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SYNERGISTIC EFFECT OF ANTIBIOTICS AND AMINO ACIDS ON *HELICOBACTER PYLORI* UREASE ENZYME PRODUCTION

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الخلاصة

هذا الاستعراض هو محاولة لتحديد استراتيجيات العلاج الأمثل لعزلات بكتريا ***Helicobacter pylori*** المقاومة للعديد من المضادات الحياتية, ولأن هناك دراسات قليلة فقط على هذه البكتيريا في العراق، بالتالي فإن الدراسة الحالية تهدف إلى تثبيط إنتاج إنزيم اليوريز في بكتريا ***H. pylori*** والذي يعتبر واحد من عوامل الضراوة الأكثر أهمية فيها عن طريق استخدام التأثير التآزري للمضادات الحياتية والأحماض الامينية. عشر عزلات ***H. pylori*** عزلت من أصل 110 نموذج خزعة نسيجية استؤصلت بواسطة جهاز الناظور من معدة المرضى الذين يعانون من مشاكل المعدة و ألاثني . خضعت الخزع للاختبارات المايكروبايولوجية التي تتضمنت: اختبار الزراعة البكتيرية و اختبار اليوريز السريع واختبار التفاعل التضاعفي لسلسلة الدنا. شملت الدراسة إجراء اختبار المضادات الحياتية لجميع عزلات ***H. pylori*** ضد ثمانية مضادات حياتية, أظهرت النتائج ارتفاع في نمط المقاومة الظاهري, حيث (100٪) من العزلات كانت حساسة تماما للتتراسيكلين، (90٪) حساسة للجنتاميسين، و (90٪) من مستوى المقاومة العالي ظهر للاموكسيسيلين, ونسبة العزلات المقاومة وصلت إلى (80٪) لكلاريثروميسين . أيضادرس تأثير مضادي (ألاموكسيسيلين, الكلاريثروميسين)، وحامض أميني واحد الكلايسين بشكل مفرد و خلطيا على البكتريا وقد حددت التراكيز المثبطة الدنيا, التراكيز تحت المثبطة الدنيا والتراكيز القاتلة الدنيا. كشفت النتائج عن وجود فروق في التراكيز المثبطة الدنيا والتراكيز القاتلة الدنيا بين العزلات , مع قدرتها على النمو في تراكيز مختلفة من المضادات . وتم التحري عن التأثير التآزري لمزج (ألاموكسيسيلين مع الكلاريثروميسين) و (ألاموكسيسيلين مع الكلايسين) على إنتاج إنزيم اليوريز في هذه البكتريا ، أظهرت النتائج زيادة واضحة في نشاط التأثير تآزري من مزج التراكيز تحت المثبطة الدنيا للاموكسيسيلين مع الكلايسين بنسبة (100٪) أعلى من مستوى التأثير ألتآزري لمزج التراكيز تحت المثبطة الدنيا للأموكسيسيلين مع كلاريثروميسين بنسبة (70٪) على إنتاج إنزيم اليوريز في عزلات ***H. pylori***.

ABSTRACT

This review is an attempt to select the optimal treatment strategies for ***Helicobacter pylori*** *isolates resistant to many antibiotics, and because there are only few studies on these bacteria in Iraq, so the present study was designed to inhibition* ***H. pylori****Urease enzyme production that consider the most important virulent factor in this bacterium by using the synergistic effect of the antibiotics and amino. Ten* ***H. pylori*** *isolates were isolated out of 110 tissue gastric biopsies specimens wereexsion from patient's stomachthat suffering from Gastroduodenal problems. These biopsies subjected to, Microbiological tests that included: bacterial Cutler test, Rapid Urease Test and Polymerase Chain Reaction test. The study included antibiogram profile test was performed for all* ***H. pylori*** *isolates against Eight antibiotics. The results showed high incidence of antibiotics resistance among* ***H. pylori****isolates, in which (100%) of the isolated were completely sensitive to Tetracycline, (90%) sensitive to Gentamycin, also, high-level of resistance to Amoxicillin (90%), while, the percentage of isolates resistance reached to (80%) forClarithromycin,The effect of Two antibiotics (Amoxicillin, Clarithromycin) and one single amino acid Glycine were investigated separately and in combination by determined Minimum Inhibitory Concentration, sub-Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. the results exhibited differences in MICs values among isolates and the* ***H. pylori*** *isolates have ability to growth in different concentrations of antibiotics And, investigation the synergistic effect of the combination of (Amoxicillin with Clarithromycin) and (Amoxicillin with Glycine) on the* ***H. pylori*** *Urease enzyme production, the results showed increase significant synergistic level from combination sub-MIC Amoxicillin with Glycine with rate (100%) higher than the synergistic effect from combination sub-MICs of Amoxicillin with Clarithromycin with rate (70%) on* ***H. pylori*** *Urease enzyme production.*

Key words: Antibacterial susceptibility profile;Urease enzyme;Anti-***H. pylori*** drugs; Glycine; and Synergistic effect.

