


# Identification and characterization of bacteria isolated from blood cultures at KAUH in Irbid, Jordan

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## ABSTRACT

Bloodstream infections are common in hospitalized patients and are associated with high morbidity and mortality. This study aims to describe the prevalence and characteristics of the bacteria isolated from positive blood cultures in patients hospitalized at King Abdullah University Hospital in Jordan, including biofilm formation tendency, antibiotic susceptibilities, and the spread of resistance genes. In this study, a total of 200 bacteria isolated from blood samples were identified and studied. About 50.5% of bacteria were Gram-negatives comprising mainly *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*), and 49.5% were Gram-positives, represented mainly by coagulase-negative *Staphylococci* (CoNS). Multidrug resistance was found in 54% of Gram-negative and 73.7% of Gram-positive isolates. Regarding antibiotic resistance genes, blaSHV was the most frequent  $\beta$ -lactamase, and blaVIM was the most frequent carbapenemase detected in Gram-negatives. *MecA* was the most frequent resistance gene found in CoNS and methicillin-resistant *Staphylococcus aureus*. Ninety-six isolates (48%) could form either strong, moderate, or weak biofilms. The minimum biofilm eradication concentration was higher than the minimum inhibitory concentration, increasing up to 1,000-fold. In conclusion, *E. coli* and *Staphylococci* represent the main causative agents for sepsis. The high frequency of antimicrobial resistance and biofilm formation in bacteremia-derived bacteria highlights the sepsis management challenge.

## 1. INTRODUCTION

Bloodstream infection (BSI) is a significant cause of morbidity and mortality in hospitalized patients. The incidence numbers vary substantially between patients, as affected by intact or impaired defense mechanisms, pathological states, and pharmacological conditions (Viscoli, 2016). The European Antimicrobial Resistance Surveillance System reports an absolute increase of 47% in BSI between 2002 and 2008 (De Kraker *et al.*, 2013). Other sources report more than 24,000 cases of BSI at

49 US hospitals over 7 years (Wisplinghoff *et al.*, 2004). Deaths associated with sepsis have been estimated to be around 5 million cases in high-income countries (Fleischmann *et al.*, 2016), and potentially higher numbers may be expected in low-income countries.

The most frequent pathogenic agents of bacteremia are *Staphylococcus* spp., *Streptococcus* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas* spp. (Al-Sweedan *et al.*, 2012; Cisterna *et al.*, 2001; Reimer *et al.*, 1997). A study conducted in 2001 at Irbid, Jordan, documented that the predominant pathogens isolated from blood specimens were Gram-positive bacteria, mainly *Streptococcus pneumoniae* and coagulase-negative *Staphylococcus* spp. The Gram-negative bacteria were mainly *E. coli*, *K. pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Yersinia* spp. (Nimri *et al.*, 2001).

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Of great concern in the management of sepsis is the ever-growing resistance to antibiotic agents, predicting poor prognosis and posing a challenge on treatment. In Gram-negative bacteria, the production of carbapenemases and extended-spectrum  $\beta$ -lactamases account for the most important causes of resistance, leading to treatment failure and further spread of resistance. For example, mortality rates of BSI caused by carbapenem-resistant *K. pneumoniae* can range between 30% and 60% and may approach 50% if colistin resistance is accompanied (Giacobbe *et al.*, 2015). Carbapenem-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* complex (*A. baumannii* cpx) have been reported to show mortality rates of 72% and 70%, respectively (Lee *et al.*, 2014, 2017). In Gram-positive agents, methicillin resistance represents the most concerning resistance factor that alters therapy. As many BSI are caused by *Staphylococcus aureus* (*S. aureus*), therapeutic choices are often reduced to vancomycin in methicillin-resistant *S. aureus* (MRSA), requiring additional monitoring to avoid toxic side effects. Although genetically coded resistance mechanisms are the main causes of antibiotic resistance, bacterial biofilm formation also shelters pathogens from therapeutic agents, making therapy regimens ineffective. Bacterial tolerance to antibiotics has been noticed to increase compared to the planktonic version of the cells (Spoering and Lewis, 2011). These are often formed on indwelling devices such as catheters or ventilators and might require the withdrawal of the device in case of being contaminated with bacterial biofilms, further complicating the therapy strategy.

As patterns of pathogens causing BSI change over the years, frequent monitoring is required for each region to provide up-to-date data to adjust infection control measures. In this work, we attempt to determine the current prevalence state of the bacteria causing BSI at King Abdullah University Hospital (KAUH), a private/referral clinical setting in Irbid, and representative for the northern region of Jordan. We assess their antimicrobial susceptibilities, along with the underlying genetic elements. We also assess their ability to form biofilms and determine minimum biofilm eradication concentration (MBEC) values in biofilm-forming strains.

## MATERIALS AND METHODS

### Bacterial isolation and identification

Two hundred bacterial isolates were collected in a 3-month period from positive blood cultures at the KAUH in Irbid, Jordan. The isolates were identified using the Vitek2 COMPACT BioMerieux system. Briefly, a 0.5 McFarland (MF) suspension of bacterial isolates was prepared using a 0.45% NaCl saline solvent. Bacterial suspensions were identified using the GN-ID and GP-ID cards to identify Gram-negative and Gram-positive isolates.

