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Investigating the effect of some physical and nutritional factors on *Klebsiella pneumoniae* biofilm formation

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ABSTRACT

One hundred clinical samples were collected from different pathological conditions. After the final diagnosis, 50 clinical bacterial isolates belonging to *Klebsiella pneumoniae* were obtained by 50%, and the percentage of isolates obtained from sputum was 23 (46%), urine 22 (44%), faeces 2 (4%) and from tracheal pus 3 (6%). The isolates were cultured and diagnosed in the conventional ways, and for final diagnosis and antibiotic sensitivity test (AST) Vitek2 system was used. All isolates of *K.pneumoniae* were highly resistant to antibiotics of 92% against Cefepime, Ceftazidime, Ciprofloxacin, Levofloxacin and ceftriaxone, while the Imipenem and Meropenem 72%, Trimethoprim/Sulfamethoxazole 68%, Tobramycin 60%, Gentamicin 56%, Amikacin 44%, Aztreonam 36%, while it was highly sensitive to 96% for each to Piperacillin, Ticarcillin/Clavulanic Acid and Piperacillin/Tazobactam were 96%. The results of the study showed the ability of *K. pneumoniae* isolates to form biofilms using the 100% Microtiter Plate method but in varying formations. 26 isolates (52%), 15 isolates (30%) and 9 isolates (18%) showed strong, medium and weak abilities respectively. Glucose, lactose, and yeast extract were among the best nutrients affecting the increase of biofilm formation. On the other hand, the temperature at 37°C and 25°C and the pH at 7, 8 and 9 had a positive effect on the production of biofilms.

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1. INTRODUCTION

Klebsiella pneumoniae belongs to Enterobacteriaceae family of Gram-negative strains and was cause of many common diseases [1, 2]. They were bacilli-shaped, nonmotile, non-spore-forming, 0.3 to 2.0 µm wide and 0.6 to 6.0 µm long, with a characteristic mucilaginous appearance on agar plates. *K.pneumoniae*, a facultative anaerobic bacterium, is able to grow in both aerobic (in the presence of oxygen) and anaerobic (in the absence of oxygen) conditions. [3]. It biochemically ferments lactose, catalase positive and oxidase negative. It biochemically ferments lactose, catalase positive and oxidase negative. An encapsulated morphology was one of the distinguishing features of *K.pneumoniae* [4]. The common opportunistic *K. pneumoniae* causes significant concern in individuals with compromised immunity or those whose immunity has been compromised by another infection. However, the highly contagious *K. pneumoniae* can infect healthy people and lead to community-acquired infections such as meningitis, endocarditis, severe pneumonia and purulent liver abscess [5]. In most cases, colonisation of the gastrointestinal tract by *K. pneumoniae* precedes the onset of nosocomial infections. It can also affect the bloodstream, respiratory system and urinary tract [6, 7]. Transformation with virulence factors that can grow on medical devices such as catheters and tracheal tubes and become a major source of infection for patients undergoing catheterization [8]. In addition, nosocomial disease caused by *K. pneumoniae* was generally prolonged due to two fundamental variables: the formation of biofilms that evade immunity in *vivo*, and the synthesis of enzymes such as beta-lactamase and broads spectrum carbapenemase that have the potential to render an organism resistant to a particular antibiotic [9, 10]. Doctors were unable to treat infected patients with an effective antibiotic due to the production of antibiotic-resistant enzymes [11, 12]. Virulence factors include the presence of lipopolysaccharides, capsule, siderophore iron and biofilm formation. The clear extracellular polysaccharide layer produced by bacteria acts as a shield against the host's innate immunity and encapsulates the cell structure [13].