Introduction

H. pylori is a Gram-negative bacterium that infects more than half of the world's population and was thought to be involved in the pathogenesis of chronic Gastritis, Peptic Ulcer Diseases (P.U.D.), and Gastric Cancer (G.C.) [1]. Eradication of ***H. pylori***infection not only improves the healing of Peptic Ulcers (P.U.) but, it also prevents its recurrence and reduces the risk of developing G.C. [2], Although the success of the treatment depends on several

factors such as patient compliance and whether the patient is a smoker, the antibiotic resistanceis was most common factor causing treatment failure [3]. Prevalence of antibiotic resistance was now increasing worldwide and varies by the geographic area [4]. In addition, the antibiotic resistance rate often parallels the antibiotic consumption rate in the population [5].

The combination of Two antibiotics can increase the success of eradication therapy and decrease the possibility of secondary antibiotic resistance [2, 6]. Several ***H. pylori*** virulence factors related to the risk of disease have been identified included Urease enzyme that was One of the most important virulence factors in this pathogen, whose presence was associated with sever local inflammation of Gastritis.It is well established an individual infected with ***H. pylori*** strains that express this virulence factor probably will plays a major role in the development P.U. D. [7]. Whoever,many studies indicated that multi-usage for antibiotics treatment of P.U.D. caused by ***H. pylori*** gave a good results in the regimen and control on the spreading but, the randomly misuse of antibiotics and without conducted the sensitivity of antibiotics test has led to appear many resistant strains for one or more antibiotics and became this resistance constitutes a serious problem of health and economic point. Because none or only few local studies [8, 9], investigated the effect of antibiotic and amino acid separately or in combination on ***H. pylori*** Urease enzyme production, so in this study, aimed to determine the Synergistic effect of antibiotics and amino acids on***H. pylori***Urease enzyme production and decreased the chances of infection by this bacterium.

Materials and Methods

Culture media: Columbia Blood Agar Base and Skirrows Selective medium.
Kits: Gas Generation Kit, ***H. pylori*** IgG- ELISA Kit and RUT kit.

Samples Collection: According on these criteria One hundred ten (110) gastric biopsies specimens were exsionfrom patient's stomachguided Upper Oesophageal Gastroduodenal Endoscopy (OGD) under consultant supervision endoscopist to evaluate determination the presence of ***H. pylori***, these patients attended to the Gastroenterology Centre in AL-Yarmook Teaching Hospital in Baghdad; whom suffering P.U. symptoms from over a period January/2015 to March/2016.With age range from 15-85 years. The biopsies subjected to RUT kit device,and Culture test for ***H. pylori*** isolation and identification [10].

Laboratory diagnosis:

- **RUT test:** RUT kit was performed strictly following the instructions of the manufacturer Kolkata Company/Indiain order to confirm the ability of bacteria to secrete Urease enzyme that change medium color with 5-10min. [11].
- **Bacteriological test:** Bacterial identification assays included biopsy specimen was suspended in one mL of sterile normal saline, gently ground in manual homogenizer. the suspension was streaked onto Skirrows Modified Selective Medium and of Colombia Agar plates, the plates were incubated for 5-6days at 37°C under microaerophilic atmosphere using GasPaks generation kit placed in an aerobic jar [12]. Several small round colonies from each patient's plate were selected and sub-cultured them 1or 2 times to obtain a pure culture according to the diagnostic procedures recommended by [13].
- **Polymerase Chain Reaction (PCR):** all ***H. pylori*** have been diagnosed using protocol standard molecular biologyof PCRtechnique with some modification based on this current study, developed by [14], also the isolates with high-level of resistant toward Amoxicillin and Clarithromycin were screening the presence of *UreA* gene.

❖ PCR Template preparation (DNA extraction and purification):The template chromosomal DNA extracted from cells of each bacterial isolates from a pure culture of ***H. pylori*** bacteria by modified Alkaline Lysis method as mentioned in [15].Using DNA Sorb-B isolation kit according to the instruction of Sacace biotechnologies/Italy Company.