### Antimicrobial susceptibility testing

#### Kirby-Bauer disc diffusion

As previously described, antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method (Schwalbe *et al.*, 2007). Briefly, Mueller-Hinton plates were seeded with 0.5 MF bacterial suspensions, followed by the addition of antimicrobial discs. Upon incubation at 37°C for 24 hours, the inhibition zones were evaluated according to the 2018

CLSI breakpoints (Wayne, 2018). Antimicrobial agents tested included erythromycin (15  $\mu$ g), gentamycin (10  $\mu$ g), levofloxacin (5  $\mu$ g), penicillin (10  $\mu$ g), oxacillin (1  $\mu$ g), ampicillin (10  $\mu$ g), piperacillin/tazobactam (100/10  $\mu$ g), meropenem (10  $\mu$ g), trimethoprim/sulfamethoxazole (1.25/23.75  $\mu$ g), clindamycin (2  $\mu$ g), rifampicin (5  $\mu$ g), doxycycline (30  $\mu$ g), vancomycin (30  $\mu$ g), cephazolin (30  $\mu$ g), cefoxitin (30  $\mu$ g), ceftazidime (30  $\mu$ g), chloramphenicol (30  $\mu$ g), azithromycin (15  $\mu$ g), and colistin (10  $\mu$ g) (Oxoid, Basingstoke, UK).

### Minimum inhibitory concentration (MIC) via e-test

Isolates exhibiting strong biofilm formation in the quantitative method were included in this test. Antibiotic MICs were determined using graded antibiotic strips (*e*-tests), as previously described (Gontijo *et al.*, 2017). Briefly, bacterial suspensions were prepared at a concentration of 0.5 MF and seeded on a Mueller-Hinton agar plate. The plates were dried for 15 minutes at room temperature, followed by the addition of the *e*-test. These were allowed to adsorb to the agar, and plates were incubated at 37°C for 24 hours. The MIC was determined as the lowest antibiotic concentration at which no bacterial growth was observed. Antibiotics tested included gentamycin, levofloxacin, piperacillin/tazobactam, meropenem, trimethoprim/sulfamethoxazole, rifampicin, doxycycline, vancomycin, chloramphenicol, and azithromycin.

### MIC via broth microdilution

The MIC was determined using the microbroth dilution method described previously (Wiegand *et al.*, 2008). The final reaction volume was set at 200  $\mu$ l, and antibiotics tested include erythromycin, ceftazidime, cefazolin, and colistin.

### Biofilm production

#### Qualitative biofilm formation testing using Congo red agar (CRA)

The isolates were tested for biofilm production using CRA (Mariana *et al.*, 2009). Briefly, CRA was composed of 37% brain heart infusion broth, 50% sucrose, 10% agar, and 0.8% Congo red stain. The bacteria isolates were streaked onto CRA and incubated at 37°C for 24 hours. Isolates growing as black or Bordeaux-colored colonies were classified as biofilm formers, while those growing as red or pink colonies were classified as biofilm nonformers. Dark-colored colonies lacking dry crystalline morphology were classified as medium biofilm formers.

#### Quantitative biofilm formation testing using a microtiter plate assay

Quantitative evaluation of biofilm formation was determined using crystal violet as previously described (Mathur *et al.*, 2006; O'Toole, 2011). Briefly, fresh bacterial suspensions were prepared in trypticase soy broth and 1% glucose. About 200  $\mu$ l of a 0.5 MF dilution was added to a microtiter plate and incubated at 37°C for 24 hours. The uninoculated broth was used as a negative control. Upon incubation, plates were inverted and washed thoroughly with phosphate buffer saline (PBS). Biofilms were stabilized with 200  $\mu$ l 30% acetic acid for 15 minutes and stained with 0.1% crystal violet for 30 minutes. On washing, the

stain was solubilized with 200  $\mu$ l 95% ethanol, and absorbance was read at OD550. The test was carried out in triplicates, and average readings were used to classify biofilm formation. The optical density cut-off value (OD<sub>c</sub>) was determined as three standard deviations above the mean optical density of the negative control. The interpretation of biofilm formation was according to the following scheme: OD  $\leq$  OD<sub>c</sub> no biofilm formation, OD<sub>c</sub> < OD  $\leq$  2  $\times$  OD<sub>c</sub> weak biofilm formation, 2  $\times$  OD<sub>c</sub> < OD  $\leq$  4  $\times$  OD<sub>c</sub> moderate biofilm formation, and 4  $\times$  OD<sub>c</sub> < OD strong biofilm formation.

#### Minimum Biofilm Eradication Concentration (MBEC)

Strong biofilm formers were further evaluated for MBEC using various antimicrobial agents. The Calgary biofilm device was used for this assay (Feng *et al.*, 2013; Schwering *et al.*, 2013). Briefly, 150  $\mu$ l of a new 0.5 MF bacterial suspension, prepared in Mueller–Hinton broth (MHB), were incubated in a microtiter plate with a peg lid at 37°C for 18–24 hours, in a humidified atmosphere. Uninoculated MHB was used as a negative control. Upon incubation, the peg lids were washed with PBS and challenged in 200  $\mu$ l of antimicrobial agents at varying concentrations for 18–24 hours at 37°C. Upon incubation, peg lids were washed with PBS, and the remaining biofilms were allowed to recover in 200  $\mu$ l MHB for 18–24 hours at 37°C. Plates were subjected to 30–40 kHz for 10 minutes in a sonicator upon recovery. The peg lids were removed, and microtiter plates were incubated at 37°C overnight. MBEC was the lowest antimicrobial concentration revealing no visible bacterial recovery growth (Ceri *et al.*, 1999). The test was performed in triplicates. Antibiotics used to determine MBEC included piperacillin/tazobactam, meropenem, levofloxacin, gentamicin, aztreonam, vancomycin, rifampicin, erythromycin, doxycycline, chloramphenicol, ceftazidime.