By preventing host clearance by phagocytosis and increasing bacterial resistance to intracellular killing, capsules are known to promote immune evasion [14, 15]. The absence of the capsule renders *K. pneumoniae* less harmful and increases the bacterial survival rate [16]. Both classical *Klebsiella pneumoniae* (cKp) and hyper virulent *Klebsiella pneumoniae* (hvKp) were present in Gram-negative bacteria with lipopolysaccharides (LPS), also known as endotoxin, in their outer membranes. Osmotic antigen, an essential oligosaccharide, and lipid A produce LPS, which acts as a barrier complement-mediated killing [17]. There were at least eight serological types of O-antigen in *K. pneumoniae*, with the O1 antigen being the most prevalent among clinical strains. There were two common types of fimbriae in *K. pneumoniae* strains [18]: Type 1 and Type 3 fimbriae, which aid in bacterial adhesion, host cell invasion, and biofilm formation, were encoded by the *fim* and *mrkABCD* operons, respectively. Siderophore-mediated iron was required for full virulence *K. pneumoniae* and essential for the bacterial growth *in vitro* and *in vivo*. The term biofilm refers to an organized community of microorganisms coated with extracellular polymers secretion (EPS) [19]. EPS, consisting mainly proteins, DNA, and polysaccharides, makes up 90% of biofilms [19]. Due to the thick EPS layers, the expression of flow pumps, and the presence of continuous cells in biofilms, bacteria are 1000 times more resistant to antibiotics than planktonic cells [20]. Biofilm formation is associated with 60-80% of bacterial infections [21]. Biofilms can protect pathogens from immune responses host of antibiotics' anti-pathogenic effects, increasing antibiotic resistance and bacterial survival, and making disease more difficult to treat [22, 23]. Despite the fact that the researchers sought to improve the conditions that *Klebsiella* isolates needed to form biofilms. Nonetheless, factors like ideal focus, the carbon nature, nitrogen, salt and amino acids sources, and the extravagance of the medium have not been totally investigated [24]. The diagnosis of bacterial isolates adopted in this study belonging to *K. pneumonia* species was confirmed using the Vitek 2 Compact System. It adopted the diagnostic kit of the device and according to the card for diagnosing these isolates and examining sensitivity to antibiotics.

2. METHOD

2.1. Bacteria isolation and purification

One hundred clinical samples were collected from different pathological conditions including (Wound, burn, urine, sputum, blood) from Ghazi Hariri Hospital, Burn Hospital, Al-Kadhimiya Hospital, Ibn Al-Baladi Hospital, Imam Ali Hospital from 30/7 and 30/9/2023. The study was conducted after receiving agreement from the participants and ethical approval from the college of education for pure science (Ibn Al-Haitham) at the University of Baghdad and the Iraqi Ministry of Health and Environment, which numbered (4166) on 25/6/2024.

The samples were cultured on plates of MacConkey agar and blood agar by a streaking method and then incubated at 24 hours and a temperature of 37°C for the purpose of distinguishing Gram-positive bacteria from Gram-negative bacteria. After that confirming the growth was cultured a single colony on the chromogen agar and eosin methylene blue agar for the purpose of diagnosing bacteria. Microscopic examination by stained the bacteria with Gram stain and biochemical tests by using indol, methyl red, Voges-Proskauer, citrate utilization, catalase, oxidase and urease. The diagnosis of the bacterial isolates adopted in this study belonging to the species *K.pneumonia* was confirmed utilizing the Vitek 2 Compact System.

2.2 Antibiotic sensitivity test

Antibiotic sensitivity tested using VITEK-2 included the following antibiotics: Cefepime, Ceftazidime, Ciprofloxacin, Levofloxacin, Piperacillin, Ticarcillin/Clavulanic acid, Ceftriaxone, Piperacillin/ Tazobactam, Meropenem, Imipenem, Trimethoprim/ Sulfamethoxazole, Tobramycin, Gentamicin, Amikacin and Aztreonam as shown in (Table 1).

Table 1. Antibiotics and their concentrations.

No	Antibiotics	Symbol and concentration (µg/mL)
1	Gentamycin	CA >=16
	Amikacin	AK >=32
	Tobramycin	Tob>=16
2	Ceftazidime	CAZ>=64
	ceftriaxone	CRI>=64
3	Cefepime	FEP>=32
4	Imipenem	IPM>=16
	Meropenem	MPN >=16
5	Aztreonam	ATM >=64
6	Trimethoprim/ Sulfamethoxazole	TEP/SMZ>=320
7	Piperacillin	PRL >=128
	Piperacillin/Tazobactam Ticarcillin	TBC>=128
	Ticarcillin/Clavulanic Acid	CLA>=128
8	Ciprofloxacin	CIP>=4
	Levofloxacin	LEV>=4

2.3. Detection of biofilm formation

The ability of bacterial isolates to form a biofilm was determined using O'Toole [25] Microliter-plate (MTP) method. Test tube was filled with 3 ml of normal saline, then a single pure colony was taken by the loop and placed in the test tube to make a bacterial suspension, and then its turbidity was measured using the Densichek Vitek 2 device to be equal to (50.0-63.0).

2.4. Effect of salt medium on formation of biofilm

0.1 g (K_2HPO_4, 0.05 g KH_2PO_4), 0.05 g ($MgSO_4.7H_2O$), 0.01 g ($FeSO_4.7H_2O$), 0.001 g ($ZnSO_4.7H_2O$) and 0.2 g ($CaCO_3$) were prepared in 100 ml of distilled water and fertilized with the autoclave and pH was adjusted to 7 [26].

