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Original Article

Investigation of Biofilm Formation and Antibiotic Resistant of Bacteria Isolated from Septic Neonates

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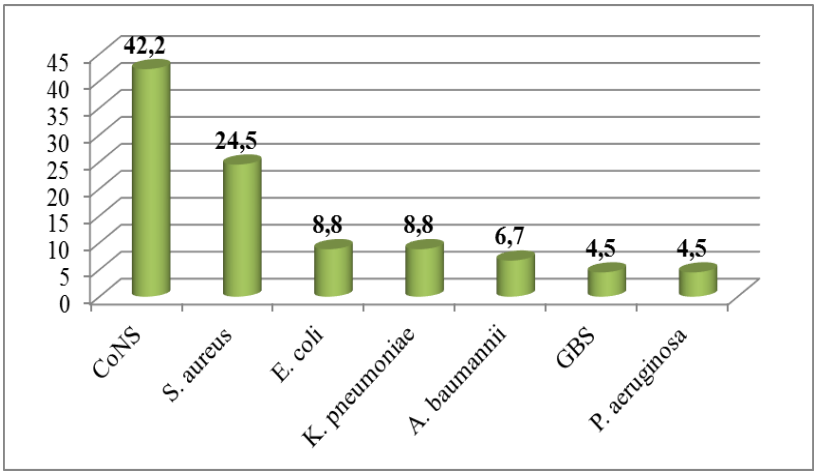
ABSTRACT

Neonatal sepsis refers to the bacterial bloodstream infections of the newborn during the neonatal period as usually the first twenty-eight days of life. The current study was done in the laboratories of AL-Batool Teaching Hospital for Gynecology and Pediatrics in Baqubah, Diyala Governorate, including 140 blood specimens collected from the neonates admitted to the hospital with suspected sepsis, the ages of the both groups was ranged from 1 day to 28 days. Out of the total cultured samples, 32.14% (45 of 140) were positive and 67.86% (95 of 140) were negative blood culture. 45 of 140 samples were negative to the blood culture chosen as control group. The results showed highest isolates were Coagulase Negative *Staphylococcus* (CoNS) 19 (42.2%), followed by *Staphylococcus aureus* 11 (24.5%), *Escherichia coli* 4 (8.8%), *Klebsiella pneumonia* 4 (8.8%), *Acinetobacter baumannii* 3 (6.7%), Group B *Streptococcus* (GBS), and *Pseudomonas aeruginosa* 2 (4.5%). Ceftazidime antibiotic has the highest resistance percentage followed by CIP, CXM, AMP, NA, C, CD, and CL among the studied bacterial isolates. Biofilm formation of isolates showed all bacterial isolates of *K. pneumonia*, *A.baumannii*, *P.aeruginosa*, and GBS by 100% can form biofilm, while the isolates of *S.aureus*, CoNS and *E.coli* were 6 (53.55%), 7 (36.84%), and 2 (50%) biofilm forming, respectively. These biofilm-forming isolates exhibited high resistance to AK, AMC, TM, CTX, NE, VAN, COT, CL, CAZ, AMP, NA, C, and CIP.

GRAPHICAL ABSTRACT



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Introduction

Sepsis in newborns is a substantial cause of morbidity and mortality. The severity of newborn sepsis varies depending on many factors. When comparing data from high-income countries with those from low-income and middle-income countries, different estimates of illness burden have been reported, and clinical symptoms range from asymptomatic infection to severe localized or systemic illness [1].

Blood cultures are the golden standards for diagnosis, but results are frequently delayed by 24 to 48 hours, and sensitivity is variable and affected by bacterial load despite improvements in the modern techniques, such as automated blood cultures. Hence, the empirical antibiotics are frequently administered to prevent the potentially fatal consequences of the untreated sepsis and the cause of this unnecessary antibiotic treatment is the negative results. The negative cultures present a difficulty to clinicians because they should distinguish between the actual sepsis and sepsis-like situations (non-infectious or viral) that do not require antibiotics. As a result, drugs should be stopped once sepsis has been ruled out [2].

