Gene expression of Combination Effects of Phage-encoded Pseudolysin Protein and Antibiotics on MDR and XDR Bacterial Isolates

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Abstract

Background and Objective: Bacterial resistance to antibiotics is a current healthy problem that has claimed millions of people in recent years. Thus, the aim of present study was to evaluate the use cell wall lysis protein (endolysin) and determine if they could replace or enhancing the action of some of the current antibiotics used in treating some bacterial infections.

Materials and Methods: Different clinical specimens were collected from patients to isolate and identify the bacterial isolates by using cultural,biochemical tests API systems and vitek-2 system followed by testing their resistance to antibiotics using antibiotic susceptibility test,then estimation the gene expression of efflux pump resistance genes when tested against antibiotic alone and with combination with prepared endolysin using real-time PCR.

Results: The results of antimicrobial activity of synthetic phage encoded endolysin (1 mg/ml) against MDR Escherichia coli,and klebsiella pneumoniae showed high efficient($p<0.05$) on the expression of efflux pump genes.On the other hand the activity of endolysin along with antibiotic showed a significant($p<0.05$) decrease in the expression of efflux pump genes. The results of gene expression for efflux pump resistance genes of Klebseilla pneumonia,and Escherichia coli gave high decrease in the expression when using endolysin, and when using endolysin with antibiotic compared with control isolates.

Conclusion:The synthetic endolysin revealed a significant effect as antimicrobial agent against MDR isolates and the effect of combination therapy of endolysin and antibiotic was synergistic effect and the expression of efflux pump genes were significantly decrease.

1. Introduction

Antibiotic resistance has been referred to as “the silent tsunami facing modern medicine” [1]. In February 2017, WHO put some pathogens on a list with the acronym ESKAPE (Staphylococcus aureus, Acinetobacter baumannii, Enterococcus faecium, Pseudomonas aeruginosa, Klebsiella pneumonia, and Enterobacter species), which were given the highest "priority status" because they pose the greatest threat to humans [2]. Among the several alternative agents present currently, bacteriophage encoded peptidoglycan hydrolases, commonly known as endolysins or lysins, are a viable alternative for treating drug-resistant bacteria. Endolysins are enzymatic proteins produced inside the bacterial host during the later stages of bacteriophage replication that can lyse cells when exposed outside [3]. Bacteriophages provide an appealing solution to combating rising antibiotic resistance because of their selectivity and limited range of action [4].

However, rather than replacing antibiotics, combining both kinds of antimicrobials may be more effective than using just one. The combination strategy's expected benefits include enhanced bacterial suppression, better penetration into biofilms, and a lower likelihood of phage resistance [5]. However, there has been observed antagonism between antibiotic and phage, choice of phage and antibiotic is experimentally established, and the influence of host variables on the efficiency is uncertain. The study looks into phage antibiotic interactions using antibiotics that have various modes of action. The findings imply that phage may reduce the working MIC for bacteria that are previously resistant to the antibiotic; however, this is dependent on the antibiotic class and pairing stoichiometry, and the host microenvironment also has a significant impact [6].

Endolysin research is a highly dynamic area., with various possible uses being researched in the medical, veterinary, and food industries. Much of this research is being driven by the present worldwide antimicrobial resistance challenge, with endolysins showing significant potential as antibiotic replacements or supplements. Endolysins with improved or novel features may be engineered to provide even more effective instruments [7].

Antibiotics have enabled the treatment of formerly incurable and lethal bacterial illnesses such as meningitis and bacteremia. Antibiotic abuse and misuse, as well as societal and economic factors, have increased the growth of antibiotic-resistant bacteria in recent decades, rendering the medical treatment ineffective. Antimicrobial resistance kills at least 700,000 people per year around the world. The World Health Organization (WHO) estimates that by 2050, this figure will have risen to ten million people due to a lack of new and better therapies. Understanding these bacteria's resistance mechanisms is a crucial step in developing new antimicrobial medications to combat drug-resistant bacteria [2].

In gram-negative bacteria, the RND (Resistance Nodulation Cell Division) efflux superfamily is most commonly linked to antibiotic resistance. This efflux pump is made up of a cytoplasmic membrane-spanning

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transporter protein that interacts with an outer membrane-specific protein (OMP) and a periplasmic protein MFP (membrane fusion protein). Antimicrobial resistance has increased as efflux pump expression has been upregulated [8]. The present study aimed to evaluate the combination of synthetic pseudolysin and antibiotics against multidrug resistant bacterial isolates(phenotypic and genotypic study).

