Antibacterial Activity of Antimicrobial Peptide Indolicidin against Multidrug-Resistant *Klebseilla pneumoniae* Isolated from Patients with Burns

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Abstract

The emergence of multidrug resistant *Klebsiella pneumoniae* has become a significant problem worldwide and also being a major threat to patients with burns infections. The therapeutic action of antimicrobial peptides derived from humans or animals or synthetic peptides attracted attention as alternatives for antibiotics in order to treat the resistant strains especially with strains isolated from burn patients. The current study investigated the role of antimicrobial peptide Indolicidin as an antibacterial agent with multidrug *K. pneumonia* isolates from burns. The collection of study samples has taken place at the period between November 2020 and completed at end of March 2021, it has included 250 clinical specimens as burn swabs from inpatients with burn infections admitted in four hospitals in Baghdad. The results of selective media, biochemical tests, and Vitek2 system identified 40 isolates (16%) as *K. pneumoniae* from all collected bacterial cultures. The results of the antimicrobial susceptibility test by using the disc diffusion method for the isolates under study showed that *K. pneumoniae* clinical isolates were moderate resistant to the majority of the antibiotics tested. The majority of *K. pneumoniae* isolates were high resistant to Erythromycin **(100%)** and Ceftazidime (85%)**,** also, it was obvious resistance to Ceftriaxone, Cefepime **and** Cefotaxime, **while the lowest percentage of resistance was for Impenem (25%) and** Meropenem (38%)**.** The results of minimum inhibitory concentrations (MICs) of indolicidine against (10) *K. pneumoniae* isolates which multidrug resistant and formed the strong biofilm, revealed that range of concentrations of indolicidin was (0.7-100 µg/ml) and it was obvious that there is a significant effect of indolicdin on the growth of *K. pneumoniae* at very low concentrations. In this study, we believe that the development of these antimicrobial peptides may become a new generation of urgently needed antimicrobials that can overcome bacterial resistance mechanisms.

Keywords*: Antibacterial, Burns, Indolicidin, Klebsiella pneumoniae*

Introduction

The high prevalence of multidrug resistant bacteria in burn units is likely a consequence of several factors, including high antibiotic pressures, high colonization pressures, need for intensive medical, surgical therapy, and a vulnerable immunocompromised patient population [1]. *Klebsiella pneumoniae* accounts for about one-third of all Gram-negative infections such as urinary tract infections, cystitis, pneumonia, surgical wound infections, burns, and septicemia

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[2]. Treating pathogens is becoming challenging because of multidrug resistance and availability of limited alternative therapies which has further confounded this problem [3]. Antimicrobial peptides (AMPs) are promising candidates as antibacterial agent against resistant bacteria, which reduce the likelihood of resistance evolving compared to the use of antibiotics. Furthermore, combinations of AMPs and traditional antibiotics with different mechanisms of action could facilitate the revival of ineffective drugs based on the enhanced or synergistic activity of the combination against human pathogens [4]. Due to the importance and wide spread of these bacteria and spread of antibiotics resistance and to achieve important of antimicrobial peptide to treatment, this study was aimed to evaluation the role of the short antimicrobial peptides indolicidin as therapeutic agents against the multidrug resistant *Klebsiella pneumoniae* isolated from burn patients. . .

Materials and Methods

Isolation and identification of *K. pneumoniae*

This study was performed at Hospitals in Baghdad, Iraq, between November 2020 and March 2021. Out of 250 burn swabs, a total of 40 isolates of *K. pneumoniae* were collected from patients with burns Infections. CHROMagar Orientation, Blood agar and McConkey agar were used for isolation *K. pneumoniae*. These isolates were identified using traditional bacteriological methods and biochemical testing, with VITEK 2 system (bioMerieux, France), according to the manufacturer's recommendations.

Antibiotic Susceptibility Test

Antimicrobial susceptibility test was conducted by using disc diffusion method. Briefly, *K. pneumoniae* overnight growth were prepared on McConkey agar and then resuspended in Mueller-Hinton broth (Oxoid).The turbidity of the suspension is adjusted to an equivalent 0.5 McFarland and this suspension was used to inoculate on Mueller-Hinton agar (Oxoid) plates. The antibiotics discs used in this study as the following: Kanamycin (K), Gentamicin (GM), Imipenem (IMI), Meropenem (MEM), Ceftazidime (CAZ), Cefotaxime (CTX), Ciprofloxacin (CIP), Tetracycline (T), Ampicillin sulbactam (SAM), Erythomycin (E), Cefepime(CPM) and Cefoxitin (FOX), (MAST, UK) were placed on the medium. The agar plates were incubated at 35 °C for 24 h. and then the inhibition zone was measured and interpreted by the percent of susceptible, intermediate, or resistant isolates as defined by CLSI breakpoint interpretative Criteria (CLSI, 2020).