#### DNA extraction for PCR

Genomic DNA was extracted using the Promega Wizard Genomic DNA Purification Kit. Briefly, 1 ml of fresh bacterial culture was pelleted and resuspended in 480  $\mu$ l 50 mM EDTA. *Staphylococcus aureus* strains were pretreated with 60  $\mu$ l 10 mg/ml lysozyme and 60  $\mu$ l 10 mg/ml lysostaphin and incubated at 37°C for 45 minutes. Upon pretreatment, extraction was performed according to the manufacturer's protocol. The extracts were eluted at 100  $\mu$ l for additional downstream applications.

#### PCR

##### Detection of carbapenemase genes

Gram-negative isolates were tested for the presence of KpC, NDM, and VIM carbapenemase genes, in a conventional multiplex PCR, using previously described primer sets (Poirel *et al.*, 2011) (Supplementary 1). The reaction mixture was composed of 12.5  $\mu$ l 2 $\times$  i-MAX II master mix (iNtRON Biotechnology, Seongnam-Si, Gyeonggi-d, Korea), 2  $\mu$ l DNA eluate, 1  $\mu$ l of each primer at a final concentration of 6.25 pmol/ $\mu$ l, and 4.5  $\mu$ l nuclease-free water. The PCR program was performed at the following conditions: 10 minutes of initial denaturation at 94°C, 36 cycles of 30 seconds at 94°C, 40 seconds at 52°C, and 50 seconds at 72°C, and a final extension step at 72°C for 5 minutes. Amplicons were detected in a 2% agarose gel electrophoresis at 130 V for 30 minutes.

##### Detection of extended-spectrum $\beta$ -lactamase (ESBL) genes

Gram-negative isolates were tested for the presence of the CTX, TEM, and SHV ESBL genes, in a conventional PCR using previously described primer sets (Supplementary 2) (Moghaddam *et al.*, 2012). The reaction mixture was composed of 12.5  $\mu$ l 2 $\times$  master mix, 3  $\mu$ l DNA eluate, 1  $\mu$ l of each primer at a final concentration of 6.25 pmol/ $\mu$ l, and 7.5  $\mu$ l nuclease-free water. The PCR program was performed under the following conditions: 5 minutes of initial denaturation at 94°C, 35 cycles of 30 seconds at 94°C; 30 seconds at 50°C, 52°C, and 56°C for blaCTX, blaTEM, and blaSHV, respectively; 30 seconds for blaCTX and blaTEM and 60 seconds for blaSHV at 72°C; and a final extension step for 5 minutes at 72°C. Amplicons were detected in a 2% agarose gel electrophoresis at 130V for 30 minutes.

##### Detection of resistance genes in *S. aureus*

*Staphylococcus aureus* strains were tested for the presence of nuc, ermA, ermB, ermC, mrs A/B, and mecA, in a conventional multiplex PCR, with previously described primer sets (Supplementary 3) (Fasihi *et al.*, 2017). The reaction mixture was composed of 12.5  $\mu$ l 2 $\times$  i-MAX II master mix (iNtRON Biotechnology, Seongnam-Si, Gyeonggi-d0, Korea), 2  $\mu$ l DNA eluate, 0.5  $\mu$ l of each primer at a final concentration of 10 pmol/ $\mu$ l, and 9.5  $\mu$ l nuclease-free water. The PCR program was performed at the following conditions: 5 minutes of initial denaturation at 96°C, 30 cycles of 45 seconds at 95°C, 2 minutes at 72°C, and a final extension step at 72°C for 5 minutes. Amplicons were detected in a 2% agarose gel electrophoresis at 130V for 30 minutes.

#### Statistical analysis

A chi-square test was used to find statistically significant levels of resistance among different bacterial BSIs. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Bacterial identification

Two hundred bacterial isolates from 200 positive blood cultures were included in this work. About 49.5% of isolates were Gram-positive and belonged to the genera *Staphylococcus* [6.5% MRSA and 43% coagulase-negative *Staphylococci* (CoNS)]. The remaining 101 isolates (51%) were Gram-negative bacteria, comprising the species *E. coli* (28%), *K. pneumoniae* (13%), *P. aeruginosa* 5%, *Enterobacter cloacae* complex (3%), and *A. baumannii* complex (1.5%) (Fig. 1).

### Antimicrobial susceptibilities

While all isolates of *P. aeruginosa* were susceptible to all tested antibiotics, higher resistance rates were observed in *A. baumannii* strains, as all were nonsusceptible to gentamicin and ceftazidime ( $p < 0.05$ ). About 67% of those were nonsusceptible to meropenem and piperacillin/tazobactam. The lactose-fermenting organisms were not susceptible to ampicillin but showed higher susceptibility percentages among the remaining antibiotics tested (Table 1) (Supplementary 4).

