anti-cancer, anti-diabetic, anti-allergic, and antibacterial activity (15). Unfortunately, curcumin faces problems such as low solubility, chemical and biological instability. Today, the use of nanotechnology has improved the biocompatibility of curcumin and the slowdown of high decomposition. On the other hand, the increase of its stability in the blood and the reduction of its toxic effects have been widely investigated (16).

2. Objectives

Regarding the effect of Curcumin on biofilm in various bacteria such as A. baumannii, this study was aimed to investigate the anti-biofilm activity of curcumin nanoparticles and its influence on Biofilm-related gene expression in A. baumannii in vitro.

3. Methods

3.1. Study Samples and Subjects

This descriptive-cross sectional research was conducted from February 2016 to December 2017. Seventy clinical specimens were included for investigation. The sample size calculation formula was:

\[ N = \frac{\left((Z_1-\alpha) + (Z_1-\beta)^2\right)p(1-p)}{d^2} \]  

(1)

With assumption of a confidence level of 95%, the power of 80%, and a precision of 10% of the sample size for a variety of strains. Various clinical specimens included tracheal, sputum, urine, burns, wounds, blood, and unknown resources were collected from patients referring to Firoozgar Hospital (Tehran, Iran).

3.2. Bacterial Culture

Different cultural and biochemical tests were used for the identification and isolation of the Acinetobacter species. Seventy A. baumannii isolates were studied. All isolates were stored at -70°C in microbank vials (Thermofisher, UK) and thawed prior to use.

3.3. Determination of Antibiotic Susceptibility by Disc Diffusion Method

Sensitivity of A. baumannii isolates to eleven antibiotics of different classes, including Piperacillin, Ceftriaxone, Cefepime, Imipenem, Polymyxin, Gentamycin, Tetacycline, Ciprofloxacin, Trimethoprim, sulfamethoxazole, Ticarcillin clavulanic prepared from Rosco company (USA) was assessed by disk diffusion method in a Mueller Hinton Agar (MCC) based on the CLSI instruction. The strains of Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 were used as the reference strains to control antibiogram discs. Ultimately, resistant strains were grouped in one of the following categories: (a) Carbapenem-resistant, (b) MDR (multidrug-resistant), (c) XDR (extreme drug-resistant), and (d) PDR (pan drug-resistant) (17).

3.4. Biofilm Formation Assay

This assay was performed according to a previously reported protocol, with some modifications (18). The level (optical density; OD) of crystal violet present in the destaining solution was measured at 570 nm using a microtiter plate reader (SeikagakuCo., Tokyo, Japan). Each assay was performed in triplicate and the mean OD570 value of the tested wells was applied to determine biofilm production ability. As a control, an uninoculated medium was used for the calibration of OD. As it is indicated in Table 1, the results of biofilm formation were classified as biofilm-negative (-), weak (+), medium (++), and strong (+++). In this study, the standard strain of E. coli ATCC 25922 was used as a positive standard control.

| Biofilm Class Status | Results |
|----------------------|---------|
| IF OD \( \leq \) OD\(_c\) | Non-adherent | - |
| IF OD\(_c\) < OD \( \leq 2 \times \) OD\(_c\) | Weakly adherent | + |
| IF OD\(_c\) < OD \( \leq 4 \times \) OD\(_c\) | Moderately adherent | ++ |
| IF OD \( \leq 4 \times \) OD\(_c\) | Strongly adherent | +++ |

3.5. Determination of Nanocurcumin Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) were determined in corning 96-well microtiter plates (Corning, Amsterdam, The Netherlands). MIC of nanocurcumin was determined against all 25 isolates of A. baumannii to nanocurcumin, the strong biofilm producer, according to the British Society of Anti-Microbial Chemotherapy (BSAC) susceptibility testing guidelines (19). Based on a previously reported instruction, nanoparticles of curcumin (nanocurcumin) were prepared by a process based...
on a wet-milling technique and were found to have a narrow particle size distribution in the range of 2 - 40 nm (20). Required volumes of curcumin stock solution were added to Mueller-Hinton agar (MHA) to achieve the final concentrations of 8, 16, 32, 64, 128, 256, and 512 µg/L. Equal volumes of A. Baumannii 105 CFU) in Iso-Sensitest broth were added to each well. After incubation at 37°C for 24 hours in air, wells were checked for turbidity and the MIC recorded as the lowest concentration where no bacterial growth was observed. All microtiter assays were performed in triplicate and mean values were presented. PAO1 strain of P. aeruginosa was used as a positive control strain. A dilution before MIC (sub-MIC) was used for real-time qPCR.