❖ DNA amplification procedure:The DNA isolated from ***H. pylori*** isolates was tested to detect *UreA* gene. The upper primer and lower primer for interested gene were designed sequence according to the information of Promega/UAS Company. PCR experiments were performed to bring the total final volume of 50µL with the concentration of each reagent in PCR mixture, and then mixer placed in a PCR Thermal cycler apparatus and adjusted the program system cycling conditions to calculate the mean number of copies of DNA directly [16]. The mixture conditions were applied quantification of ***H. pylori*** by employing to 35 repeated cycles were used for amplification *UreA* gene, and each cycle consisted of One min. Denaturation at 95°C, min. annealing at 45°C and One min. Extension at 72°C. After 35 cycles, the reaction was further extended of one cycle for Ten min. at a temperature of 72°C to final extension replicated DNA strand. Determine the bacterial load {The mass of the ***H. pylori*** genome was approximately 1.65 Mb which translates to approximately 1.78×10^{-6} ng} in the tissue biopsy by extrapolating the total number of copies in the total volume isolated from the DNA isolation procedure based on the number of copies in the 25µL used for the

reaction. Divide this number by the tissue mass to obtain number of copies per mg tissue that ranging from 100 to 10^7 copies according to [17].

❖ Detection amplified PCR products (Gel electrophoresis):Successful amplified PCR products were analyzed by Agarose gel electrophoresis exposing to UV light using UV transilluminator at 336nm to visualized band of DNA a crossing with Ethedium bromide dye and photographed using digital camera. Then compared DNA fragments with DNA ladder that was used to assess PCR product size as a guide reference to [18].

Results and discussion

The 110 patientswith P.U.D. problems under this study was subjected to various investigations for diagnosis the ***H. pylori***infection including (Cutler, RUT, and PCR) tests.

Antibiotics susceptibility testing against *H. pylori* isolated from patients with P.U.D:

In the present work the sensitivity test of Ten ***H. pylori*** isolated from patients with P.U.D. was evaluated against Eightlocal Antibioticsincluded [Amoxicillin (AMX), Clarithromycin (CLR), Erythromycin (E), Gentamycin (CN), Levofloxacin (LEV), Metronidazole (MNZ), Rifampicin (R), and Tetracycline (TET)]. (Figure 1) that showed inhibition zones of some antibiotics against ***H. pylori*** resistance isolate calculated by the diameter in mm.

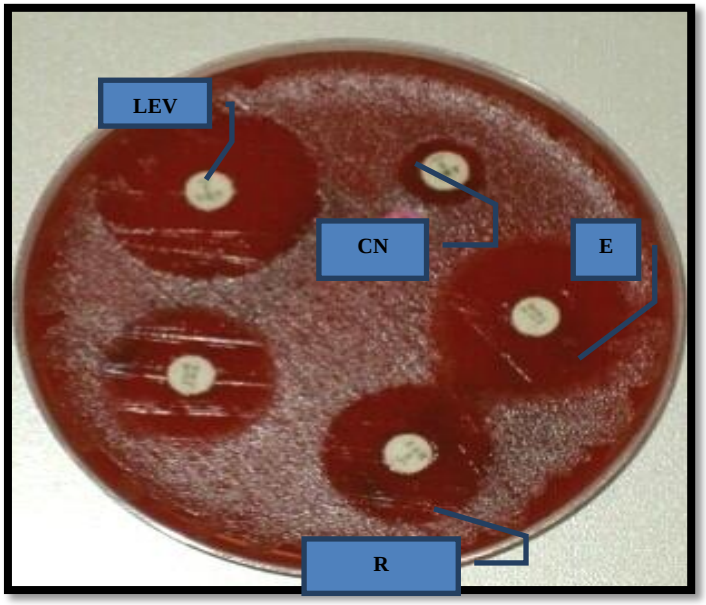

Figure 1: Results of inhibition zones mastered with diameter in mm for Four antibiotics against *H. pylori* resistance isolate by using Disc Diffusion method.

Depending on current data the highest resistance rate was found with AMX and CLR (90%), (80%) respectively among all ***H. pylori***isolates, as tabled in (Table 1) indicating that there are an increased resistance which was worrying about it.