All isolated Gram-positive organisms were susceptible to vancomycin, doxycycline, and chloramphenicol but were tested nonsusceptible to penicillin. All *S. aureus* strains were tested as nonsusceptible to oxacillin in the double agar diffusion test,

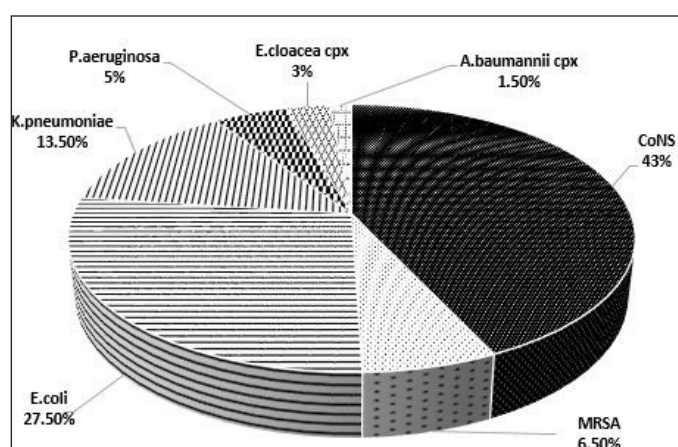
thus identifying MRSA (Table 2). CoNS were more resistant to erythromycin than MRSA ( $p < 0.05$ ).

Multidrug resistance (MDR) was observed in 94 Gram-positive isolates (94.9%), testing as nonsusceptible to three or more antimicrobial agents belonging to different categories, compared to 65 Gram-negative isolates (50.5%).

### Biofilm formation

Biofilm formation was not observed in 155 (77.5%) isolates tested using the CRA screening method. Among the remaining 45 biofilm producers (22%), CoNS and *E. coli* shared the highest rates with 27% and 29%, respectively (Fig. 2).

More detailed results were seen in the quantitative measurements as 31 of all isolates (15.5%) were identified as strong, 32 (16%) as moderate, and 33 (16.5%) as weak biofilm



**Figure 1.** Percentage of bacteria isolated from BSI. BSI, bloodstream infection; CoNS, coagulase-negative Staphylococci; MRSA, methicillin-resistant Staphylococcus aureus.

formers. Among the remaining 104 isolates (52%) unable to form biofilms in quantitative testing were mainly CoNS 43.2%, followed by *E. coli* (36.5%) (Table 3).

### MBEC

Among the 31 isolates, 14 CoNS, 1 *S. aureus*, 7 *K. pneumoniae*, 4 *P. aeruginosa*, 3 *E. cloacae cpx*, and 2 *E. coli* strains were quantitatively defined as strong biofilm formers, and assessed for their MBEC. Nine CoNS strains (64% of the CoNS tested) required a vancomycin MBEC  $\geq 500$   $\mu\text{g/ml}$  while exhibiting susceptible MIC values. Similar results were noticed with chloramphenicol, rifampicin, doxycycline, gentamycin, and levofloxacin, requiring a similar concentration in 5 (35.7%), 3 (21.4%), 3 (21.4%), 1 (7.1%), and 1 (7.1%) isolates, respectively. Only two isolates (14.2%) resistant to gentamycin, required MBEC values  $\geq 500$   $\mu\text{g/ml}$ . Similarly, high concentrations for biofilm eradication were only noticed with one strain nonsusceptible to levofloxacin (7.1%). All CoNS strains required an MBEC of  $\leq 0.5$  using erythromycin. All MBEC values  $\geq 500$   $\mu\text{g/ml}$  had susceptible MIC values to the corresponding antimicrobial agent (Table 4).

In 42 times, Gram-negative isolates required  $\geq 500$   $\mu\text{g/ml}$  of piperacillin/tazobactam, meropenem, levofloxacin, gentamicin, aztreonam, doxycycline, chloramphenicol, cefazolin, and ceftazidime to eradicate biofilms, while isolates grown as planktonic were susceptible to the corresponding antibiotic agent. Only in nine tests of 3 *K. pneumoniae* isolates, did a nonsusceptible MIC value corresponded to an MBEC value of  $\geq 500$   $\mu\text{g/ml}$  using aztreonam and doxycycline (Table 5).

### PCR

The prevalence of tested resistance genes in Gram-negative and Gram-positive bacteria is shown in Table 6 and Figure 3.

**Table 1.** Antimicrobial susceptibilities of Gram-negative isolates.

Antibiotics tested	<i>Escherichia coli</i>		<i>Klebsiella pneumoniae</i>		<i>Paeruginosa aeruginosa</i>		<i>Enterobacter cloacae cpx</i>		<i>Acinetobacter baumannii cpx</i>	
	N = 56		N = 26		N = 10		N = 6		N = 3	
	S	S%	S	S%	S	S%	S	S%	S	S%
AMP	56	100	26	100	-	-	6	100	-	-
TZP	10	18	9	35	0	0	1	17	2	67
CZ	31	55	12	46	-	-	6	100	-	-
MEM	4	7	1	4	0	0	0	0	2	67
GEN	13	23	16	62	0	0	3	50	3	100
AZM	12	21	3	12	-	-	0	0	-	-
DO	17	30	4	15	-	-	2	33.3	-	-
LEV	26	46	9	35	0	0	1	17	1	33.3
SXT	35	63	5	19	-	-	4	67	-	-
C	6	11	2	8	-	-	2	33.3	-	-
CT	-	-	-	-	0	0	-	-	0	0
CA	-	-	-	-	0	0	-	-	3	100

