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Research article

Effect of combination of D-glycin and antibiotics on biofilm formation by clinical, food and environmental isolates of *Escherichia coli*

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ABSTRACT

The ability of many bacteria to adhere to surfaces and form biofilm makes the treatment of infections so difficult and has major consequences in a variety of industries such as food industry. Biofilm generates a persistent cause of contamination, thus, in the present study; inhibition of biofilm formation efficacy of the combination of D-glycin with antibiotics was compared with the antibiotic alone against *Escherichia coli* biofilm formation. It was found that the synergetic action of antibiotics with D-glycin was effective on inhibition of biofilm formation. The isolates ability to form biofilm was assayed using the tissue culture plate and Congo-red agar methods and findings of this study were source related; since food origin isolates were more biofilm producers when the Congo-red agar used as compared with tissue culture plate method. Minimum inhibitory concentration (MIC) of D-glycin, imipenem and ceftriaxone were determined. The combinations of D-glycin with antibiotics inhibited bacterial biofilm formation more than the antibiotics imipenem and ceftriaxone separately. Susceptibility test to eight antibiotics: Imipenem, ceftriaxone, ampicillin, amoxicilin, cephalexin, clarithromycin, gentamycin and tetracycline; was performed on planktonic cells, and the cells exhibited different sensitivity patterns to them depending on their source.

Keywords: Biofilm, Ceftriaxone, D-glycin, *Escherichia coli*, Imipenem

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INTRODUCTION

Biofilms are defined as microbial derived sessile communities characterized by the cells that are irreversibly stacked to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they had produced, and exhibit a unique phenotype with regard to growth rate and gene transcription [1]. The ability to attach to surfaces and to engage in a multistep process leading to the formation of a biofilm is almost abundant among microorganisms. Therefore, the presence of bacterial biofilms within wounds is quoted as a significant factor sharing in the chronicity and pathogenesis of wound infections [2, 3]. Biofilm formation has substantial inclusions also; in fields extending from industrial processes like oil drilling, paper manufacture and food processing, to medical fields [4]. The fundamental cellular mechanisms to microbial biofilm formation and behavior are beginning to

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be assumed and are targets for novel specific intervention approaches to sequester problems caused by biofilm formation in these diverse fields. Food spoilage and deterioration not only results in vast economic losses, food safety is a major urgency in today's globalizing market with worldwide transference and consumption of raw, fresh and minimally processed foods.

Antibiotic is widely used to defend the infectious diseases and more uses of antimicrobial agents are thought to heighten resistance of bacteria and it may contribute to antimicrobial resistance in humans acquired through the food chain [5]. The present study aims to find the effects of antibiotics and combination D-Glycin with antibiotics on biofilm formation.

MATERIALS and METHODS

Specimens' collection

Fifteen clinical specimens were collected from patients, the specimens were included urine and stool, and twenty five food samples while the others were from environment included sewage, and identification was carried out by using biochemical tests according to Bergey's Manual [6], Api20E system and VITIK complete system.

Antibiotic stock solution preparation

Solutions of imipenem and ceftriaxone were prepared at final concentration of 1mg/ml by dissolving 0.01 gm of imipenem, ceftriaxone in 10 ml of normal saline followed by sterilization by filtration using 0.22 mμ membrane filter.

Antibiotic susceptibility test

Modified Kirby-Bauer method was used. The diameter of inhibition zone for individual antimicrobial agent was translated in terms of sensitive, intermediate and resistant categories by comparison with the standard inhibition zone [7,8] according to Clinical Laboratories Standards Institute (CLSI, 2011) [9].