The causative agent may be attributed to acquisition from the mother's flora, intrauterine infection, or postnatal acquisition from the community or hospital. The bacterial biofilms are intricate collections of one or more different kinds of bacteria, extracellular polymeric substances (EPS) bind them and keep them connected to the surfaces including live tissue, medical equipment, food, and industrial machinery [3, 4]. Biofilms are organized groups of different types of bacteria that are responsible for the majority of chronic and recurring infections, such as sepsis in infants. Infections caused by biofilm occur in approximately 65 to 80% of cases, and the bacteria associated with biofilm are usually antibiotic resistant [3].

In this regard, the present study sought to isolate and identify the bacterial species from the blood samples of neonates with sepsis, detect both the susceptibility of isolates to antibiotics, and the phenotypic of biofilm formation.

Materials and methods

The current study was done in the laboratories of AL-Batool Teaching Hospital for Gynecology and Pediatrics in Baqubah, Diyala Governorate, including 140 blood specimens collected from the neonates admitted to the hospital with suspected sepsis, their ages ranging from 1 day to 28 days. The blood specimens were collected from November 2021 to March 2022.

Five ml of blood were drawn from the infants and divided into three parts, 2 ml were injected into an EDTA tube to do a complete blood count (CBC) test, 1 mL was placed in a gel tube to assess the C-reactive protein, and the last 2 ml were injected into the blood culturing vial, and then incubated in the BacT/Alert instrument for 7 days. The growth of positive blood culture was sub-cultured on MacConkey agar, chocolate agar, and blood agar. After the growth of bacteria, the isolates were identified by microscopic examination, biochemical tests, analytical profile index 20E (API), and VITEK-2 system, by using the Kirby-Bauer method, the isolates' susceptibility to the specific antibiotics was evaluated [4-7].

Detection of biofilm formation

The tissue culture plates assay was the most widely used and was considered as standard test for the detection of biofilm formation. The effect of media composition on biofilm formation was also studied, so two media were used to assess the production of biofilms, tryptone soya broth (TSB), and TSB with 1% glucose.

A bacterial isolate suspension equivalent to the McFarland No. 0.5 turbidity standard was inoculated in TSB and incubated for 18 hours at 37°C in stationary condition before being diluted 1:100 with fresh TSB and TSB supplemented with 1% glucose.

To check for sterility and non-specific medium binding, 0.2 mL aliquots of the diluted cultures were placed in each well of a sterile, 96-well, flat-bottomed polystyrene tissue culture plate. The tissue culture plates were kept at 37 °C for a 24-hour incubation period. By gently tapping the plates, the contents of each well were gently removed. To get rid of bacteria that were floating

freely, the wells were then rinsed three times with phosphate-buffered saline (pH 7.2). Crystal violet (0.1%) and 2% sodium acetate were used to fix and stain the adherent organisms. Deionized water was used to remove any excess discoloration, and the plates were stored to dry. By using a micro-ELISA auto reader at a wavelength of 570 nm, the optical density (OD) of stained adherent biofilm was measured; the experiment was performed in triplicate and repeated three times.

The IBM SPSS version 28.0 was used to analyze the data statistically, the data expressed as mean, standard error of mean for parametric data, and the probability calculated by student's t-test, ANOVA table. While the non-parametric data are expressed as frequency and frequency percentages, the probability was calculated by using Pearson's chi-square. The probability was significant when it was less than 0.05.

Results and Discussion

The blood samples of total 140 patients were collected and cultured after inclusion and exclusion criteria. Out of the total cultured samples, 32.14% (45 of 140) were positive for blood culturing.

Culture identification

After the isolation, the characteristics of isolates were studied by culturing them on the differential media. All isolates were confirmed by microscopic examination stained with Gram stain. The results appeared that 71.11% (32 of 45) were Gram-positive, and 28.89% (13 of 45) were Gram-negative

All findings revealed that the most and the highest frequency percentage of the bacterial isolates were coagulase negative *Staphylococcus* (CoNS) (42.2%) followed by *Staphylococcus aureus* (24.5%), *Escherichia coli* (8.8%), *Klebsiella pneumonia* (8.8%), *Acinetobacter baumannii* (6.7%), GBS (4.5%), and *Pseudomonas aeruginosa* (4.5%), as displayed in Figure 1.

