MICROBIOLOGICAL AND MOLECULAR STUDIES OF SOME GRAM NEGATIVE PATHOGENS CAUSING BLOOD STREAM INFECTIONS IN INTENSIVE CARE UNITS

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ABSTRACT

Bloodstream infections (BSIs) and especially central line associated bloodstream infections (CLABSIs), are increasing globally as an important cause of morbidity and mortality. The condition can be life threatening in critically ill patients in the intensive care units (ICUs) of the hospitals. There are serious concerns about the rapid increase of multidrug resistant Gram negative bacteria (MDR-GNB) such as *Escherichia coli*, *Klebsiella pneumonia* and *Acinetobacter baumannii* as the major causes of bloodstream infections. The aim of the study was to emphasize on the significance of MDR-GNB, determine their antibiotic susceptibility pattern, genotypic identification as well as the detection of some virulence genes. Blood specimens were collected from 231 bacteremic patients admitted to different ICUs at three tertiary care hospitals in Egypt. Recovered bacterial isolates were subjected to biochemical identification using conventional methods. Antimicrobial susceptibility testing was performed on Gram negative isolates using both Kirby-Bauer disc diffusion method and Vitek-2 system. Thirty MDR-GNB isolates were selected and screened for the presence of *16S-23S rRNA, UIDA* and *OMPA* genes using PCR assays to confirm their identification. Also, the thirty isolates were screened for the presence of *East-1*, *Cnf-1*, *IutA* and *FyuA* genes. A total of 185 clinical isolates were recovered from 185 positive blood specimens. Laboratory examination of the positive blood culture specimens revealed that 120 (64.4%), 56 (30.2 %) and 9 (4.8%) were Gram negative, Gram positive and Candida spp., respectively. Thirty MDR-GNB were selected and their antimicrobial susceptibility was compared using the conventional and the Vitek-2 system. For the *K. pneumoniae* isolates 100% Categorical agreement (CA) was detected for Ceftriaxone, Cefepime, Gentamicin, Amikacin and Tetracycline. For the *E. coli* CA of (100%) was seen for Ampicillin+Sulbactam, Cefepime, Amikacin, Ciprofloxacin and Tigecycline. categorical agreement (100%) was seen with the tested antibiotics for *A. baumannii*. Genotypic confirmation of the selected MDR isolates indicated the presence of genes used for identification in all of them. As for the harboring of virulence genes, *EAST-1* gene has only been found in 13.3% of the isolates. *CNF-1* gene has been identified in 66.6% of the isolates, *IutA* gene has been found in 96.6% of the isolates and FyuA has been found in 90% of the isolates. Gram negative pathogens were found to be the major bacteria causing bloodstream infection. Vitek-2 system provide accurate antimicrobial susceptibility testing results. CNF-*1* toxin production related gene was found to be

prevalent in most of the isolates. *IutA* and *FyuA* siderophore formation related genes were found to be prevalent in most of the isolates.

Keywords: central line associated bloodstream infections**,** *Escherichia coli***,** *Klebsiella pneumonia, Acinetobacter baumannii,* Vitek-2 system,

Introduction

Central line associated bloodstream infection (CLABSI) is a primary laboratory confirmed bloodstream infection (BSI) occurring in patients who had a central venous line within 48 hours' period before the development of bacteremia. Patients who are admitted to intensive care units (ICUs) require management of life threatening conditions. The treatment and the procedures that patients in ICU need are very complex making central venous lines (CVLs) essential. Parenteral nutrition, high volume intravenous fluids, medication administration, cardiovascular measurements and blood infusions all require the use of CVLs. **(Rode *et al.*, 2017).**

There has been a huge increase in the number of infections caused by antibiotic-resistant strains of Gram negative bacilli in the past years and this has had an important impact on the outcomes of BSIs and are becoming an important cause of community acquired infections. These organisms are resistant to many of the antimicrobial agents usually recommended for the treatment of these infections **(Tumbarello *et al*.,2010).**

Escherichia coli, *(E. coli*), *Klebsiella pneumonia* (*K. pneumonia*) and *Acinetobacter baumannii* (*A. baumannii*) are capable of causing serious infections in hospitalized patients and also have been linked with cases of septicemia, meningitis, endocarditis, skin infections, urinary tract infections and respiratory tract infections. The dissemination and prevalence of these bacteria is related to their features such as the ability to survive dehydration and persist for long periods of time in the hospital environment, inheritance of the ability to acquire antibiotic resistance genes and large rate of nosocomial transmission. The presence of virulence factors (VF) is associated with pathogenesis in bacteria. Virulence factors help bacteria to colonize on the epithelium, getting nutrients from the host and producing toxins to inhibit the host's immune response **(Momtaz *et al.*,2015)**.

A great variety of virulence factors are encoded in these virulence genes (VGs), including adhesions or fimbriae, toxins, iron-acquisition systems (called siderophores). All of these factors are responsible for pathogenicity of Gram negative bacilli **(Palm *et al*., 2016)**. The aim of this study was comparing the antimicrobial susceptibility testing of GNB isolated from blood specimens using the conventional method and the Vitek-2 system. As well as, the molecular identification and screening of some virulence genes among some of the recovered MDR-GNB.

Materials and Methods

A-Materials

The majority of the culture media was provided by HiMedia, India. The antimicrobial discs were provided by Bioanalyse, Turkey. DNA purification kit by Thermoscientific, Lithuania. PCR primers were provided by Willowfort, UK. The thermocycler was 2720, applied bio systems, life technologies, USA.The ladder was Thermo Fischer Scientific, UK. The master mix was COSMO Thermo Fischer Scientific, UK and the Vitek-2 system was provided by BioMerieux, France.

B-Methods

Clinical specimens and patient data

Blood specimens were collected from 231bacteremic patients admitted to the ICU from three tertiary care hospitals in Egypt over the 34-month period from May 2018 to March 2021. The three hospitals were Sayed Galal hospital (SGH), Ain Shams university hospitals (ASUH) and International medical center (IMC). Patients were of different age groups from infants of 1 year to older patients up to 76 years. These patients were admitted to different ICUs such as medical ICUs, surgical ICUs, neurological ICUs, burn ICUs and cardiac care units.