2. Materials and Methods

Samples collection

During the period from September 2021 To February 2022, A total of 180 patients (males &females) of different ages whom admitted from different clinical wards in Al-Diwaniyah Teaching Hospital and Burn center in Al-Diwaniyah city. The samples include swabs from Naso-gastric tube, Sakar tube from intensive care unit patients, burns, oral endoscope, and foley catheter. A portable container was used to transfer all samples to the laboratory. After labeling, the swabs were streaked on MacConkey's, then incubated at 37°C for 24-48 h in an aerobic environment to perform the diagnosis, according to the standard microbiological procedure [9].

Antibiotic Susceptibility Testing

For the antibiotic susceptibility test, the antibiotic discs supplied by Bioanalyze company (Turkey). According to Clinical and Laboratory Standards Institute recommendations, the antibiotic susceptibility of the isolates was evaluated using the Kirby–Bauer disk diffusion technique.Each isolate's suspension was smeared on the surface of Mueller-Hinton agar (HiMedia/India) using sterilized glass rods. The antibiotic discs were then put aseptically on the surface of the Mueller Hinton agar plate that had been inoculated. The plate was then incubated for 18 hours at 37°C. The diameters of the inhibition zones were measured according to CLSI (2021) to establish antimicrobial susceptibility. The Endolysin used antibacterial like-substance in this work was supplied from laboratory of zoonotic disease research unit / University of Al-Qadisiyah/ College of Veterinary Medicine/ Iraq.

Primers

This work used the NCBI-Genbank database and the online primer3 plus tool for designing PCR primers. The Macrogen Company in South Korea supplied these primers, which are listed in table (1)

Table (1): The specific primers for target genes and their sequences.

Primer		Sequence (5'-3')	Product Size	Genbank
Klebsiella pneumonia - AcrAB	F	CGAAAGTGGAGCTGGTGACT	113bp	MK106181.1
	R	GTAGCGTGATTGAGCCGGT		
Escherichia coli TolC	F	ATTAATGAAGCGCGCAGTCC	82bp	FJ768952.1
	R	TTAACTGCAAGGACGCACTG		
Klebsiella pneumonia - recA housekeeping-gene	F	TAACGCGCTGAAGTTCTACG	125 bp	DQ859859.1
	R	GCGCGGCAATCTTGTTTTTC		
Escherichia coli HKG-gapA housekeeping gene	F	AGGCGAAATGAAAGGCGTTC	77 bp	EU014639.1
	R	AAGTGCAAACTTCGCCGTTG		
*F =Forward ; *R= Reversed				

Real-Time PCR

Table (2). shows the Real-Time PCR expression Kits that are used in the present work & their manufacturing company and the country of origin.

Table (2): PCR kits content used in this work with their remarks.

PCR Kit	Companies	origin
easy-BLUE™ Total RNA Extraction Kit	iNtRON	South Korea
Trizol reagent 100ml		
DNase I enzyme kit	Promega	United state
DNase I enzyme		
10x buffer		
Free nuclease water		
Stop reaction solution		
Accu Power® Rocket ScriptTM RT PreMix	Bioneer	South Korea
Rocket Script Reverse Transcriptase (200U)		
5X reaction buffer		
dNTP 250µM		
DTT 0.25Mm		
RNase Inhibitor (1U)		
RealMODTM Green SF 2X qPCR mix	iNtRON	South Korea
RealMODTM Green SF 2X qPCR mix (1ml)		

