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Evaluation of Antimicrobial Peptide LL-37 Role in the Treatment of Antibiotic-Resistant Bacteria in haematological patients

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Abstract: Haematological diseases are disorders in the structure and content of the blood. Multi-drug resistant (MDR) bacteria increase rates of morbidity and mortality; therefore, our study focuses on detection of resistant bacteria and role of peptide LL-37 in their treatment. One hundred different clinical specimens were cultured and isolated, and the identification was done by using the Vitek2 system. The minimum inhibitory concentrations (MICs) of peptide LL-37 were calculated by using the microdilution technique. the results showed that, females were more than males, and the most haematological diseases were Acute Myeloid Leukemia (AML) at 20%, followed by Acute Lymphoblastic Leukemia (ALL) and non-Hodgkin Lymphoma (NHL) at 18%. Most specimens were sputum and blood, followed by wound swabs, and then others. Antibiotic susceptibility testing showed all investigated bacteria to Benzylpenicillin, Ampicillin, and Ticarcillin were resistance 100%, In contrast, all isolates investigated to Linezolid, Tigecycline, Ertapenem, and Minocycline were sensitive 100%. Most the investigated isolates for Colistin antibiotic were intermediate (89.47%) and variable susceptible for the other antibiotics. All investigated MDR isolates were sensitive to the antimicrobial peptide LL-37 at concentrations ranging from 31.25 to 125 µg/ml. in conclusion it is necessary to develop this antimicrobial peptide for particularly in haematological disease patients.

Keywords: Peptide LL-37, multi-drug resistant (MDR), Minimum inhibitory concentrations (MICs).

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Introduction

Hematological diseases are diseases that affect the functioning and composition of blood, red blood cells, white blood cells, platelets, and plasma. These disorders may be inherited or acquired and can cause serious complications if not treated. Blood disorders are usually classified into four main types which: anemia, leukemia, hemophilia, and thrombocytopenia are the most common types of blood disorders. Anemia, Erythrocytosis. Neutropenia, Abnormal white cells. Thrombocytopenia, Abnormal platelets. Coagulation disorders (1,2). The most serious blood disorders are blood cancers or leukemia, and they account for 2.5% of cancers and 3.1% of cancer deaths. Chemotherapy is the mainstay treatment for cancer and is usually non-specific and toxic to all rapidly proliferating cells. Typical toxicities result in a highly increased risk of infection through depletion of circulating immune cells, in the

particular neutrophils, and the breakdown of barrier defenses, including the gut mucosa. Neutropenic sepsis has been reported in more than 50% of leukemia patients with 20% mortality (3). Opportunistic pathogen are mainly causes for hospital infections especially in immunocompromised individuals (4). There is a global rise of multi-drug-resistant bacteria, posing a significant challenge to antibiotic treatment (5). The presence of multi-drug resistant (MDR) bacteria has been linked to higher rates of morbidity and mortality in patients with blood disorders (6). The WHO has named antibiotic resistance one of the three most important public health threats of the 21st century (7). such, reducing unnecessary antibiotic use is a core intervention to lower AMR. Antibiotic stewardship programs based on current knowledge (8), evolved to address the rising calls to regard antibiotics as a non-renewable resource, still a possible counter to the global plight of bacterial resistance to antibiotics lies in antimicrobial peptides (AMP), such as the human cathelicidin LL-37. The LL-37 peptide acts as a strong antimicrobial agent against multi-drug-resistant present-day bacterial strains (9). LL-37 combination with some antibiotics is more effective against MDR bacteria (10).

Materials and methods

Sample Collection

One hundred different clinical haematological patients' samples were collected from June 2024 to January 2025. Each sample was recovered from unique clinical specimens at the following medical centers: Blood Diseases and Bone Marrow Transplant Center in the medical city in Baghdad capital. Primary diagnosis was made on clinical examination by hematologist physicians, peripheral smear examinations, bone marrow aspiration, molecular detection and immunophenotyping if available. Sets of cultures were taken: samples, sputum, blood, urine, wound, or any suspected site to identify the organisms.