Identification and antimicrobial susceptibility testing for Gram negative isolates

Identification of the isolates was performed according to Bergey's manual of determinative bacteriology 9th edition **(Holt *et al*., 1994)**. The antibiotic susceptibility was analyzed by the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute 2020 (CLSI). The concentrations of the used antibiotic discs (expressed in μg) were: Amikacin (30), Ampicillin/Sulbactam (10/10), Ceftriaxone (30), Cefepime (30), Ciprofloxacin (5), Gentamicin (10), Tetracycline (30), Meropenem (10), Ertapenem (10), and Tigecycline (15). The zones of inhibition determined whether the microorganisms were susceptible, intermediately sensitive or resistant to each antibiotic. The standard strains of *K. pneumonia* ATCC 700603, E. *coli* ATCC 25922, and *A. baumannii* ATCC 19606 were used for the quality control of antimicrobial susceptibility tests. Final confirmation of suspected Gram negative isolates along with antibiotic susceptibility profiles was performed with Vitek-2® system (BioMerieux® -France). Interpretative values for susceptibility testing obtained by Vitek-2 were compared to those obtained with the conventional methods.

Genotypic confirmation of Gram negative isolates by polymerase chain reaction method

The polymerase chain reaction (PCR) was done for the verification of some of, *K. pneumonia, E. coli and A. baumannii* strains by targeting *16S-23S ribosomal RNA* (*16S-23S rRNA*), *UIDA* and *OMPA* genes respectively. The bacterial genomic DNA was extracted from overnight cultures using a DNA purification kit (Thermo Fisher Scientific, United States) according to manufacturer's protocol. After extraction the

PCR reaction was carried in a final volume of 20 µL having 2 µL of each primer (forward and reverse), 10 µL of PCR master mix (Willow fort, United Kingdom) and 4 µL of a DNA extract. All primers used and the description of identification genes are listed in **Table (1)**. PCR thermo cycling conditions are described in **Table (2)**.

Table (1). Primers used for targeting *16S-23S rRNA, UIDA and OMPA* genes.

Identification Gene	Description	Primer sequence (5′ → 3′)	Amplicon size (bp)	source or reference
16S-23S rRNA	16S-23S ribosomal RNA	(F) ATTTGAAGAGGTTGCAAACGAT (R) TTCACTCTGAAGTTTTCTTGTGTTC	130	**(Derakhshan *et al*.,2016)**
UIDA	Beta-glucuronidase A	(F) TGGTAATTACCGACGAAAACGGC (R) ACGCGTGGTTACAGTCTTGCG	162	**(Joy *et al* .,2021)**
OMPA	Outer membrane protein A	(F) TCTTGGTGGTCACTTGAAGC (R) ACTCTTGTGGTTGTGGAGCA	85	**(Bardbari *et al*.,2017)**

Table (2). PCR thermo cycling conditions.

Gene	Initial Denaturation °C / Time	Denaturation °C / Time	Annealing °C / Time	Extension °C / Time	Final extension °C / Time	Number of Cycles	References
16S-23S rRNA	94°C / 5 min	95°C /1 min	58°C /1 min	72°C /1 min	72°C /7 min	35	**(Derakhshan *et al*.,2016**
UIDA	94°C /3 min	94°C /30 s	52°C /30 s	68°C /30 s	68°C /5 min	30	**(Joy *et al* .,2021)**
OMP A	94°C /5 min	94°C /1 min	50°C /1 min	72°C /45 s	72°C /5 min	30	**(Bardbari *et al*.,2017**

Molecular detection of virulence genes

PCR was used to detect genes as *EAST-1, CNF-1, IutA and FyuA* in *K. pneumonia, E. coli and A. baumannii* isolates. All primers used and the description of virulence genes are listed in **Table (3)**. PCR thermo cycling conditions are described in **Table (4)**.

Table (3). Primers used for detection of *EAST-1, CNF-1, IutA and FyuA* genes.

Virulence Gene	Description	Primer sequence (5′ → 3′)	Amplicon size (bp)	source or reference
Toxins				
EAST-1	Enteroaggregative heat-stable enterotoxin-1	(F) ATGCCATCAACACAGTATAT (R) GCGAGTGACGGCTTTGTAGT	110	**(Palma *et al*., 2016)**
CNF-1	Cytotoxic necrotizing factor-1	(F) AAGATGGAGTTTCCTATGCAGGAG (R) CATTCAGAGTCCTGCCCTCATTATT	498	**(Palma *et al*., 2016)**
Siderophore				
IutA	Iron uptake transport receptor	(F) GGCTGGACATCATGGGAACTGG (R) CGTCGGGAACGGGTAGAATCG	300	**(El Fertas-Aissani *et al*., 2013)**
FyuA	ferric yersiniabactin uptake receptor	(F) TGATTAACCCCGCGACGGGAA (R) CGCAGTAGGCACGATGTTGTA	880	**(Momtaz *et al*.,2015)**

F: Forward **R:** Reverse **bp:** Base pair

Table (4). PCR thermo cycling conditions.

Gene	Initial Denaturation °C / Time	Denaturation °C / Time	Annealing °C / Time	Extension °C / Time	Final extension °C / Time	Number of Cycles	References
EAST-1	95°C /4 min	94°C /50 s	58°C /1.5 min	72°C /1.5 min	72°C /10 min	35	**(Boisen *et al.*, 2012)**
CNF-1	95°C /4 min	95°C /50 s	58°C /1 min	72°C /45 s	72°C /8 min	30	**(Momtaz *et al.*, 2015)**
IutA	94°C /4 min	94°C /30 s	63°C /40 s	72°C /1 min	72°C /10 min	30	**(El Fertas-Aissani *et al.*, 2013)**
FyuA	95°C /4 min	95°C /50 s	58°C /1 min	72°C /45 s	72°C /8 min	30	**(Momtaz *et al.*, 2015)**

Results

Microbial isolation, identification and antimicrobial susceptibly pattern

231 blood specimens were collected by nurses from the peripheral vein. Blood culture bottles (Oxoid Signal®) were prepared for inoculation before taking the blood specimens. Aseptically, a maximum volume of 1 ml of venipuncture blood were injected into the bottle. The blood was thoroughly mixed with the broth in the bottle and sent to the laboratory for cultivation and subsequent processing. The inoculated bottles were placed in an incubator at 36 ± 1°C then removed from the incubator and the rubber stopper of the bottles was disinfected by alcohol swabbing. The growth indicator device was placed on to the neck of the blood culture bottle. Examination of the system for a positive result was carried out at least twice daily. The total incubation period was at least seven days. A positive blood culture, indicating growth of micro-organisms was recognized by the appearance of the blood/broth mixture in the transparent growth indicator device above the level of the locking sleeve.

A total of 185 blood specimens were shown to be positive for growth. Preliminary isolation was done from the blood culture bottles that have shown positive results. This was performed by inoculating 2 to 3 loopfulls of blood on different culture media. The media used for isolation were Mueller Hinton agar and blood agar as enriched media and MacConkey agar as a selective media appropriate for isolation of GNB.The specimens were inoculated on the corresponding culture medium by swabbing and streaking for isolation. The plates were then incubated at 37º C from 24-48 hours and after incubation the plates were examined. This is repeated again for the last time after 7 days before blood culture is considered free of microorganisms (negative).

