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Effect of β-defensin 2 on biofilm formation in pathogenic *E. coli* bacteria isolated from clinical samples

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Abstract: Biofilms are formed by bacteria as part of their survival processes, and they are so common in nature. Biofilms are made up of microbial communities that are encased in a layer of self-produced extracellular polymers. Biofilms form on both abiotic and biotic surfaces and are important in a variety of settings, including aquaculture, the food industry, and the clinical field as a factor in antimicrobial drug resistance**.** Several studies on the mechanisms of interaction between defensins and cell membranes have revealed that positively charged defensin residues interact with negatively charged components (lipopolysaccharides or phospholipids) in microbial membranes to disrupt the cell membrane as the first step in killing bacteria. The goal of this study is to demonstrate the effect of beta-defensin 2 on biofilm formation by *E. coli*. To examine the biofilm formation of 67 *E. coli* isolates, microtiter plate method was used. After that, the mic of beta-defensin 2 was determined, then an anti-biofilm test was conducted to find out the extent of its effect on biofilm formation. The results of examining the ability of bacteria to produce biofilm showed that all isolates were biofilm producers in varying degrees. β-defensin 2 was shown to be effective in eliminating biofilms formed by *E. coli.*

Key words: *E. coli*, Biofilm, β-defensins2, antibiofilm

Introduction

Biofilms are communities of microorganisms with more than one cell that are held together by a substance made by the cells themselves. Microbes can always, everywhere, and in a constantly changing way make biofilm. This dynamic process of biofilms sets up an important way for organisms to deal with harsh environments and antimicrobial agents. (1). Bacterial biofilms are one of the Earth's oldest and most common multicellular life forms, and they're becoming more important in fields like industrial fouling, medicine, and biotechnology. The main obstacles to obtaining definitive experimental results are time-varying biofilm properties, structural and chemical heterogeneity, and, most importantly, their high sensitivity to environmental cues (2). Extrinsic resistance occurs when antimicrobials are unable to penetrate the biofilm's cells, and intrinsic resistance occurs when the cellular envelope, which is a target for many antimicrobials, is altered within the biofilm to prevent antimicrobial action (3). Biofilm formation and persistence are responsible for 75% of all human bacterial infections(4). *E. coli* has also been shown to persist and regrow after antibiotic exposure due to a variety of factors, including reduced antibiotic penetration in biofilms and/or drug cellular extrusion (5). Several investigations have found that 50–70% of isolates taken from patients with recurrent infections develop biofilm (6). Several studies on the mechanisms of interaction between defensins and cell membranes have revealed that positively charged defensin residues interact with negatively charged components (lipopolysaccharides or phospholipids) in microbial membranes to disrupt the cell membrane as the first step in killing bacteria (7,8). Defensins have been described as killing bacteria directly through membrane destruction and decomposition, or as causing cell death by altering the cytoplasmic membrane's permeability and energy state, as well as attacking internal targets such as negatively charged DNA or RNA (9,10). Defensins are a class of low-molecular-weight antimicrobial peptides (AMPs) that are secreted by organisms and can be used as potential alternatives for novel therapeutic drugs due to their broad-spectrum activity against pathogens. These peptides also play an important role in both innate and adaptive immunity (11,12).

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Materials and Methods

Sample collection: Sixty-seven isolates of *E. coli* were obtained from two hundred and fifty specimens collected from different infections in humans: ear infection, urinary tract infection, sputum, burns, wounds and feces, which were collected from patients of different ages for the period from November 2021 to January2022, in Al Fallujah General Hospital and Fallujah Women and Children Hospital. Samples were diagnosed by Vitek 2 System and 67 isolates of *E. coli* were obtained.

Biofilm formation: According to a recent study (13) ,a microtiter plate test was performed to determine the capacity of isolates to produce biofilms:(i) Bacterial suspension was created by inoculating overnight culture isolates in Brain Heart Infusion broth added with 1% glucose and adjusted for comparable 0.5 McFarland (5×10^5) CFU/ml.(ii) Twenty microliters (20µl) of bacterial suspension were transferred to wells containing 180 µl of Brian Heart Infusion broth with 1% glucose and cultured for 24 hours at 37 ºC. (only BHI broth was used as control). (iii) The contents of the wells were discarded after incubation, and they were rinsed three times with distilled water before drying at room temperature. Two hundred microliters (200 µl) of methanol solution were applied to the wells to fix the attached film and left for 15-20 minutes before removing and drying. For 15 minutes, attached bacteria were dyed with 200 µl of 1% crystal violet. The dishes were washed three times with distilled water to eliminate any remaining stain. The plates were dried by leaving them at room temperature for around 30 minutes to ensure that they were completely dry. Finally, the stain was fixed with ethanol 95% The absorbance was measured using an ELISA reader at 630 nm.

