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Research article

Hydrogen peroxide controls biofilm formation by *Klebsiella pneumoniae e in vitro*

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ABSTRACT

The biofilm formation of bacteria in medical devices especially indwelling urinary catheters considers one of the hospital complications of infections. Here, the anti-biofilm effect of hydrogen peroxide (H_2O_2) against biofilm produced by *Klebsiella pneumoniae* on urinary catheters was evaluated. In the present study, 2the five isolates of *K. pneumoniae e e* were isolated from urine samples of patients suffering from urinary tract infections (UTIs). The biochemical and VITIK technology was used to identify the bacterial isolates. The ability of *K. pneumoniae* to adhere and form biofilm onto the indwelling urinary catheter was measured. Moreover, the negative effect of different concentrations of H_2O_2 (0.01%, 0.02%, 0.04%, 0.08%, and 0.16%) on the biofilm formation of K. pneumoniae on the pieces of the indwelling urinary catheter was evaluated in test tubes (in vitro). The result showed that all isolates produced a high level of biofilm. Three isolates were considered moderated biofilm producers and 2 were considered strong biofilm producers. All the used concentrations of H_2O_2 were effect-negative ($P<0.05$) on *K. pneumoniae* (K.p.3). The present study confirmed the anti-biofilm effect of H_2O_2 on the biofilm formation by K. pneumoniae on the indwelling urinary catheter *in vitro*.

Keywords: Biofilm, H_2O_2, indwelling urinary catheter, *Klebsiella pneumoniae.*

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1. INTRODUCTION

Klebsiella pneumoniae e is a Gram-negative bacterium that can form biofilms on various surfaces, including urinary catheters [1, 2]. These biofilms contribute to persistent infections and are often resistant to antibiotics [3]. The use of hydrogen peroxide (H_2O_2) has been investigated for its potential to control different Gram-negative bacteria such as *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp biofilm formation [4]. While specific research on the effects of hydrogen peroxide on *K. pneumoniae e* biofilms on urinary catheters is limited, studies on biofilm-related infections and the broader antimicrobial properties of hydrogen peroxide provide some insights. A previous study examined the effects of hydrogen peroxide on biofilm formation by *K. pneumoniae e* [4]. The researchers found that hydrogen peroxide treatment significantly inhibited biofilm formation and reduced the metabolic activity of *K. pneumoniae e*. They suggested that hydrogen peroxide exerted its effects by disrupting the biofilm matrix and interfering with bacterial adhesion to abiotic surfaces [4].

The researchers observed that the combination of hydrogen peroxide with certain antibiotics enhanced the anti-biofilm activity against different bacterial species [5,6]. They suggested that hydrogen peroxide acted synergistically with antibiotics to disrupt biofilm structures and increase bacterial susceptibility Hydrogen peroxide has been shown to be effective against *K. pneumoniae e* biofilms in vitro and in vivo. In one study, hydrogen peroxide was able to reduce the size of *K. pneumoniae e* biofilms by up to 90%. In another study, H_2O_2 was shown to be effective in preventing the formation of *K. pneumoniae e* biofilms in mice [6].

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The above studies indicate that hydrogen peroxide has the potential to inhibit *K. pneumoniae e* biofilm formation and reduce the viability of existing biofilms. However, further research is needed to optimize the concentration, exposure time, and compatibility of hydrogen peroxide with urinary catheter materials to ensure effective biofilm control without compromising patient safety. That is why, the current study was built to evaluate the role of low concentrations of hydrogen peroxide on the adhesion of *K. pneumoniae e* on urinary catheters and biofilm formation in vitro.

2. MATERIALS and METHODS

2.1. Bacterial isolation and identification

The urine samples (67 urine samples) were collected from patients suffering from urinary tract infections (UTIs), the patients visited the Baghdad education hospital, Baghdad, Iraq from the 1st of October 2018 to the 23rd of December 2018. The samples of urine were cultured on MacConkey agar (Hi-Media Laboratories Pvt. Ltd., India). The experiments were approved by the biomedical ethical committee of the College of Science, University of Baghdad, Baghdad, Iraq (617, Date: 5/07/2018). The standard biochemical test [catalase test (positive), oxidase test (negative), motile (negative), indole (positive), citrate (negative), urease test (positive), and TSI (triple sugar iron) test is with A/A with gas production.