From the positive blood specimens, 185 microbial isolates were recovered. For Gram negative bacteria, isolates were cultured on several growth media as MacConkey agar, Eosin methylene blue (EMB) and CHROMagar media. Appearance of pink or yellow colonies on MacConkey agar differentiated our isolates into lactose fermentor and non-lactose fermentor. Presence of green metallic sheen colonies on EMB confirmed presence of *E. coli* isolates and the presence of pink colonies confirms *K. pneumoniae* isolates. CHROMagar media inhibits the growth of most bacteria and employs a color-change identification method that allows *A. baumannii* to appear as red colonies. These Laboratory examinations of the positive blood isolates along with other

biochemical tests revealed that out of the 185 collected isolates, 120 were Gram negative. The major organisms identified among the 120 recovered gram negative isolates along with final conformation using the Vitek-2 system were *K. pneumonia* (51;27.5%) followed by *E. coli* (46; 24.8%) and *A. baumannii* (11; 5. 9%).

Antibiotic susceptibility pattern of 30 selected Gram negative isolates was performed using the conventional method and the Vitek-2 system. Ten selected *K. pneumoniae* isolates revealed that Tigecycline had maximum susceptibility by (100%). On the other hand, the highest resistance patterns were to Ceftriaxone and Cefepime (100%), followed by Gentamicin, Amikacin, Tetracycline and Ciprofloxacin (90%).Vitek-2 showed that Ampicillin+Sulbactam, Ceftriaxone, Cefepime, Ertapenem and Amikacin had the highest resistance (100%).When comparing the results of the conventional method and the Vitek-2 system, the *K. pneumoniae* isolates showed CA of (100%) for Ceftriaxone, Cefepime, Gentamicin, Amikacin and Tetracycline and (90%) CA seen for Ciprofloxacin. mE of (30%) was seen for Meropenem followed by (20%) for Ampicillin+Sulbactam and Ertapenem **(Tables 5 and 6)**.

The antimicrobial susceptibility analysis of the 10 *E. coli* isolates done manually showed uppermost resistant pattern (100%) to Ampicillin+Sulbactam, Cefepime and Ciprofloxacin. As for the Vitek-2, the *E. coli* were resistant (100%) to all tested antibiotics except for Amikacin which was (90%). Oppositely, Tigecycline showed the highest susceptibility pattern by (80%) in both the conventional and Vitek-2 system. When comparing the results of both conventional and Vitek -2 system, CA of (100%) was seen for Ampicillin+Sulbactam, Cefepime, Amikacin, Ciprofloxacin and Tigecycline. (90%) for Ceftriaxone, Ertapenem, Gentamicin and Tetracycline. mE (10%) was seen for Ceftriaxone, Meropenem and Tetracycline. ME of (10%) seen for Ertapenem, Meropenem and Gentamicin **(Tables 7 and 8)**.

For the 10 *A. baumannii* isolates, Cefepime, Gentamicin, Amikacin and Tetracycline showed (100%) resistance in both the conventional and Vitek -2 system. No minor error (mE), major error (ME) or very major error (VME); with 100% categorical agreement (CA) was seen with the tested antibiotics for *A. baumannii* **(Tables 9 and 10)**.

Table (5). Antimicrobial susceptibility patterns of *K. pneumoniae* isolates.

Antimicrobial agent	Susceptibility pattern of *K.pneumoniae* done using conventional methods(N=10)						Susceptibility pattern of *K.pneumoniae* done using the Vitek-2 system(N=10)					
	Sensitive (S)		Intermediate (I)		Resistant (R)		Sensitive (S)		Intermediate (I)		Resistant(R)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ampicillin+ Sulbactam	0	0	2	20	8	80	0	0	0	0	10	100
Ceftriaxone	0	0	0	0	10	100	0	0	0	0	10	100
Cefepime	0	0	-	-	10	100	0	0	-	-	10	100
Ertapenem	0	0	2	20	8	80	0	0	0	0	10	100
Meropenem	0	0	4	40	6	60	0	0	1	10	9	90
Gentamicin	1	10	0	0	9	90	1	10	0	0	9	90
Amikacin	1	10	0	0	9	90	0	0	0	0	10	100
Tetracycline	1	10	0	0	9	90	1	10	0	0	9	90
Ciprofloxacin	1	10	0	0	9	90	1	10	0	0	9	90
Tigecycline	10	100	0	0	0	0	10	100	0	0	0	0

Table (6). Correlation between antibiotic susceptibility test results of *K. pneumoniae* between Vitek-2 and conventional method.

Antimicrobial agent	Susceptibility Number and Percentage(%)							
	CA		mE		ME		VME	
	No.	%	No.	%	No.	%	No.	%
Ampicillin+Sulbactam	8	80	2	20	0	0	0	0
Ceftriaxone	10	100	0	0	0	0	0	0
Cefepime	10	100	0	0	0	0	0	0
Ertapenem	8	80	2	20	0	0	0	0
Meropenem	7	70	3	30	0	0	0	0
Gentamicin	10	100	0	0	0	0	0	0
Amikacin	9	100	0	0	1	10	0	0
Tetracycline	10	100	0	0	0	0	0	0
Ciprofloxacin	9	90	0	0	1	10	0	0
Tigecycline	10	100	0	0	0	0	0	0

VME: Very major error, **ME:** Major error**, mE:** Minor error**, CA:** Categorical agreement

Table (7). Antimicrobial susceptibility patterns of *E. coli* isolates.

Antimicrobial agent	Susceptibility pattern of *E.coli* done using conventional methods(N=10)						Susceptibility pattern of *E.coli* done using the Vitek-2 system(N=10)					
	Sensitive (S)		Intermediate (I)		Resistant (R)		Sensitive (S)		Intermediate (I)		Resistant (R)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ampicillin+ Sulbactam	0	0	0	0	10	100	0	0	0	0	10	100
Ceftriaxone	0	0	1	10	9	90	0	0	0	0	10	100
Cefepime	0	0	-	-	10	100	0	0	-	-	10	100
Ertapenem	1	10	0	0	9	90	0	0	0	0	10	100
Meropenem	1	10	1	10	8	80	0	0	0	0	10	100
Gentamicin	1	10	0	0	9	90	0	0	0	0	10	100
Amikacin	1	10	0	0	9	90	1	10	0	0	9	90
Tetracycline	0	0	1	10	9	90	0	0	0	0	10	100
Ciprofloxacin	0	0	0	0	10	100	0	0	0	0	10	100
Tigecycline	8	80	0	0	2	20	8	80	0	0	2	20

Table (8). Correlation between antibiotic susceptibility test results of *E. coli* between Vitek-2 and conventional method.