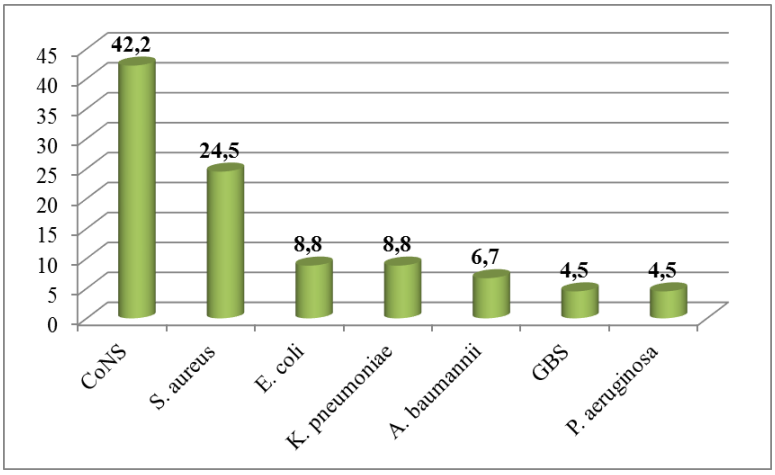

Figure 1: Distribution of pathogenic bacterial isolates from blood culture

Antibiotics susceptibility test

Concerning the antibiotic susceptibility test, Gram-positive bacterial isolates showed a high resistance for Chloramphenicol and Ceftazidime (78.1% for both), followed by Cefuroxime (71.8%), Ampicillin (62.5%), and showed a high sensitivity to Co. Trimoxazole (65.6%) followed by Netilin (59.3%), as listed in Table 1.

While, the results of antibiotic susceptibility for Gram-negative bacterial isolates showed a high resistance to Clindamycin (100%) followed by Clarithromycin and Ceftazidime (92.3% for both), Chloramphenicol and Ampicillin (84.6% for both), cefuroxime (69.2), and Amoxicillin-clavulanic acid (61.5%). In addition, it showed sensitivity to Amikacin (77%) followed by Co. Trimoxazole (61.5%), as presented in Table 2.

Biofilm detection

In addition, the result of biofilm formation frequency percentage showed that the bacterial isolates of GBS, *K. pneumonia, A. baumannii*, and

P. aeruginosa were 100% biofilm forming. While *S. aureus* isolates were 53.55% biofilm forming, CoNS isolates were 36.84% biofilm forming, *E. coli* isolates were 50% biofilm forming, as depicted in Figure 2.

The results in Figure 3 revealed the frequency percentage of the bacterial isolates according to the biofilm formation grade. The obtained results showed that 33.33% of the isolates have the ability to form strong biofilm, 24.44% were moderate and 42.22% were none or weak biofilm forming. In addition, the strong biofilm formation percentage of GBS was 100%, followed by *S. aureus* and CoNS (27.2% and 26.3%, respectively). While in the negative-gram bacterial isolates, *P. aeruginosa* and *A. baumannii* isolates were 100% strong biofilm forming, followed by *E. coli* and *K. pneumonia* isolates (50% for both).

Table 1: Antibiotic susceptibility frequency percentage of Gram-positive bacteria isolated from blood culture

Isolates		AMP	AK	C	CIP	NET	VAN	TE	CL	AMC	NA	COT	CD	TR	OF	CFX	CAZ	CXM
S. aureus	R	100	45.4	63.6	72.7	27.2	18.1	9	81.8	36.3	54.5	18.1	36.3	45.4	18.1	27.2	72.7	54.5
	I	0	27.2	0	0	9	36.3	27.2	9	18.1	18.1	9	18.1	18.1	9	27.2	27.2	36.3
	S	0	27.2	36.6	27.2	63.6	45.4	63.6	9	45.5	27.2	72.7	45.4	36.3	72.7	45.4	0	9
GBS	R	0	50	100	100	0	100	0	100	50	100	0	100	0	100	0	100	100
	I	50	0	0	0	0	0	0	0	0	0	50	0	0	0	50	0	0
	S	50	50	0	0	100	0	100	0	50	0	50	0	100	0	50	0	0
CoNS	R	47.3	26.3	52.6	63.1	26.3	10.5	26.3	73.6	52.6	73.6	26.3	52.6	47.3	63.1	36.8	78.9	78.9
	I	21.1	10.5	15.7	10.5	21	78.5	36.8	10.5	36.8	10.5	10.5	31.5	21	0	31.5	5.2	15.7
	S	31.6	63.1	31.5	26.3	52.6	31.5	26.8	15.7	10.5	15.7	63.1	15.7	31.5	36.8	31.5	15.7	5.2
Total	R	62.5	34.3	59.3	68.7	25	18.7	18.7	78.1	46.8	68.7	21.8	50	43.7	50	31.2	78.1	71.8
	I	15.6	15.6	9.3	6.2	15.6	46.8	31.2	9.3	28.1	12.5	12.5	25	18.7	3.1	31.2	12.5	21.8
	S	21.8	50	31.2	25	59.3	34.3	50	21.8	25	18.7	65.6	25	37.5	46.8	37.5	9.3	6.2
Total %		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 2: Antibiotic susceptibility frequency percentage of Gram-negative bacteria isolated from blood culture