Isolation and Identification

In the laboratory of the hospital under aseptic conditions, starting liquid specimens (blood and body fluid (BF)) inoculated into broth bottles of BacT/Alert FA plus (aerobic) (10 ml), FN plus (anaerobic)(10 ml), and PF plus (pediatric) bottles (4 ml); (blood specimens taken from different vein punctures). In two different sites, blood was taken simultaneously and incubated in BacT/ALERT® 3D system (BioMe'rieux, Durham, North Carolina, USA). Segment culture on blood agar plate, MacConkey agar plate, and chocolate agar under aerobic and anaerobic induced from an inoculum raised from the positive flagged culture bottles and all other specimens streaking directly on these plates and kept in an incubator at 37°C for 24 hours. Morphological characteristics were observed, and Biochemical tests were performed and Gram staining was performed for the characterization of the colony of sub-cultured organisms. Identification was performed based on the two editions of Bergey's manual of systematic bacterial, and confirmation of identification was performed using the Vitek 2 compact system. Per CLSIM100 instructions from last edition (11).

Minimum Inhibitory Concentrations (MICs) of Peptide LL-37.

Peptide LL-37 source

LL-37 (CAP-18) 10 mg peptide used in this assay was purchased from (PEPTIDE SHOP Online, USA Made, with a purity grade of 99%). which was chemically synthesized according to standard methods.

Preparation of LL-37 stock solution

5 ml of phosphate buffered saline (PBS),were added to 10 mg of peptide LL-37 vial to become the final concentration 2000 µg/ml, then Vortex until completely dissolved and prepare aliquots and store stock solution at -20°C.

Peptide LL-37 MIC Procedure

Minimum inhibitory concentration (MIC) is the lowest antimicrobial concentration that prevents visible growth of microbe after a defined period of incubation. They were assessed by the microdilution method (Microtiter Plate Assay) using the dye resazurin in accordance with CLSI for the range of (1000 – 3.90) µg/ml. MICs were determined by brain heart broth (18–24 h at 37°C) for each of the MDR and most frequently recurrent species. Positive controls were wells without Peptide LL-37, and negative controls were wells without bacteria. After a 24-hour incubation at 37°C, wells were checked visually for growth; after satisfactory growth, 20µl of resazurin was added to each well, and the mixture was incubated at 37°C for 2–4 hours longer and a pink color shift was noted (indicating viability). The MIC (minimum inhibitory concentration) was measured as the concentration at which the color unchanged occurred.

Statistical Analysis

The Statistical Packages of Social Sciences-(SPSS) (2019) software was used to analyze the effect of different groups or factors on study parameters. The Chi-square test was used to significant compare percentage, with significance levels test at P≤ (0.05 and 0.01) (12).

Results and Discussion :

One hundred from haematological diseases patients, were enrolled in our study. The number of female's patients was 55 (55%), while males was 45 (45%). From a hundred collected specimens, 47 bacterial isolates isolated from 43 specimens were as positive culture. According age groups from (11-20, 21-30, 51- 60) year were the most blood disease patients whose were suspected of bacterial infections, and the samples were collected. (Table 1).

Table (1): Distribution of sample study according to Gender and Age groups

Gender number (percentage)		Age group	number (percentage)
Female:	55 (55%)	0- 10:	0 (0%)
		11-20:	18 (18%)
		21-30:	18 (18%)
		31 -40	8 (8%)
		41 -50:	15 (15%)
Male:	45 (45%)	51- 60:	18 (18%)
		61- 70:	17 (17%)
		71-80:	5 (5%)
		81- 90:	1 (1%)
P-value = 0.317 NS		P-value = 0.0001 **	
** (P<0.01).			

The site of infection was distributed to different sites of the body where specimens were taken. Sputum and blood specimens were the most common, followed by wound swabs specimens, urine specimens, skin specimens, pleural fluid specimens, and then nasal secretion specimens (Table 2). In another study on haematological patients, the respiratory tract infections were the most 30–40% specimens, bloodstream 15–20%, urine 10–15%, skin8–10%, intestinal tract 5–8%, and other sites 10–15 (13).

Table (2): Distribution of sample study according to specimen positive to bacteria

Specimen	Number (Percentage)
Blood stream	11 (25.58 %)
Urine	6 (13.95 %)
Wound	7 (16.27 %)
Sputum	14 (32.55 %)
Skin	2 (4.65 %)
Nasal	1 (2.32 %)
Pleural fluid	2 (4.65 %)
Total	43 (100%)
P-value	0.0001 **
** (P<0.01).	

The most common haematological diseases distributed in the target patients were acute myeloid leukemia (AML) 20 (20%) followed by Acute lymphoblastic leukemia (ALL) 18 (18%) and non-Hodgkin lymphoma (NHL) 18 (18%) followed by others. (**Table 3).** In another study, Acute lymphoblastic leukemia (ALL) is the most common blood cancer that occurs in pediatrics and adults (14). In another study non-Hodgkin lymphoma (NHL) is the most common haematological malignancy, and NHL is the seventh most prevalent cancer and has the sixth highest mortality among cancers in the USA (15).