Minimum inhibitory concentrations (MIC).

MIC was determined using the microdilution method (Microtiter Plate Assay with Resazurin Dye) as described by the Clinical and Laboratory Standards Institute (CLSI, 2020). Briefly, 1:2 serial dilutions of Indolicidin peptides in Mueller Hinton Broth (MHB) were placed in a 96-well round-bottom plate at concentrations ranging from 100 to 0.7 µg/ml. The bacterial inoculum was prepared from a subculture of *K. pneumoniae* in LBB incubated for 18–24 hours at 35 ± 2°C before to the test. The bacteria suspension was diluted to $1x10^8$ colony forming units (CFU)/mL, to obtain a turbidity equivalent to 0.5 on the McFarland scale, confirmed by spectrophotometry upon reaching an absorbance between 0.08–0.1 at a wavelength of 625 nm; then a 1:200 dilution in MHB was performed to obtain a final concentration of $5x10^5$ CFU/mL. The diluted bacterial suspension was added to the 96-well plate containing the serially diluted peptides. The final volume of 200 µL per well consisted of 100 µl of the compound and 100 µL of diluted bacteria suspension. Negative and positive growth controls were performed by adding only MHB or *K. pneumoniae* with MHB to the wells, respectively. After incubation for 24 h at 37 °C, resazurin (0.015 %) was added to all wells (30 µl per well), and further incubated for 2–4 h for

the observation of colour change. On completion of the incubation, columns with no colour change (blue resazurin colour remained unchanged) were scored as above the MIC value. At the end of the incubation time, MIC was determined as the lowest compound concentration at which no bacterial growth was observed.

Results and Discussion

Isolation and characterization of *K. pneumoniae*

The two most important distinguishing characteristics of *Klebsiella* spp. are positive lactose fermentation on MacConkey agar medium and the viscosity of the colonies. *Klesbsiella* spp. isolates (such as *K. pneumonia*, and *K. oxytoca*) showed positive result on the MacConkey agar after 24-48 hours of incubation at 37^{o}C, as shown in figure (1).

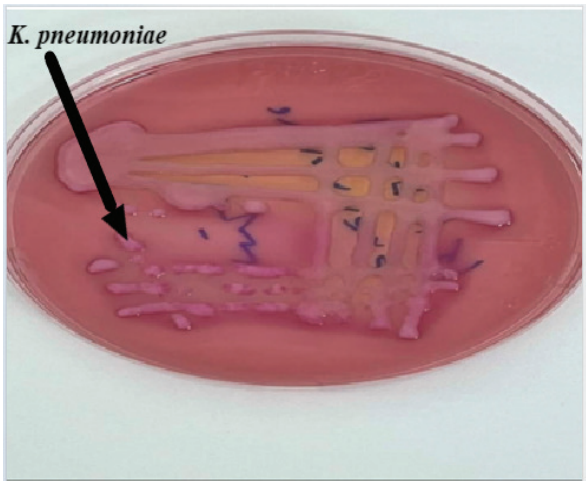


Figure (1): *Klebsiella* spp. on MacConkey agar plate.

All suspected *Klebsiella* colonies were detected by culturing on blood agar medium (supplemented with 5% human blood) showing large shiny, mucoid, whitish-grey and round colonies with no hemolysis[5]. Moreover, as described in Figure (1), mucous colonies of *K. pneumonia* were touched with a standard inoculating loop and the loop was lifted vertically from the surface of the agar plate, mucoid isolates adhered to the loop and stretched more than 5 mm in length as it was lifted from the plate [6].