Antimicrobial agent	Susceptibility Number and Percentage(%)							
	CA		mE		ME		VME	
	No.	%	No.	%	No.	%	No.	%
Ampicillin+Sulbactam	10	100	0	0	0	0	0	0
Ceftriaxone	9	90	1	10	0	0	0	0
Cefepime	10	100	0	0	0	0	0	0
Ertapenem	9	90	0	0	1	10	0	0
Meropenem	8	80	1	10	1	10	0	0
Gentamicin	9	90	0	0	1	10	0	0
Amikacin	10	100	0	0	0	0	0	0
Tetracycline	9	90	1	10	0	0	0	0
Ciprofloxacin	10	100	0	0	0	0	0	0
Tigecycline	10	100	0	0	0	0	0	0

VME: Very major error, **ME:** Major error**, mE:** Minor error**, CA:** Categorical agreement

Table (9). Antimicrobial susceptibility patterns of *A. baumannii* isolates.

Antimicrobial agent	Susceptibility pattern of *A.baumannii* done using conventional methods(N=10)						Susceptibility pattern of *A.baumannii* done using the Vitek-2 system(N=10)					
	Sensitive (S)		Intermediate (I)		Resistant (R)		Sensitive (S)		Intermediate (I)		Resistant (R)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ceftriaxone	0	0	1	10	9	90	0	0	1	10	9	90
Cefepime	0	0	-	-	10	100	0	0	-	-	10	100
Meropenem	1	10	1	10	8	80	1	10	1	10	8	80
Gentamicin	0	0	0	0	10	100	0	0	0	0	10	100
Amikacin	0	0	0	0	10	100	0	0	0	0	10	100
Tetracycline	0	0	0	0	11	100	0	0	0	0	11	100
Ciprofloxacin	1	10	1	10	8	80	1	10	1	10	8	80

Table (10). Correlation between susceptibility test results of *A. baumannii* between Vitek-2 and conventional method.

Antimicrobial agent	Susceptibility Number and Percentage(%)							
	CA		mE		ME		VME	
	No.	%	No.	%	No.	%	No.	%
Ceftriaxone	10	100	0	0	0	0	0	0
Cefepime	10	100	0	0	0	0	0	0
Meropenem	10	100	0	0	0	0	0	0
Gentamicin	10	100	0	0	0	0	0	0
Amikacin	10	100	0	0	0	0	0	0
Tetracycline	10	100	0	0	0	0	0	0
Ciprofloxacin	10	100	0	0	0	0	0	0

VME: Very major error, **ME:** Major error**, mE:** Minor error**, CA:** Categorical agreement

Identification of selected Gram negative isolates using PCR

The *16S-23S ribosomal RNA* gene –directed PCR has confirmed the identification of the ten selected isolates of *K. pneumoniae* by detection of the expected band of 130 base pair (bp) corresponding to expected length of the *16S-23S rRNA* gene **(Figure 1)**. Also, the *UIDA* gene- directed PCR has confirmed the identification of the ten selected isolates of *E. coli* by detection of the expected band of 162 bp corresponding to expected length of the *UIDA* gene **(Figure 2)**. Finally, the *OMPA* gene–directed PCR has confirmed the identification of the ten selected isolates of *A. baumannii* by detection of the expected band of 85 bp corresponding to expected length of the *OMPA* gene. **(Figure 3)**.

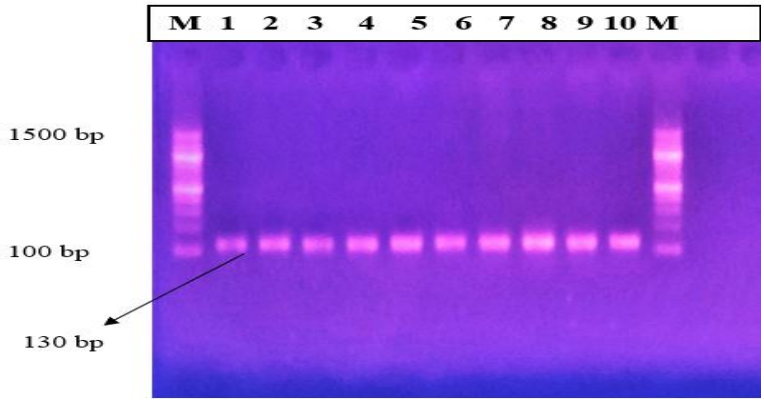


Figure (1). PCR amplification with *16S-23S rRNA* gene primers for identification of *K. pneumoniae* strains. A 130 bp of *16S-23S rRNA* gene. Lane (M), DNA molecular size marker (l500 bp ladder), Lanes (1-10) show positive result with positive bands of 130 bp.

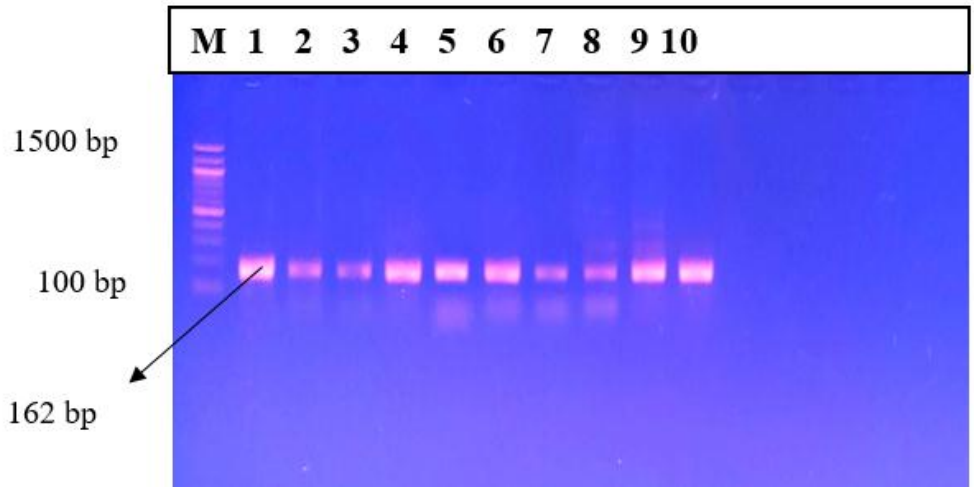


Figure (2). PCR amplification with *UIDA* gene primers for identification of *E. coli strains*. A 162 bp of *UIDA* gene. Lane (M), DNA molecular size marker (l500 bp ladder), Lanes (1-10) show positive result with positive bands of 162 bp.