Specifically, it is important to determine whether the increased resistance to AMX and CLR were a result of their increased use or due to the ethnic differences of the populations described herein. The same data were reported in a study carried out in Iraq by [19] on the ***H. pylori*** isolates that recorded totally resistance (100%) rates to this antimicrobial agent. Also, in a study conducted by [20] recorded that drug sensitivity of the isolates were (20%), and (80%) to CLR, and AMX respectively, and revealed that high percentage (80%) of ***H. pylori*** isolates were sensitive to AMX due to the activities of this antibiotic against this bacteriumdepend on the height pH therefore, they a raise pH.

On the other hand the results completely incompatible with the study of [21] whom noted that isolates have a highest sensitivity to AMX, CLR (96-100%), and (90%) respectively, and clarify that these antibiotics are the most effective drugs used in the eradication of ***H. pylori*** infection worldwide.

Furthermore, several published studies focused primarily on***H. pylori***resistance to this drug are either very rare or non-existent [22]. Our approach not correlates with the report by [23] whom indicating that AMX has been considered as a potent drug against ***H. pylori*** isolates and the rates of resistance were still generally low distribution at less than (2%) in all countries, but, local isolates of ***H. pylori*** showed high degree of resistance (90%), may be related to the established new isolates have ability to tolerance or changing in the nature of this antibiotic during the storage or cooling process. This controversy may be related to variations in the environmental factor which Iraqi people exposed to particularly during the last years, and the effects of wars which enhance the disease development bythis pathogen. Unfortunately, the resistance rate for CLR also very high occurrence among***H. pylori***isolates was observed.

The data in (Table1) had been investigate that most isolates increasing frequency of resistance to CLR comparing with the literatures of [24] in Iran, that notifies a low rate of resistance for CLR (30%) susceptibility against this antibiotic since, drug is not currently used in Iran, emerging resistance to this antibiotic is unexpected. And other investigator found that resistance rate of CLR is strongly different to around (10%) where Macrolides

have rarely been used so the level of ***H. pylori*** resistance remains very low [25]. While, the data of [26] from India in their studies have revealed that resistance to CLR are predominating, because this antibiotic often appears at low levels by acquiring of an initial resistance-conferring mutation in gene, in which acquired resistance of subsequent mutations leads to higher levels resistance of bacteria, the study of [27] confirmed that when showed the resistance of ***H. pylori*** against antibiotics associated closely with prior used treatment of other medical cases. The observation in this review found that most resistance ***H. pylori*** isolates was seen with R and E to reached to (50%) and this become constant with results of [28].

The bactericidal effect of R happened by disabling the protein expression system universally conserved by all bacteria. The resistant to this antibiotic occurs because it's targeted the DAN synthesis [29].Whereas, other study of [30] showed the only (10%) of ***H. pylori*** isolates was resistant to R because the resistance for this antibiotic can be acquired but, the rate is very low.

Table 1: Percentage results for antibiotics susceptibility test of Eight antibiotics against Ten local *H. pylori* isolates.

No.	Antibioticclass	Antibiotics Code	Disc potenc µg/disc	Results%	
				Resistan	Sensitive
1	β-lactamases	AMX	20	90	10
2	Macrolides	CLR	15	80	20
3	Macrolides	E	15	50	50
4	Aminoglycosides	CN	10	10	90
5	Fluorquinolones	LEV	5	30	70
6	Nitroimidazoles	MNZ	5	20	80
7	Ansamycines	R	5	50	50
8	Trtracyclines	TET	30	0	100

According to the E ***H. pylori*** resistance isolates appeared with (50%) percentage may attributed to main resistance mechanism of this Macrolides thatlinked to chromosomes mediated as well as, the modification in a target location of 23S rRNA in 50S–subunit in bacterial ribosome that led the production of functionally useful proteins [31]. Our result was higher than [9] results that showed the (25%) of ***H. pylori*** isolates were resistant to E.

However, the result did not agreed with findings of [32] that arise to reach (76%) of isolates resistant to E. Additionally, to these results some isolates have denoted the lowest prevalence rates of resistance against LEV and MNZ to reach (30%) and (20%) respectively of these antimicrobial agents. In this study it seems to be the LEV and MNZ were longer effective antibiotics to treat ***H. pylori*** and the bacteria are rarely resisted. The resistance mechanisms of MEZ have been extensively investigated and new information has been obtained. In developing countries resistance to MEZ can be as high as (80-90%) as reported by [33] whom they obtained that there a trend toward an increased resistance rate in developed countries may be attributed to widespread over-using frequently of this drug to treat not only ***H. pylori*** infection but, also other many infections such asintestinal parasites, gynecological diseases, and dental infections. that facilitate resistance organism can easily to emerge, in which the mechanism action of Nitroimidazolesclass of a MNZ in resistance ***H. pylori*** isolates is able to subsequent damage the nucleic acid helicoidal structure and inhibit DNA synthesis in the ***H. pylori*** cells [34].

