Higher Biofilm Formation by Multi-Drug Resistant *K. pneumoniae* and *K. rhinoscleromatis* Strains and Effects of Lemon and Ginger Essential Oils on Biofilm Formation

Gulcan Sahal*, Nermin Hande Avcioglu, Isil Seyis Bilkay
Biotechnology Section, Biology Department, Faculty of Sciences, Hacettepe University, Beytepe, 06800, Ankara, TURKEY.

ABSTRACT

**Background:** Klebsiella strains are opportunistic pathogens forming biofilms on many surfaces. Nowadays some resistance traits’ acquirements via gene transfer mechanisms in biofilm environments cause an urgency to discover new substances targeting biofilm inhibition. **Objective:** The focus of this study is to examine different *K. pneumoniae* and *K. rhinoscleromatis* strains’ clinical information and antibiotic resistance results according to their biofilm formation levels and to determine lemon and ginger essential oils’ effects on biofilm formation. **Methods:** Biofilm formation of different *Klebsiella* strains and effects of ginger and lemon essential oils on biofilm formation were determined by Crystal Violet Binding assay. **Results:** Strong biofilm forming *K. pneumoniae* strains were more frequently observed to be isolated from blood sample. 80% of multi-drug resistant *K. pneumoniae* strains were strong biofilm forming and all multi-drug resistant *K. rhinoscleromatis* strains were intermediate biofilm formers. 170 μl/mL of lemon essential oil caused 48.3% (±13.7) and 83.4% (±7.2) decreases in biofilm formation amounts of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains respectively. **Conclusion:** According to our results; multi-drug resistant *K. pneumoniae* and *K. rhinoscleromatis* strains form higher amount of biofilms and lemon essential oil might be used as a new anti-biofilm agent, targeting the inhibition of biofilm formation of *K. pneumoniae* and *K. rhinoscleromatis* strains.

Key words: Anti-Biofilm, Biofilm Formation, Ginger Essential Oil, *Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*, Lemon Essential Oil.

INTRODUCTION

*Klebsiella* species, which exist as commensal microorganisms in human upper respiratory and human gastrointestinal tracts, are also known as opportunistic pathogens causing; bacteremia, neonatal sepsis, urinary tract and wound infections especially in immuno compromised individuals and within them, while *Klebsiella pneumoniae* is known as one of the most frequent agents of catheter-associated urinary tract infections (CAUTIs), *Klebsiella rhinoscleromatis* strains exist as an etiological agent of rhinoscleroma disease and rarely as an agent of bacteremia. Additionally, as a result of including huge amount of extracellular DNA molecules and providing distinct microorganisms to come together and stay immobile inside them, biofilm environments also promote genetic acquisition of antimicrobial resistance genes for several microorganisms. In this respect, the possibility of some resistance traits’ acquirement via gene transfer within biofilms causes an urgency to discover new substances targeting the inhibition of biofilm formation.
The aim of this study is to examine different K. pneumoniae and K. rhinoscleromatis strains’ clinical information and antibiotic resistance results according to their biofilm formation levels and to determine lemon and ginger essential oils (EOs) effects on biofilm formation.

**MATERIALS AND METHODS**

**Bacterial strains**

In this study, K. pneumoniae and K. rhinoscleromatis strains which were obtained from different clinical materials and identified in Urkmez (2009)’s previous study were used. All isolated strains were inoculated in to the Brain Heart Infusion Broth media including 10% glycerol and stored at -20°C.

**Biofilm formation**

The determination of biofilm formation in K. pneumoniae and K. rhinoscleromatis strains was performed by Crystal Violet Binding Assay described by O’Toole with some modifications. Briefly, bacterial cells corresponding to a 2.0 McFarland optical density standard were inoculated into Brain Heart Infusion Broth medium and were incubated at 37°C for 24 h. After incubation, the culture was 1:100 diluted into a fresh BHI medium and the wells of a polystyrene plate were filled with 1 ml of the diluted inoculum. Then, the plates were incubated for 24 h at 37°C. Following this, the wells were gently washed and stained with 1% crystal violet for 45 min at room temperature. After washing the wells again, bound crystal violet in each well was solubilized by Ethanol (96.6%) solution and solubilized crystal violet for each well was read by a spectrophotometer at 540 nm. Klebsiella strains, having an OD value ≥ 0.4, were evaluated as biofilm producers and classified into three categories as follows:

- 0.4 ≤ OD < 0.8 Weak Biofilm Former (WBF)
- 0.8 ≤ OD < 1.2 Intermediate Biofilm Former (IBF)
- OD ≥ 1.2 Strong Biofilm Former (SBF)

**Effects of ginger and lemon essential oils on biofilm formation of Klebsiella pneumoniae and Klebsiella rhinoscleromatis strains**