3.6. Quantitative qPCR

RNA was extracted from strong biofilm-producing strains treated with Sub-MIC of curcumin (64 µg/mL) by high pure RNA isolation kit (Roche, Switzerland), according to the manufacturer’s instructions. RNA quantity and quality was measured by nanodrop and the RNA integrity was assessed by gel electrophoresis. For cDNA synthesis, 1 µL of the reverse transcriptase enzyme (4 Unit/µL), 2 µL of 10X RT-PCR buffer, 1 µL of mixer master, 11 µL of distilled water, and finally 5 µL of the RNA were mixed to reach a final volume of 21 µL. The reaction mixtures were then incubated for 31 minutes at 39°C. Expression assay of CsuE gene was performed by using SYBR Green real-time qPCR inRotor gene 2000 (Qiagen, USA) using SYBR® Premix Ex Taq™II mastermix (Clontech, Takara, Japan) according to the following program: holding at 95°C for 10 minutes, and 40 cycles of 95°C for 20 seconds, 58°C for 20 seconds, and 72°C for 20 seconds. Moreover, 16s rRNA and a reaction without cDNA were used as the reference and negative controls, respectively. The expression of CsuE in the treated samples was calculated in comparison to untreated samples. The primer sequences for CsuE gene were forward: CATCTCTTATTTTCGGTCCC and reverse: CCGTCTGAGCATTGGTAGT and the primer sequences for 16s rRNA were forward: CAGCTCCTGTCTGAGAT and reverse: CGTGAGGCAGTACGACTTT. A melt curve analysis was also performed after any reaction to confirm specific amplification.

3.7. Statistical Analysis

Data analysis was performed using statistical package for the social sciences (SPSS) Software version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was assessed via the Pearson χ² test or the Fisher exact test for categorical variables and Student t-test or Mann-Whitney U test for continuous variables.

4. Results

4.1. Specimens and Bacterial Culture

In this descriptive study, 91 samples were successfully taken from patients at three to seven days after admission in Firoozgar Hospital (Tehran, Iran). Seventy isolates of A. baumannii bacteria were successfully obtained, of which 38 isolates had a female source and 32 isolates had a male source. These samples were obtained from the age group of 2 - 75 years. The distribution of A. baumannii isolates collected from different clinical sources are shown in Figure 1.

4.2. Antibiotic Susceptibility

Antimicrobial resistance assay of isolated bacteria showed high resistance to most tested antibiotics, except for polymyxin B (Figure 2). The results also showed that about 20% of the isolates were also sensitive to Tetracycline. These results showed that 92.95% of the A. baumannii isolates were MDR and 86.53% of the isolated were XDR, but there were no PDR-positive isolates.

4.3. Biofilm Formation

Using a standard qualitative plate microtiter method, the biofilm-producing capacity of 70 isolates was calculated. Two isolates showed no biofilm-producing capacity, 13 isolates were weak, 31 isolates were medium, and 25 isolates were strong to form biofilms (Figure 3). Fifty-two isolates (94.23%) out of a total of 55 medium or strong biofilm-forming isolates were MDR.

Figure 1. The distribution of A. baumannii isolates collected from different clinical sources is shown

Figure 2. Antimicrobial susceptibility

Figure 3. Biofilm formation
4.4. CsuE Expression Results: A Measure of Curcumin Inhibitory Potential

The sensitivity of 25 strong biofilm-producing isolates of A. baumannii was determined based on the minimum inhibitory concentration (MIC) of growth using broth microdilution method to determine the susceptibility to curcumin. The MIC for all of the isolates was 128 µg/mL of the nanoparticle. All of the isolates were treated with a Sub-MIC concentration of 64 µg/mL of curcumin nanoparticles. Twenty-three isolates treated with curcumin (compared with untreated isolates) showed down-regulation and only 2 isolates showed up-regulation in CsuE gene. Totally, the fold change of the CsuE gene, a biofilm-producing-related gene, in the treated samples with curcumin was equal to 0.31 in comparison to the untreated samples (Figure 4).