2.5. The effect of some factors on biofilm formation

Bacteria were cultured on Heart and Brain Infusion Broth (BHIB) for 24 hours, 50µl of the broth containing bacteria was taken and 1000µl of the prepared broth salt medium containing the different carbon and nitrogen sources was inoculated, and distributed to the wells by adding 200 microliters per well of microtiter plate and effect of nutrients was studied. One gram yeast extract, NH_4Cl glucose sugar, lactose sugar were added to the salt medium in each experiment, then inoculated with diluted bacteria in the same medium, were incubated after adding diluted bacteria [27].

2.6. Statistical Analysis

The data was tabulated in a datasheet of IBM SPSS version 25.0, which was utilized to do the statistical analysis [28]. The mean and standard errors of continuous variables were reported, and significant differences were tested using the analysis of variance (ANOVA) test, followed by the least significant difference (LSD) test. Statistical significance was defined as a probability value ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1. Isolation and diagnosis of bacteria

After the final diagnosis, 50 clinical bacterial isolates belonging to *K. pneumoniae* were observed (50%), and the percentage of isolates obtained from sputum were 23 (46%), urine 22 (44%), stool 2 (4%) and tracheal pus 3 (6%) as shown in (Figure 1).

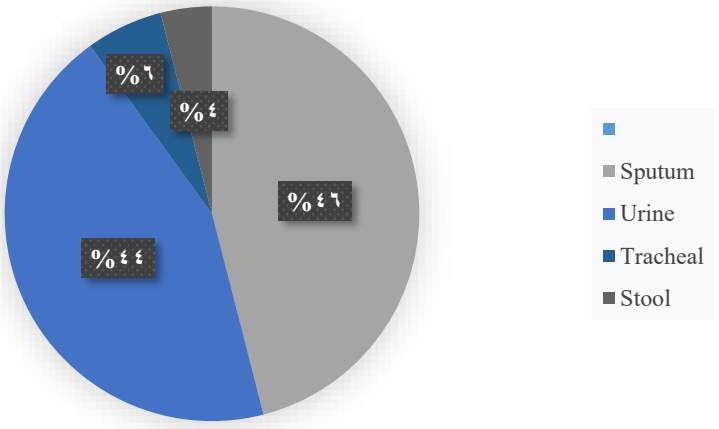

Figure 1. Percentages of sources *isolating K. pneumoniae*

The results of this study showed that the occurrence of *K. pneumoniae* bacteria isolated from sputum (46%) and this result is similar to the study of Alnaqeeb and Gergees, [29] and Hao *et al.,* [30] who they found that the percentage of 50%, the reason may be due to hospital contamination and transmission of infection between outpatients and inpatients, while the percentage of bacteria isolated from urine was 44% and this result is similar to the results of Taha *et al*., [31] and Prasada Rao *et al.,* [32] who found that the majority of individuals (50%) were exposed to urinary tract infections due to changes in various anatomical and physiological characteristics. The percentage of bacteria isolated from wounds and tracheal pus was 6% and this result is similar to the study of Taha *et al.*, [31] and Puca *et al*., [33] who found the percentage 6.25%, while the percentage of bacteria isolated from stool was 4%, and this percentage was similar to Alwan and Abass [34] and Moon *et al.,* [35] who found that percentage were 2% and 3% respectively.

The specimens from the sputum lavage played the dominant role in current study because of *K. pneumoniae* was an important causative agent of several clinical diseases, including urinary tract infection, pneumonia, skin and soft tissue infections, and bacteremia septicemia, which are associated with high morbidity and mortality [36, 37].

3.2. Determination of the minimum inhibitory concentration (MIC) of antibiotics using the VITEK-2 system

The *K.pneumoniae* isolates showed high resistance of 92% to Cefepime, Ceftazidime, Ciprofloxacin and Levofloxacin and high sensitivity of 96% to Piperacillin, Ticarcilllin/Clavulanic acid and Piperacillin/Tazobactam, while their resistance to ceftriaxone was 72%. The results of this study also showed high resistance of *K. pneumoniae* to Imipenem and Meropenem by 72%, Trimethoprim/ Sulfamethoxazole recorded 68% resistance. The Tobramycin had a resistance rate of 60%, Gentamicin had a resistance rate of 56%, the Amikacin had a resistance percentage of 44%, and the bacteria were least resistant to Aztreonam at 36% (Table 2 and Figure 2). These results were consistent with the studies by Adeosun *et al.*, [38] and Kadum and Al Rubaeye, [39].