Isolates		AMP	AK	C	CIP	NET	TE	CL	AMC	NA	COT	CD	TR	OF	CFX	CAZ	CXM
E. coli	R	75.0	0.0	50	100	0	50	100	50	75	50	100	25	50	25	100	100
	I	25.0	25.0	0	0	0	0	0	50	0	0	0	50	0	50	0	0
	S	0.0	75.0	50	0	4	50	0	0	25	50	0	25	50	25	0	0
K. pneumonia	R	100.0	25.0	100	50	75	75	75	75	50	75	100	75	50	50	100	50
	I	0.0	0.0	0	0	0	0	0	25	0	0	0	0	0	25	0	0
	S	0.0	75.0	0	50	25	25	25	0	50	25	0	25	50	25	0	50
A. baumannii	R	66.6	0.0	100	100	66.6	66.6	100	66.6	100	0	100	33.3	66.6	66.6	100	66.6
	I	33.3	0.0	0	0	33.3	0	0	33.3	0	0	0	33.3	33.3	33.3	0	33.3
	S	0.0	100.0	0	0	0	33.3	0	0	0	100	0	33.3	0	0	0	0
P. aeruginosa	R	100.0	50.0	100	50	0	100	100	50	50	0	100	50	0	50	50	50
	I	0.0	0.0	0	50	0	0	0	50	0	0	0	50	0	0	50	0
	S	0.0	50.0	0	0	100	0	0	0	50	100	0	0	100	50	0	50
Total	R	84.6	15.4	84.6	77	38.4	69.2	92.3	61.5	69.2	38.4	100	46.1	46.1	46.1	92.3	69.2
	I	15.4	7.6	0	7.6	7.6	0	7.6	38.4	0	0	0	30.7	7.6	30.7	7.6	7.6
	S	0.0	77.0	15.4	15.4	53.8	30.7	7.6	0	30.7	61.5	0	23	46.1	23	0	23
Total %	Total %	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

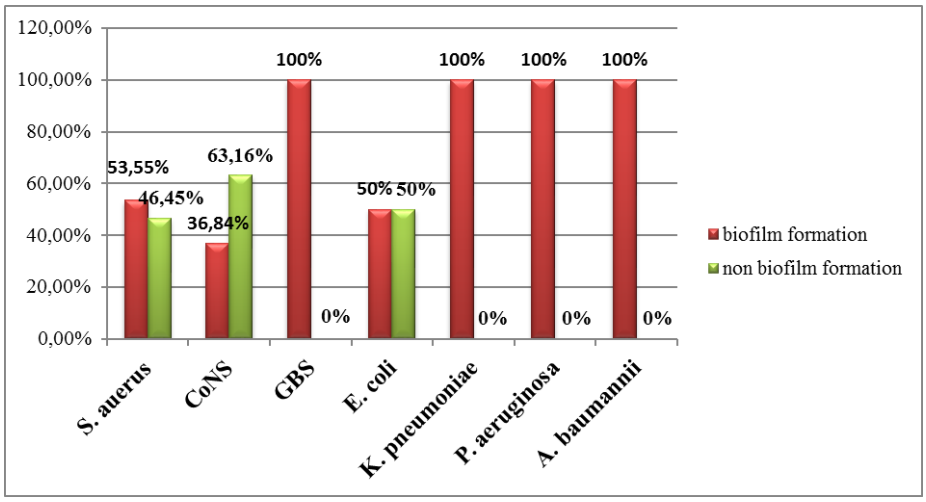

Figure 2: Biofilm forming percentage according to the biofilm and non-biofilm forming bacterial isolates

