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ORIGINAL ARTICLE

Histopathological Study for Biofilm-Forming *Klebsiella pneumoniae*

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ABSTRACT

The goal of this investigation was to ascertain if biofilm-forming Klebsiella pneumoniae isolates may cause histopathological alterations. Thirty-eight K. pneumoniae isolates were obtained from patients who visited two hospitals in Baghdad; each isolate was identified using biochemical, microscopic, and cultural characteristics. Polystyrene microtiter plates were used to assess the K. pneumoniae isolates' capacity to produce biofilm. The degree of biofilm thickness was reflected in the optical density (OD), which was measured at 630 nm. Approximately 21.05% of the tested isolates were weakly adhering, 73.6% were moderately adherent, and 5.26% were strongly adherent, according to the results. Isolate K22 was comparatively biofilm-forming, while isolate K20 was poor. Eighteen male Swiss white mice (Mus musculus) were used to test these two isolates intranasally. Weak biofilm former K. pneumoniae K20 caused less lung damage than moderate biofilm former K. pneumoniae K22, with pathology scores of 9 and 13, respectively, according to histopathological abnormalities in mice's lungs.

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1- INTRODUCTION

Instead of existing as individual planktonic cells, the majority of bacteria are found in intricate, dense colonies known as biofilms that are attached to surfaces. Numerous health issues are often linked to biofilms [1]. Because biofilm-associated bacteria are far more resistant to host immune responses, medications, and hydrodynamic shear stresses than their comparable planktonic counterparts, biofilm-linked illnesses are especially troublesome. Biofilm-associated disorders are very difficult to cure due to all these factors. Biofilm-associated bacteria often and inconveniently spread the infections after treatment [2]. Since the germs were brought into hospital settings and became a major cause of nosocomial infections in the early 1970s, Klebsiella pneumoniae has drastically altered its epidemiology and range of disorders [3]. Numerous traits affect the pathogenicity of Klebsiella species. They have two different kinds of antigens on their cell surface: capsular and lipopolysaccharide antigens. The taxonomy, pathogenicity, and epidemiology of Klebsiella species are significantly influenced by both antigens [4].

Examining the histopathological consequences of biofilm-forming Klebsiella pneumoniae isolates was the aim of the current study.

2- MATERIALS AND METHODS

2.1 Specimens collection, Bacterial isolation and identification

Patients of both sexes who visited Educational Al-Karama Hospital and Educational Al-Yarmouk Hospital in the Baghdad governorate provided urine samples in sterile containers. These samples were previously gathered in order to determine the distribution and prevalence of Klebsiella species. From these previously collected clinical specimens, 38 isolates of K. pneumoniae serotype K2 were obtained. These isolates were identified using biochemical tests and morphological characteristics based on Forbes et al. (2007) [6] and Bergey's Manual of Systematic Bacteriology, 2nd edition [5], unless otherwise noted. The Presto TM Mini gDNA bacteria kit technique was used to extract DNA from K. pneumoniae isolates.

2.2 Biofilm assay

We used the approach outlined in Sanchez et al. (2013) [7]. To sum up, bacterial isolates were cultivated in Brain Heart Infusion (BHI) broth and incubated for eighteen hours at 37 degrees Celsius. Lastly, the bacterial culture was adjusted in accordance with MacFarland tube 0.5 and diluted with phosphate buffer saline (PBS). Pre-sterilized 96-well polystyrene microtiter plates were filled with an aliquot (150 µl) of the bacterial culture and incubated for a full day at 37°C. To get rid of any detached cells, each well was cleaned with PBS twice or three times after incubation. Each well was then filled with 150 µl of 0.5% crystal violet. To assist the colorant sink to the bottom of the well, the plates were shook three times. Each well was cleaned with sterile PBS to get rid of the planktonic cells and stain the ones that weren't sticking to the well after ten minutes at room temperature. The surface of the well was only preserved by the adhering bacteria that formed the biofilm. For ten minutes, 150 µl of methanol was added to each well. After that, the methanol was extracted from the wells without being cleaned. Next, 200 µl of ethyl alcohol was used to remove the crystal violet that had been attached to the biofilm. Lastly, a microplate reader was used to measure absorbance at 630 nm; the result was called OD. Every test was run three times.
Crystal Violet was used as a control in wells that had recently been exposed to the bacterial-free culture mix. For a microtiter plate, the cut-off optical density (ODc) is three standard deviations higher than the negative control's mean OD. According to Liu et al. (2015), Table 1 shows how the various adherence strengths are categorized. [8].