Total RNA were extracted from MDR bacterial after growing in the three groups using antibiotics (Klebsiella pneumonia: cefotaxime /Escherichia coli: ciprofloxacin) and endolysin 1 mg (according to recommendations of previous studies) by used easy-BLUE™ Total RNA-Extraction Kit and follow the manufacturer's instructions.The total RNA that was extracted was analyzed using Nanodrop(Thermo-Scientific-NanoDrop-Lite–UV-Visible Spectrophotometer /USA) determined the concentration of the RNA in units of ng/L and evaluated the purity of the RNA in terms of absorbance at 260/ 280 nm. The RNA that had been extracted was then treated with the DNase-I enzyme to remove any traces of genomic DNA that might have been present in the eluted total RNA. This was done with the help of samples from a DNase I enzyme kit and was carried out following the method outlined in the instructions provided by the Promega company(USA), DNase treated total RNA samples were employed in the cDNA synthesis process from mRNA transcripts by use (Accu-Power® RocketScriptTM RT PreMix), this kit was performed following the instructions provided by the manufacturer Then, the RT mix components that were listed in the table above were put in the strip tubes of the Accu-Power® Rocket-ScriptTM RT PreMix kit. These strip tubes included all of the other components that were required for cDNA synthesis, such as primers and terminators (Reverse Transcriptase, 5X-Reaction Buffer, DTT, dNTP, and RNase Inhibitor). After that, all of the strip tubes were placed in an Exispin vortex centrifuge at 3000 rpm for 3 minutes, and then they were incubated in a Thermocycler (BioRad, USA) according to the protocol for the thermocycler conditions. The qPCR master mix was made by using a kit called RealMODTM Green SF 2x qPCR mix Kit, which was based on the amplification of SYBER green dye in a Real-Time PCR system. After that, the components of the qPCR master mix were transferred to qPCR white plate strip tubes, exposed to an Exispin vortex, and centrifuged for a total of five minutes. Finally, they were inserted into a MiniOpticon Real-Time PCR system. The qPCR thermocycler conditions were carried out following the instructions provided in the qPCR kit, and Optimase ProtocolWriterTM was used online for primer annealing calculation purposes. The data results of qPCR were obtained for both the target genes and the housekeeping genes, and the expression analysis (fold change) was performed by using the(ΔCT methods using a reference gene) which was published by Livak and Schmittgen [10], and the following equation describes the method:

Ratio(reference /target) = 2 CT (reference) – CT (target)

Statistics analysis

The results of the current study were statistically analyzed using the Statistical Package for Social Science SPSS Twenty-Third Edition, and the statistical program was used according to the data of the study results. The Chi-square test was applied for this purpose, and significant differences less or equal to 0.05 were determined [11].

3. Results and Discussion

Isolation and identification of bacterial isolates

The results revealed that out of 180 isolates, only 153 (85%) gave bacterial positive growth and showed that Gram-negative bacteria comprised a high ratio of 114 (74.50%) which included *klebseilla pneumoniae* 30 (19.60%), *Escherichia coli* 37(24.18%), *Pseudomonas aerugenosa* 19 (12.41%), *Enterobacter* spp were 20 (13.07%), A *cintobacter bumani* 3 (1.96%) and *Citrobacter* spp 5 (3.26%).

Antibiotics susceptibility profile for isolated bacteria

Bacterial isolates were tested for their antibiotic's susceptibility toward 20 antibiotics using Kirby-Bauer disc diffusion method. Screening for antibiotic susceptibility were done according to CLSI (2021). The antimicrobial potency of selected antibiotics against the isolates are shown in figure (1).

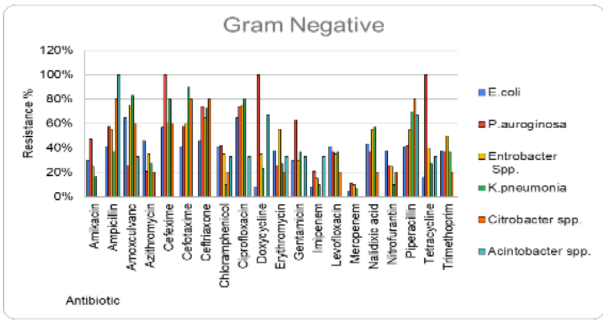

Figure (1): Antibiotic resistance for gram negative bacteria.

The findings of this research indicated that 55 (36 %) of the isolates were MDR (meaning that the bacteria in the isolate are resistant to at least one in three or more classes of the tested antibiotics). Also, the result revealed that isolates percentage 5(3%) as XDR (bacterial isolate still sensitive to one or two class of antibiotics). On the other hand, the findings indicated that PDR bacteria was not present (bacterial isolate resist all types in all classes of antibiotics). The results showed that 55(36%) of the bacterial isolates were MDR, while 5(3%) were XDR and no isolates were PDR (resistant to all antibiotic groups). The results were nearly similar to a study done by Baiou et al. whom found Klebsiella pneumoniae MDR with ratio (23.5%), Enterobacter cloacae MDR (18.4%),E.coli MDR (12.12%),and Pseudomonas spp MDR were (7.7%) [12].While Shah et al. found that MDR isolates percent were 60.3% for Staph aureus and 33% for Acintobacter [13]. While As found that isolates of MDR were (71.23%) in his study [14].