Determination of minimum inhibitory concentration of D-glycin

D-glycine was prepared to determine the minimum inhibitory concentration (MIC) for planktonic cells. A stock solution of 1 M of the amino acid was prepared in distilled water. The stock solution was filtered through 0.45 mμ membranes (Billerica, MA. USA). This was prepared to achieve different molarities of amino acid starting with 100mM and serially diluted with the medium to the end point concentrations. MIC tests were performed in 96 flat bottom microtiter plates (TPP, Switzerland). Each test well was filled with 100 µl double strength Muller-Hinton broth. A sample 100 µl of the stock solution was added to the first test well and mixed. A series of dilutions was then prepared across the plate. Each microtiter plate well was inoculated with 10 µl of bacteria to achieve a final inoculum size of $1x10^8$ CFU/ml well with overnight culture. Nutrient broth and bacterial inoculum but without amino acid treatment were considered as positive growth controls, whereas negative controls were the wells that treated with D-amino acid but without inoculum. All control wells were filled and incubated under the same experiment conditions; plates were incubated for 24 h at 37Cº. By naked eye the wells were examined for microbial growth. The lowest D–amino acid concentration that inhibited ≥ 80% of microbial growth conducted MIC value; microbial growth in the test wells was detected as turbid in relative to the negative and positive controls. MIC determination was carried out in triplicate [10].

Biofilm formation assays

Congo-red agar method

Bacterial ability to produce slime layer and biofilm formation was detected using prepared Congo-red agar medium (CRA) [11]. CRA was inoculated with bacterial isolates and the plates were incubated aerobically for 24-48 h at 37C°. A positive result was indicated by black colonies with dry crystalline consistency while pink colonies considered as non-slime producers. A darkening of the colonies but with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

Tissue culture microtiter plate method

Tissue culture microtiter plate method is a quantitative test described by previous study [12]. It is considered as the gold standard technique to detect the biofilm. The bacterial isolates isolated from fresh agar plates were inoculated in 10 ml of trypticase soy broth (TSB) with 1% glucose w/v and incubated at 37Cº for 24 h. The culture was then diluted 1:100 with fresh medium; individual wells of sterile 96 well- flat bottom polystyrene tissue culture plate. Negative control wells filled with sterile broth only. The plates were incubated at 37Cº for 24 h, after incubation, gentle tapping was performed to remove the content of each well. The wells were washed with sterile distilled water once to eradicate free floating bacteria. Biofilm formed by adherent bacteria to the wells were stained by crystal violet (0.1%) w/v. Excess stain was removed using distilled water and plate were left for drying. Micro ELISA auto reader (model 680, Biorad, UK) at wavelength 630 nm was used to obtain the optical density (OD) of stained adherent biofilm. The experiment was performed in triplicate and repeated three times (**Table 1**).

Table 1. Interpretation of biofilm production. Optical density cut- off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control [1].

Average OD value	Biofilm production
≤ OD /ODc < ~ ≤ 2x ODc	Non / weak
2x ODc < ~ ≤ 4 x ODc	Moderate
> 4x ODc	Strong

Study the effect of D-amino acids on biofilm formation

The protocol described by Goh and colleagues [13] with minor modifications was used to assay biofilm formation; it was performed using 96 well microtiter plate. Briefly, TSB was inoculated with *E. coli* overnight the growth culture was diluted to 1:100 (TSB + 1% w/v glucose). Each well of microtiter plate was loaded with 100 µl of medium and 100 µl of 50,100 mM of D-glycin, while the control well without amino acid. Each concentration for the D-amino acid tested

was evaluated as triplicate. The plate then incubated at 37Cº for 24 h. The wells were washed with sterile distilled water once to remove planktonic bacteria. Then 0.1% w/v crystal violet solution was added to each well and the plate was kept to stain for 10 min at room temperature. Crystal violet solution was removed by immersing the plate in a water tray. The plate was then inverted on paper towels to eliminate excess liquid and left to air dry. A treatment with 96% v/v ethanol for 10 min at room temperature was applied on the stained wells to solubilize the dye. The bacterial suspension in each well was mixed well and its optical density was measured in a micro ELISA auto reader at 630 nm.

Statistical analysis

One-way analysis of variance (ANOVA) in factorial experiment with complete randomized design was used. Difference between means was analyzed by least significant difference (LSD) at $p<0.05$ using (SPSS) program 2010 and excel application to find results.