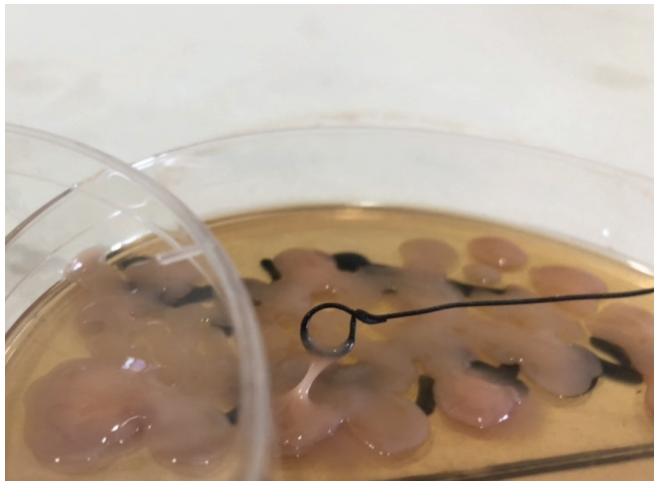


Figure (2): String test for *Klebsiella pneumonia* mucoid colonies.

CHROMagar Orientation medium was used for specific isolation of urinary tract pathogens. On CHROMagar, isolates of Klebsiellaappeared as metallic blue colonies at 37°C for 24 hours as shown in Figure (3) this medium also has selectivity for other Urinary tract pathogens with specific color for each bacterial genus, where the colonies of Escherichia coli appeared as pink red colonies

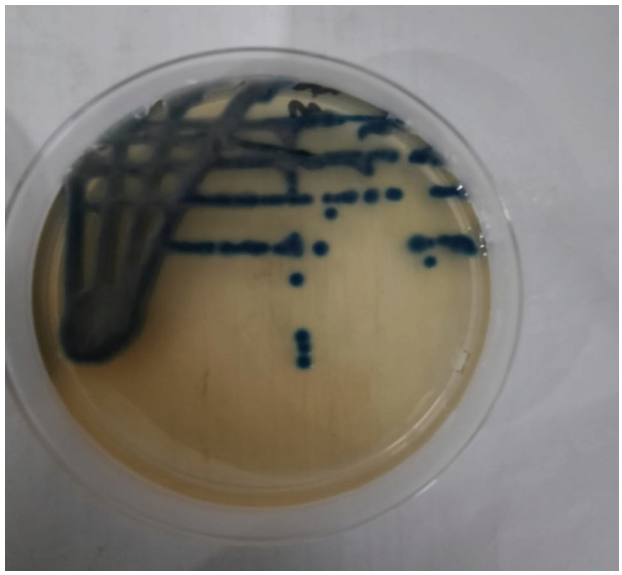


Figure (3): Metallic blue colonies of *K. pneumoniae* on chrome agar surface after 24 h at 37o C of incubation (Selective tes).

Chromogenic agars are reliable media for the detection aerobic Gram negative bacteria by easier

recognition of different colonies on these media. CHROMagar Orientation medium is preferred medium because of the high accuracy and the rapid identification with very low false positive rates [7]. The use of CHROMagar Orientation medium reduces the need for further reagents and extra confirmatory tests suggesting that CO medium is a cost-effective replacement for conventional urine culture methods and its significantly reduced workload in the microbiology laboratory compared to that for Blood agar and MacConkey agar, and should be considered as an alternative to conventional culture methods for detecting and reporting uropathogens [8]. *Klebsiella* isolates found to be non-motile which differentiate them from other motile Enerobaceriaceae genus. All isolates gave negative results for oxidase test and positive results for catalase and urease tests. Glucose was fermented with the production of acid and gas, H_2S was not produced. Most of clinical *K. pneumoniae* isolates obtained in presented study exhibited haypermucoviscosity by forminga string ≥ 5 mm in length [9]. The results of biochemical test were summarized in table 1.