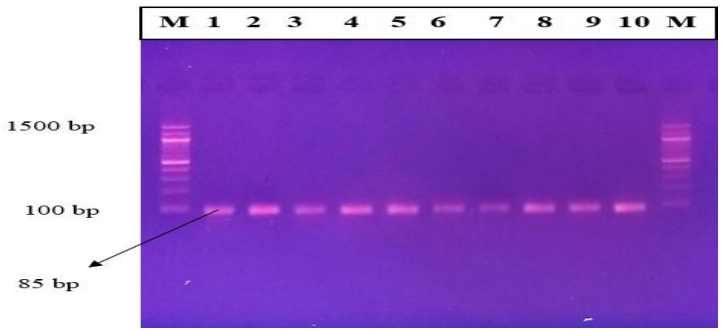


Figure (3). PCR amplification with *OMPA* gene primers for identification of *A. baumannii strains*. A 85 bp of *OMPA* gene. Lane (M), DNA molecular size marker (l500 bp ladder), Lanes (1-10) show positive result with positive bands of 85 bp.

Molecular detection of some virulence genes

EAST-1 gene has only been found in 4 isolates, 2 isolates of *K. pneumoniae* and 2 isolates of *E. coli* which represents (13.3%). *CNF-1* gene has been identified in 20 isolates (66.6%) of all the tested 30 isolates **Figures (4 and 5)**.

As for genes responsible for Siderophore formation. I*utA* has been found in 29 isolates (96.6%). *FyuA* has been found in 27 isolates (90%) **Figures (6 and 7)**.

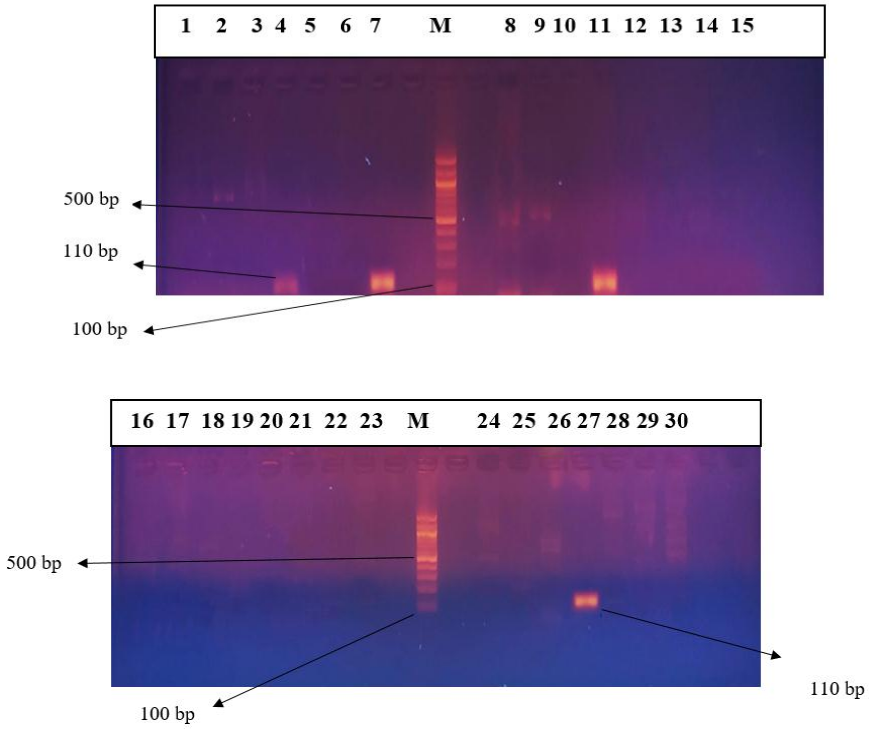
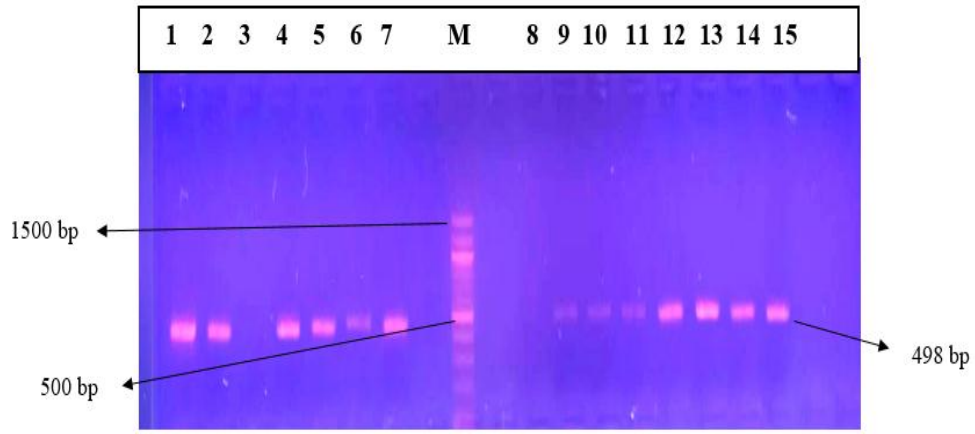


Figure (4). PCR amplification with *EAST-1* gene primers. A 110 bp of *EAST-1* gene. Lane (M), DNA molecular size marker (l500 bp ladder), Lanes (4), (7), (11) and (27) show positive result with positive bands of 110 bp. Other Lanes show negative result.



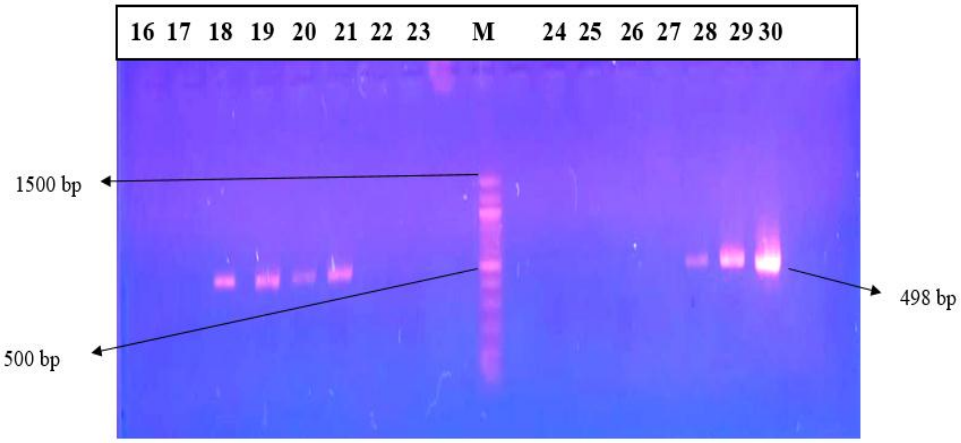


Figure (5). PCR amplification with *CNF-1* gene primers. A 498 bp of *CNF-1* gene. Lane (M), DNA molecular size marker (l500 bp ladder), Lanes (1-2), (4-7), (9-15) (18-21) and (28-30) show positive result with positive bands of 498 bp. Other Lanes show negative result.