The high resistance of *K.pneumoniae* isolates to anti carbapenems and cephalosporins (Cefepime, Imipenem, Ceftazidime, Meropenenm, and Ceftriaxone) may be due to the widespread use and misuse of β-lactams has led to the present of resistance against these antibiotics. There were several mechanisms responsible for this resistance including modifying the target site (mutation or expression of alternative PBPs), reducing the regulation of purine, leading to a decrease in cells, efflux pump and modification of enzymes [40].

While the antibiotics resistance to some isolates of Sulfonamides group may be due to the presence of genes associated with sulfonamides resistance, and new genetic contexts have been described for *bla GES*-16 gene (carbapenimase gene). A range of mechanisms that can contribute to antibiotic resistance have also been found, commonly discovered in *Klebsiella*, including chromosomal mutations, flow systems, proteins, and regulators [41].

As for the reason for *Klebsiella* resistance to the Monobactams group, a significant association was observed between MDR phenotype and ESBL production, and the majority of samples were able to produce biofilms. All the efflux genes and biofilms investigated were detected at high frequencies. In addition, strong associations have been observed between efflux pump genes and biofilm-related genes [42].

Table 2. Minimum inhibitory concentration (MIC) of *K. pneumoniae* by using Vitek-2 device.

Antibiotics	PDR			MDR			XDR		
	No	%	MIC	No	%	MIC	No	%	MIC
Ceftazidime	46	92 %	64،32،16>=				4	8%	4،1،0.12>=
Cefepime	46	92 %	64،32>=				4	8%	1،0.12>=
Ceftriaxone	36	72%	64>=	7	14%	0.12>=	7	14%	0.12>=
Ciprofloxacin	46	92 %	4،1>=	2	4%	1	2	44 %	0.25،0.06>=
Levofloxacin	46	92 %	48،>=	1	2%	1	3	6%	0.25،0.06>=
Meropenenm	36	72 %	16،8>=	1	2 %	2*	13	26 %	0.25<=
Imipenem	36	72 %	16،4>=				14	28 %	1،0.5،0.25<=
Trimethoprim/ Sulfamethoxazole	34	68 %	320>=				16	32%	4020،<=
Tobramycin	30	60 %	16،8>=				20	40 %	<=1
Gentamicin	28	56 %	16	1	2 %	8	27	54 %	<=1
Amikacin	22	44 %	64				28	56 %	4،2،1<=
Aztreonam	18	36 %	64،16،4<=				32	64 %	<=1
Ticarcilllin	2	4 %	128				48	96 %	
Ticarcilllin/ Clavulanic Acid	2	4 %	16،8<=				48	96 %	<=4
Piperacillin	2	4 %	64				48	96 %	8
Piperacillin/ Tazobactam	2	4 %	<=128				48	96 %	16, 4 <=

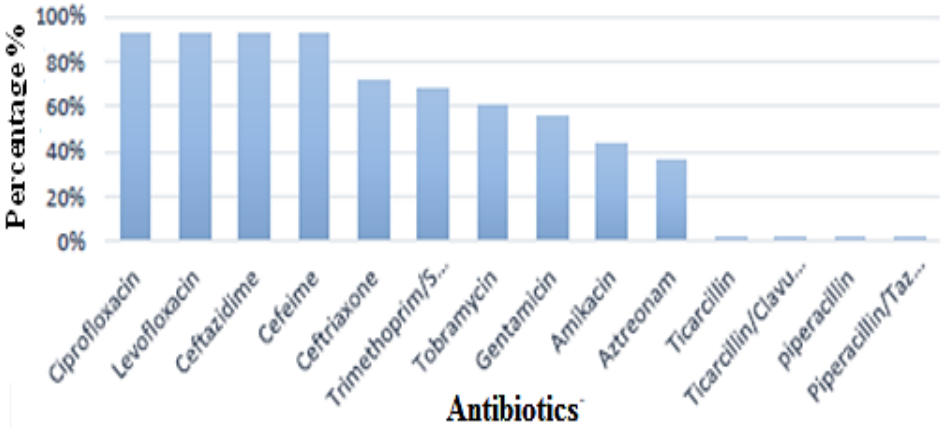

Figure 2. Percentages of minimum inhibitory concentration (MIC) of *K. pneumoniae*.