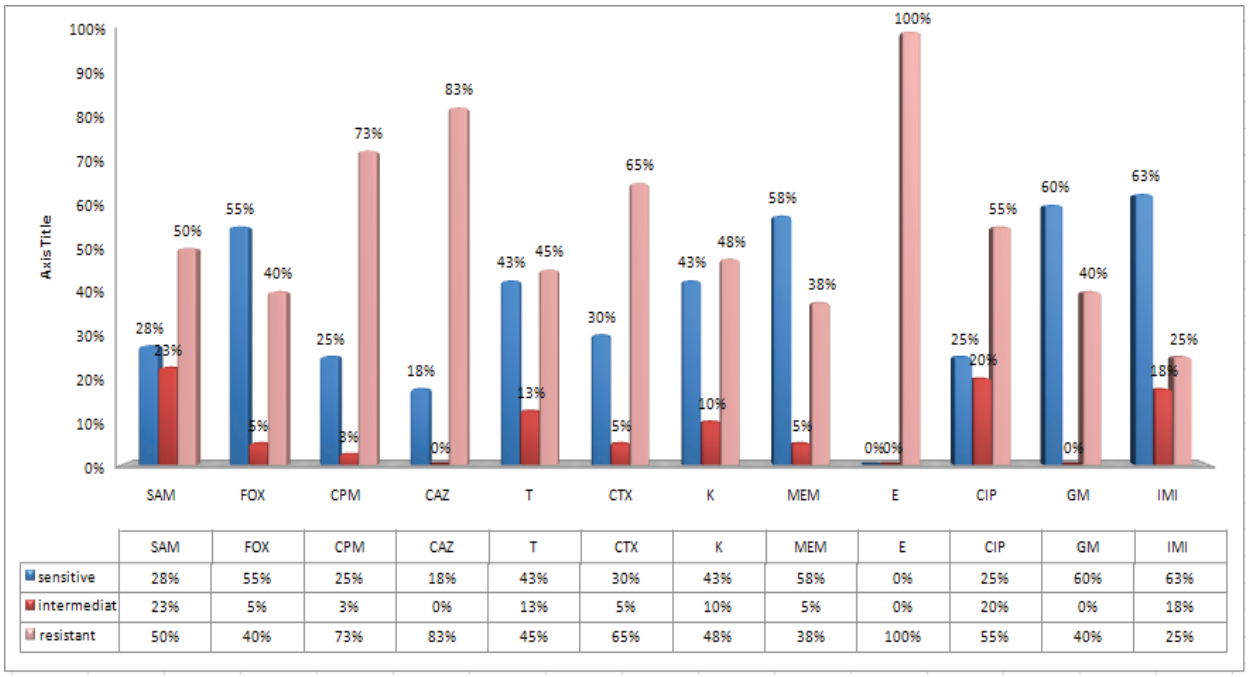
Table (1): The results of some biochemical test of *Klebsiella pneumoniae* and others bacteria.

ID	Biochemical tests		K. pneumoniae
1	Gram stain		Negative
2	Motility		Negative
3	indole		(-)ve
4	Simmon citrate test		(+)ve
5	Urease test		(+)ve
6	Triple sugar iron	H2S	(-)ve
7		CO2	(+)ve
8		Acid	A/A
9	Methyl Red test		(-)ve
10	Voges Proskauer(VP)		(+)ve
11	Catalase Test		(+)ve
12	oxidase		(-)ve
13	String		Positive (≥ 5mm)

The VITEK 2 system was used to confirm a final diagnosis of *K. pneumoniae.* This system was detected bacteria faster, efficient and away from the contamination that may prevent detection of the pathogen. Results of the tests used in this system confirmed the results obtained from morphological, biochemical, so all isolates (40) that previously identified as *Klebsiella* spp. are proved to be *Klebsiella pneumoniae*.

Antibiotic Susceptibility of *Klebsiella pneumoniae*

The antibiotics resistance and sensitivity of *K. pneumoniae* isolates using disc diffusion method was evaluated for all 40 isolates with 12 antibiotic discs as shown in Figure (3).



	SAM	FOX	CPM	CAZ	T	CTX	K	MEM	E	CIP	GM	IMI
sensitive	28%	55%	25%	18%	43%	30%	43%	58%	0%	25%	60%	63%
intermediat	23%	5%	3%	0%	13%	5%	10%	5%	0%	20%	0%	18%
resistant	50%	40%	73%	83%	45%	65%	48%	38%	100%	55%	40%	25%

Figure (4): Antibiotic susceptibility results for 40 *K. pneumoniae* isolates with 12 antibiotics. (Kanamycin (K), Gentamicin (GM), Imipenem (IMI), Meropenem (MEM), Ceftazidime (CAZ), Cefotaxime (CTX), Ciprofloxacin (CIP), Tetracycline (T), Ampicillin sulbactam (SAM), Erythomycin (E), Cefepime(CPM) and Cefoxitin (FOX)).