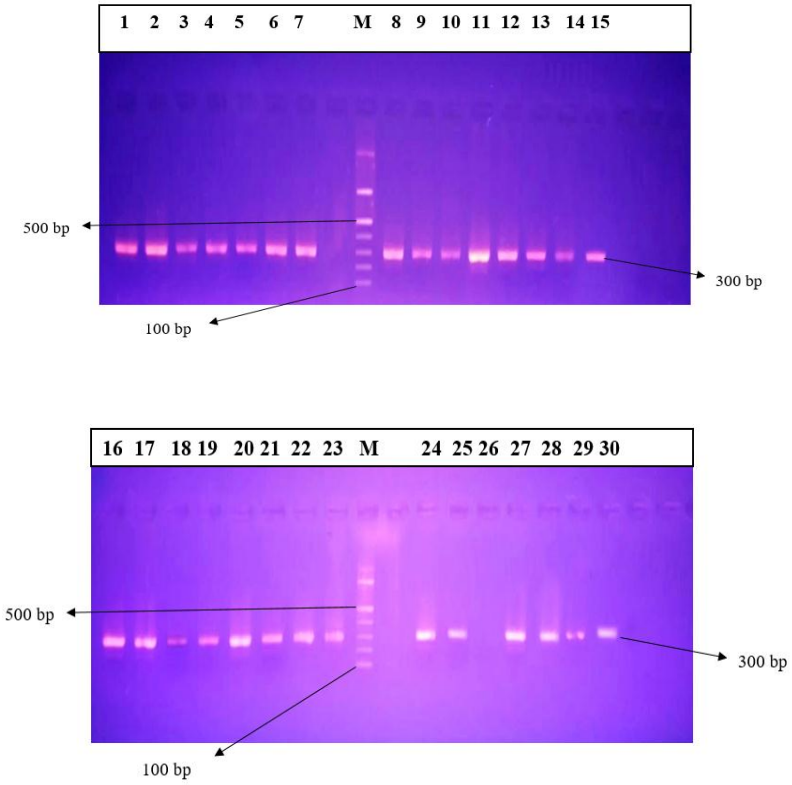


Figure (6). PCR amplification with *IutA* gene primers. A 300 bp of *IutA* gene. Lane (M), DNA molecular size marker (l500 bp ladder), Lanes (1-25) and (27-30) show positive result with positive bands at 300 bp. Lane (26) show negative result.

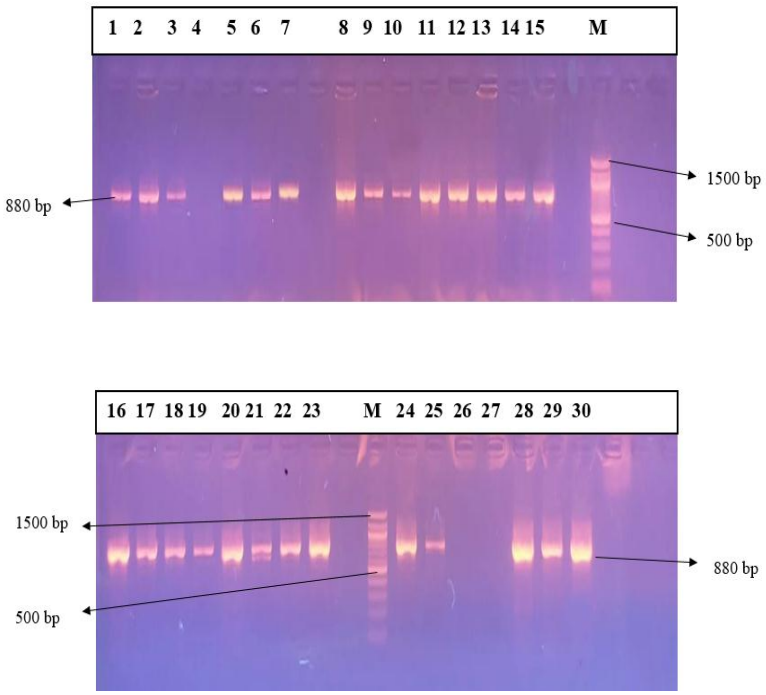


Figure (7). PCR amplification with *FyuA* gene primers. A 880 bp of *FyuA* gene. Lane (M), DNA molecular size marker (l500 bp ladder). Lanes (1-3), (5-25) and (28-30) show positive result with positive bands at 880 bp. Lanes (4) and (26-27) show negative result.

Discussion

Bloodstream infection (BSI) is among the leading acquired and life-threatening condition in hospital settings. Critically ill patients are particularly predisposed to the acquisition of BSI, which occur in approximately 7% of all patients within the first month of hospitalization in ICUs. During the last few years, an increasing number of BSI caused by resistant bacteria in the ICU setting, which presents a major public health problem worldwide **(Bassetti *et al*., 2017)**.

In the present study, a total of 231 blood specimens were collected from patients admitted to ICUs. The collected blood specimens were from patients within wide range of age (1-76). **Khawa (2021)** reported the factors associated with increased risk of early-onset BSI which included; low birth weight infants, prematurity, neonates in need of mechanical ventilation, total parenteral nutrition administration and longer placement of catheter. While **Leibovici-Weissman *et al*. (2021)** demonstrated the risk factors for BSI in elderly as higher rates of comorbidities, Immunosenescence, malnutrition, reduced functional capacity and mainly, higher likelihood of healthcare exposure and instrumentation.

Our results revealed that the higher prevalence was for the Gram negative isolates (64.8%) causing BSIs compared to the Gram positive isolates (30.2%). Among the recovered Gram negative isolates, the major organisms were *K. pneumonia* (27.5%), *E. coli* (24.8%), and *A. baumannii* (5.9%). The epidemiology of BSIs towards GNB has

been observed in previous literature such as a study conducted by **Bajaj *et al*. (2019)** where the percentage of Gram negative isolates recovered from blood specimens was (65.8%), while the percentage of Gram positive isolates was (30.83%). Another study has also recorded the higher prevalence of GNB in BSI (57.3%), while the Gram positive bacteria (40.8%) **(Kuo *et al*., 2017).** This could be attributed to being in the hospital for more than 7 days with a catheter in situ for more than 3 days, having undergone surgery and having received antimicrobial treatment with beta-lactams and Linezolid **(Ripa *et al*., 2018)**. The predominance of *K. pneumonia* and *E. coli* has been reported in several studies. In Egypt, a study was conducted by **Fahim (2021)** has reported *K. pneumoniae* and *E. coli* with isolation rates of 47.5% and 27.9% respectively. **Menzo. *et al* (2015)** declared that is due to the ability of *E. coli* to colonize the human gastrointestinal tract; As it is the most common bacterial species found in human fecal flora. Thus, it is not surprising that it also represents the more frequent cause of Gram-negative BSI in patients. Also, *K. pneumoniae* is the primary species of genus *Klebsiella* associated with illness in human beings. It is found in the gastrointestinal tract and is frequently involved in health-care and ICU associated infections. Infections with *K. pneumoniae* are usually hospital-acquired, sustained by MDR strains and occur primarily in patients with impaired host defenses.