Table (1): Bacterial adherence classification using the microtiter plate technique [8]

Mean OD_{630}	Compliance Formation of Biofilms
OD ≤ ODc	Non-compliant
ODc < OD ≤ 2*ODc	Inadequately devoted
2*ODc < OD ≤ 4*ODc	Moderately devoted
OD < 4* ODc	Extremely devoted

1.3 Histopathological study

2.3.1 Laboratory animals

Eighteen male Swiss white mice (Mus musculus) weighing twenty to twenty-five grams at eight weeks of age were supplied by Al-Nahrian University/Biotechnology Center. Each of the six groups (A through E) had three animals. Although each animal had its own cage, they all had unrestricted access to the same food and water..

2.3.2 Bacterial suspension

Several well-isolated colonies from an overnight BHI plate were cultivated for 24 hours at 37°C after being injected in BHI broth. After that, a suspension was created and the concentration was raised to MacFarland standard no. 0.5.

2.3.3 Inoculation protocol

Animal groups were injected with corresponding bacterial suspensions, as follows: Each mouse was pentobarbitone anesthetized according to (Satoh *et al.,* 1984) and held inverted with the nose up. After that, the inoculum (20 µl) was injected intranasally with a 0.6 mm in diameter catheter. Another group was administered PBS as a control group. After 3 days of injection, mice were sacrificed, and the left lung was aseptically removed, and fixed with 10% formalin.

Table (2): Animal groups with the corresponding bacterial isolates and injection type

Animal group	***Klebsiella pneumoniae* isolate**	**Biofilm degree**	**Type of injection**
D		Weak	Intranasally
E		Moderate	Intranasally
F*	-	-	Intranasally

*** Group F was injected with normal saline to be control group**

After the lungs were fixed in 10% formalin, the tissues were cleaned with tap water for a few minutes before being subjected to escalating ethanol concentrations (50, 60, 70, 80, 90, and 100%, respectively) for two hours. The samples were fixed in paraffin wax at 60°C for three hours after an hour of xylol cleaning. A microtome was then used to cut the paraffin blocks to a size of around 5 µm. The slices were stained with hematoxylin for five minutes, rinsed with tap water, stained with eosin for one minute, and then cleaned with distilled water after being dehydrated for two days at 37°C in an incubator. Following two minutes of exposure to increasing ethanol concentrations (70%, 90%, and 100%, respectively), they were administered drops of Canada balsam and coated with slides prepared for light microscope examination [9].

3- RESULTS AND DISCUSSION

3.1 Isolation and Identification

Numerous local studies have found Klebsiella in urine and other clinical samples. Klebsiella species accounted for 54.16% of all isolates from clinical specimens, with K. pneumoniae making for 79.12% of these isolates [10]. K. pneumoniae was present in 36.13% of Klebsiella isolates [11]. Furthermore, the most frequent source of Klebsiella isolates was urine (42.16%), followed by wounds (31.25%) and sputum (28.57%). The most prevalent nosocomial Gram-negative bacterium found in all of these local studies was Klebsiella. It is important to treat this nosocomial bacterium carefully.

3.2 Biofilm assay

The capacity of K. pneumoniae isolates to generate biofilm was evaluated using pre-sterilized 96-well polystyrene microtiter plates. By measuring absorbance at 630 nm, the degree of biofilm for the isolates under investigation that stuck to the surface of the microtiter well was subsequently ascertained; the findings are shown in Table 3. The results showed that each isolate had a unique ability to form biofilm even under the same experimental conditions.

Table (3): Absorbance of *Klebsiella pneumoniae* biofilm at 630nm

Isolate code	[a]OD_{630}	Standard deviation	Biofilm degree	Source
K1	0.210	0.09	moderate	Al-yarmouk hospital
K2	0.165	0.04	moderate	Al-yarmouk hospital
K3	0.249	0.11	moderate	Al-yarmouk hospital
K4	0.152	0.02	moderate	Al-yarmouk hospital
K5	0.161	0.02	moderate	Al-yarmouk hospital
K6	0.108	0.004	weak	Al-yarmouk hospital
K7	0.232	0.03	moderate	Al-yarmouk hospital
K8	0.221	0.03	moderate	Al-yarmouk hospital
K9	0.152	0.02	moderate	Al-yarmouk hospital
K10	0.151	0.01	moderate	Al-yarmouk hospital
K11	0.191	0.01	moderate	Al-karama hospital
K12	0.148	0.03	moderate	Al-yarmouk hospital
K13	0.229	0.03	moderate	Al-yarmouk hospital
K14	0.225	0.04	moderate	Al-yarmouk hospital
K15	0.206	0.04	moderate	Al-yarmouk hospital
K16	0.155	0.01	moderate	Al-yarmouk hospital
K17	0.152	0.01	moderate	Al-yarmouk hospital
K18	0.152	0.005	moderate	Al-yarmouk hospital
K19	0.142	0.01	moderate	Al-yarmouk hospital
K20	0.127	0.003	weak	Al-yarmouk hospital