RESULTS and DISCUSSION

Susceptibility test to eight antibiotics; Imipenem, ceftriaxone, ampicillin, amoxicilin, cephalexin, clarithromycin, gentamycin and tetracycline was performed on planktonic cells, and the cells exhibited different sensitivity patterns to them depending on their source as shown in **table 2**. It was demonstrated that there was one *E. coli* isolate isolated from sewage resisted to imipenem, while one *E. coli* isolate that isolated from stool resisted to amoxicillin antibiotic. From urine one isolate was sensitive to gentamycin, while food origin isolates were resistant to most tested antibiotics, only one of them was sensitive to tetracycline and all of them were imipenem sensitive. It was previously reported that the isolated *E. coli* were highly resistant to tetracycline (15.6%), streptomycin (12.5%), ampicillin (10.4%), nalidixic acid (9.4%) and ticarcillin (9.4%) [14]. MICs of the amino acid D-glycin and the antibiotics imipenem and ceftriaxone that suppressed *E. coli* strains were determined as 50mM of D-glycin, and 4µg/ml for both antibiotics.Imipenem is one of the group of carbapenems, which are fused β-lactam antibiotics; that used to treat infections caused by multidrug resistant gram negative bacteria even those producers of extended spectrum β-lactamases, so the resistance to these antibiotics due to the production of carbapenemases [15].
The ability of isolates to form biofilm was assayed using the tissue culture plate and Congo-red agar (CAR) methods. In CRA method the highest strong biofilm formation ratio was by food origin isolates (83.33%) while in TCP method clinical isolates were all of them biofilm producers; nevertheless both of food and stool origin isolates produced the same ratio (66.66) [**Table 3, Fig 1**]

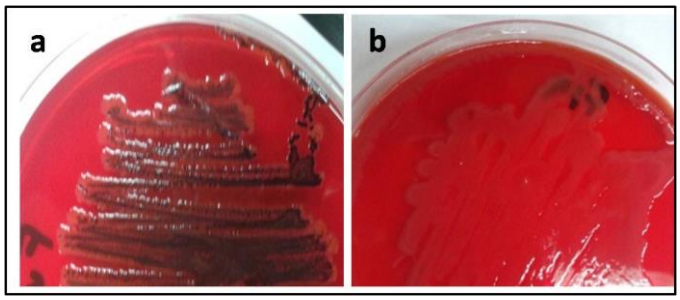

Fig 1. Congo red agar inoculated by *E. coli* isolates; a, Black glistening colonies (strong biofilm producers); b, Red colonies (weak biofilm producers) after 24 h incubation at 37˚C at aerobic condition.

In a previous study compared the results obtained by the CRA and microtiter plate method. Using microtiter plate method is the gold standard, and the results revealed that the sensitivity and the specificity as compared with CRA test were 86% and 100%, respectively [16]. Biofilm formation in food environment by pathogenic or spoilage microorganisms causes a negative influence on food quality and safety thereby acting as a microbial contamination persistent source, leading to food spoilage or transmission of diseases; food spoilage may end in economic losses [17,18].

Table 2. Antibiotic susceptibility of *E. coli* strains according to their source of isolation. I, Intermediate ; R: Resistant ; S: Sensitive

Isolate No.	Source	Antibiotics							
		IPE	CN	AX	CRO	CLR	KF	AM	TE
1	Food	S	I	R	R	R	R	R	R
2	Food	S	R	R	R	R	R	R	R
3	Food	S	R	R	R	R	R	R	R
4	Food	S	I	R	R	R	R	R	S
5	Food	S	I	R	R	R	R	R	I
6	Food	S	R	R	R	R	R	R	R
7	Sewage	S	R	R	R	R	R	R	R
8	**Sewage**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
9	Sewage	S	I	R	R	R	R	R	R
10	Urine	S	S	R	R	R	R	R	R
11	Urine	S	R	R	R	R	R	R	R
12	Urine	S	R	R	R	R	R	R	R
13	Stool	S	R	S	R	R	R	R	R
14	Stool	S	R	R	R	R	R	R	R
15	Stool	S	I	R	R	R	R	R	R

Table 3. Biofilm formation ratio by clinical, food, and environmental *E. coli* isolates using two assays TCP and CRA.