Calculated the ability of biofilm formation by:
Biofilm degree=OD630 of tested bacterial _OD630 control

Determination of minimum inhibitory concentration of beta defensins2: Microtiter plates were employed for Resazurin techniques, and a Resazurin-based Microtiter Dilution Assay (RMDA) with various modifications was used to determine MIC, all wells of microtiter plates were filled with 100 µl of BHI broth, then 100 µl of Beta defensins2 were added to the first row, transferring 100 µl to the next well to obtain a serially concentrations (1/2,1/4,1/8,1/16,1/32,1/64). 10 µl of bacterial suspension was obtained and added to each well. The plates were incubated for 24 hours at 37° C. After incubation, add 15 µl of resazurin solution to each well as an indicator and incubate for another 1 hours.

Antibiofilm activity of B defensins2 by using micro-titer plate method: The anti-biofilm test was carried out in the same way in which biofilm formation was detected except for step No.(ii), where 168 µl of BHI medium and 20 µl of bacterial suspension were added and 12 µl of sub-inhibited concentrate(sub-mic) of beta-defensins2 was added. Percentage of inhibition of bacterial adhesion by application of the equation described by (14).

$$\% \text{ of inhibition of biofilm formation} = 1 - \frac{\text{O.D of treatment}}{\text{O.D of control}} \times 100$$

Results

Biofilm formation

The biofilm composition of 67 *E. coli* isolates was detected. The results showed that a high percentage of *E. coli* isolates were biofilm-producing, and 67 bacterial isolates belonging to *E. coli* had the ability to produce biofilm in different degrees, in comparison with the negative control. There were 35 bacterial isolates capable of strong adherent biofilm production, 21 bacterial isolates capable of moderately adherent biofilm production, and 11 bacterial isolates capable of weakly adherent biofilm production as shown in figure (1).

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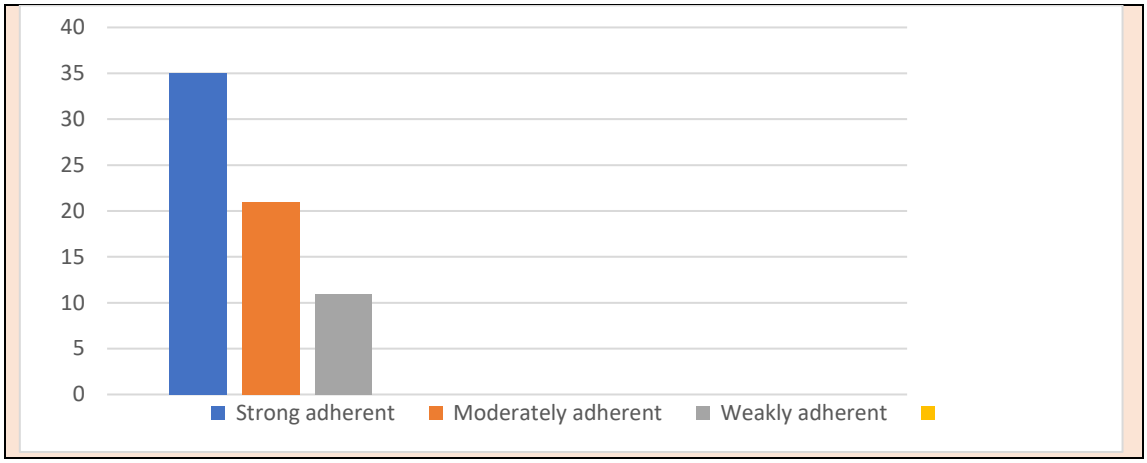

Figure (1) Biofilm production by *E. coli*

The mean of the three replicates for each isolate was calculated. The results of the microtiter-plate reader were then analyzed. According to the table (1), biofilm formation is characterized as none, weak, moderate, or strong.

Table (1): Classification of biofilm production abilities by micro titter plate technique.

Cut-off value calculation	Biofilm production abilities
OD ≤ (ODc)	**None**
(ODc) < OD ≤ (2×ODc)	**Weak**
(2×ODc) < OD ≤ (4×ODc)	**Moderate**
OD > (4×ODc)	**Strong**

OD: mean of sample absorption at 630 nm.
ODc: mean of control absorption at 630 nm.