Isolate code	OD_{630}	Standard deviation	Biofilm degree	Source
K21	0.188	0.004	moderate	Al-yarmouk hospital
K22	0.209	0.02	moderate	Al-yarmouk hospital
K23	0.212	0.01	moderate	Al-yarmouk hospital
K24	0.223	0.02	moderate	Al-karama hospital
K25	0.228	0.10	moderate	Al-karama hospital
K26	0.308	0.03	strong	Al-karama hospital
K27	0.257	0.03	moderate	Al-yarmouk hospital
K28	0.331	0.04	strong	Al-yarmouk hospital
K29	0.176	0.07	moderate	Al-yarmouk hospital
K30	0.141	0.01	moderate	Al-yarmouk hospital
K31	0.105	0.005	weak	Al-yarmouk hospital
K32	0.085	0.01	weak	Al-karama hospital
K33	0.084	0.01	weak	Al-yarmouk hospital
K34	0.141	0.02	moderate	Al-yarmouk hospital
K35	0.237	0.06	moderate	Al-yarmouk hospital
K36	0.093	0.007	weak	Al-yarmouk hospital
K37	0.078	0.009	weak	Al-yarmouk hospital
K38	0.132	0.004	weak	Al-yarmouk hospital
[b]Control	0.067	0.002	-	-

[a]Each datum is a mean of triplicates, OD_{630} denotes to optical density at 630 nm

[b]Control represents bacteria free medium. $P = 4.6 \times 10^{-12}$, $LSD_{0.05} = 0.067$

Of the isolates under study, 21.05% showed poor adherence, 73.6% showed moderate adhesion, and 5.26% showed high adhesion to the surface of the microtiter. The variations in biofilm thickness were caused by variations in the quantity and quality of auto-inducers (quorum sensing signaling molecules) produced by each isolate, variations in the isolates' ability to form biofilm, and variations in the primary number of cells that were successful in sticking[12].

3.3 Histopathological study

Two isolates, the weak (K20) and the moderate (K22) biofilm formers, were injected intranasally to be tested in vivo, as mentioned previously in Table 3.

3.3.1 Laboratory animals with Intranasal injection

Three groups of mice (D, E, and F) were injected intranasally, and groups D and E were injected with two bacterial isolates, the weak (K20) and the moderate (K22) biofilm formers, respectively, as mentioned before in Table 2. Group F was injected with normal saline only and considered as a control group. The structure of the lung tissues appeared normal, as illustrated in Figure 1.