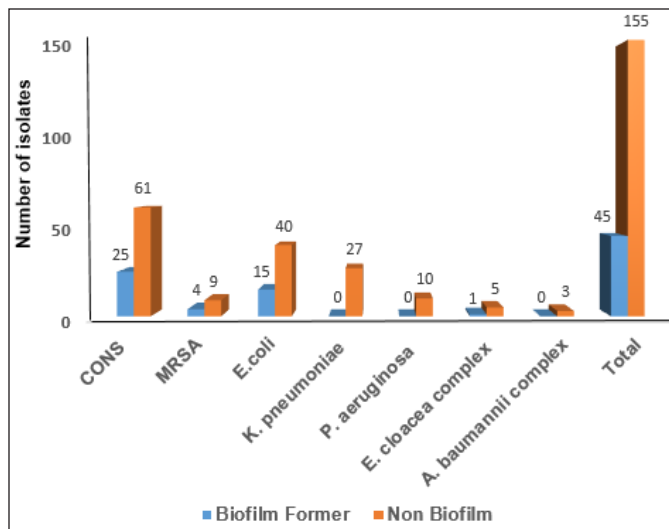
N, Number of tested isolates; S, number of susceptible isolates; S (%), percentage of susceptible isolates; AMP, Ampicillin (10  $\mu\text{g}$ ); TZP, Piperacillin-Tazobactam (100/10  $\mu\text{g}$ ); CZ, Cefazolin (30  $\mu\text{g}$ ); MEM, Meropenem (10  $\mu\text{g}$ ); GEN, Gentamicin (10  $\mu\text{g}$ ); AZM, Azithromycin (15  $\mu\text{g}$ ); DO, Doxycycline (30  $\mu\text{g}$ ); LEV, Levofloxacin (5  $\mu\text{g}$ ); SXT, Trimethoprim-Sulphamethoxazole (1.25/23.75  $\mu\text{g}$ ); C, Chloramphenicol (30  $\mu\text{g}$ ); CT, Colistin (10  $\mu\text{g}$ ); CAZ, Ceftazidime (30  $\mu\text{g}$ ).

(-), not tested.

**Table 2.** Antimicrobial susceptibility of Gram-positive bacteria isolated from blood.

Isolates	N	P		OX		VA		GEN		E		DO		LEV		DA		SXT		C		RD		FOX	
		S	S%	S	S%	S	S%	S	S%	S	S%	S	S%	S	S%	S	S%	S	S%	S	S%	S	S%	S	S%
CoNS	86	86	100	83	95.3	0	0	39	45.4	80	93	1	1.16	42	48.8	29	33.7	36	41.7	17	19.8	20	23.3	76	88.4
<i>Staphylococcus aureus</i>	13	13	100	13	100	0	0	1	7.7	7	53.8	0	0	2	15.4	1	7.7	1	7.7	0	0	1	7.7	11	84.6

N, Number of tested isolates; S, number of susceptible isolates; S (%), percentage of susceptible isolates; MRSA, methicillin-resistant *Staphylococcus aureus*; CoNS, coagulase-negative staphylococci; P, Penicillin (10 units); OX, Oxacillin (1 µg); VA, Vancomycin (30 µg); GEN, Gentamicin (10 µg); E, Erythromycin (15 µg); DO, Doxycycline (30 µg); LEV, Levofloxacin (5 µg); DA, Clindamycin (2 µg); SXT, Trimethoprim-Sulphamethoxazole (1.25/23.75 µg); C, Chloramphenicol (30 µg); RD, Rifampicin (5 µg); FOX, Cefoxitin (30 µg).



**Figure 2.** Qualitative biofilm formation test using Congo red agar.

The β-lactamase gene detected most frequently was SVH, found in 54 isolates (53.4%), mainly in *K. pneumoniae* and *E. coli*, in 21 (20.7%) and 23 (22.7%), respectively. Twenty-four isolates (23.7%) were positive for TEM mainly found in *E. coli* isolates, and 14 (13.8%) for CTX mainly found in *E. cloacae*. Carbapenemases were less frequently detected as only 15 isolates (14.8%) tested positive for VIM, mainly *K. pneumoniae*, followed by KPC and NDM, detected in 9 (8.9%) and 3 (2.9%), respectively. In CoNS, *mecA*, *ermA*, *ermB*, *ermC*, *nuc*, and *mrsA/B* were detected in 40 (46.5%), 11 (12.7%), 3 (3.4%), 24 (27.9%), 12 (13.9%), and 0 isolates, respectively. In MRSA, these were found in 7 (53.8%), 0, 0, 3 (23%), 1 (7.6%), and 0 isolates, respectively.

**DISCUSSION**

This work included a total sample volume of 200 bacterial species, isolated from positive blood cultures, derived from the KAUH. Gram-negative bacteria were isolated in 50.5% of the total sample size and Gram-positive ones in 49.5%. The most frequently isolated bacterial species were CoNS isolated in 43% of the times. This is consistent with previously described numbers in other regions (Gheybi *et al.*, 2008; Kirchoff and Sheagren, 1985) and with previous investigations conducted in the same clinical setting between 2003 and 2008, recovering CoNS from positive blood cultures as the most frequent species in 59% of the time (Al-Sweedan *et al.*, 2012). CoNS comprise multiple *Staphylococcus* species, such as *S. epidermidis*, *S. hominis*, *S. capitis*, *S. haemolyticus*, and others, and are acquired during sample withdrawal from the skin. Their recovery from blood cultures is often considered a contamination, of no etiological importance than an actual infection, with no further identification and therapy conducted. While this is true for most CoNS species, more attention has to be paid to *S. epidermidis*, as it is reported to be the causative agent of various bacterial infections, including BSI, especially in patients carrying implant devices (Namvar *et al.*, 2014). Therefore, further identification of *S. epidermidis* should be considered when isolating CoNS, accurately identifying the underlying cause of infection and allowing for appropriate therapy