Table (3): Distribution of sample study according to type of the disease

Blood disease	Number (Percentage)
Acute lymphoblastic leukemia (ALL)	18 (18%)
Chronic myeloid (CML)	8 (8%)
chronic lymphocytic leukemia (CLL)	2 (2%)
Acute myeloid leukemia (AML)	20 (20%)
Myelodysplastic syndrome (MDS)	6 (6%)
Hodgkin lymphoma (HL)	7 (7%)
Non -Hodgkin lymphoma (NHL)	18 (18%)
Aplastic anemia (AA)	5 (5%)
Multiple myeloma MM	11 (11%)
Hairy cell leukemia (HCL)	1 (1%)
Immune thrombocytopenic purpura (ITP)	2 (2%)
Marginal zone lymphoma (MZL)	1 (1%)
Deep vein thrombosis (DVT)	1 (1%)
P-value	0.0001 **
** (P<0.01).	

According to the prevalent of bacterial species, Gram-negative bacteria were 33 (70.21 %), *Escherichia coli* 9 (19.14%), *Pseudomonas aeruginosa* 11 (23.40%) and *Klebsiella pneumoniae* 8 (17.02%) were the most prevalent, and *Pseudomonas fluorescens, Acinetobacter baumanii, Acinetobacter calcoaceticus, Enterococcus faecalis,* and *Klebsiella oxytoca* were 1(2.12%) for each one. While Gram-positive bacteria were 14 (29.79 %) all of which were from *staphylococcus species.* P-value was (0.0001). **(Figure 1 and 2),** the most frequent pathogens in another study were methicillin-resistant *Staphylococcus aureus* (MRSA, 11 patients), methicillin-sensitive *Staphylococcus aureus* (MSSA, 6 patients), *Klebsiella spp*., and *Staphylococcus epidermidis.* (16)