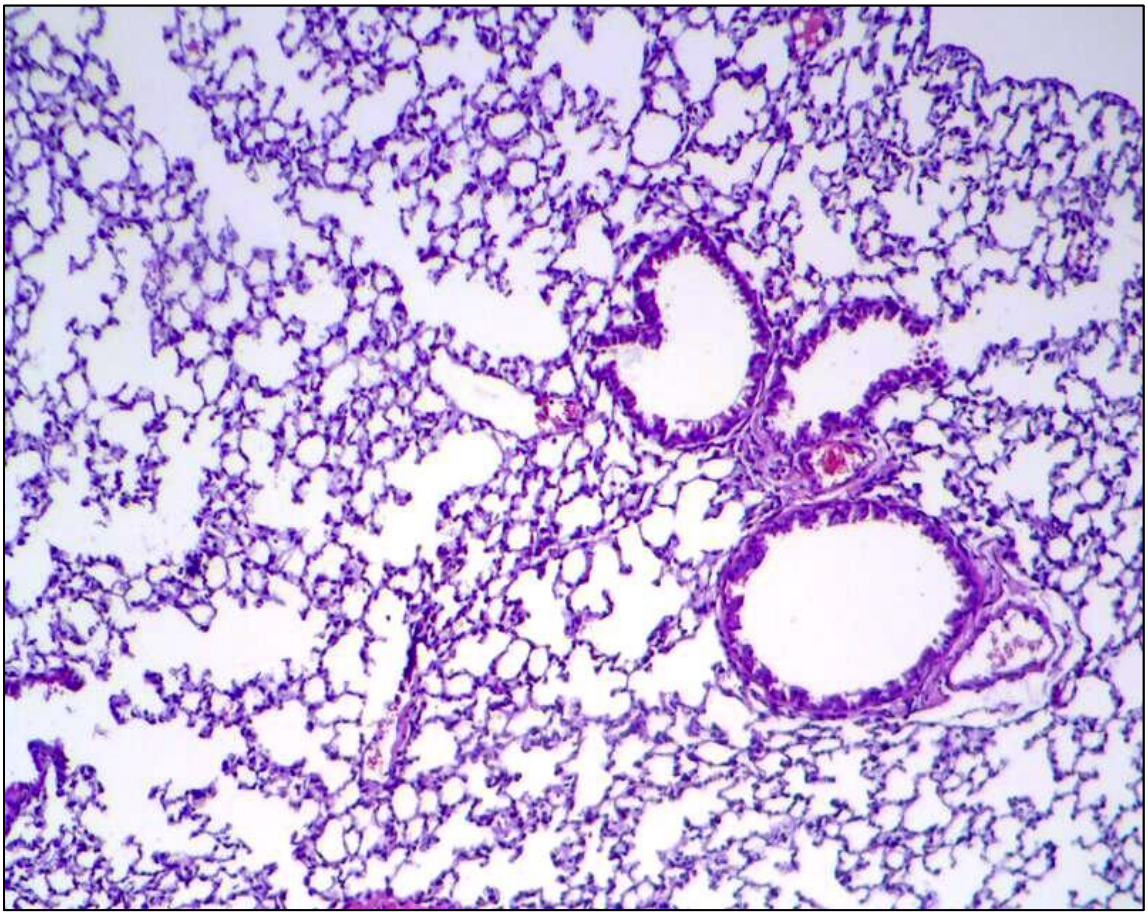

Figure (1): Lung tissue seems normal in a section of a rat lung that was intranasally injected with normal saline (control group).

Group D showed mild inflammatory cell infiltration and mild thickening of alveolar septa, as shown in Figure (2 A). Also, injection of group D showed hemorrhage with destruction of alveoli septa and its congestion of capillary. The destruction of alveoli led to the formation of emphysematous changes, as shown in Figure (2 B).