A very big challenge associated with bacterial infections is the spread of antimicrobial resistance which resulted from practices such as; the inappropriate use of antimicrobial agents in the management of conditions that are not caused by bacteria, the use of antimicrobial agents as growth enhancers in animals' feed and the inadequate use of antimicrobial agents to manage bacterial infections **(Chandra *et al*., 2020)**.

In this study, *K. pneumoniae* isolates were fully resistant to Ceftriaxone and Cefepime (100%) followed by Gentamicin. Amikacin, Tetracycline and Ciprofloxacin (90%), both Ampicillin+ Sulbactam and Ertapenem were (80%) and Meropenem (60%). In contrast, these isolates showed highest susceptibility to Tigecycline by (100%). Our results were in agreement with **Zhang *et al*. (2018)**, reporting the highest resistance pattern to Ampicillin+ Sulbactam (98.2%) followed by Ceftriaxone (78.8%) also Tigecycline was shown to be susceptible by (96.2%) making it the first option for treatment. **Olson *et al*. (2006)** recorded that Tigecycline has activity against a wide range of GNB including strains with well-defined resistance mechanisms. In addition, Tigecycline possesses activity against clinical isolates resistant to tetracyclines that carry TET genes encoding either efflux or ribosomal protection resistance mechanisms. Tigecycline has a different mechanism of inter-action with the bacterial ribosome, which clearly differentiates Tigecycline from the structurally related Tetracyclines and contributes to the enhanced clinical effectiveness of this new agent.

For the *E. coli* isolates, the highest resistance was to Ampicillin + Sulbactam, Cefepime and Ciprofloxacin (100%). Additionally, Ceftriaxone, Ertapenem, Gentamicin, Amikacin and Tetracycline showed high resistance patterns (90% for each). These results of resistance came with close agreement with the study conducted by **Santimaleeworagun *et al*. (2021)** which reported highest resistance to Ciprofloxacin (92.3%), while Ceftriaxone, Cefepime and Gentamicin showed resistance of (76.9%) for each. Also, the same study showed the highest susceptibility to Tigecycline (100%).

Regarding the *A. baumannii* isolates, antimicrobial resistance rate was very high and the majority of these isolates were resistant to multiple antimicrobial agents including Cefepime, Gentamicin, Amikacin and Tetracycline (100% for each). As well, Ceftriaxone also showed a high resistance pattern (90%) followed by Meropenem and Ciprofloxacin (80%) for each. These results came with close agreement with **Kim *et al*. (2017)** study were resistance to Ciprofloxacin was (85.2%) and Gentamicin (80%).Additional study recorded highest resistance of *A. baumannii* isolates to Ciprofloxacin (95.5%), then Cefepime by (92.2%), and finally Amikacin by (90%) **(Ballouz *et al*., 2017)**. Vitek-2 system can be repurposed to provide highly accurate antimicrobial susceptibility testing results directly from positive blood cultures with average time of 9 hours, which may be rapid enough to enable same-day reporting for the cultures that turn positive in early morning hours and allow clinical teams to promptly act on these results. This represents an innovative, pragmatic and low-cost repurposing strategy of Vitek-2 system. The turnaround time for inoculum preparation and Vitek-2 set-up is approximately 10 minutes, which can be relatively easily integrated into the workflow of low-to-medium volume laboratories. Importantly, Vitek-2 antimicrobial susceptibility testing can be initiated without the organism identification **(Hogan *et al*., 2019)**

In the present study, confirmed identification of thirty phenotypically identified bacterial isolates was carried out using PCR amplification of certain partial genes sequences. In a study by **Ghasemnejad *et al*. (2019)**, PCR amplification of *16S-23S ribosomal RNA* gene was a sensitive and a fast method for *K. pneumoniae* identification. Another study by **Ali *et al*. (2021)** confirmed the identification of *E. coli* bacteria by PCR amplification of *UIDA* gene. Finally, the identification of *A. baumannii* bacteria was confirmed by PCR amplification of *OMPA* gene **(Hassannejad *et al*., 2019)**.

The use of PCR based technology to identify virulence factor genes has become widely adopted. The development of gel based PCRs for identification of virulence factor genes has made the process simpler and very rapid **(West *et al*., 2007).** Our study involved detection of 4 virulence genes in all 30 isolates which have been genotypically confirmed by PCR amplification method. In our research, only 2 isolate of *K. pneumoniae* (20%) and 2 isolates of *E. coli* (20%) harbored the *EAST-1* gene responsible for Toxin production. **Palma *et al.* (2016)** and **Mirzarazi *et al*. (2015)** studies showed nearly the same results of our study by reporting that only (10.8%) and (9.5%) respectively of the tested *E. coli* isolates had the *EAST-1* gene. This could be attributed to that the *EAST-1* virulent gene detected in our study has not been reported previously in the *K. pneumoniae* and *A. baumannii* isolates. However, DNA search in the GenBank database carried out using BLAST (Basal Local Alignment Research Tool) records the presence of the gene's sequence in several *K. pneumoniae* strains as (https://www.ncbi.nlm.nih.gov/nucleotide/1890420180) and (https://www.ncbi.nlm.nih.gov/nucleotide/1567146795) but not for *A.baumannii* .The *CNF-1* gene again responsible for toxin production has been found in (60%) of *K. pneumoniae* isolates, (50%) of *E. coli* isolates and (90%) of *A. baumannii* isolates in our research. In a related study by **Lateef *et al*. (2012)** identified the *CNF-1* gene in (57.1%) of *K. pneumoniae* collected isolates which shows a significant agreement to our study. Studies by **Guiral *et al*. (2011)** and **Sheikh *et al*. (2019)** has reported has detected the

gene in the collected *E. coli* isolates but at lower frequencies (30%) and (22.8%) respectively. As for *A. baumannii* isolates, it was reported by **Darvishi *et al*. (2016)** and **Al-Kadmy *et al*. (2018)** that *CNF-1* gene was detected in (50%) and (47.6%) respectively in their obtained isolates.