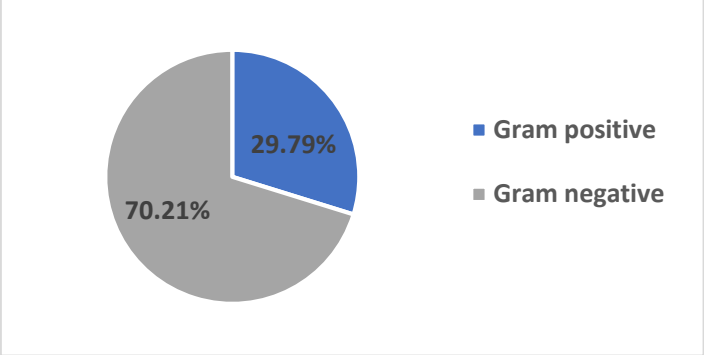


Figure (1): Distribution of isolates according to Gram stain

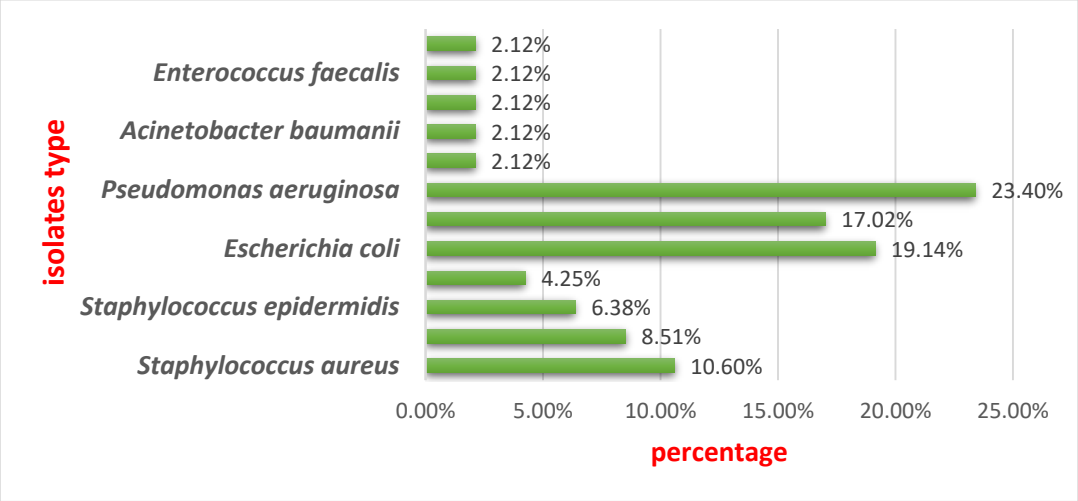


Figure (2): Distribution of sample study according to bacterial species

The results of the antibiotic susceptibility test, including all investigated bacterial isolates for Benzylpenicillin, Ampicillin, and Ticarcillin were resistant as following, 13 (100%), 10 (100%), 6 (100%) respectively, while all investigated isolates for each of Linezolid, Tigecycline, Ertapenem, Minocycline, were sensitive as following, 12 (100%),25 (100%),2 (100%), 1 (100%) respectively. Most investigated isolated for Colistin antibiotic were Intermediate 17 (89.47%). Most investigated isolates were variable sensitivity for the other antibiotics, **Table 4.** In another study in 2024, among the tested bacteria, 611(69.5%) were resistant to ciprofloxacin, 347(59.4 %) were resistant to levofloxacin. While, 177(70.8 %) were sensitive to moxifloxacin, and 220 (84.6 %) were sensitive to rifampicin (17).

Table (4): Percentages of antibiotic sensitivity for bacterial isolates.

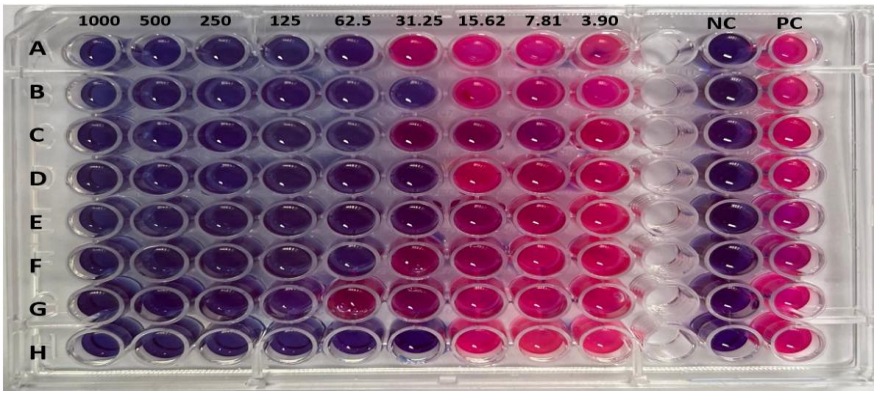
N	Antibiotic	Sensitive	Intermediate	Resistant
1	Benzylpenicillin	0 (0.00%)	0 (0.00%)	13 (100%)
2	Ampicillin	0 (0.00%)	0 (0.00%)	10 (100%)
3	Oxacillin	2 (14.28%)	0 (0.00%)	12 (85.71%)
4	Imipenem	16 (55.17%)	0 (0.00%)	13 (44.82%)
5	Gentamicin	30 (65.21%)	0 (0.00%)	16 (34.78%)
6	Ciprofloxacin	12 (31.57%)	1 (2.63%)	25 (65.78%)
7	Moxifloxacin	5 (33.33%)	4 (26.6%)	6 (40%)
8	Azithromycin	2 (16.66%)	0 (0.00%)	10 (83.3%)
9	Erythromycin	2 (16.66%)	0 (0.00%)	10 (83.3%)