3.3. Detection of biofilms

The results of the study showed the ability of *Klebsiella* isolates to form biofilm based on the microtiter plate (MTP) method according to changes in optical density of the ELISA. All isolates tested were 100% biofilm forming, but with varying of formation intensity when compared to negative control reading rate. The results of detection this study showed that 26 isolates (52%) had strong biofilm formation, 15 isolates (30%) had moderate biofilm formation, and only 9 isolates (18%), were classified as having weak biofilm formation. The results of our study were similar to those of Swedan and Aldakhily [33] who recorded percentages of 56.3%, 25.7%, and 10.2% for strong, moderate, and weak formation respectively. While the results of Karimi *et al*., [43] were slightly different they recorded 20.4% for strong, 21.6% for moderate, and 32.5% for weak biofilm formation (as shown in Figure 3).

K.pneumoniae that form biofilms were partially protected from immune defenses. The matrix blocks the arrival of antibiotics and antibacterial peptides and reduces the efficiency of complements and phagocytosis. It is also possible that there were mechanisms reduce immunity towards reducing inflammatory responses and establishing chronic infections. The biofilms were most famous for is high-level resistance to antibiotics. Bacteria adapt to lack of food and low oxygen leading to stunted growth which in turn reduces the efficiency of antibiotics that target metabolically active and dividing cells [44].

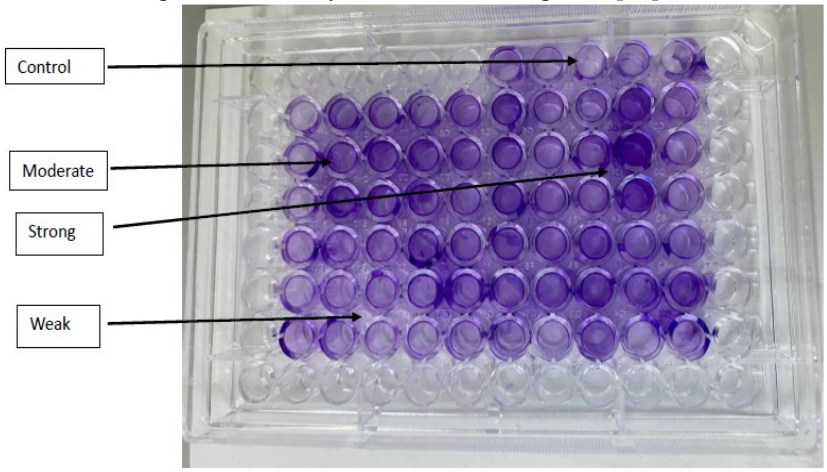

Figure 3. Heterogeneity of formation biofilms in *K. pneumoniae* by Microliter Plate method, as the deep purple wells indicate the highly formed isolates by producing the biofilm.

3.4. Factors effecting the formation of biofilm

Eight isolates (4, 6, 8, 9, 10, 13, 14, 17) of strong *K. pneumoniae* biofilm formation were selected for to study the physical and nutrient effect on biofilm formation.

3.4.1. Effect of the culture medium

Biofilm productivity on the nutrient soy broth medium was better than on the salt medium. The salt medium gave lower proportions than the first medium. EPS production was influenced by nutrient content of the growth medium, which increases with carbon availability, and decreasing nitrogen, potassium and phosphate promoting EPS synthesis [45, 46]. The nutritious soy broth medium was an easy source of energy because the medium rich in elements and nutrients, unlike salt medium, which was a poor source of nutrients, because there was no carbon or nitrogen source.

3.4.2. Effect of Temperature

The results of temperature effect on biofilm formation showed that highest productivity at temperatures of 37°C and 25 °C. Mirkar *et al*. [47] indicated that the optimum temperature for biofilm formation was 37°C. While the study of Alabdullatif [48] indicated a weak biofilm formation at a temperature 4 °C, and this result agrees with the results of our study (as shown in Table 3 and Figure 4).

Table 3. Effect the different temperatures on biofilm formation of *K. pneumonia*.

Isolates	Control Mean±S.E	4°C Mean±S.E	25°C Mean±S.E	37°C Mean±S.E
K4	0.469±0.008^{a}	0.163±0.001^{b}	0.203±0.001^{c}	0.278±0.003^{d}
K6	0.470±0.003^{a}	0.147±0.003^{b}	0.222±0.001^{c}	0.287±0.002^{d}
K8	0.454±0.002^{a}	0.153±0.001^{b}	0.296±0.004^{c}	0.279±0.003^{d}
K9	0.436±0.002^{a}	0.151±0.001^{b}	0.233±0.001^{c}	0.275±0.005^{d}
K10	0.475±0.001^{a}	0.124±0.001^{b}	0.257±0.003^{c}	0.278±0.003^{d}
K13	0.449±0.001^{a}	0.174±0.001^{b}	0.243±0.001^{c}	0.297±0.002^{d}
K14	0.462±0.005^{a}	0.186±0.002^{b}	0.238±0.003^{c}	0.285±0.002^{d}
K17	0.436±0.002^{a}	0.168±0.003^{b}	0.219±0.003^{c}	0.299±0.003^{d}
P-Value	0.001*			

