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Effect of Zinc Ferrite Nanoparticles on Some Virulence Factors of *Klebsiella Pneumonia*

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ABSTRACT

The ability of *K.Pneumonia* isolates to form biofilm was tested using the calibration plate method. The results showed that all isolates of which 50 (100%) have the ability to form biofilm, as 40 isolates (80%) were strong for biofilm formation, while 8 isolates (16%) were medium and 2 (4%) were weak for biofilm formation.

The antibiotic effect of zinc nanoparticles on biofilm formation showed that out of 50 *K. Pneumoniae* isolates, the biofilm of 14 isolates was inhibited by (28%), and the highest inhibition rate of biofilm was at a concentration of 64 µg /ml.

The antibiotic showed that the effect of zinc nanoparticles, $ZnFe_2O_4$ on the production of urease enzyme for 50 isolates of *K. Pneumoniae* (10 isolates) was inhibited by 20% on the production of urease enzyme, and 40 isolates by 80% were urease-producing.

INTRODUCTION

The wrong use of antibiotics against bacteria has led to the emergence of antibiotic-resistant strains that pose a threat to infection control, which may lead to death or lead to prolonged treatment. The difficulty of finding new antibiotics with different targets and mechanisms of action which requires high cost and a decade to launch it as a commercial drug urging the innovation of new treatment methods for infections bacteria (Johnson and Abramovich, 2017).

Nanotechnology is used to produce materials of different sizes starting from 1-100 nanometers, which is equal to 109, and it is obtained in multiple ways. To a large extent, the solvents and chemicals used, hence the importance of biotechnology, which is one of the most environmentally friendly technologies in the manufacture and production of nanomaterials and its use. It has recently been used to control types of pathogenic bacteria at different concentrations (Nasrollahzadeh *et al*., 2019).

Determining the effectiveness of zinc ferrite nanoparticles in inhibiting some virulence factors for bacteria represented in the production of biofilm formation and the production of urease enzyme.

MATERIALS AND METHODS

Samples were taken from the infected wound site such as caesarean section, nephrectomy, appendectomy, cholecystectomy, and hernia using a sterile cotton swab containing a vector medium, and patients who taking antibiotics were excluded. The primary determination tests included the phenotypic characteristics of bacterial growth on different media that had been previously inoculated, such as Maconkey agar media, blood agar media, and chrome agar media. Observable by colony shape, colony structure, coloration, and edges (Brooks *et al*.,2013). Biochemical tests were also conducted to check the properties of the isolate bacteria, and these tests included the indole test to verify the production of indole, the methyl red test to check the fermentation sugar and acid production, the Vogus-Proscauer test to detect the acetone compound. The citrate test to verify the Consumption of citrate as a single carbon source and formation of sodium carbonate, urease test indicating hydrolysis to form urea and ammonium, oxidase test to verify production of cytochrome c, catalase and fermentation tests for sugars (Brown and Smith, 2017).

Solutions that were used in the investigation of biofilm production were prepared such as phosphate buffer saline, crystal violet, and ethanol alcohol 96%.

Investigation of Biofilm Production Using Microtiter Plates:

The ability of *Klebsiella pneumonia* to form biofilm was revealed by following the method of (Babapoure *et al.*,2016). The method of accurate calibration dishes (Microtter Plate 96) was used in the following steps:

1. A bacterial suspension was prepared from BHI broth, as the sample was diluted and compared with McFarland standard solution, then incubated at 37°C for 24 hours. hour.
2. 200 microliters of BHI broth containing 2% glucose without bacteria were placed in the first three wells of the calibration dish, and it was considered to be negative control.
3. (150) microliters of BHI broth containing 2% of glucose were distributed into the holes of the microtter plate, then the holes were inoculated by taking 50 microliters of the bacterial suspension prepared in the first section, and then three Duplicates for each isolate.
4. Close the calibration dish, then cover it with Parafilm to prevent contamination, and incubate at a temperature of 37°C for 24 hours. After incubation, the contents of the pits were removed, then washed three times with PBS with pH 7.2 to get rid of bacterial cells, then left to dry at laboratory temperature.
5. The adherent live cells were fixed by adding 200 microliters of absolute methanol to each hole and left for 15 minutes, after which the contents of the holes were removed and left to dry.
6. 200 microliters of Crystal Violet at a concentration of 1 was added to each hole and left for 20 minutes, after which the dye was removed and washed three times with PBS to get rid of the remaining dye, then left to dry in laboratory temperature.
7. Add 200 microliters of 96% ethanol to each well.
8. The Optical Density (OD) was evaluated using the ELISA instrument at a wavelength of (630) nm.

The efficiency of the isolates on adhesion and biofilm formation was determined by comparing their readings It was obtained according to the following equations:

1. The isolate is considered non-adherent if the control optical density is greater than or equal to the isolate's optical density ($OD > ODc$).
2. The isolate is considered to be adherent weakly biofilm-forming if the optical density of the isolate is greater than the optical density of the control or equal to or less than twice the optical density of the control ($ODC < OD < 2xOD_C$).