N	Antibiotic	Sensitive	Intermediate	Resistant
10	Clindamycin	7 (53.84%)	0 (0.00%)	6 (46.15%)
11	Linezolid	12 (100%)	0 (0.00%)	0 (0.00%)
12	Teicoplanin	10 (71.42%)	0 (0.00%)	4 (28.57%)
13	Vancomycin	11 (78.57%)	0 (0.00%)	3 (21.42%)
14	Tetracycline	10 (66.66%)	0 (0.00%)	5 (33.33%)
15	Tigecycline	25 (100%)	0 (0.00%)	0 (0.00%)
16	Fusidic Acid	6 (50%)	0 (0.00%)	6 (50%)
17	Rifampicin	10 (76.92%)	0 (0.00%)	3 (23.07%)
18	Trimethoprim/Sulfameyhoxazole	16 (48.48%)	0 (0.00%)	17 (51.51%
19	Tobramycin	9 (47.36%)	0 (0.00%)	10 (52.63%)
20	Levofloxacin	5 (27.77%)	1 (5.55%)	12 (66.66%)
21	Nitrofurantoin	10 (58.82%)	4 (23.52%)	3 (17.64%)
22	Ticarcillin/Clavulanic Acid	4 (30.76%)	1 (7.69%)	8 (61.53%)
23	Piperacillin/Tazobactam	16 (51.61%)	0 (0.00%)	15 (48.38%)
24	Ceftazidime	8 (26.66%)	1 (3.33%)	21 (70%)
25	Cefepime	14 (45.16%)	0 (0.00%)	17 (54.83%)
26	Meropenem	11 (52.38%)	0 (0.00%)	10 (47.61%)
27	Amikacin	20 (71.42%)	0 (0.00%)	8 (28.57%)
28	Minocycline	3 (50%)	0 (0.00%)	3 (50%)
29	Colistin	1 (5.26%)	17 (89.47%)	1 (5.26%)
30	Ampicillin/Sulbactam	1 (20%)	0 (0.00%)	4 (80%)
31	Cefotaxime	1 (20%)	0 (0.00%)	4 (80%)
32	Ticarcillin	0 (0.00%)	0 (0.00%)	6 (100%)
33	Aztreonam	1 (25%)	0 (0.00%)	3 (75%)
34	Ceftazidime/Avibactam	5 (83.33%)	0 (0.00%)	1 (16.66%)
35	Ceftolozane/ Tazobactam	5 (83.33%)	0 (0.00%)	1 (16.66%)
36	Cefoxitin	5 (71.42%)	0 (0.00%)	2 (28.57%)
37	Ceftriaxone	2 (22.22	0 (0.00%)	7 (77.77%)
38	Etrapenem	2 (100%)	0 (0.00%)	0 (0.00%)
39	cefazolin	2 (20%)	1 (10%)	7 (70%)
40	Piperacillin	2 (15.38%)	0 (0.00%)	11(84.61%)
41	Minocycline	1 (100%)	0 (0.00%)	0 (0.00%)

The result of Minimum inhibitory concentrations of the antimicrobial peptide LL-37 for investigated MDR isolates, were from (31.25-125) µg/ml. **Table 5 and Figure 3.** In a similar study, peptide LL-37 MICs of the most bacterial isolates were from (128 ->256) µg/ml (18). In another study in Iraq on MDR *Pseudomonas aeruginosa* isolates, MICs of Peptide LL-37 were from (15.62- 250) µg/ml (19). The variation between bacterial strains is a main reason for variation in peptide LL-37 MICs, where Gram- positive and Gram-negative bacteria differ in cell wall structure and defense mechanisms, such as efflux pumps, proteases, or biofilms, which can affect peptide LL-37 efficacy. where biofilm formation increases bacterial resistance (20).

Table(5): Minimum inhibitory concentrations test of the antimicrobial peptide LL-37 for investigated MDR isolates, from (1000-3.90) µg/ml.

	Peptide LL-37	Antimicrobial activity (MICs µg/ml)
A	*Staph hominis*	62.5
B	*E. coli*	31.25
C	*Staph aureus*	62.5
D	*Staph haemolyticus*	31.25
E	*Pseudomonas aeruginosa*	31.25
F	*Acinetobacter calcoaceticus*	62.5
G	*Klebsiella pneumoniae*	125
H	*Staph epidermidis*	31.25



Figure(3): Minimum inhibitory concentrations test of the antimicrobial peptide LL-37 for investigated MDR isolates, from (1000-3.90) µg/ml.

Conclusion

most haematological diseases patients suffer from weakened immunity due to medication or illness, make them frequent with infection by nosocomial bacteria. The rise in antibiotics- resistant bacteria leads to increase in morbidity and mortality rates. Bacterial infections commonly occur in the blood and respiratory system because these areas provide a favorable environment for bacteria to grow, and the use of ventilators and the implantation of cannulas can introduce bacteria into the respiratory system or blood, increasing the risk of infections. Antibiotic resistance among bacteria varies based on genetic factors, bacterial characteristics, and exposure methods for antimicrobial agents. The severity of hospital-acquired infections is influenced by the local environment, healthcare practices, and adherence to infection control measures, highlighting the need for strict antimicrobial stewardship and preventive strategies. Therefore, it is crucial to provide them with unique and high-quality healthcare. The antimicrobial peptide LL37 has a potent activity against most MDR bacterial strains, even in low concentrations. This creates a need for studies and development of this antimicrobial peptide, particularly in blood disease patients.

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