5. Discussion

Strains belonging to the Acinetobacter baumannii-Acinetobacter calcoaceticus cluster are emerging as problematic opportunistic pathogens due to a rapid increase in multidrug or pan-drug resistance (13). The present study aimed to investigate the effect of curcumin nanoparticles on biofilm-related CsuE gene expression in A. baumannii in vitro. In this study, antibiotic resistance and frequency of MDA and XDR A. baumannii isolates, biofilm formation capacity, as well as the MIC for curcumin nanoparticles were determined. Our data showed that this bacterium had high resistance to most antibiotics, except for polymyxin B. Twenty-five strains showed high biofilm formation capacity. MIC for curcumin was 128 µg/mL in all 25 strains. Expression results indicated that CsuE gene was downregulated to 0.31 fold in curcumin-treated samples compared to untreated samples.
The findings of the current study revealed that all isolates were susceptible to at least one antibiotic, but most isolates were resistant to most antibiotics. Similar to our data, Bassetti and Shakibaie studies showed that different isolates of *A. baumannii* were resistant to most antibiotics, and 70% of *A. baumannii* isolates were resistant to 3 or more antibiotics (9, 21). According to various reports, the majority of *A. baumannii* isolated in Iran were resistant against the first-line drugs of Aminoglycosides, Ceftazidime, Fluoroquinolones, Imipenem, Meropenem (22, 23). MDR-positive *A. baumannii* isolates have been reported in different studies, wherein, the frequency varies from 40% to 80% in general (24). In the present study, the percentage of MDR-positive *A. baumannii* isolates was 95.92%. These results indicate that the number of MDR-positive *A. baumannii* is rapidly increasing in Iran. Therefore, studying the resistance of the *A. baumannii*, might provide enough information for physicians to select appropriate therapies against infections caused by this organism.

Bacterial biofilms are associated with problems in the prevention and treatment of *A. baumannii* infection. Curcumin is an unstable, reactive, nonbioavailable compound, while it is an established therapeutic agent and is effective against various strains of Gram-negative and Gram-positive pathogens. Its mode of action at the molecular level has not been established, but it is thought to disrupt bacterial membranes (14). On the other hand, the formation of biofilms has been indicated that can be controlled by coating surfaces with nanoparticles (25). Our findings showed that most of the isolates possess a medium strength of biofilm production. These findings were almost consistent with previous studies in which the percentage of biofilm-producing bacteria was reported as % 63.6 (26), 62% (27), and 60% (28). The findings of this study also showed that there is a significant relationship between the biofilm-producing strength and the resistance to various antibiotics; thus more than 90% of the biofilm-producing bacteria had multidrug resistance properties, which was in accordance with the published reports (26, 28).

In this study, the mean curcumin MICs were determined in 25 strong biofilm-producing isolates and a sub-MIC concentration of 64 µg/mL was used for the treatment of strong biofilm-producing isolates. Of the 25 strong biofilm-producing isolates examined, 23 treated isolates compared with untreated isolates were downregulated in terms of *CsuE* gene. This can be explained that the addition of the curcumin nanoparticle promotes the decreased expression of *CsuE* in comparison to untreated samples. Studies have shown that the ability of *Acinetobacter* strains to form pili and attach to create a biofilm on living surfaces depends on the expression of the *CsuE* gene (29). In-activation of *CsuE* has been shown to lead to the inability of pilus production and the formation of biofilms (13). It could be concluded that downregulation of *CsuE* gene by nanocurcumin highlights the anti-biofilm activity of this compound that can be helpful in decreasing/eliminating of antibiotic resistance by providing new and more effective therapies. There are several limitations to the present work, especially no assessment of antibacterial properties of curcumin by disk diffusion method. Besides, the examination of the effects of other nanoparticles on the formation of biofilm, investigation the toxicity of curcumin nanoparticles, study of the curcumin effects on the animal models, and the evaluation of the curcumin effects on other biofilm-producing-associated genes are suggested for future studies.

### 5.1. Conclusions

The ability of *A. baumannii* to form biofilms can lead to a high level of antibiotic resistance and survival in the environment. Considering the inhibitory effect of curcumin nanoparticles against the biofilm-associated *CsuE* gene, it is suggested to use this nanoparticle as a complementary therapeutic agent against *A. baumannii* to likely inhibit its virulence gene.

### Acknowledgments

The authors received no specific funding for this work. We thank our colleagues who provided insight and expertise that greatly assisted the research; however, they may not agree with all of the interpretations/conclusions of this paper.

### Footnotes

**Authors’ Contribution:** Atefeh Hosseini contributed to laboratory works, drafting of the manuscript, and data analysis. Taher Nejadsattari contributed to laboratory works and data analysis. Mohsen Zargar contributed to study design, data analysis, and manuscript writing.