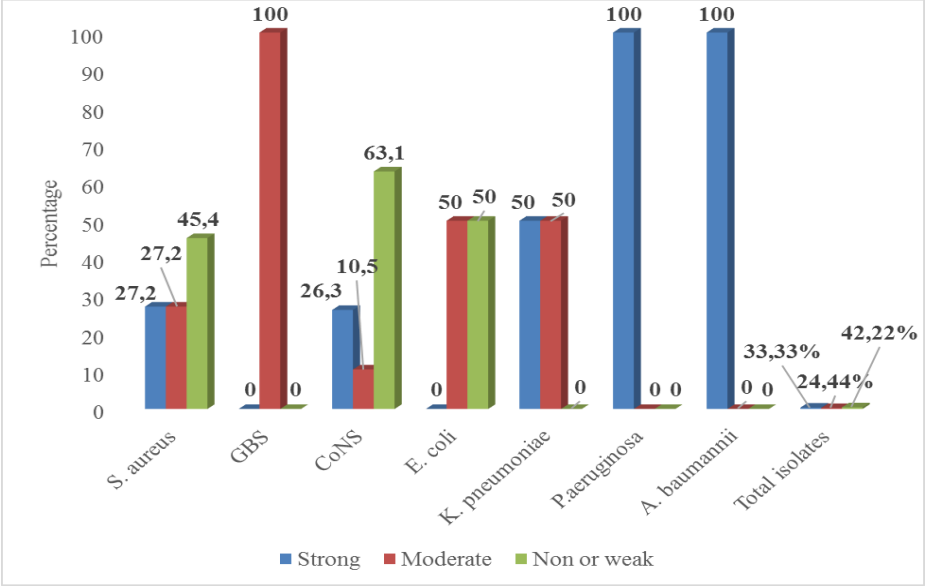

Figure 3: Biofilm formation grade by bacterial isolates

Also, Figure 4 depicted the results of antibiotics resistance for the biofilm forming bacterial isolates.

The results in Table 3 indicated the frequency percentage of antibiotics susceptibility for the biofilm formation bacterial isolates.

The current results showed that the most and highest isolates were CoNS which followed by *Staphylococcus aureus*. These results were resembled the studies conducted in Saudi Arabia showed that CoNS was the most prevalent bacterial isolates [9]. In contrast, many studies' results differed from the present results and demonstrated that the main causative pathogens of neonatal septicemia were Gram-negative bacteria, and it is increasingly common, especially in low- and middle-income countries along with rising worries about antibiotic resistance [10, 11]. Neonatal sepsis-causing microbes have evolved through time and differ noticeably from area to area. The etiology of the neonatal sepsis has been documented to have changed as a result of prematurity, the frequent catheter use, the use of complete parenteral feeding, and frequent antibiotic resistance to target the most likely bacteria for quick therapy when an infection is suspected [12]. The results in the current study of antibiotic susceptibility for the Gram-positive bacteria were in agreement with study in Erbil conducted the isolated of Gram-negative bacteria were highly resistant to ampicillin [11].