3. The isolate is considered moderately biofilm-forming if the average optical density of the isolate is greater than twice the optical density of the control or equal to or less than four. fold control (xODc < OD < 4xOD2).
4. The isolate is considered highly biofilm-forming Adherent Strong if the average optical density of the isolate is more than four times that of the control (OD > 4xOD).

Effect of Zinc Ferrite Nanoparticles on Biofilm Production:

According to Namasivayam *et al*., (2012). the effect of zinc ferrite nanoparticles on the formation of biofilms by microtiter plates method is as follows:

1. *K.Pneumoniae* bacteria were cultured in the heart-brain infusion medium at 37 °C for 24 hours.
2. A bacterial suspension of 20 microliters was added to all plates (wells 96) containing 80 microliters of BHI with 2% sucrose and 100 microliters of different concentrations of zinc nanoparticles, mixed well, then incubated for 24 hours at a temperature of 37 °C.
3. After incubation, the content of the Pits and washed three times with 7.2 phosphate buffer solution, and then Left to dry at room temperature.
4. Add 200 microliters of crystal violet to the pits for 10 minutes.
5. The adherent bacterial cells were washed three times with PBS (PH7.2) solution and left to dry at room temperature.
6. Then it was removed twice with 200 microliters of ethanol 95 and the absorbance of each hole was measured by the ELISA device at a wavelength of 630 nm.
7. The inhibition rate of biofilm was calculated using nano-zinc ferrite according to the following equation:

Inhibition rate %=OD of control-OD of treated/OD of control x100.

The Effect of Zinc Ferrite Nanoparticles on The Urease Enzyme:

The examination was carried out according to Khadim *et al*., (2019). (0.1) of sub-MIC was placed at a concentration of 1 µg / ml of the nanomaterial $ZnFe_2O_4$ on the surface of the urea agar plate, then the plate was left at room temperature until it dried completely. After that, the surface of the agar was inoculated with the bacteria under study *K.Pneumoniae*, and it was placed in the incubator at a temperature of 37 ^{O}C for 24 hours. Urease enzyme production was detected by observing the change in the color of the yellow urea medium to pink. The lack of production of the urease enzyme was detected by the continued appearance of the yellow color of the medium (Saghalli *et al*., 2016).

RESULTS AND DISCUSSION

Testing the Ability of *K.Pneumonia* Isolates to Form Biofilms:

The ability of *K. pneumonia* isolates to form a biofilm was tested using the calibration dishes method, where the ability of the bacteria to form a biofilm was determined by comparing the obtained readings with the optical density of the control as mentioned in the methods section. The results showed that all isolates of which 50 (100%) have the ability to form biofilm, as 40 isolates (80%) were strong for biofilm formation, while 8 isolates (16%) were medium and 2 (4%) were weak for biofilm formation, as shown in the table.

Table 1: Number and rates of isolates that biofilm-producin.

Isolates no. percentage	Biofilm formation
40 (80%)	Strong
8 (16%)	Moderate
2 (4%)	Weak
50(100%)	Total

The biofilm formation process has a role in the structure and regulatory genes and affects colony aggregation by different mechanisms in addition to altering the synthesis of transcription factors and the regulation of extracellular polysaccharide production (Alzubaidy *et al.*, 2019). Our results were consistent with some local results (Al-Rubyaie, 2021), which indicated that 100% of clinical *Klebsiella pneumoniae* isolates were biofilm-producing. Al-Timimi (2021) also noted that 50 *Klebsiella isolates* were 23: isolates with a ratio of (46) were weak biofilm producers, while the other isolates (14)28 were moderate, (3)6 were strong producers, and (10)20 were non-biofilm producers

An important sign of biofilms is that bacterial cells can survive at high levels of antibiotics. The biofilm is a major virulence factor for gram-negative bacteria, especially *K.pneumoniae*, which can lead to chronic infections. The bacteria present in biofilms are One thousand times more resistant to antibiotic treatment than plankton (Sharma *et al*., 2019). Abdelraheem *et al*., (2020) published that the bacteria that generate biofilms are more resistant to antibiotics than isolates that are not productive. Piperaki *et al*., (2017) indicated that the biofilms of *K.pneumoniae* are the most famous due to their high level of antibiotic resistance.

Effect of Zinc Ferrite Nanoparticles on the Formation of Biofilms of *k. pnumoniae*:

The antibiotic effect of zinc ferrite nanoparticles on 50 isolates of *K.pneumoniae* showed that the biofilm of 14 isolates was inhibited by (28%), and the highest inhibition percentage of biofilm was at a concentration of 64 µg/ml. It was observed that the percentage of inhibition increased with increasing concentration of nanoparticles as shown in the Table 2.

Table 2: The effect of nanoparticles on biofilm inhibition of *K. pneumoniae*.