Treatment of bacterial strains with H2O2

The bacterial isolate of *K. pneumoniae e* was cultured in Luria Bertani broth (Hi-media, India), and the number of bacteria was adjusted to 10^6 CFU/ml. The bacterial suspension was exposed to different concentrations (0.01, 0.02, 0.04, 0.08, and 0.16 %) of H_2O_2 for 5 h.

2.2. Biofilm assay

The method of Zgair and Chhibber, (2011) was followed. Briefly, 200 µl of sterile Tryptic soy broth (TSB) was added to the different sterile test tubes. The indwelling urinary catheter (Hollister catheter) was cut into several pieces of 1cm aseptically. In each tube, one piece of an indwelling urinary catheter was added. Five microliters of overnight growth of *K. pneumoniae e* (K.p1, K.p.2, K.p.3, Kp.4, and K.p.5) were washed five times with sterile phosphate buffer saline (0.1 M, pH 7.2) using centrifugation at 14000 g for 10 min (Beckman, USA). The final number of bacteria suspensions was $5x10^6$ c.f.u. /ml. 5 µl of bacterial suspension was added to each tube and then the tubes were incubated at 37°C overnight. The TSB media were discarded, and the indwelling urinary *catheter* pieces were washed five times with sterile distilled water. The quantity of biofilm formation was measured by the spectrophotometric method. The biomass of biofilm of five isolates of *K. pneumoniae e* was dried and fixed at 60 oC for 30 min. Then, 1 ml of Hucker crystal violet (0.4%) was added to each tube (containing the indwelling urinary *catheter* pieces) and incubated for 10 min at 21 °C. The pieces were washed four times with distilled water and dried for 30 min at 37°C. Two ml of acetone: ethanol (30:70) was added to the tubes (containing one piece of an indwelling urinary catheter). The absorbency was measured at a wavelength of 570 nm using a spectrophotometer (Beckman, USA). The experiment was repeated three times [7]. The following criteria were used for biofilm gradation in clinical isolates [8].

ODcut = ODavg of negative control + 3 × standard deviation (SD) of ODs of the negative control.

OD ≤ ODcut = Non-biofilm-former (NBF)

ODcut < OD ≤ 2 × ODcut = Weak biofilm-former (WBF)

2 × ODcut < OD ≤ 4 × ODcut = Moderate biofilm-former (MBF)

OD >4 × ODcut = Strong biofilm-former.

Effect of different concentrations of H_2O_2 on biofilm formation

The method of biofilm formation of explain above was followed to measure the biofilm formation of bacterial isolates that were exposed previously to different concentrations of H_2O_2 (0.01, 0.02, 0.04, 0.08, and 0.16 %). The microtiter titer plate method and the amount of biofilm were measured by the spectrophotometric method after being stained with 0.4% of Hucker crystal violet [7].

2.3. Statistical analysis

The statistical analysis was done by using Origin 8 software. The data were expressed as means ± SE. The differences were evaluated by using a student t-test and one-way ANOVA. The relationship was signed by using Pearson's correlation coefficient. A value of $P<0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Bacterial isolates

In the current study, five isolates of *K. pneumoniae e* were isolated from 67 urine samples collected from patients suffering from UTIs. Gram stain and biochemical tests were used to identify the bacterial species. The VITIK 2 Technology proved that the five isolates were *K. pneumoniae e*.