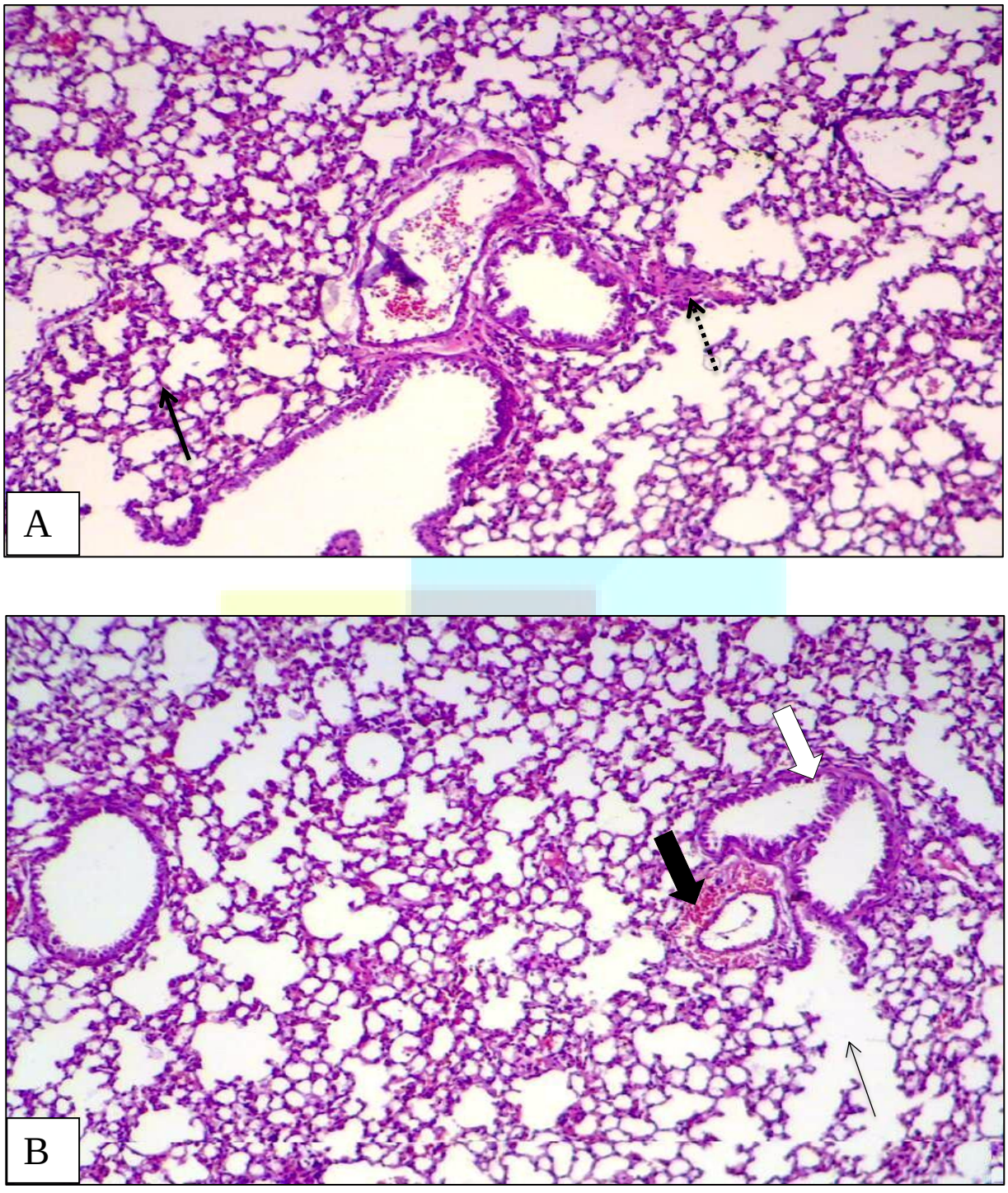

Figure (2): Section of mouse lung injected with 1× 10^8 CFU/ml of *Klebsiella pneumoniae* K20 showing (A) mild thickening of alveolar septa (solid arrow) with mild infiltration of inflammatory cells (dashed arrow), (B) Congestion (black block arrow) and haemorrhage (white block arrow), with destruction of alveoli septa (thin arrow), (100 X), H&E

Group E showed a wild area of destruction of alveolar tissue with abundant inflammatory cell infiltration, which destroyed the alveolar bronchiole, emphysematous changes, and also, congestion with haemorrhage were noticed, as shown in Figure (3A) and Figure (3B).