Besides that, there have been very few surveys concerning resistance to of LEV as Quinolones class, many studies recommended the use of LEV for ***H. pylori*** eradication is increasing thorough the world because of its role in ‘rescue therapy’ regimens following the failure of CLR-based treatments [35].But, researcher [36] reported resistance to of LEV is usually to be less than (1%) and this data was highly less than our result when recorded that resistance rate to LEV was (30%) which ass close to the results of [37] that has been obtained the primary resistance of ***H. pylori*** to LEV (22%) in different countries.

Resistance to LEV is easily acquired, and the resistance rate is relatively elevated in countrieswith a high consumption of these drugs [38]. LEV as bactericidal drug exert their antimicrobial activity by binding to the sub-unit A of DNA gyrase (topoisomerase II), lead inhibiting the function of this enzyme which is an essential for relax the supercoiled DAN strand to allow it's replication during ***H. pylori*** cell division [39]. Finally, in our pilot study the results of antibiotic susceptibility test shown that approximately similar trends were

observed for sensitivity pattern for both TET and CN antibiotics to record (100%) and (90%) respectively as tabled in (Table 1).

There is a clear impact of resistance on the cure of the infection caused by ***H. pylori***, and partly overcome through the addition of these effective antibiotics. Our findings revealed there was no resistance ***H. pylori*** isolates to TET as illustrate in (Figure 2) this drug is broad spectrum antibiotics, so, the bacterium does affected by the addition of TET.

Our reviews are little bit higher than the literature of other investigators [40] whom they found the sensitivity result to TET higher than (90%) to in most studies. In contrast the researches of [41] disagree with our results because they showed that TET resistance was only (25%).

The interpretation of this minor difference may be related to the variations in the laboratory conditions and timing.Based on the selection criteria, these patients had no prior exposure to antibiotics nor had previous ***H. pylori*** eradication treatment, our result compared with same published study of [22], leading that TET become drug of choice for treat ***H. pylori*** infection.With regard to CN used to treat many types of bacterial infections, particularly those caused by gram-negative organisms.

The bactericidal effect of this antibiotic inhibit ribosome functionsby its work irreversibly through specific bind to bacterial 30S ribosomal subunit leaving the bacterium unable to synthesize proteins, vital to its growth [42] . The lack of resistance to TET and low rate resistance to CN indicates that ***H. pylori*** resistance to these agents is probably exceptional and may related to the smaller sample size or the type of population enrolled. It seems that, in Iraq these two drugs almost give to patient with ***H. pylori*** complications, and may possibly be employed as deterrents for ***H. pylori*** also, can control it's infections, due to their have been potent activity that are clinically important for treating P.U.D. [43]. Moreover, both antibiotics are easy absorption, not toxic and highly efficiency, that made them the most widely antibiotics used in the treatment, and for to increase their effectiveness; they used with the P.P.I. such Omeprazole [44].

These differences in resistancy of some antibiotics may be occurred in some ***H.pylori*** species depending on the drug of choice that has been treated and the time of using in addition to the nature of the isolates and the habitat.