3.2. Biofilm formation

The present study showed that the highest level of biofilm formation was seen in the case of *K. pneumoniae e* (K.p.3) followed by K.p.2, while the lowest level of biofilm formation was found in the case of K.p.4. That is why, the K.p.3 that gave the highest level of biofilm was used in further experiments (figure 1). The five isolates were divided into three groups depending on the amount of biofilm formation. The present study showed that three isolates were classified as strong biofilm producers (K.p.3, K.p.2, and K.p.5), and two isolates were considered moderate biofilm producers (K.p.1 and K.p.4).

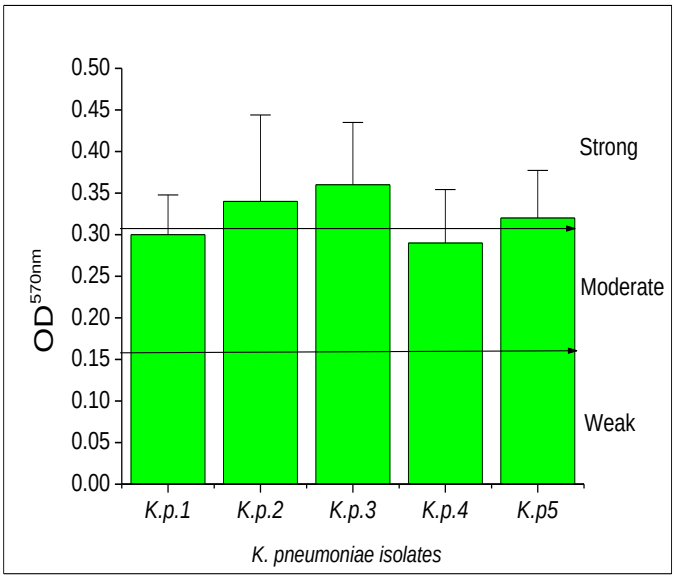

Fig.1 The biofilm formation in terms of the optical density (570nm) of five isolates of *K. pneumoniae e* that were isolated from urine samples of the patient suffering from UTI. The five isolates were divided into three categories dependent on the level of biofilm formation.

3.3. Effect of H2O2 on the biofilm formation

In the current study, the bacterial isolate that produced the highest amount of biofilm was exposed to different concentrateions of H2O2 before checking its biofilm formation in vitro. The bacteria were exposed to five percentages (concentrations) of H2O2 (0.01%, 0.02%, 0.04 %, 0.08%, and 0.16 %). The results showed that the exposing to H2O2 reduced the biofilm formation significantly at all the concentrations used in the current study. The decreasing of biofilm formation was in concentration-dependent manure (Fig. 2). Figure 3 showed a negative correlation between the H2O2 concentrations and biofilm formation by K.p.3 isolates (r: -0.95, P<0.01). .

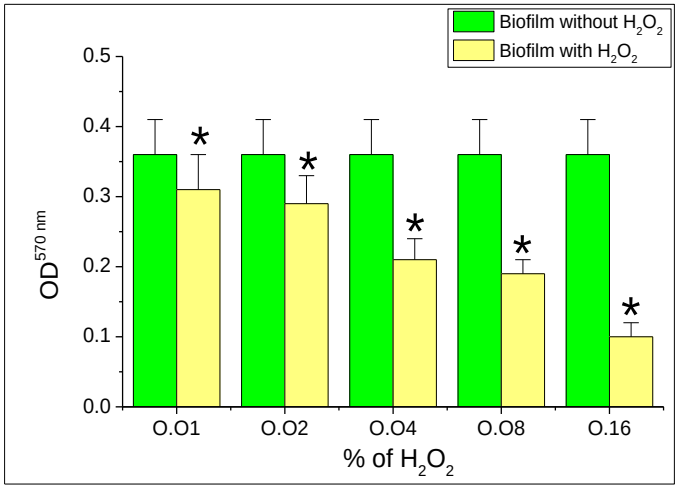

Fig. 2 Effect of different concentrations of H_2O_2 (0.01%, 0.02%, 0.04 %, 0.08%, and 0.16 %) on the biofilm formation of K.p.3 isolates. *,P<0.05.