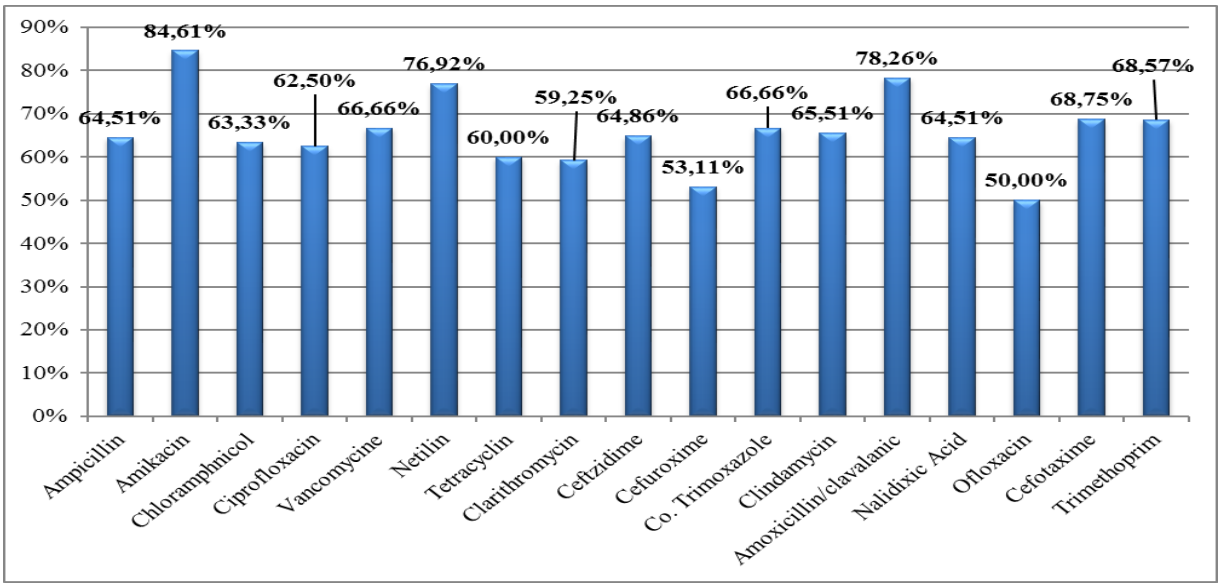

Figure 4: Antimicrobial resistance frequency percentage in the biofilm forming isolates

Table 3: The frequency percentage of antibiotics susceptibility according to the biofilm formation bacterial isolates

Antibiotic		Biofilm-forming bacterial isolates					
	S. aureus	GBS	CoNS	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*A. baumannii*
Ampicillin	100%	0%	57.1%	100%	100%	100%	66.6%
Amikacin	66.6%	50%	57.1%	0%	25%	50%	0%
Chloramphenicol	66.6%	100%	28.5%	100%	100%	100%	100%
Ciprofloxacin	100%	100%	57.1%	100%	50%	50%	100%
Vancomycin	16.6%	100%	14.2%	0%	0%	0%	0%
Netilin	50%	0%	28.5%	0%	75%	0%	66.6%
Tetracycline	0%	0%	14.2%	50%	75%	100%	66.6%
Clarithromycin	33.3%	50%	42.8%	100%	75%	100%	100%
Ceftazidime	100%	100%	85.7%	100%	100%	50%	100%
Cefuroxime	83.3%	100%	42.8%	100%	50%	50%	66.6%
Co. Trimoxazole	33.3%	0%	28.5%	50%	75%	0%	0%
Clindamycin	33.3%	100%	57.1%	100%	100%	100%	100%
Amoxicillin/clavalanic	50%	50%	85.7%	100%	75%	50%	66.6%
Nalidixic Acid	100%	100%	57.1%	100%	50%	50%	100%
Ofloxacin	16.6%	100%	42.8%	50%	50%	0%	66.6%
Cefotaxime	50%	0%	28.5%	50%	50%	50%	66.6%
Trimethoprim	50%	0%	85.7%	0.0	75%	50%	33.3%

Multidrug resistant (MDR) bacteria included both Gram-negative and Gram-positive strains, the resistance emergence of these bacteria as a result of indiscriminate antibiotic usage. There are numerous factors contributing to it, for example, many antibiotic prescriptions are written in clinical settings without the initial identification of the infectious germ or running an antibiotic sensitivity test. In addition, patients frequently do not take their medications exactly as prescribed, even when they begin to feel better. As a result, the bacteria are more likely to develop drug resistance. Because the newborn sepsis epidemiology varies greatly, it is challenging to compare antibiotic resistance across nations. Studies of multidrug-resistant bacteria that cause newborn sepsis, particularly in neonatal intensive care units (NICUs), are growing in developing countries [13, 14].

Regarding the results of biofilm-forming, it appeared that 53.55% of *S. aureus* isolates were biofilm-forming. This result approached the results of studies which showed the strong biofilms were created by approximately 50% of *S. aureus* isolates [15, 16]. While other studies demonstrated that 86%-99.2% of *S. aureus* isolates could form the biofilm [17, 18]. All of these findings indicated that the biofilms development is influenced by numerous variables, including environmental conditions, because *S. aureus* isolates are very sensitive to the environmental factors, such as environment, and availability of nutrients like the amount of glucose or glucosamine available for the matrix formation [19].