The results of this study showed that the highest percentage of sensitvity of antibiotic against *K.pneumoniae* **was for impenem (63%),** Gentamicin **(60%) and** Meropenem **(58%), while the lowest percentage was for** Erythromycin **(0.00%) and** Ceftazidime **(18%). In case of intermediate, the highest percentage of intermediate activity of antibiotic against** *K.pneumoniae* **was for ampicilln-sulbactam** Ampicilln sulbactam (23%) **,** Ciprofloxacin(20%), **impenem (18%) and** Tetracycilln **(13%), while the lowest percentage was for** Erythromycin, Gentamicin, Ceftazidime **(0.00%),** Cefepime(3%), Cefoxitin, Cefotaxime **(5%) and** Kanamycin **(10%). Finally, the highest percentage of antibiotic resistance by** *K.pneumoniae* **was for** Erythromycin **(100%)** Ceftazidime(85%), **,**Cefepime **(73%)and** Cefotaxime (65%) **while the lowest percentage was for impenem (25%) and** Meropenem (38%)**.**

The antibiogram results showed that a significant resistance to the most of antibiotics used in this study. In a local study on *K. pneumoniae* isolates

from inpatients with and burns infections in Al-Kufa hospital in Al-Najaf province, Iraq. A total of 43 *K. pneumoniae* strains were isolated. The highest resistance rate was observed for amoxicillin, and amoxicillin+clavulanic acid (97.67%) while the lowest resistance rate was observed for imipenem (9.30%) [10].

One of the studies about the antimicrobial susceptibility patterns of *Klebsiella isolates* from burn patients. Out of 883 isolates from 1294 patients 195 were found to be *Klebsiella* spp. Based on the biochemical properties 153 isolates were *Klebsiella pneumoniae*,. In this study it was found that 54% of the *Klebsiella isolates* were multidrug resistant as they were resistant to at least one antibiotic of three or more different groups of antibiotics[11] . Two hundred and seventy-two wound swabs from burnt patients were collected from Burn Intensive Care Unit of Eastern India, out of which 62.8% (n = 185) were revealed as positive for the presence of bacteria. *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii* and *E. coli* were discovered to be the most common organisms in patients. Isolated bacteria were least resistant to tigecycline and colistin. All the *K. pneumoniae* isolates were resistant to ampicillin, cefuroxime, ceftriaxone and cefepime [12].

Minimum Inhibitory Concentrations (MICs) of Indolicidin against *K. pneumoniae* isolates

By using Microtiter Plate Assay with Resazurin Dye , Minimum Inhibitory Concentrations (MICs) of Indolicidin against *K. pneumoniae* isolates were detected as shown in Figure (6) and Table (3).

Table (2): The Minimum Inhibitory Concentrations (MICs) of AMP (indolicidin) against *Klebsiella pneumoniae* Isolates.

The Isolate cod	Minimum Inhibitory Concentration (MIC) (µg/ ml)							
	100	*50*	*25*	*12.5*	*6.25*	*3.125*	*1.5*	*0.7*
K 3	+	+	+	+	+	+	+	+
K 5	+	+	+	+	+	+	+	+
K 6	+	+	+	+	+	+	+	+
K 19	+	+	+	-	-	-	-	-
K 20	+	+	+	+	+	-	-	-
K 21	+	+	+	+	+	-	-	-
K 24	+	+	+	+	+	-	-	-
K 25	+	+	+	+	+	-	-	-
K 30	+	+	+	+	-	-	-	-
K 33	+	+	+	+	+	+	+	+