4. DISCUSSION

Biofilm formation is a process by which microorganisms, such as bacteria, adhere to surfaces and form a protective community of cells encased in a matrix of extracellular polymeric substances (EPS). This matrix provides a protective barrier for the microorganisms, making it difficult for antibiotics and the immune system to reach and eliminate the bacterial cells [9]. The biofilm formation in medical devices is important for the persistence of many bacterial infections [10] which complicates the process of treating bacterial infections that accompany adhesion on medical equipment, such as indwelling urinary catheters and prosthetic valves [11, 12]. In the present study, five isolates of *K. pneumoniae e* were isolated from urine samples collected from patients suffering from UTI. All these isolated were moderate to strong biofilm-forming bacteria. The treatment of *K. pneumoniae e* (K.p.3) with H_2O_2 reduced the biofilm formation significantly and it was in a concentration-dependent manner.

Hydrogen peroxide reduces biofilm formation because H_2O_2 is a strong oxidizing agent, and when it comes into contact with bacteria in a biofilm, it can induce oxidative stress. that can damage bacterial cells by targeting their DNA, proteins, and lipids, disrupting their normal functioning and leading to cell death [13]. H_2O_2 can degrade EPS components, such as polysaccharides and proteins, destabilizing the biofilm structure. By breaking down the matrix, H_2O_2 weakens the biofilm and makes it more susceptible to removal [14]. Furthermore, can penetrate the biofilm and kill the bacterial cells within it. This antimicrobial effect is due to the ability of H_2O_2 to generate reactive oxygen species, such as hydroxyl radicals, which are highly reactive and can damage bacterial cells [13]. The H_2O_2 can promote the dispersal of cells within a biofilm. Biofilm cells are often highly resistant to antimicrobial agents and can form a protective barrier against them [15].

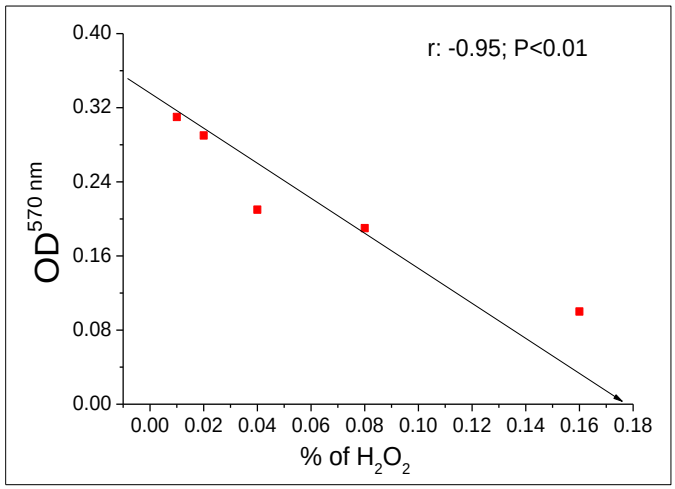

Fig.3 Relationship between biofilm formations in terms of optical density at 570 nm (OD570nm) by *K. pneumoniae e* (K.p. 3 produced the highest level of biofilm) and the percentages (concentrations) of H2O2). The negative correlation was seen, r: Pearson correlation coefficient.

It's worth noting that the effectiveness of H_2O_2 in reducing biofilm formation can vary depending on factors such as the concentration of H_2O_2, the type of microorganism involved, and the specific characteristics of the biofilm. In some cases, combining H_2O_2 with other antimicrobial agents or physical cleaning methods may further enhance its effectiveness in biofilm removal. The role of H_2O_2 in penetrating the biofilm body sparks the idea of the possibility of using H_2O_2 in increasing the affectivity of antibiotics.

5. Conclusion:

From the present study, it can be concluded that the anti-biofilm effect of H_2O_2 was in a dose-dependent manner.

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Conflict of interest

The authors declare that they have no conflict of interests.

Ethical statement

The study was conducted following approval from the Biomedical Ethics Committee of University of Baghdad, Baghdad, Iraq (Reference number 617, Date: 5/07/2018).

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