Regarding GBS, the present results showed all isolates were moderately formed the biofilm. The findings of the study supported the potential of GBS strains to produce biofilms; they reported that about 4% of the studied strains developed strong biofilms [20], while more than half of them produced the moderate ones. However, a study showed that 16.6% of GBS isolates formed biofilm only [21]. The stable colonization of pathogenic bacteria typically entails the development of biofilm and pili, it was interesting to investigate the potential role of pili in the colonization and biofilm development in GBS strains, because GBS is covered by pili, which leads to the advanced bacterial aggregation and adhesion to the host surfaces. A high-risk factor for neonatal meningitis and sepsis is maternal vaginal mucosa colonization with GBS that is not symptomatic [22].

While, the results of CoNS were similar to the study that indicated 42.1% of CoNS isolates produced biofilms. On the other hand, another previous study revealed a high percentage ratio of biofilm formation by CoNS isolates [23]. Accordingly, there are a number of elements that could contribute in biofilm production, including the environment, nutrition, sub-inhibitory concentrations of specific antibiotics, and stress (temperature, osmolarity), which could explain why different clinical isolates produce biofilms at diverse rates [24]. From another veiwpoint, the difference in the type of sample may play a role in the different rates of biofilm formation, as indicated by this study, showed that urinary isolates demonstrated a much higher percentage of the high biofilm production than that from the blood sample [25].

Concerning *K. pneumonia* isolates, the current findings were corroborated with the findings of study by [26] found about the ability form the biofilm from all isolates, while another study showed that from 75% of *K. pneumonia* isolates 20% were strongly biofilm formation [27], the presence of the polysaccharide capsule, fimbriae, and pili, iron metabolism, and diverse bacterial species, are some of the elements leading to the development of biofilms formation [28].

In addition, in the current results about *P.aeruginosa* showed that all isolates were strong biofilm forming, these results were similar to Iraqi study done by [29], who showed that all of *P. aeruginosa* isolates were strongly biofilm producer, and also matched the Iranian studies done by [30] and [31] who mentioned that all the isolates of *P. aeruginosa* were biofilm producers, while the other studies showed lower percentage of biofilm formation by *P. aeruginosa* [32, 33]. Therefore, the strains in the current study appeared to be more pathogenic, and produced more robust biofilms. This may be due to the type of sample or the random using of antibiotics. Furthermore, due to the insufficient sterilization of the medical devices, it led to the environmental selection in the strains that preferred the robust biofilm formation.

Finally, concerning *A. baumannii* isolates, the results showed that all isolates were strongly biofilm producer, the environmental survival of *A. baumannii* through the number of ways for extending antibiotic resistance and living on inanimate things, and resistance to environmental stress, in addition to its ability to endure in a hostile environment survive, the dormancy of bacterial cells was deep within the biofilm. Thus, *A. baumannii* has been designated as a human pathogen of red alert, because of its capacity to develop the resistance to all antimicrobial substances that are currently used for treatment [34].

Regarding the results of antibiotics susceptibility biofilm-forming isolates demonstrated a high resistance to antibiotics. The biofilm plays a significant role in pathogenicity by forming the mucous layer and the host's proteins, which provides the ideal environment for the growth of bacteria and their resistance to treatment [35]. The high percentages of the bacterial production of the biofilm in the current study may explain the high rate of bacterial resistance to most antibiotics. Therefore, the association between biofilm determinants and genes associated with antibiotic resistance or the development of antibiotic tolerance brought on by physiological changes in the bacterial cells brought on by the presence of the biofilm matrix, biofilm formation is typically associated with decreased antibiotic sensitivity [36].

Conclusion

The current study concluded that the frequency percentage of Gram-positive bacterial isolates was higher than the Gram-negative isolates. Regarding biofilm formation, GBS, *K. pneumonia*, *A. baumannii*, and *P. aeruginosa* were 100% strongly biofilm-forming compared with the other biofilm-forming bacterial isolates, and these biofilm forming isolates demonstrated a high resistance to the antibiotics.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

The author declared that they have no conflict of interest.

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