Essential oils of ginger and lemon were purchased from NU-KA Define Essencia, TURKEY. Bacterial cells corresponding to a 2.0 McFarland optical density standard were inoculated into Brain Heart Infusion Broth medium and were incubated at 37°C for 24 h. 1:100 diluted inoculums of strongest biofilm forming K. pneumoniae and K. rhinoscleromatis strains were incubated with 170 µl/mL and 1.7 µl/mL concentrations of ginger and lemon EOs for 24 h at 37°C and the biofilm formations were analyzed via “Crystal Violet Binding Assay” which has been described previously. The wells which does not include any concentration of essential oils, were evaluated as controls. Increases (%) or decreases (%) in biofilm formation were calculated by the following formula:

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\text{% of Decrease} = \frac{(OD \text{ Control} - OD \text{ Treatment}) \times 100}{OD \text{ Control}}
\]

\[
\text{% of Increase} = \frac{(OD \text{ Treatment} - OD \text{ Control}) \times 100}{OD \text{ Control}}
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**Statistical Analysis**

All experiments were done in triplicate and data were compared using Student’s t-test, accepting p<0.05 as statistically significant.

**RESULTS**

In this study, K. pneumoniae and K. rhinoscleromatis strains which were obtained from different clinical materials and service units were classified as strong, intermediate and weak biofilm formers, according to their biofilm formation levels. When the occurrences of K. pneumoniae and K. rhinoscleromatis strains in different clinical materials are examined, all K. pneumoniae strains isolated from clinical materials of tracheal aspirate and blood were SBF (Figure 1) and SBF K. pneumoniae strains were more frequently observed to be isolated from blood sample (Figure 1). In addition; all K. pneumoniae strains isolated from; service units of otorhinolaryngology, internal medicine and surgical intensive care and all K. rhinoscleromatis strains isolated from urology unit were SBF (Figure 2).

Antibiotic resistance patterns of K. pneumoniae and K. rhinoscleromatis strains have been determined previously in Urkmez’s study\(^{11}\) and when the multi-drug resistant K. pneumoniae and K. rhinoscleromatis strains are examined according to their biofilm formation levels, 80% of MDR K. pneumoniae strains were observed as SBF and all MDR K. rhinoscleromatis strains were IBF (Figure 3). In the last part of this study, effects of lemon and ginger essential oils on biofilm formation of strongest biofilm forming K. pneumoniae and K. rhinoscleromatis strains were determined. According to our results, 170 µl/mL and 1.7 µl/mL concentrations of ginger EO did not cause any significant decrease/increase on biofilm formations of strongest biofilm forming K. pneumoniae and K. rhinoscleromatis strains (Figure 4 and 5). When the effect of 170 µl/mL concentration of lemon EO is examined; 48.3% (± 13.7) and 83.4% (± 7.2) decreases in biofilm
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Figure 1: Percentage of (a) SBF, IBF and WBF *Klebsiella pneumoniae* strains (b) SBF, IBF and WBF *Klebsiella rhinoscleromatis* strains in different clinical materials


Figure 2: Percentage of (a) SBF, IBF and WBF *Klebsiella pneumoniae* strains (b) SBF, IBF and WBF *Klebsiella rhinoscleromatis* strains, in different service units


Figure 3: Percentages of Multi-Drug Resistant *K. pneumoniae* and *K. rhinoscleromatis* strains according to their biofilm formation levels

formation amounts of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains were observed respectively (Figure 4, 5). Furthermore, lemon EO’s 1.7 μL/mL concentration also caused a significant decrease in biofilm formation amount of strongest biofilm forming *K. rhinoscleromatis* strain (Figure 5).

**DISCUSSION**

Biofilms, which are known as organized matrix-enclosed communities adhering to particular living and non-living surfaces, play a major role in development of many nosocomial infections. By means of providing an inert surface for the attachment of diverse microorganisms and thereby enhancing microbial colonization; indwelling biomaterials are especially known to favor biofilm formation of many pathogens such as different *Klebsiella* species. Biofilm formation by these pathogenic bacteria is believed to inhibit the effectiveness of antibiotic treatment and facilitate bacterial communication leading to expression of some other virulence determinants both on particular tissues of immuno compromised hosts and on diverse indwelling biomaterials. Therefore; determining biofilm formations of different *K. pneumoniae* and *K. rhinoscleromatis* strains, examining their biofilm formations according to their clinical information and

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**Figure 4:** Effects of lemon and ginger essential oils on biofilm formation of strongest biofilm forming *K. pneumoniae* strain
* indicates a significant difference (Student’s t-test; *P* < 0.05).

**Figure 5:** Effects of lemon and ginger essential oils on biofilm formation of strongest biofilm forming *K. rhinoscleromatis* strain
* indicates a significant difference (Student’s t-test; *P* < 0.05).
anti-microbial resistances, and investigating natural substances that can able to inhibit their biofilm formation will provide development of more comprehensive preventing strategies against Klebsiella related biofilm infections both in immuno compromised and indwelling biomaterial using individuals.