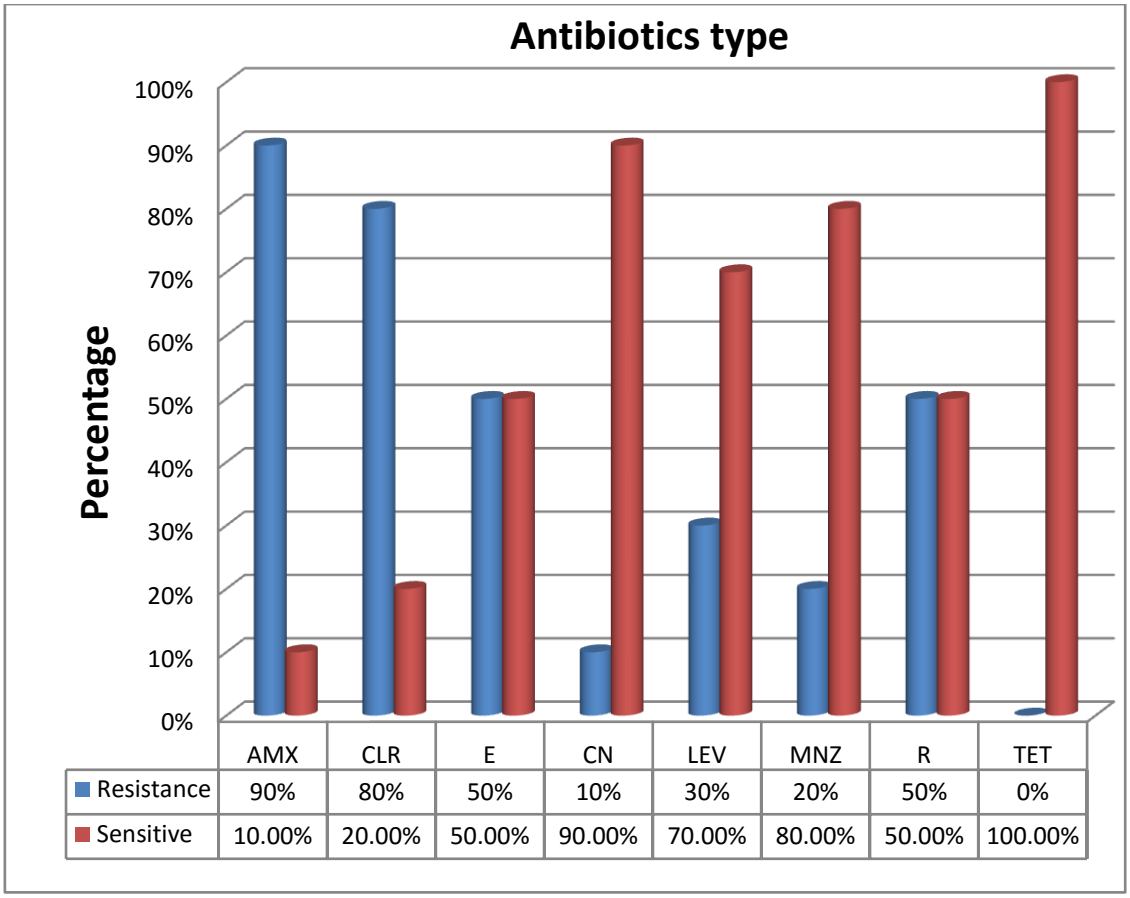

Figure 2: Percentage of sensitivity *H. pylori* isolates againstEightdifferent antibiotics.

Determination of MICs and MBCs for antibiotics against *H. pylori*:

Serial laboratory experiments were achieved to investigate the effect of MICs, sub- MICs and MBCs for Two locally available antibacterial compounds (AMX, CLR) and One amino acid Glycine (GLY) antimicrobial agent on the growth of Ten ***H. pylori*** isolates that are available for determinating the distribution of the MICs and MBCs values successfully measuredbased on universally Modified Serial Broth Macrodilutiontechniqueto verify the resistance pattern in these isolates according to breakpoint of the NCLSI guidelines [45].

In view data the MICs values for AMX were recorded highly resistant ***H. pylori***rangefrom 1-4 µg/mL with breakpoint greater than MIC 0.12µg/mL as seen in (Table 2).

Table 2: The distribution of MICs (µg/mL) values of the Two antibiotics (AMX, CLR) and single amino acid GLY against Ten *H. pylori* isolates.

Antibiotics and ami Acid with MIC resistance breakpoi	MIC (µg/mL)									
	***A* 1**	***A* 2**	***A* 3**	***A* 4**	***A* 5**	***A* 6**	***A* 7**	***A* 8**	***A* 9**	***A* 10**
*AMX >0.12µg/mL	1	1	1	4	2	1	2	1	2	1
CLR > 0.5 µg/mL	32	16	32	32	32	8	32	32	32	32
GLY None	512	1024	512	1024	2048	512	512	512	512	512

* There is low significance difference($P < 0.001$).

To discuss the results of AMX MICs values against ***H. pylori***, the percentage of resistance was very high and fully resistant toward andoffered (100%) resistances to this antibiotic, this mean AMX was the not active enough for the inhibition of growth ***H. pylori***. So it is necessary to seek a resistance mechanism. It can be hypothesized that the usually increase wide use of AMX to specifically treat ***H. pylori*** infection probably will cause elevate the resistance rate. It then mandatory to have a surveillance of ***H. pylori*** resistance to β-Lactam [46].