Source	Percentage of the Biofilm formation (%)					
	Congo Red Agar (CRA)			Tissue Culture Plate (TCP)		
	Strong	Intermediate	Weak	Strong	Intermediate	Weak
Food	83.33	—	16.66	66.66	—	33.33
Sewage	33.33	66.66	—	33.33	66.66	—
Stool	66.66	33.33	—	66.66	33.33	—
Urine	66.66	33.33	—	100	—	—

Environments of food processing confer food-interaction surfaces come in contact with fluids holding various levels of food components. Under such circumstances conditioning is one of the first occasions to occur via the adsorption of food particles to surface. Surface conditioning and growth media both were found to affect the attachment of bacterial cells to stainless steel [19]. Microbial efficacy of D-glycin plus imipenem, D-glycin plus ceftriaxone, imipenem and ceftriaxone alone separately against biofilm producing *E. coli* isolated from different sources was determined was demonstrated in **tables 4** and **5**.

Table 4. Relationship between biofilm formation and Imipenem antibiotic susceptibility alone and with D-Glycin. Different letters represents a significant different ($P \leq 0.05$).

Source	Treatments	Imipenem Versus Imp.+D.glycine	
		Mean ± S.d.	Sig.
Biofilm formatoin	Urine	0.156 ± 0.047	a
	Stool	0.113 ± 0.051	b
	Sewage	0.154 ± 0.061	a
	Food	0.133 ± 0.059	ab
LSD P ≤ 0.05		0.030	n1=18, n2=18
LSD P ≤ 0.05		0.026	n1=18, n2=36
	Impinim	0.164 ± 0.049	a
	Imp.+D.glycine	0.112 ± 0.052	b
LSD P ≤ 0.05		0.019	
Interactions	Urine Imp	0.187 ± 0.0 34	ab
	Urine Imp+Gly	0.125 ± 0.036	cd
	Stool Imp	0.150 ± 0.044	bc
	Stool Imp+Gly	0.077 ± 0.023	e
	Sewage Imp	0.211 ± 0.017	a
	Sewage Imp+Gly	0.097 ± 0.017	de
	Food Imp	0.137 ± 0.049	c
	Food Imp+Gly	0.129 ± 0.069	cd
LSD P ≤ 0.05		0.042	n1=9, n2=9
LSD P ≤ 0.05		0.037	n1=9, n2=18
LSD P ≤ 0.05		0.030	n1=18, n2=18

Synergetic effect of D-glycin with impinem in the inhibition of biofilm formation was more than the antibiotic alone. Biofilm rise the opportunity of gene transfer between the microorganisms and can convert a previously commensal organism to an extremely virulent pathogen. The superior efficiency of gene relocation in biofilms also facilitates the blowout of antibiotic resistance and virulence factors [20]. D-glycin with cephtriaxon combination, greatly affected the biofilm formation by *E.coli* isolates, this effect was more than of the cephtriaxon alone. This synergetic inhibitory effect may be due to inhibition of some extracellular expressed proteins as reported by Chaudhary and Payasi [21].

It is obvious from **fig 2** and **3** that the antibiotics with D-glycin were more effective in suppressing biofilm than the antibiotic alone despite the result that imipenem inhibitory effect was so clear. Goh and colleagues showed that glycine inhibited the formation of the biofilm and the extent of inhibition was concentration-dependent [13]. Another studies showed the same effect of D-glycin on biofilm form-

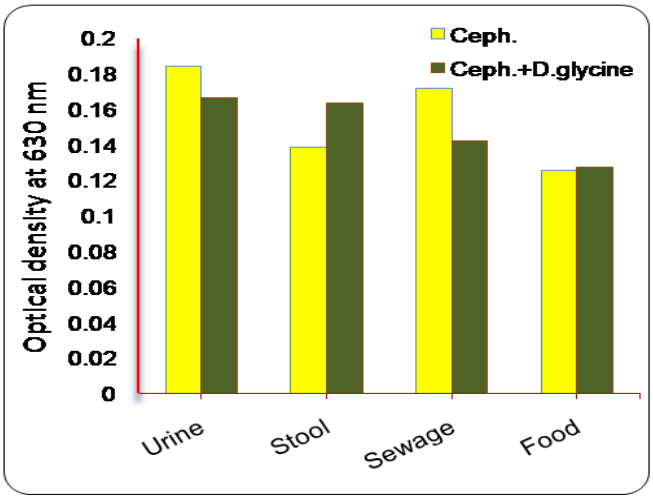

Fig 2. A comparative scheme shows the combination effect of Cephtriaxon with D-Glycin and mere Cephtriaxon on biofilm formation in terms of absorbance means for strains from four sources of isolation.