*($P \leq 0.05$) indicates statistical significance.
Different small letter indicated present significant differences in same rows

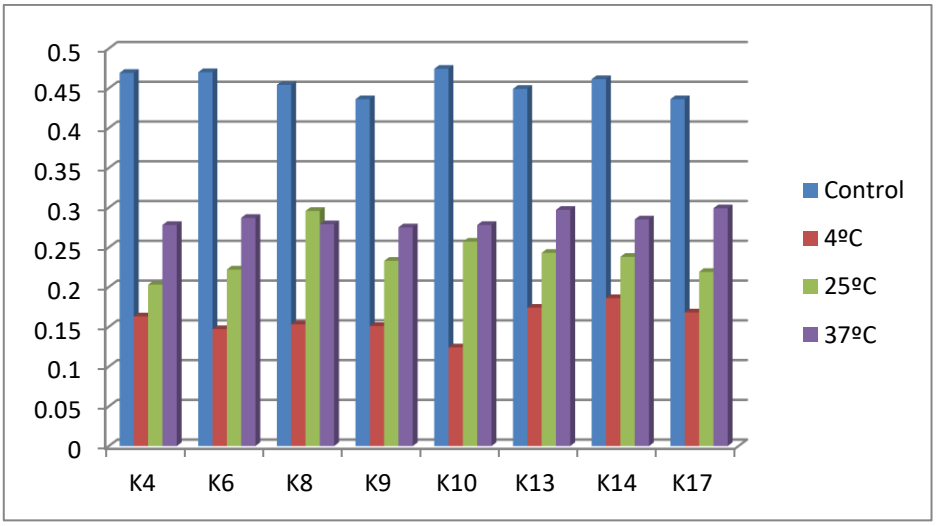

Figure 4. Effect of temperature on biofilm formation.

3.4.3. Effect of pH

The results showed that the maximum biofilm formation occurred at pH 7, 8, 9, and decreased at pH 5, 6. The biofilm was a means of protecting bacteria from the surrounding environmental factors and the pH affects the formation of the biofilm by the effect on changing the properties of the nutrient medium and the solubility and readiness of nutrients. pH 7, which approaches the pH of body fluids, has been shown to enhance virulence and biofilm formation, so bacteria will be more effective in causing infection (as shown in Table 4 and Figure 5). A low pH value can lead to DNA damage, when the pH drops to between 3-5, polysaccharide production and protein concentration decrease rapidly due to bacterial damage. However, polysaccharides and protein concentrations were milder at high pH than at low pH [49]

Table 4. Impact of pH levels on biofilm development..

Isolates	Control Mean±S.E	pH 5 Mean±S.E	pH 6 Mean±S.E	pH 8 Mean±S.E	pH 9 Mean±S.E
K4	0.469 ± 0.008^{a}	0.159 ± 0.003^{b}	0.236 ± 0.002^{c}	0.565 ± 0.002^{d}	0.562 ± 0.004^{d}
K6	0.470 ± 0.003^{a}	0.165 ± 0.002^{b}	0.259 ± 0.003^{c}	0.507 ± 0.002^{a}	0.581 ± 0.005^{d}
K8	0.454 ± 0.002^{a}	0.327 ± 0.151^{b}	0.240 ± 0.004^{c}	0.604 ± 0.001^{d}	0.642 ± 0.002^{d}
K9	0.436 ± 0.002^{a}	0.151 ± 0.001^{b}	0.218 ± 0.003^{c}	0.613 ± 0.001^{d}	0.630 ± 0.004^{d}
K10	0.475 ± 0.001^{a}	0.174 ± 0.001^{b}	0.206 ± 0.002^{b}	0.471 ± 0.004^{a}	0.509 ± 0.003^{a}
K13	0.449 ± 0.001^{a}	0.179 ± 0.003^{b}	0.238 ± 0.003^{b}	0.599 ± 0.003^{c}	0.504 ± 0.002^{a}
K14	0.462 ± 0.005^{a}	0.163 ± 0.001^{b}	0.247 ± 0.002^{c}	0.588 ± 0.003^{d}	0.596 ± 0.002^{d}
K17	0.436 ± 0.002^{a}	0.161 ± 0.001^{b}	0.251 ± 0.002^{c}	0.423 ± 0.005^{a}	0.499 ± 0.003^{d}
P-Value			0.001*		

*($P \leq 0.05$) indicates statistical significance.
Different small letter indicated present significant differences in same rows
Similar small letter indicated non-significant differences in same rows.