These results may compatible with the result of [47] that foundthe MICs for 12 ***H. pylori*** strains were ≥ 1µg/mL. But, [48] reported the occurrence of AMX resistance in ***H. pylori*** strains isolated from patients in the United States with MIC, 8µg/mLand were apparently associated with a marked reduction in treatment efficacy. Whereas, the results of [49]investigated that ***H. pylori***AMX-resistant was MIC 2mg/mL while, AMX-sensitive was MIC 0.06 mg/mL, and their results indicated that *pbpA* point mutations are responsible for MIC increases resistance in ***H. pylori*** isolates.

Our results disagree within performed studies, of [21] that recorded the AMX has been used almost exclusively to treat ***H. pylori*** infection, and it is included in most current therapeutic regimens, due to they found a few sensitive isolates with reduced susceptibility in which very low MICs most attractive in range of 0.06 µg/mL against this bacterium.When comparing different sets of data, it is important to note that variations in rates may arise due to the effects of inter-laboratory reproducibility, caused by the lack of standardized testing protocols or regional prescribing practice. Moreover, because ***H. pylori***showed variable degrees of

resistance after comparing the results with that in [45], the AMX MICs of concentration in 8, 16 and 32µg/mL were chosen, then***H.pylori*** viability evaluated by determining viable count and estimating the possible MBCs concentration for eradication ***H. pylori*** as tabled in (Table 3).In which the MBCs for AMXin concentration 8µg/mL had effect on reducing the viable count of ***H. pylori*** cells to $70X10^{\wedge 3}$, while, the MIC in 16µg/mL decreased ***H. pylori*** cell count but still left about $20X10^{\wedge 3}$. And MIC in 32µg/mL was substantial reductions a viabilities of ***H. pylori*** cells following 24hr. to obtained significantly decreased to $3X10^{\wedge 2}$ CFU/mL but, did not eliminate the bacteria. Regarding, AMX, it was relatively bacteristatic agent against ***H. pylori***at a very high concentration 23µg/mL MIC, since still left 300 CFU/mL after pre-treatment.

These results are disagreement with those recorded by [34] whom showed that AMX MIC at 125µg/mL had a limit effect on ***H. pylori*** after 24hr. of exposure, but the results agree with study of [47] when reported that 32X MBC of AMX were effective against ***H. pylori***which support previous findings.Generally, amongthe Macrolides available, the only CLR used in ***H. pylori*** regimens which act against the ribosome with low MICs.The patterns of CLR MICs in this study were similar high resistance range between 8-32 µg/mLwas associated with a decreased eradication.

Table 3: Possible minimum *H. pylori* cell eradication concentration (MBCs) of Tow antibiotics (AMX, CLR) and single amino acid GLY against Ten *H. pylori* isolates.

Antibiotics and amino acid	Control (average)	No. Of viable cells (CFU/mL) in antibiotic and amino acid under MIC (µg/mL) concentration		
AMX	150X $10^{\wedge 3}$ without	$70X10^{\wedge 3}$ in 8 µg/mL	$20X10^{\wedge 3}$ in 16µg/mL	$3\ X10^{\wedge 2}$ in 32µg/mL
CLR		$140X10^{\wedge 3}$ in 64g/mL	$50X10^{\wedge 3}$ in 128 µg/mL	$1\ X10^{\wedge 3}$ in 256µg/mL
GLY		$130X10^{\wedge 3}$ in 4096µg/mL	$90\ X10^{\wedge 3}$ in 8192µg/mL	0 in 16384µg/mL

Our data noticingthat ***H. pylori***isolates increase-level of resistance towardCLR in which the highest titer of CLR MICwas 32µg/mL as a maximum rate 8 (80%) of ***H. pylori***resistant

isolates while, the breakpoint for this antibiotic is only 0.5µg/mL. This percentage of CLR-resistant in ***H. pylori*** isolates was observed with significantly highdifference ($P < 0.001$), because may be having frequently been used in the treatment of various diseases such as respiratory tract Infections and otolaryngology fields in many clinical practice [27].