**Table 3.** Quantitative biofilm formation test using microtiter plate.

	Strong biofilm former (S)	Moderate biofilm former (M)	Weak biofilm former (W)	Total number of biofilm former	Nonbiofilm former
<i>CoNS</i>	14	14	13	41	45
<i>Staphylococcus aureus</i>	1	1	3	5	8
<i>Escherichia coli</i>	2	5	10	17	38
<i>Klebsiella pneumoniae</i>	7	10	3	20	7
<i>Paeruginosa aeruginosa</i>	4	1	1	6	4
<i>Enterobacter cloacae cpx</i>	3	0	0	3	3
<i>Acinetobacter baumannii</i>	0	1	1	2	1

**Table 4.** MIC and MBEC results for Gram-positive strong biofilm formers.

Isolate	Antimicrobial agents tested													
	VAN		GEN		DO		LEVO		C		REF		E	
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
21.CoNS	0.5	≥500	0	0	0.38	25	0	0	1.5	≥500	0.006	≥500	0	0
27.CoNS	1	2.5	0	0	4	5	0.047	0.05	2	2.5	0	0	0	0
30.CoNS	0.75	≥500	4	≥500	0.38	25	0	0	0	0	0	0	0	0
33.CoNS	1	≥500	0	0	1	5	0	0	0	0	0	0	0	0
36.CoNS	0	0	0	0	0.25	2.5	0	0	4	25	0	0	0	0
98.CoNS	0.75	≥500	1.5	≥500	0.19	≥500	0	0	3	5	0.016	5	0	0
113.CoNS	0.5	0.5	0.75	5	0	0	0	0	1.5	5	0.008	5	0	0
119.CoNS	1.5	≥500	0	0	0.19	50	0	0	1.5	≥500	0.012	50	0	0
142.CoNS	1	5	0.125	5	0.19	≥500	0.094	0.5	3	5	0.012	≥500	0	0
145.CoNS	0.75	≥500	0	0	0.25	25	8	≥500	2	≥500	0.016	50	0	0
149.CoNS	0.75	2.5	0.5	2.5	1.5	2.5	0	0	2	50	0.002	0.005	0	0
150.CoNS	2	≥500	0.064	≥500	0.38	≥500	0.094	≥500	2	≥500	0.012	≥500	0	0
178.CoNS	0.5	≥500	0	0	0.19	50	0	0	2	≥500	0.008	50	0	0
197.CoNS	1	≥500	0	0	1	50	0	0	3	5	0	0	0	0
200.S. aureus	0.5	≥500	0.19	5	0.19	50	0.094	≥500	3	≥500	0.016	0.5	0.25	≥500

0, nonsusceptible; V, Vancomycin; GEN, Gentamycin; DO, Doxycycline; LEVO, Levofloxacin; C, Chloramphenicol; REF, Rifampicin; E, Erythromycin.

adjustment. Among the Gram-negative bacteria isolated was *E. coli*, the most identified species, comprising 27.5% of the total sample size, followed by *K. pneumoniae* with 13.5%. The most common Gram-negative bacteria recovered from blood cultures used to be *K. pneumoniae* in this region (Al-Sweedan *et al.*, 2012).

Antimicrobial susceptibility profiles revealed that piperacillin-tazobactam was effective in 64.3% of Gram-negative isolates. Chloramphenicol was effective in 69.3%, and azithromycin in 70.2% of these bacilli. Meropenem, empirical therapy for infections caused by Gram-negative bacteria, was effective in 92% of these isolates. Ampicillin, on the other hand, was ineffective in all lactose-fermenting isolates. Particular attention was paid to colistin, as global resistance concerns to this antibiotic have been reported in various regions (Zafer *et al.*, 2019). In our sample size, no resistance to colistin was observed in any of the nonfermenting lactose isolates. An alarming concern was the observed resistance to methicillin in 100% of *S. aureus* strains since previous values had been 36% in the same hospital during the past decade (Al-Sweedan *et al.*, 2012). The reason for optimism was given by the susceptibilities observed to vancomycin

and doxycycline, as all MRSA isolates were susceptible to these agents. MDR, on the other hand, defined according to previously set criteria (Magiorakos *et al.*, 2012), was observed in 64.3% of all Gram-negative isolates, along with 94.9% in Gram-positive isolates. Numbers that come in concordance with previous reports in which ESBL-producing Enterobacteriaceae were the most common MDR, followed by MRSA (Capsoni *et al.*, 2019).

When examining the underlying resistance genes in MRSA, 53.8% harbored the *mecA* gene, responsible for the production of penicillin-binding protein 2a, conferring resistance to methicillin. Methicillin resistance in the remaining MRSA isolates could be driven by the commonly reported homolog of *mecA* and *mecC* (García-Álvarez *et al.*, 2011; Shore *et al.*, 2011), emphasizing the need to screen for both genetic elements when defining MRSA. Among the resistance genes found in Gram-negative isolates,  $\beta$ -lactamases were more frequently found than carbapenemases, with *blaSHV* being the predominant gene found in 53.4% of tested species, followed by *blaTEM* in 23.7% of these isolates. An outcome achieved from other countries such as Saudi Arabia, South India, China, and Korea listed *blaTEM* as the predominant  $\beta$ -lactamase

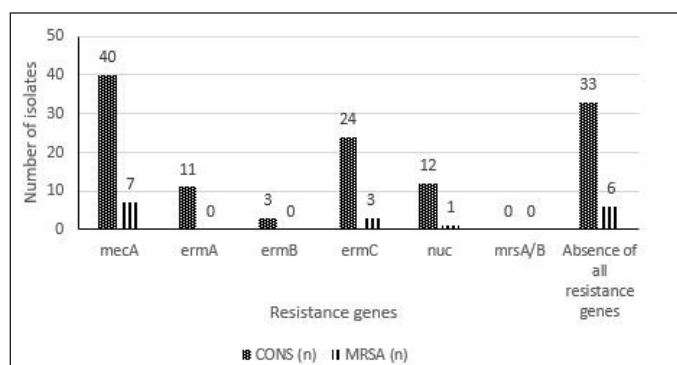
**Table 5.** MIC and MBEC results for Gram-negative strong biofilm formers.