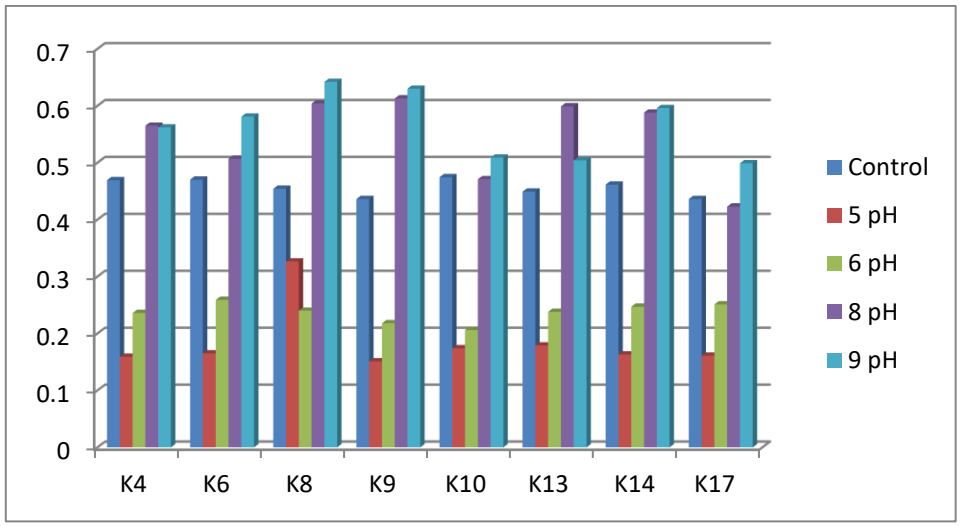

Figure 5. Effect of pH on biofilm formation.

3.4.4. Effect of carbon sources

Carbon sources (glucose, lactose) were used to study the productivity of the biofilm, and the results showed that glucose and lactose were two of the best carbon sources for biofilm formation. They gave a high productivity of the biofilm compared to other carbon sources (fructose, sucrose and starch) which had a low productivity at pH 7 and temperature of 37°C [39] (as shown in Figure 4). The accumulation of biofilms and the density of bacteria in biofilms is higher in the presence of carbon-rich nutrients than in their absence. Elkheloui *et al*. [50] noted that its strong effect of glucose on EPS production and its ability to form biofilms. Glucose can be a source of carbon signal by influencing the expression of many genes and those involved in carbon use. Glucose stimulates multicellular cell aggregation and biofilm formation. Glucose stimulates multicellular cell aggregation and biofilm formation. Glucose is the most abundant simple sugar and was used for energy in some metabolic processes by living cells that produce vital molecules as well as encourage the growth of bacteria in low-nutrient environmental conditions [51].

3.4.5. Effect of nitrogen sources

The results of using yeast extract showed a higher biofilm formation and this result was identical to the study of Alotaibi and Bukhari [52] who observed a higher biofilm formation using yeast extract and explained that this may be due to the availability of nutrients yeast extract contains different types of nutrients, such as minerals and vitamins. Yeast extract provides vitamins, nitrogen, amino acids, minerals and carbon to the culture medium. Nitrogen and vitamins are important components yeast extract for its nutritional properties Milic *et al*. [53]. The results of study by Al-Sa'adi [26] indicated a high biofilm formation in the presence of yeast extract (organic sources) compared to inorganic sources such as $NaNO_3$. The results of the use of ammonium chloride in this study showed a higher biofilm formation and this result was identical to the study of Sandt *et al*., [54] (as shown in Table 5 and Figure 6).

Table 5. Effect the different carbon and nitrogen sources on biofilm formation.