- = Growth; + = Inhibition

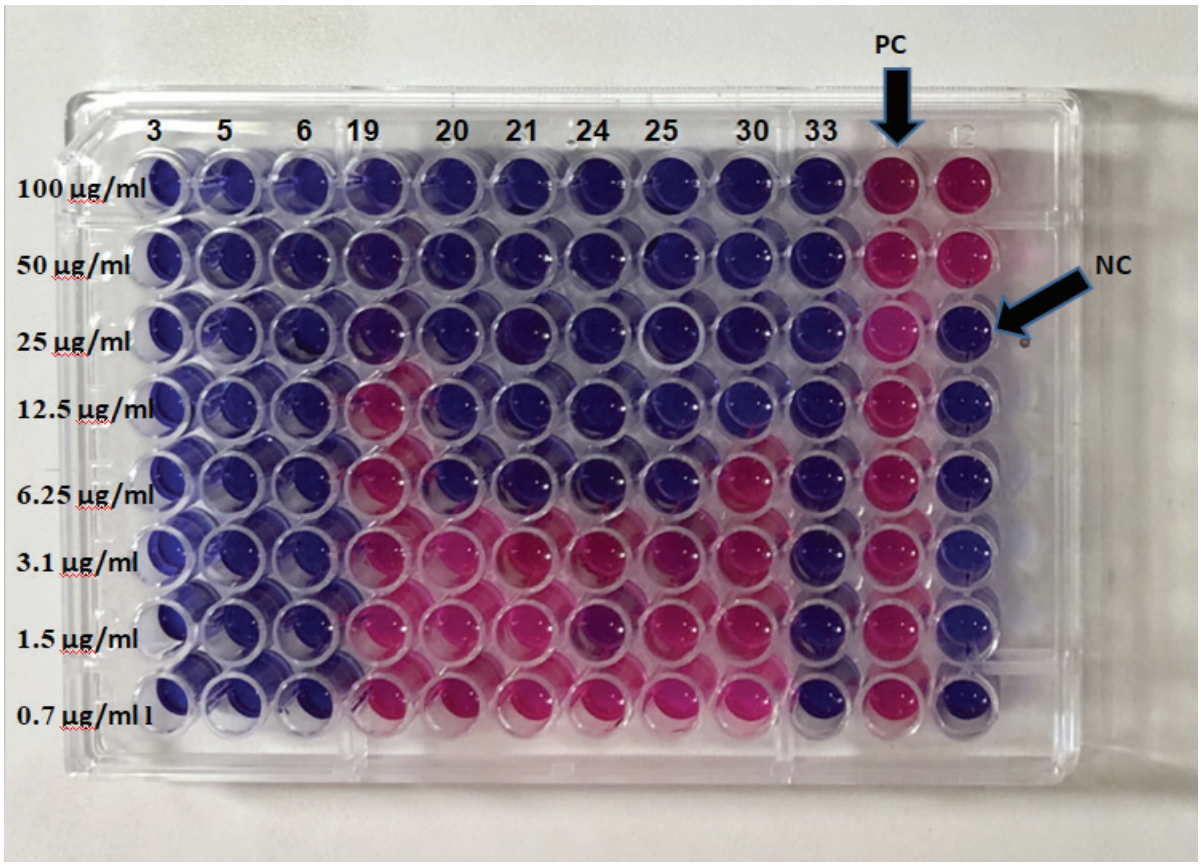


Figure (4: The minimum Inhibitory Concentrations (MICs) of AMP (indolicidin) at the Concentrations (0.7-100 µg/ml) Against *klebsiella pneumoniae* Isolates by Microtiter Plate Assay with Resazurin Dye.

The present study demonstrated that MIC was different from strain to another, where it was found that the isolates K3, K5 and K6 were very resist to the Indolicidin concentrations used in this study (0.7-100).

The minimum inhibitory concentration of the isolates K20, K21, K24 and K25 was 6.25.125, while for the isolates K19 and K33 was 25, and 12.2 was recorded for the isolate K3.It was reported that the production of a hybrid molecule composed of AgNPs and indolicidin had antibacterial activity, where the AgNP antibacterial activity was evaluated versus oral Gram-positive and Gram-negative bacteria. This study found that the coated nanoparticles' antibacterial activity strongly inhibited the growth of microorganisms, with very low minimum inhibitory concentration (MIC) values in the range of 5–12.5 µg/mL, and this effect depended on the specific characteristics of the metal surface coated with indolicidin [13].The cytoplasmic membrane was the site of action of indolicidin as assayed in *E. coli* by the unmasking of cytoplasmic beta-galactosidase due to membrane permeabilization. The mechanism for this activity was shown to be the ability of the peptide to cause an increase in the transmembrane current of planar lipid bilayers. The small size and unique composition of indolicidin, it was capable of killing Gram-negative bacteria by crossing the outer membrane and causing disruption of the cytoplasmic membrane by channel formation [14].

In a previous study, Among these 15 antimicrobial peptides (AMPs), melittin, indolicidin and mastoparan showed good activity against both colistin-susceptible

and colistin-resistant *A. baumannii*, where Indolicidin showed MICs of 8 and 16 mg/L for colistinsusceptible *A. baumannii* and colistin-resistant *A. baumannii*, respectively [15]..Indolicidin was used as a positive control since it is known to be active against S. aureus, The MIC of indolicidin was determined to be 16µg/ml (MSSA) and 32µg/ml (MRSA),and Indolicidin possesses a broad antimicrobial activity against a range of Gram-positive and Gram-negative bacterial strains due to its high affinity for lipopolysaccharides and membrane proteins [16].

Conclusion

The potent activity of antimicrobial peptide Indolicidin against antibiotic-resistant strains of *K. pneumoniae*, suggests this peptide could be a critical advancement in the development of new treatments for *K. pneumoniae* infection, especially in burn and wounds.

Conflict of Interest: None

Funding: self

Ethical Clearance: Not required

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