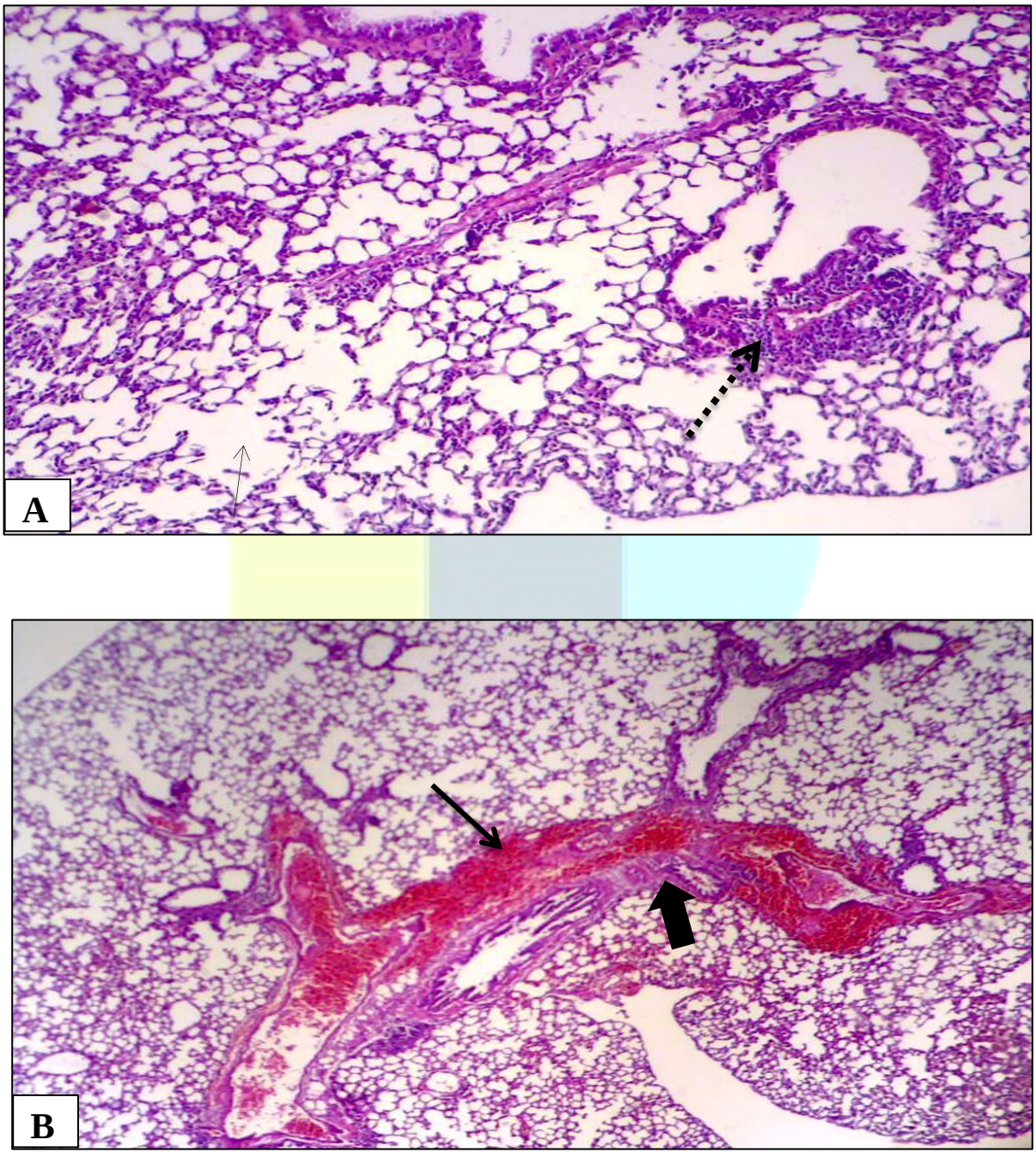

Figure (3): Section of mouse lung injected with 1× 10^8 CFU/ml of *Klebsiella pneumoniae* K22 showing (A) wide area of destruction of alveolar tissue with abundant inflammatory cells infiltration (dashed arrow), and (B) with congestion (black block arrow), with hemorrhage (solid arrow), (100X), H&E

The choice to use an intranasal inoculation rather than an intratracheal one was made due to a variety of factors; the intranasal inoculation lessens procedural stress and more closely mimics a natural route of infection. Additionally, as tracheal integrity is not affected during the inoculation, this tissue may be used as an alternative location for evaluating bacterial growth and persistence [13].

3.3.2 Pathological score

Concerning the lung, the scores were 9 and 13 for weak and moderate biofilm formers, respectively (Table 4).

Table (4): Pathological score of intranasal injection

Tissue section	Biofilm former	Inflammatory cells infiltration	Congestion	Hemorrhage	Thickening of alveolar septa	Destruction of alveolar septa	Score
Lung	Weak	2	2	1	2	2	9
Lung	Moderate	3	3	2	2	3	13

Upon results depicted in previous Figures, in addition to the pathological score summarized in Table 3, we can conclude that the damage caused by weak biofilm former *K. pneumoniae* K20 is less than that produced by moderate biofilm former *K. pneumoniae* K22 to lung.

According to studies, mice infected with serotypes K1/K2 had greater mortality rates because they were unable to get rid of germs, which led to bacterial overgrowth in many organs and peritonitis. This resulted in exposure to high levels of bacterial endotoxins, which in turn caused multiple organ dysfunction syndrome and fast death [14].

One of the three K. pneumoniae strains (serotypes K1, K2, and K25) was given intratracheally to female BALB/c mice using the murine method. The K. pneumoniae strain with serotype K25 is much less virulent than the strains with serotypes K1 and K2. The blood and lungs of mice showed the highest levels of host colonization, followed by the liver and spleen. [15].

5- CONCLUSION

All of the K. pneumoniae isolates from the present study produced biofilm using the microtiter plate test. The wzy gene may be used to swiftly and reliably detect K. pneumoniae serotype K2 molecularly. The present study found that the moderate biofilm isolate was more harmful to the lungs than the weak biofilm isolate.

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