Isolates	Control	Yeast medium	Glucose	Lactose	Salt medium	NH_4Cl
K4	0.469 ± 0.008^{a}	0.308 ± 0.001^{b}	0.388 ± 0.003^{c}	0.371 ± 0.002^{d}	0.275 ± 0.010^{e}	0.247 ± 0.003^{f}
K6	0.470 ± 0.003^{a}	0.439 ± 0.003^{b}	0.398 ± 0.002^{c}	0.398 ± 0.005^{c}	0.285 ± 0.002^{d}	0.332 ± 0.001^{e}
K8	0.454 ± 0.002^{a}	0.375 ± 0.001^{b}	0.393 ± 0.005^{c}	0.464 ± 0.003^{a}	0.275 ± 0.002^{d}	0.365 ± 0.005^{b}
K9	0.436 ± 0.002^{a}	0.375 ± 0.001^{v}	0.536 ± 0.002^{c}	0.465 ± 0.006^{d}	0.271 ± 0.001^{e}	0.349 ± 0.005^{f}
K10	0.475 ± 0.001^{a}	0.380 ± 0.005^{b}	0.368 ± 0.001^{b}	0.336 ± 0.006^{c}	0.276 ± 0.002^{d}	0.316 ± 0.001^{e}
K13	0.449 ± 0.001^{a}	0.476 ± 0.003^{b}	0.382 ± 0.001^{c}	0.387 ± 0.006^{c}	0.295 ± 0.002^{d}	0.372 ± 0.001^{c}
K14	0.462 ± 0.005^{a}	0.311 ± 0.002^{b}	0.395 ± 0.004^{c}	0.311 ± 0.003^{b}	0.281 ± 0.001^{d}	0.361 ± 0.003^{e}
K17	0.436 ± 0.002^{a}	0.406 ± 0.001^{b}	0.396 ± 0.025^{b}	0.334 ± 0.002^{c}	0.298 ± 0.003^{d}	0.325 ± 0.003^{c}
P-Value	0.001*					

**($P \leq 0.05$) indicates statistical significance.
Different small letter indicated present significant differences in same rows
Similar small letter indicated non-significant differences in same rows.

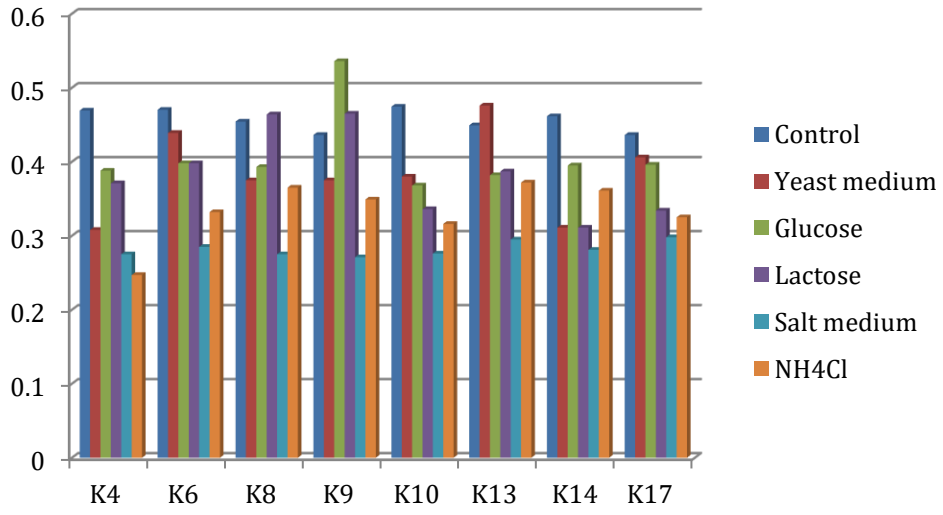

Figure 6. Effect of carbon and nitrogen sources on biofilm formation.

4. CONCLUSION

The study concluded that *K. pneumoniae* isolates obtained from sputum was 23(46%), urine 22 (44%), stool 2(4%) and tracheal pus 3(6%), as well as all bacteria studied were highly resistant to Cefepime, Ceftazidime, Ciprofloxacin, Levofloxacin and ceftriaxone, followed by Imipenem and Meropenem then Trimethoprim/Sulfamethoxazole, Tobramycin, Gentamicin, Amikacin, Aztreonam and high susceptibility to Piperacillin, Ticarcillin / Clavulanic Acid and Piperacillin /Tazobactam. Must isolates showed strong biofilm formation (26 isolates (52%) had strong biofilm formation, 15 isolates (30%) had moderate biofilm formation, and only 9 isolates (18%), were classified as having weak) and the best nutrients to enhance biofilm formation were glucose, lactose and yeast extract. There was also a strong effect at temperature (37°C, 25°C) and pH (7, 8,9) on biofilm formation. The best nutrients and physical condition to enhance biofilm formation were glucose, lactose and yeast extract. At temperature (37°C, 25°C) and pH (7, 8, 9). Recommendations studying the effect of other carbon and nitrogen sources (organic and inorganic) on biofilm formation, studying other factors that may inhibit biofilm formation and studying genes and gene expression that encoding biofilm.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical Clearance

The samples were obtained according to Local Research Ethics Committee approval in Iraqi Ministry of Health No. 42161 in 18/7/2023.

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