Determination of MIC of β-defensins2

The results showed in this test that the concentration (3µg/ml) is the MIC for β-defensins2. For isolates that were strong in biofilm production, moderate and weak. As shown in the figure (1)

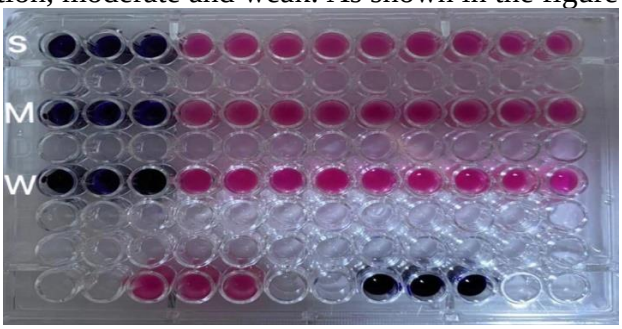

Figure (1) Determination of the mic of β-defensins2: s (strong biofilm), M (moderatebiofilm (weak biofilm)

Determination of beta defensins2 antibiofilm activity against *E. coli* biofilms (inhibition of biofilm)

The study used the Sub-MIC (1.5 µg/mL) of beta-defensins 2. During this experiment, it was found that beta-defensins2 has an effective activity in eliminating the membranes formed by the *E. coli* bacteria. The results of these experiments revealed that the optical density of wells containing β- defensins 2 concentration approach to the optical density of wells containing no bacterial isolates. This shows that beta defensins2 inhibited bacterial growth, resulting in the absence of a biofilm in these wells. The inhibition rate for strong biofilm 77.9%, Moderate biofilm 83.6%, weak biofilm 84.1%. As shown in the figure (2)

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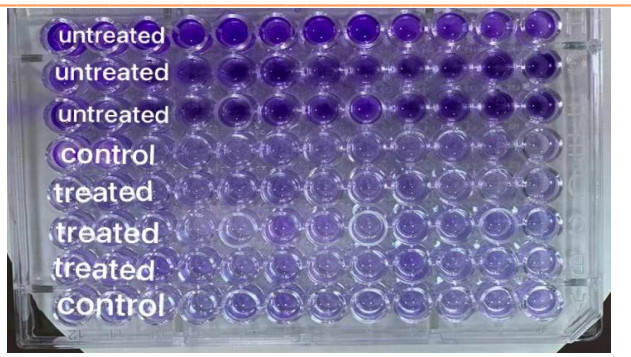

Figure (2) Shows the effect of beta-defensins2 on biofilm production by *E. coli*

Discussion

The results of the current study on the ability of bacteria to form biofilms agree with the findings of the researcher (15), which showed that the percentage of bacterial isolates that have the ability to produce biofilm was(100%),and it is also close to the results of the study conducted by the researcher (16) in Egypt, and the results reached by researcher (17) in India, in which the percentage of bacterial isolates that have the ability to produce biofilm reached (76.5%) and (80%) respectively. While the results of the study to researcher (18) in India and findings by (19) in Nepal showed that the proportion of bacterial isolates that had the ability to produce bioactivity films were (10%) and (39.6%), respectively. The ability of *E. coli* to produce biofilm is one of the most important factors of bacterial virulence conferred by bacteria that are resistant to most antibiotics (20). Biofilm-producing bacteria are 1,000 times more resistant to antibiotics than bacteria non-produce biofilm, for several reasons, including the lack of diffusion of antibiotics through the adhesive interface of the biofilm, and the transfer of resistance genes within the biofilm environment between bacterial cells, whether by plasmid or gene transposons. Or because of random mutations that increase the resistance of cells to toxins and antibiotics (21). The results showed that beta-defensins2 has an effective activity against bacteria, and this matches what was mentioned in (22). Yasir *et al.*, (2018) was found that AMPs contain a range of active anti-biofilm mechanisms that could be used to remove biofilms in therapeutic settings. It's evident that AMPs have a lot of potential as an active anti-biofilm agent, especially in high-risk situations like hospitals. To eliminate biofilms, AMPs could be employed alone or in conjunction with other antimicrobials (23).

Conclusion

There is a need for more studies on biofilm-forming bacteria and the extent to which this biofilm affects the resistance of bacteria to antibiotics. β-defensins2 showed anti-biofilm and antibacterial activity in isolates that produced moderate, strong and weak biofilm isolates

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