Relative Gene expression of resistance efflux pump genes

The reverse transcription Real-Time PCR (RT-qPCR) was done in order to molecularly detection and quantify the levels of gene expression for the efflux pump antibiotic resistance gene (AcrAB and TolC) in Multi-drug isolates. It performed a relative expression analysis by using the ΔCT (using reference gene) method. This approach was used for the comparison of expression levels (levels of mRNA transcripts) of target genes with a housekeeping gene that was suitable. In this research it used (recA and HKG-gapA) respectively. The analysis results of relative gene expression for efflux pump resistance genes for tested bacteria(Klebseilla pneumonia and Escherichia coli)showed the highest decrease (fold change) in the gene expression for AcrAB and TolC-gapA, respectively when tested with endolysin as antibacterial-like substance compared with control isolates (fig.2)

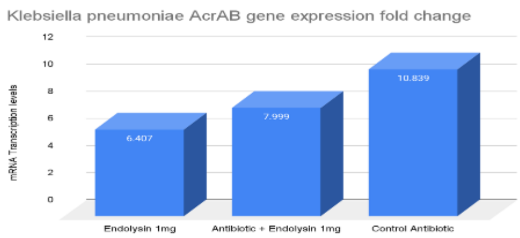

Figure (2):Relative Gene expression of resistance efflux pump in K.pneumonia under different treatment.

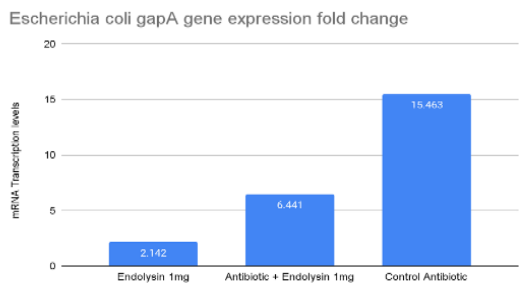

Figure (3): Relative Gene expression of resistance efflux pump in E.coli under different treatment.

Combination effect of Endolysin and Antibiotics

Table (3) shows the comparisons of relative gene expression of tested bacterial isolates with three treatments (endolysin 1mg; antibiotic +endolysin,and antibiotic alone).

Table (3):Gene expression of combination effect of endolysin and antibiotics.

Bacteria	**mRNA Transcription levels**		
	Endolysin 1mg	Antibiotic + Endolysin 1mg	Antibiotic As control
K.pneumoniae	6.4±3.2a	7.9±1.4 a	10.9±1.6 b
E.coli	2.1±0.2 a	6.4±2.2 a	15.4±5.8 b

This table revealed the presence of a significant decrease ($p \leq 0.05$) in transcription levels of mRNA of efflux pump gene in all tested bacterial isolates when used the combined of antibiotic and endolysin (1mg) in comparison with antibiotic alone. Also there was a significant decrease ($p \leq 0.05$) in transcription levels of mRNA of efflux pump gene when compare the used of antibiotic alone with endolysin.

The previous studies observed that endolysin and antibiotic can work together in a synergistic manner. For example, Combining colistin with LysABP-01 endolysin, which is produced as an A. baumannii phage, resulted in significant growth suppression and a synergistic result. Combining LysABP-01 endolysin with other antibiotics [15]. Through a minimum inhibitory concentration (MIC) and a time-kill assay, it was shown that the muralytic activity of ElyA1 endolysin in a variety of MDR strains had an elevated level of activity when used in combined with colistin. This activity was seen in a variety of A. baumannii and P. aeruginosa strains, but not in Klebsiella pneumoniae [16]. Both Gram-positive and Gram-negative bacteria were shown to benefit from the synergistic effects that antibiotics and endolysin have on one another. Chimeric endolysin Cpl-711 was shown to have a synergic effect with a number of different antibiotics in the treatment of several drug-resistant strains of S. pneumonia [17].

The use of endolysins as an external therapy for Gram-negative bacteria was limiting because of the presence of the outer-membrane, which blocks access to the peptidoglycan layer. In the process of creating medicines based on endolysin, one of the most significant challenges is overcoming this protective barrier. The majority causes of nosocomial infections (Acinetobacter baumannii and Pseudomonas aeruginosa). Which both are Gram-negative and have the ability to form biofilms,and resistant to many drugs (MDR), So, certain endolysins have the ability to intrinsically pass through the outer membrane [7].