Table 5. Relationship between biofilm formation and Cephtriaxon susceptibility alone and with D-Glycin. Different letters were significantly different ($P \leq 0.05$).

Biofilm formatoin	Treatments Source	Cephtriaxon Versus Cep.+D.glycine	
		Mean ± S.d.	Sig.
	Urine	0.175 ± 0.047	a
	Stool	0.151 ± 0.052	ab
	Sewage	0.157 ± 0.031	a
	Food	0.126 ± 0.054	b
LSD P ≤ 0.05		0.032	n1=18, n2=18
LSD P ≤ 0.05		0.028	n1=18, n2=36
	Cephtriaxon	0.149 ± 0.052	a
	Cep.+D.glycine	0.145 ± 0.051	b
LSD P ≤ 0.05		0.023	
Interactions	Urine Cep.	0.184 ± 0.0 29	a
	Urine Cep.+Gly	0.166 ± 0.061	ab
	Stool Cep.	0.139 ± 0.032	c
	Stool Cep.+Gly	0.163 ± 0.066	abc
	Sewage Cep.	0.172 ± 0.033	ab
	Sewage Cep.+Gly	0.142 ± 0.023	abc
	Food Cep.	0.125 ± 0.063	c
	Food Cep.+Gly	0.127 ± 0.044	c
LSD P ≤ 0.05		0.045	n1=9, n2=9
LSD P ≤ 0.05		0.039	n1=9, n2=18
LSD P ≤ 0.05		0.032	n1=18, n2=18

ation inhibition [22,23]. D-amino acids did not impede the initial cells attachment to surface but obstructed the following foci growth into superior assemblies of cells [24]. Exogenous adding of the D-amino acids caused disrupted preformed biofilms and was also vigorous in preventing biofilm formation by *S. aureus* and *P. aeruginosa* [25].

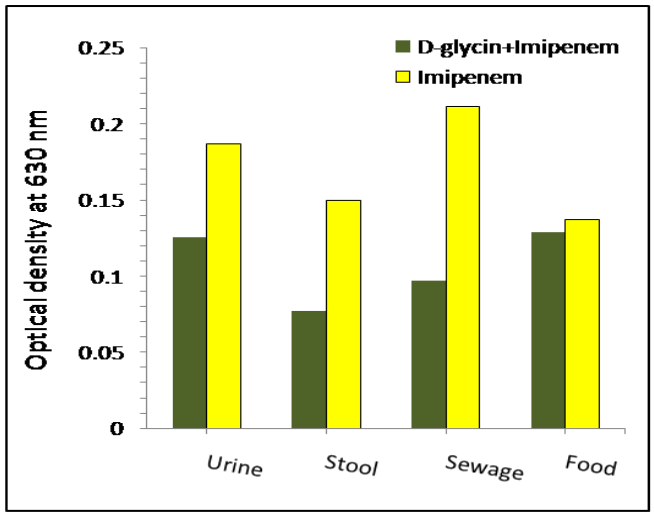

Fig 3. A comparative scheme shows the combination effect of Imipenem with D-Glycin and mere Imipenem on biofilm formation in terms of absorbance means for strains from four sources of isolation.

Cava and colleagues clarified that biofilm disassembly was done by D-amino acids via disrupting adhesive fiber interactions [26]; while another study revealed that D-amino acids inhibit S. aureus biofilm formation by preventing protein localization to the cell surface [24]. In this research, a relationship between the biofilm formation and source of isolates was observed. Our results suggest that D-amino acids provide potential to prevent the formation of biofilm and it may be a vital combination therapy against *E. coli* infections and to minimize or sequester contamination in food environment. Since biofilm has the main impact in the progression of infection and in the contamination complications in food industry and environment. Food origin isolates affected by combination therapy (D-amino acid + antibiotic) more than the other isolates this may be due to the different mechanisms participate in biofilm formation. The outbreak of bacteria that resisted to imipenem in food, environmental as well as clinical *E. coli* isolates indicates uncontrolled antibiotic usage and it is a threat that is reducing the effectiveness of antibiotics against pathogens.

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