According to our results, similar with one of the recent studies, all K. pneumoniae strains isolated from blood samples were SBF and blood was the most frequent clinical material for SBF K. pneumoniae isolation (Figure 1). When the studies applied on blood stream infections is examined; immuno suppression and extended duration of catheter placement are indicated as main risk factors for nosocomial bloodstream infections. Therefore, by means of providing a favorable surface for bacterial attachment, Central Venous Catheters (CVCs) might be one of the reasons of biofilm related blood stream infections. Apart from this, a research applied in United States indicates that; most frequent nosocomial infection sites (blood stream, urinary, and respiratory tract) in medical-surgical intensive care units (MS ICUs) were almost always found to be associated with use of an indwelling device. Therefore, by means of being responsible from blood stream infections, indwelling devices also may emerge as reason of many nosocomial infections in surgical intensive care units. Hence, our finding which shows that all K. pneumoniae strains isolated from service unit of surgical intensive are SBF; may also be the result of enhanced indwelling biomedical device usage.

When the prevalence of K. rhinoscleromatis strains according to different clinical materials are examined; it was surprising to observe the isolation of these common respiratory tract infection (Rhinoscleroma) agents from sample of urine in higher frequency and 50% of these K. rhinoscleromatis strains which were isolated from urine were SBF.

Nowadays, many investigations on correlation between biofilm production and multiple drug resistance in clinical isolates of different strains are being applied. Because, by means of including huge amount of extra cellular DNA and providing too many distinct bacterial cells to stay in close contact and immobile, biofilm environments are known to favor genetic exchange of some anti-microbial resistance genes. Therefore, they are suggested to increase bacterial virulence and contribute to the development of multi resistant phenotypes. Confirming these, in our study; 80% of MDR K. pneumoniae strains were observed as SBF and all MDR K. rhinoscleromatis strains were IBF (Figure 3).

Today, the usage of new synthetic derivatives or synthetic antibiotics is regarded as unsafe by means of their side effects and biofilm mode of growth causes an urgency to discover new substances targeting biofilm inhibition. Thus, new antimicrobials like medicinal plants which have little or no toxic effect are being considered as potential sources for the inhibition of biofilm associated infections. Essential oil of lemon which is extracted from Citrus limonum and known to pose an antioxidant activity was observed to inhibit biofilm formations of K. pneumoniae and K. rhinoscleromatis in our study. Similar to our finding, one of the recent studies shows that, essential oil of Citrus limonum displays highest biofilm inhibition activity and greatest AI-2 (Quorum Sensing mediating molecule) reduction activity (96%) in Campylobacter jejuni strains. Additionally, Citrus limonum is also indicated to reduce the levels of expression of two genes (fla A and fla B) which are crucial for bacterial pathogenesis and play an important role in biofilm formation, in highest level.

CONCLUSION

In conclusion, this study shows that; MDR K. pneumoniae and K. rhinoscleromatis strains display higher biofilm formation and essential oil of lemon might be used as a new anti-biofilm agent, targeting the inhibition of biofilm formation of K. pneumoniae and K. rhinoscleromatis strains in the future. Therefore, this study not only throws light on many studies carried out on transformation of harm-less commensal human natural colonizers into multi-drug resistant nosocomial pathogens but also exhibits a new plant substance, that inhibits biofilm formation of K. pneumoniae and K. rhinoscleromatis strains.

CONFLICTS OF INTEREST

The authors declare that, there are no conflicts of interest.

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PICTORIAL ABSTRACT

SUMMARY

- Strong biofilm forming K. pneumoniae strains were more frequently observed to be isolated from blood sample.
- 80% of multi-drug resistant K. pneumoniae strains were strong biofilm forming and all multi-drug resistant K. rhinoscleromatis strains were intermediate biofilm formers.
- 170 µl/mL of lemon essential oil caused 48.3% (±13.7) and 83.4% (±7.2) decreases in biofilm formation amounts of strongest biofilm forming K. pneumoniae and K. rhinoscleromatis strains respectively.
- 1.7 µl/mL of lemon essential oil caused 54.1% (±18.6) decrease in biofilm formation amount of strongest biofilm forming K. rhinoscleromatis strain.
About Authors

Gulcan Sahal: Is a doctoral student at Hacettepe University, where she graduated in Bachelor of Biology and Master of Biotechnology. Her doctoral research focuses on biofilm formation on different surface materials and anti-biofilm effects of different plant-derived natural substances on biofilm formation. She has done some of her Ph. D. researches in Biomedical Engineering Department of University Medical Center Groningen (UMCG), Netherlands in 2014-2015.

Nermin Hande Avcioglu: Is a post doctoral researcher at Hacettepe University, where she graduated in Bachelor of Biology, Master of Biotechnology and Ph. D. of Biotechnology. She worked on typing of antibiotic resistance and plasmid profiles of Klebsiella strains as a master project and her doctoral research focused on biodegradation of cyanide. She continues working on biodegradation of various toxic compounds.

Assoc. Prof. Isil Seyis Bilkay: Obtained her Ph. D. degree in 2004 from Faculty of Sciences, Hacettepe University, Ankara, Turkey. Currently, she is positioned as an associate professor at the Biotechnology Division of Biology department of Hacettepe University, Ankara (Turkey).

Dr. Seyis Bilkay is working on nosocomial infections, biodegradation of various toxic compounds, industrial enzymes and medical microbiology.