ID # / isolate type	Antimicrobial agents tested																							
	PTZ		MEM		LEVO		GEN		AZM		DO		C		CZ		CAZ							
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC				
1. <i>E. cloacae</i> cpx	6	≥500	0.047	≥500	0.75	≥500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
34. <i>E. cloacae</i> cpx	0	0	0.047	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
61. <i>E. cloacae</i> cpx	1.5	≥500	0.047	0.05	0.032	0.05	0.25	≥500	12	≥500	0.5	≥500	1.5	≥500	1	≥500	0	0	0	0	0	0		
122. <i>E. coli</i>	2	≥500	0.032	≥500	0.5	≥500	0.38	≥500	0.032	≥500	8	≥500	2	≥500	0	0	0	0	0	0	0	0		
128. <i>E. coli</i>	0	0	0.047	≥500	0	0	0	0	24	≥500	0	0	3	≥500	0	0	0	0	0	0	0	0		
19. <i>K. pneumoniae</i>	2	≥500	0.032	≥500	0.032	≥500	0.19	≥500	6	≥500	1.5	≥500	1.5	≥500	0.25	≥500	0	0	0	0	0	0		
23. <i>K. pneumoniae</i>	0	0	0.047	25	0.75	≥500	0	0	8	≥500	0	0	3	50	0	0	0	0	0	0	0			
24. <i>K. pneumoniae</i>	3	≥500	0.047	≥500	0.5	≥500	0.25	≥500	0	0	4	≥500	3	≥500	0.25	25	0	0	0	0	0	0		
81. <i>K. pneumoniae</i>	1.5	2.5	0.047	25	0.032	2.5	0.25	0.5	12	0.5	0.5	2.5	1.5	2.5	≤1	2.5	0	0	0	0	0	0		
114. <i>K. pneumoniae</i>	0	0	0.032	≥500	0.38	2.5	0	0	2	≥500	24	≥500	1.5	≥500	0	0	0	0	0	0	0	0		
124. <i>K. pneumoniae</i>	2	≥500	0.047	25	0.047	2.5	0.25	50	8	≥500	0.5	5	1.5	2.5	0.25	50	0	0	0	0	0	0		
138. <i>K. pneumoniae</i>	3	5	0.064	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
22. <i>P. aeruginosa</i>	2	≥500	0.047	≥500	0	0	0.25	≥500	12	≥500	0	0	0	0	0	0	0	0	0	0	0	0		
131. <i>P. aeruginosa</i>	1.5	≥500	0.064	≥500	0.25	≥500	0.5	≥500	0	0	0	0	0	0	0	0	0	0	0	0	2	≥500		
151. <i>P. aeruginosa</i>	0	0	0.125	0.5	0	0	0.5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5		
152. <i>P. aeruginosa</i>	4	≥500	0.125	50	1	≥500	0.5	50	0	0	0	0	0	0	0	0	0	0	0	0	2	≥500		

0, nonsusceptible; PTZ, Piperacillin-Tazobactam; MEM, Meropenem; LEVO, Levofloxacin; GEN, Gentamicin; AZM, Azithromycin; DO, Doxycycline; C, Chloramphenicol; CZ, Cefazolin; CAZ, Cefazidime.

**Table 6.** The frequency of beta-lactamase gene and carbapenemases genes among Gram-negative bacteria.

	<i>Escherichia coli</i> (n 55)	<i>Klebsiella pneumoniae</i> (n 27)	<i>Paeruginosa aeruginosa</i> (n 11)	<i>Enterobacter cloacae cpx</i> (n 6)	<i>Acinetobacter baumannii</i> (n 3)	Total
blaSHV	23	21	7	1	2	54
blaTEM	20	2	0	2	0	24
blaCTX	4	5	0	5	0	14
blaKPC	0	6	1	2	0	9
blaNDM	0	1	1	1	0	3
blaVIM	0	6	3	4	2	15
Absence of all beta-lactamase genes	11	4	3	1	1	20
Absence of all carbapenemase genes	55	18	7	4	1	78

**Figure 3.** The frequency of methicillin resistance *S.aureus* nuc, mecA and mrsA/B and erythromycin and clindamycin-resistant genes ermA, ermB and ermC.

gene found (Al Sheikh *et al.*, 2014; Men *et al.*, 2013; Rameshkumar *et al.*, 2016; Shin *et al.*, 2009). Among the carbapenemases detected was blaVIM, the most frequent gene detected in 14.8% of all Gram-negative isolates, followed by blaKPC seen in only 8.9% of these isolates. Although different from other regions where KPC accounts for one of the most carbapenemases found, the lower prevalence of this resistance gene could be explained by our lower incidences of *K. pneumoniae*, as KPC is found mainly in this species found, the lower prevalence of this resistance gene could be explained by our lower incidences of *K. pneumoniae*, as blaKPC is found mainly in this species (Han *et al.*, 2020).