IutA gene in the present study is responsible for siderophore formation and has been found in (90%) of the *E. coli* isolates and (100%) in both *K. pneumoniae* and *A. baumannii* isolates. (80%) of *K. pneumoniae* isolates did contain the *IutA* gene as recorded in a study by **(Candan and Aksöz 2015)**. Another study **Yan *et al*. (2016)** has also reported a high frequency of *IutA* gene detection in the isolates of *K. pneumoniae* by demonstrating that (86.6%) of the isolates in the study had the virulent gene. **Darvishi (2016)** and **Momtaz *et al*. (2015)** have both reported the presence of *IutA* gene in *A. baumannii* isolates but with a lower frequency (25%) and (19%) respectively**.** *FyuA* gene also responsible for siderophore formation has been detected in (90%) of *K. pneumoniae, E. coli* and *A. baumannii* isolates respectively**. Kumar *et al*. (2020)** have recorded the *FyuA* gene which is also play a role in siderophore formation to be present by (37.6%) in *K. pneumoniae* isolates which is considered a lower frequency than that of our study. Also, another study by **Gołębiewska *et al*. (2019)** recorded the gene to be present by (47.4%) in *K. pneumoniae* isolates. As for *E. coli* isolates to do contain the *FyuA* gene, a study in Mexico by **López-Banda *et al*. (2014)** recorded the *FyuA* gene in (44.4%) of the *E. coli* isolates. In another study by **Pompilio *et al*. (2018**) have reported the *FyuA* gene in (86.7%) of *E. coli* isolates which has a close agreement with our result. In the end, **Hetta *et al*. (2021)** demonstrated the presence of *FyuA* gene in *A. baumannii* by recording that (48.2%) of the collected isolates harbored the gene.

Conclusion

Gram negative pathogens were found to be the major bacteria causing BSIs. We found that the Vitek-2 system provide accurate antimicrobial susceptibility testing results. Concerning toxin production and siderophore formation, *CNF-1* toxin production related gene was found to be prevalent in most of the isolates. Moreover, *IutA* and *FyuA* siderophore formation related genes were found to be prevalent in most of the isolates.

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الدراسات المكروبيولوجية والجزيئية لبعض مسببات الأمراض السالبة الجرام التي تسبب عدوى تدفق الدم بين المرضى في وحدات العناية المركزة

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المقدمة تتزايد عدوى مجرى الدم وخاصة عدوى مجرى الدم المرتبطة بالخط المركزي عالميًا كسبب مهم للممراضة والوفيات. يمكن أن تكون الحالة مهددة للحياة في المرضى المصابين بأمراض خطيرة في وحدات العناية المركزة في المستشفيات. هناك مخاوف جدية بشأن الزيادة السريعة في البكتيريا سالبة الجرام المقاومة للأدوية مثل كلبسيلا الالتهاب الرئوي، الايشيريشيا كولاى والراكدة البومانية باعتبارها الأسباب الأكثر انتشارًا لعدوى مجرى الدم. **الهدف من الدراسة** هو التأكيد على خطورة البكتيريا سالبة الجرام المقاومة للأدوية للمضادات الحيوية، وتحديد النمط الجيني وكذلك الكشف عن بعض جينات الضراوة. **الطريقة** تم جمع عينات الدم من ٢١٣ مريضاً بالبكتيريا تم إدخالهم إلى وحدات العناية المركزة المختلفة في ثلاثة مستشفيات للرعاية المتخصصة في مصر. تم إخضاع العزلات البكتيرية المستعادة للتعريف البيو كيميائي بالطرق التقليدية. تم إجراء اختبار الحساسية لمضادات الميكروبات على عزلات سالبة الجرام باستخدام كل من طريقة نشر قرصKirby-Bauer ونظام Vitek-2. تم اختيار ثلاثين عزلة من البكتيريا سالبة الجرام المقاومة وفحصها لوجود جينات 16S-23S rRNA وUIDA وOMPA باستخدام فحوصات PCR لتأكيد تحديد هويتهم. كما تم فحص الثلاثين عزلة لوجود جينات East-1 وCnf-1 وIutA وFyuA. **النتائج** تم انتشال ١٨٥ عزلة إكلينيكية من ١٨٥ عينة دم إيجابية. أظهر الفحص المخبري لعينات مزرعة الدم الإيجابية أن ١٢٠ منهم (٤. ٦٤٪)، ٥٦ (٣٠.٢٪) و٩ (٤.٨٪) كانوا سلبية الجرام، إيجابية الجرام ومبيضات على التوالي. تم اختيار ثلاثين عزلة من البكتيريا سالبة الجرام المقاومة للأدوية للمضادات الحيوية وتمت مقارنة حساسيتهم لمضادات الميكروبات باستخدام النظام التقليدي ونظام Vitek-2. لعزلات كلبسيلا الالتهاب الرئوي تم الكشف عن اتفاق قاطع ١٠٠٪ لكل من سيفترياكسون وسيفيبيم وجنتاميسين وأميكاسين وتتراسيكلين. بالنسبة للايشيريشيا كولاى تم الكشف عن اتفاق قاطع (١٠٠٪) مع أمبيسلين + سولباكتام، سيفيبيم، أميكاسين، سيبروفلوكساسين وتيجيسيكلين. لوحظ اتفاق قاطع (١٠٠٪) مع المضادات الحيوية المختبرة مع الراكدة البومانية. أشار التأكيد الوراثي لعزلات المقاومة المختارة إلى وجود الجينات المستخدمة لتحديد الهوية في كل منهم. أما بالنسبة لإيواء جينات الضراوة، فقد تم العثور على جين EAST-1 فقط في ٣. ١٣ ٪ من العزلات. تم التعرف على جين CNF-1 في ٦. ٦٦٪ من العزلات، وتم العثور على جين IutA في ٦. ٩٦٪ من العزلات وتم العثور على FyuA في ٩٠٪ من العزلات. **الاستنتاج** وجد أن مسببات الأمراض سالبة الجرام هي البكتيريا السائدة المسببة لعدوى مجرى الدم. كما يوفر نظام Vitek-2 نتائج دقيقة لاختبار الحساسية لمضادات الميكروبات. تم العثور على الجينCNF-1 المرتبط بإنتاج سموم سائدًا في معظم العزلات. تم العثور على الجينات المرتبطة بتكوين حاملة الحديد IutA وFyuA سائدة في معظم العزلات.

الكليمات المفتاحية : عدوى مجرى الدم المرتبطة بالخط المركزي ، كلبسيلا الالتهاب الرئوي، الايشيريشيا كولاى ،الراكدة البومانية ، نظام Vitek-2