**Conflict of Interests:** All the authors declare that there is no conflict of interest.

**Funding/Support:** The authors declare that there is no specific funding for this work.

### References

1. Antunes LC, Visca P, Towner KJ. Acinetobacter baumannii: Evolution of a global pathogen. Pathog Dis. 2014;71(3):292–301. doi: 10.1111/2049-632X.12125. [PubMed: 24376225].

Arch Clin Infect Dis. 2019;14(4):e83263.
The biology of Acinetobacter: Mechanisms of virulence and resistance. Int J Antimicrob Agents. 2010;35(3):219-26. doi: 10.1016/j.ijantimicag.2009.10.024. [PubMed: 20047818].

Lin YC, Sheng WH, Chang YY, Wang LH, Lin HC, Chen ML, et al. The biology of Acinetobacter baumannii: Taxonomy, clinical importance, molecular biology, physiology, industrial relevance. 57. Springer Science & Business Media; 2013.

Bassoetti M, Repetto E, Righi E, Boni S, Diverio M, Molinari MP, et al. COL-1, a family of CRISPR/Cas systems in Acinetobacter baumannii, Acinetobacter genospecies 3 and Acinetobacter genospecies 5 in Taiwan. Int J Antimicrob Agents. 2010;35(5):439-43. doi: 10.1016/j.ijantimicag.2009.11.020. [PubMed: 20066315].

Navidinia M. The clinical importance of emerging ESKAPE pathogens in nosocomial infections. J Paramed Sci. 2016;7(1):34-57.

Lee CR, Lee JH, Park M, Park KS, Bae IK, Kim YB, et al. Comparison of planktonic and biofilm cells in Acinetobacter baumannii isolates from Mainland China. Front Microbiol. 2012;3:273-8. doi: 10.3389/fmicb.2012.00057. [PubMed: 22743053].

Rao RS, Karthika RU, Singh SP, Shashikala P, Kanungo R, Jayachandran S, et al. The use of an anti-inflammatory supplement in patients with chronic kidney disease. J Complement Integr Med. 2013;10. doi: 10.1515/jcim-2012-0011. [PubMed: 23828329].

Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of curcumin. Adv Exp Med Biol. 2007;595:305-25. doi: 10.1007/978-0-387-46401-5_1. [PubMed: 17569207].

Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966;45(4):493-6. doi: 10.1093/ajcp/45.4.493.

O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp. 2012;47. doi: 10.3791/2437. [PubMed: 23078831]. [PubMed Central: PMC3862663].

Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48 Suppl 1:15-46. doi: 10.1093/jac/dag121. [PubMed: 11420343].

Basiwaii RK, Buttar HS, Jain VK, Jain N; Bhawana. Curcumin nanoparticles: preparation, characterization, and antimicrobial study. J Agric Food Chem. 2011;59(5):2056-61. doi: 10.1021/jf104402i. [PubMed: 21322613].

Aziz O, Shakhbaie MR, Badmasti F, Modarresi F, Ramazanazadeh R, Mansouri S, et al. Class 1 integrons in non-clonal multidrug-resistant Acinetobacter baumannii from Iran, description of the new blaMP-5 allele in In1241. J Med Microbiol. 2016;65(9):928-36. doi: 10.1099/jmm.0.000315. [PubMed: 27430533].

Wang SH, Sheng WH, Chang YY, Wang LH, Lin HC, Chen ML, et al. Healthcare-associated outbreak due to pan-drug-resistant Acinetobacter baumannii in a surgical intensive care unit. J Hosp Infect. 2003;53(2):57-102. doi: 10.1053/jhin.2002.1348. [PubMed: 12586567].

Mak JK, Kim MJ, Pham J, Tapsall J, White PA. Antibiotic resistance determinants in nosocomial strains of multidrug-resistant Acinetobacter baumannii. J Antimicrob Chemother. 2009;64(1):47-54. doi: 10.1093/jac/dkn454. [PubMed: 18988680].

Asif M, Alvi IA, Rehman SU. Insight into Acinetobacter baumannii: Pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. Infect Drug Resist. 2015;8:249-60. doi: 10.2478/idr-2015-0016. [PubMed: 26744448].

Ghosemanian E, Naghoni A, Rahvar H, Kialla M, Tabarase B. Evaluating the Effect of Copper Nanoparticles in Inhibiting Pseudomonas aeruginosa and Listeria monocytogenes Biofilm Formation. Jundishapur J Microbiol. 2015;8(5):e17430. doi: 10.5812/jjm.8(5)2015.17430. [PubMed: 26290685]. [PubMed Central: PMC4537522].