Lai et al. recombinantly expressed LysAB2 from AB2,and then apply it to A. baumannii. An amphipathic α-helix may be found at the C-terminus of LysAB2. This helix interacts with the negatively charged components of the outer membrane, which in turn helps to facilitate the creation of a transmembrane pore. This makes it possible for the N-terminus catalytic domain to react with the peptidoglycan layer and lyse the cell, which ultimately results in antibacterial activity [18]. The primary findings of Gu Liu et al. explained the following: (a) the invention of a novel high-throughput platform for rapidly assessing the effects of different phage and antibiotic concentrations on the bacterial growth, a process termed "synography "(The resulting data represent as synogram); (b) Synograms show a broad ranges of circumstances in which combinatorial therapy is synergistics, additive, or antagonistic, with all three occurring in same analysis at times; (c) phage may exhibit extremely efficient killing or inhibition when paired with particular class of antibiotics,but not with another; (e) that phage may restore antibiotic competence even with bacteria that encoded resistance elements against the antibiotic of choice, a result we call "phage adjuvation" since phage adjuvate, or make the antibiotic better; (f) that greatly genetically similar phages produce significantly different synograms even if combining them with the same group of antibiotics; (f) that phage-antibiotic synergy can avoid resistance, but just if the concentration of the antibiotic is increase; (g) that host-like condition have a significant effect on PAS and synogram profiles in general, highlighting needing for test antibacterial effect under conditions that more reliably simulate the host environment. PAS seems to be dampened in this situation because of slower growth rate when the bacteria is in urine or blood [19].

The present results showed that the endolysin has a significant activity ($p \leq 0.05$) against selected Gram-negative bacterial isolates. In similar manner, the LysAB54 (100g/ml) was reported to kill logarithmic A.baumannii with 0.6 logs of decrease at the first minute of incubation, and more than 4 logs of reduction in the bacterial number after a 10-minute incubation [3]. The findings revealed that Lys AB54 had both robust and quick bactericidal action, as well as strong bactericidal activity against all A.baumannii, E. coli, and K.pneumoniae isolates examined. Furthermore, LysAB54 susceptibility was discovered in 8 out of 10 P. aeruginosa strains. The variation in log reduction's antibacterial efficacy against different clinical strains might be attributed to differences in the bacterial outer membrane's molecular architecture. Notable Reductions in 4 logs (from 4.2 to 0), 2.17 logs (from 5.77 to 3.60), 2 logs (from 4.22 to 2.16), and 2.33 log (from 3.86 to 1.53) were observing in A.baumannii, P.aeruginosa, E.coli, and K.pneumoniae. These results suggested that LysAB54 has a broad range

of antibacterial activity against multi-drug resistant Gram-negative microbes.
Also Park et al. studied recombinant AP 50-31 and Lys B4, which displayed broad bacteriolytic activity against all the Bacillus spp [20]. Oliveria et al. produced a recombinant endolysin (Abgp 46) from Acinetobacter-phage vb_Aba P_CEB1, and several multidrug-resistant A.baumannii strains were suppressed. They combined endolysin with a membrane-permeabilizing components to provide antibacterial action against Gram-negative bacteria including P. aeruginosa and Salmonella typhimurium [21].
On the other hand, Guo et al. observation was that Lys PA26 recombinant endolysin, expected to relate to the lysozyme-like domain family and encoded by the Pseudomonas-bacteriophage JD 010, demonstrated bactericidal effect against exponentially growing P.aeruginosa as a functions of concentration, peaking activity at 500 µg/mL -1 without outer membrane-permeabilizers. P. aeruginosa cells were eliminated 100% at a concentration of 500 µg/mL -1, compared to 20% at a concentration of 50 µg/mL -1. Furthermore, after adding LysPA26 up to 50 µg, P. aeruginosa biofilm was dramatically decreased [19].
Finally, in this research an attempt was done to enhance the activity of endolysin by adding specific antibiotics to view the combination effects when using them together,and concluded that there are a significant effect on bacterial isolate represented as change in the fold of gene expression of resistance efflux pump in each bacteria. This may help as in elimination of MDR gram negative bacteria and may also used as a treatment strategy in the future.

Ethic Consideration

This study in accordance with ethic committee of Al-Diwaniayah teaching hospital,Iraq.Verbal agreement was obtained from participants in the study and the relatives pre-taking samples.
Conflict of interest: No known conflict of interest correlated with this publication.
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Availability of data and materials: The data used and/ or analyzed throughout this study are available from the corresponding authors on reasonable request.
Consent for publication: Not applicable.
Competing interest:The authors declared that they have no competing interest.
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