Isolate NO		2 g/mlµ	4 g/mlµ	8 g/mlµ	16 g/mlµ	32 g/mlµ	64 g/mlµ	Inhibition average
1	Od	0.015	0.011	0.010	0.005	0.003	0.001	92.5 A
	Inhibition rate	85	89	90	95	97	99	
2	Od	0.011	0.007	0.005	0.003	0.002	0.001	95.16 A
	Inhibition rate	89	93	95	97	98	99	
3		0.019	0.015	0.012	0.010	0.003	0.002	89.83 BC
	Inhibition rate	81	85	88	90	97	98	
4	Od	0.020	0.015	0.007	0.005	0.003	0.001	91.5 B
	Inhibition rate	80	85	93	95	97	99	
5	Od	0.022	0.019	0.012	0.009	0.006	0.002	88.33 C
	Inhibition rate	78	81	88	91	94	98	
6	Od	0.025	0.016	0.016	0.011	0.006	0.004	87.00 C
	Inhibition rate	75	84	84	89	94	96	
7	Od	0.020	0.013	0.010	0.008	0.003	0.001	90.83 BC
	Inhibition rate	80	87	90	92	97	99	
Control	Od	0.100	0.100	0.100	0.100	0.100	0.100	0.0 D
	Inhibition rate	0	0	0	0	0	0	
conc Average	Od	71.00E	75.50 D	8.80 Cd	81.12bc	84.25ab	86.00a	P–Value=<0.05
	P–Value=<0.05							

We note from the results of the above table that there was a strong inhibition of the biofilm of bacteria by a large percentage. The highest inhibition rates were for isolate No. (1,2) with an inhibition rate of (95.16), (92.5) %. Sharma *et al*., (2022) reported that the outstanding efficiency of ZFe_2O_4 nanoparticles in an anti-biofilm study by inhibiting biofilms up to 81.76% and reducing mature biofilms up to 56.22% at 75 µg/mL minimum concentration value.

Also, in the Sharma *et al*., (2022) study of nano zinc ferrite coated with chitozan CT- $ZnFe_2O_4$, where its inhibitory effect on biofilm formation and removal of immobilized biofilms was evaluated. The results showed that $ZnFe_2O_4$ inhibits biofilm formation by more than 65% and reduces confirmed biofilms up to 50% at the lowest inhibitory concentration. Several studies indicated that nanoparticles have an effective effect in inhibiting biofilms. Sharma *et al*., (2015) found how silver impairs the formation of biofilms when silver nanoparticles move into the cell and interact with proteins and enzymes, which are necessary for sensing microbial development or adhesion. Which led to a decrease in the functioning of the biofilms.

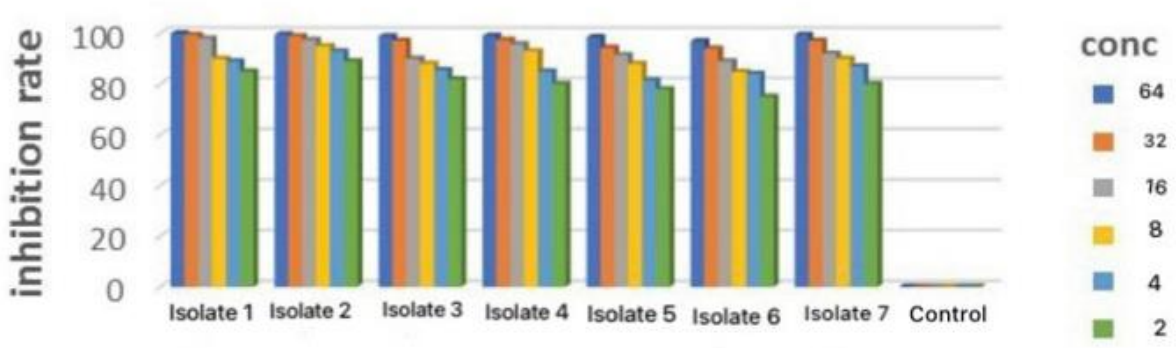

Fig. 1: **The effect of nanoparticles on biofilm inhibition of *K. pneumoniae***

The Effectiveness of the Nanomaterials against the Production of the Urease Enzyme:

Urease is an unstable hydrolytic enzyme whose role is represented in the catalytic degradation of urea to carbomate and ammonia. Some urease inhibitors contain heavy metals such as zinc, copper, and mercury. It is considered a promising and potential method for the treatment and elimination of urease-producing bacteria that cause diseases and eliminate them (Kafarski and Talma 2018).

The results showed the effect of $ZnFe_2O_4$ nanoparticles at a concentration of 1 µg/ml Sub-MIC on 50 isolates of *Klebsiella pneumoniae* (10 isolates) was inhibited by 20% on the production of urease enzyme (inhibition occurred). The other isolates, 40 isolates with a rate of 80%, were urease-producing. (No inhibition occurred). The results were observed by changing the color of the medium from pink to yellow, which indicates inhibition of the production of urease enzyme.

The survival of the pink color of the medium was an indication of the lack of inhibition of the enzyme.

Fig. 2: Effect of the $ZnFe_2O_4$ against the production of the urease enzyme.

a. Urease positive +, b. Urease negative−

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