Additional resistance due to biofilm production was assessed by determining the ability of all isolates to form biofilms, using quantitative and qualitative methods, with the subsequent measurement of antibiotic concentrations required to eradicate those biofilms. While only 22.5% of biofilm formers were detected quantitatively using CRA, more accurate results were achieved by the microtiter plate method. A result that might be contributed to the media's nutritious conditions in the microtiter plate method as previously described (Reisner *et al.*, 2006). About 48% were identified as either strong, moderate, or weak biofilm formers with almost equal proportions. CoNS were the predominant species that formed biofilms, followed by *E. coli*. When comparing the MIC values with MBEC values of biofilm formers, data indicate that concentrations required to eradicate biofilms are higher than the MIC. In 66% of all biofilm-forming *Staphylococci*, a vancomycin concentration >500 µg/ml was required, despite susceptible MIC values. A similar increase in MBEC values was noticed in

Gram-negative isolates in all tested antibiotics. The result is not surprising, as some reports estimate a 10–100-fold concentration of antibiotics needed to eradicate bacteria in biofilms (Ceri *et al.*, 2001).

## CONCLUSION

This work provides an up-to-date state on the main causative agents for BSI, emphasizing the ever-growing issue of antibiotic resistance and the therapy challenge posed by biofilm-forming bacteria in a clinical setting sample in Irbid, Jordan. It highlights the need for novel therapy and disinfection strategies to eradicate these organisms and minimize the consequences posed by such pathogens. Although some of our results appeared to be unchanged compared to previous values, others showed some variation in prevalence in different regions and the same clinical setting a decade later. Again, constant monitoring of clinically relevant microorganisms and virulent characteristics is emphasized on, as infection control measures can only be successful with accurate numbers.

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## AUTHOR CONTRIBUTIONS

Nid'a Alshraiedeh has substantially contributed to conceptualization, data curation, design of study, analysis and interpretation of the data, funding acquisition, project administration, resources, supervision, and writing the original draft and revising it critically for intellectual contents.

Majd Alzedan has substantially contributed to the design of the work, material preparation, acquisition of data, analysis, interpretation of data, and writing the original draft.

RashaBani Salameh has substantially contributed to analysis, interpretation of data, and writing the original draft.

Rawan Alsharedeh has substantially contributed to the conception, design of the work analysis, interpretation of data, and writing the original draft.

Majed Masadeh has substantially contributed to the conception and critical revision of draft for important intellectual content.

Enas Alrafayah, Toga Zghoul, and Farah Atawneh have substantially contributed to analysis, interpretation of data, and critical revision of the draft for important intellectual content.



All authors have reviewed and approved the final version of the manuscript. In addition, all authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest for this manuscript.

## ETHICAL APPROVALS

The study was approved by the institutional review board (IRB) of the Jordan University of Science and Technology and KAUH. Informed consent form was waived by the IRB of the Jordan University of Science and Technology and KAUH (IRB ref: 24/124/2019, date 21.5.2.19). All methods were carried out in accordance with approved guidelines and regulations.

## DATA AVAILABILITY

All data generated and analyzed during this study are included in this published article and its supplementary information files.

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## SUPPLEMENTARY MATERIALS

**Table 1.** Primers used for amplification of carbapenemase genes s (Poirel *et al.*, 2011).

Gene	Primer	Primer sequence 5'-3'	PCR product size (bp)
<i>blaKPC</i>	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	798
	KPC-Rm	CTTGTCATCCTTGTTAGGCG	
<i>blaNDM</i>	NDM-F	GGTTTGGCGATCTGGTTTTC	621
	NDM-R	CGGAATGGCTCATCACGATC	
<i>blaVIM</i>	VIM-F	GATGGTGTTTGGTCGCATA	390
	VIM-R	CGAATGCGCAGCACCAG	

**Table 2.** Primers used for amplification of ESBL genes (Moghaddam *et al.*, 2012).

Gene	Primer	Primer sequence 5'-3'	PCR product size (bp)
<i>blaTEM</i>	TEM-F	ACATGGGGGATCATGTAACT	421
	TEM-R	GACAGTTACAATGCTTACT	
<i>blaSHV</i>	SHV-F	ATGCGTTATATTCGCCTGTG	859
	SHV-R	AGCGTTGCCAGTGCTCGATG	
<i>blaCTX</i>	CTX-MU1	ATGTGCAGYACCAGTAARGT	593
	CTX-MU2	TGGGTRAARTARGTSACCAGT	

**Table 3.** Primers used for amplification of resistance genes in Gram-positive isolates (Fasihi *et al.*, 2017).

Gene	Primer	Primer sequence 5'-3'	PCR product size (bp)
<i>nuc</i>	F	GCG ATT GAT GGT GAT ACG GTT	279
	R	AGC CAA GCC TTG ACG AAC TAA AGC	
<i>mecA</i>	F	TCC AGA TTA CAA CTT CAC CAG G	162
	R	CCA CTT CAT ATC TTG TAA CG	
<i>ermA</i>	F	TAT CTT ATC GTT GAG AAG GGA TT	139
	R	CTA CAC TTG GCT TAG GAT GAA A	
<i>ermB</i>	F	CTA TCT GAT TGT TGA AGA AGG ATT	142
	R	GTT TAC TCT TGG TTT AGG ATG AAA	
<i>ermC</i>	F	AAT CGT CAA TTC CTG CAT GT	297
	R	TAA TCG TGG AAT ACG GGT TTG	
<i>mrs(A/B)</i>	F	GCA AAT GGT GTA GGT AAG ACA ACT	402
	R	ATC ATG TGA TGT AAA CAA AAT	