Likewise, these results were agree with those of [50] who reported that the ***H. pylori***isolate were resistance to CLR when MIC values >1µg/mL, Six isolates with MIC values which equal to the breakpoint 0.5µg/mL, while only 2 isolates were susceptible to this antibiotic when the MIC were less than the breakpoint. And, our data came approach with results of [47] that recorded the 14 ***H. pylori***strains were categorized as resistant to CLR with MIC between 0.6 to 32µg/mL, meaning that the occurrence of CLR resistance had increased at breakpoint of 0.5µg/mL. Moreover, in the case of CLR concentrations of 64, 128 and 256µg/mL MICs were chosen to determine the possible MBCs concentration for eradication ***H. pylori***. In which CLR at concentrations 64µg/mL MIC had no remarkable effect on the viability of the ***H. pylori*** cells and the number was, $140X10^{\wedge 3}$, even when 128µg/mL MIC were used still about 50.000 CFU/mL were detected, but, when used concentration 256µg/mL led to be reduction the viable cell count of ***H. pylori*** cells from $50X10^{\wedge 3}$ to $1X10^{\wedge 3}$ CFU/mL, and this probably because MICs were attractive in that range to inhibit the***H. pylori*** in liquid cutler media to 1000 CFU/mL, although the isolates are able to grow in the previously concentrations less than0.5 µg/mL breakpoints. While, other study [51] recorded increase level of resistance ***H. pylori*** cellsafter exposure to a very high concentration ≥256 µg/mL MIC of CLR, the cells were remained in the broth about $60X10^{\wedge 3}$ and reported in their research CLRwas insufficient to eradicate the ***H. pylori***. Generally, increased resistance to a wide range of antibiotics from different classes, was alarming as a big problematic thus, making the treatment of infections with these bacteria very difficult issue.

Determination of MICs and MBCs forGlycine against *H. pylori*

Clearly, the official breakpoint for GLY resistance has not been established for ***H. pylori*** isolates by the NCLSI. Therefore, any provisional breakpoint to GLY was assigned, and the resistance breakpoints of MIC values >2048µg/mL of GLY was accepted according the study of [52]. And as a result the GLY has no breakpoint so, all Ten***H. pylori*** showed complete sensitive (100%) to the new used of GLY. Potential reasons for the absence of GLY resistant ***H. pylori*** isolates in this study may attribute to the type of population were enrolled

based on the selection criteria; these patients had no exposure to previous GLY. Thus, it could be said that the best and the highest activity for GLY in concentration greater than 2048µg/mL.

The effect of anti- ***H. pylori*** activities for GLY against ***H. pylori*** clinical isolates was examined in which the majority of GLY MICs values were ranged in concentrations from 512 to 2048 µg/mL as seen in (Table 2). In our findings GLY with the concentrations 4096, 8192 and 16384 µg/mL MICs were chosen to determine the possible MBCs against ***H. pylori*** eradication. GLY MIC at 4096µg/mL concentration is responsible for a significantly higher risk of failure to inhibition ***H. pylori*** cells growth and the available cell countstill high detected in BHI broth with GLY to $130X10^{\wedge 3}$CFU/mL after 24 hr. of exposure.while, when exposureMIC of GLY in concentration 8192 µg/mL resulted in a dramatic decrease in the number of viable cells to 90 $X10^{\wedge 3}$. Whereas, only in concentration 16384 µg/mL MIC of GLY had the highest anti-***H.pylori*** activity against ***H. pylori***treated following 24 hr. of exposurehad considerably reduced from $90X10^{\wedge 3}$ to reach a zero CFU/mL as outlined in (Table 3).

The reasonable results described above, proposed that the addition of GLY resulted in a significant killing was seen for bacterial cells at the highest effective concentrationwhich is considered to be an extremely severe condition for the bacteria. Furthermore, administration GLY is acted as higher active agent than AMX and CLR to suppressing number of available ***H. pylori*** cells depending different concentrations in all tests performed, due to its possessed strong anti-***H. pylori*** activities with high level of toxicity for this bacteria.

These datawere somewhat similar to the results obtained by [53] who was found that GLYhas a high specificity because it's exhibited antibacterial activity against ***H.pylori***, and showed a slight or no activity against other bacterial species examined. From the other hand the results disagree with studies of [54] that proposed that GLY possesses a very narrow antibacterial spectrum against ***H. pylori*** when added to the medium.

The combination effect of sub-MICs for AMX with CLR and of sub-MICs for AMX with GLY against *H. pylori* Urease enzyme production:

Our assays involving investigate the effect of thesub-MICs for AMX, CLR and GLYseparately as well as, sub-MICs of combination AMX with CLR and AMX with GLY on ***H. pylori***Urease enzyme activity and the effects were evaluated by determination of

synergism percentage, after have been Ten ***H. pylori*** isolates were tested on Urea Agar Slant Medium and by RUT device kit to confirms positive Urease enzyme production before treatment with antibiotics or amino acid,the results showed all isolates (100%) hydrolysis urea to ammonia and raises the pH from acidic to alkaline indicated by change the medium to pink color, in order to identify the ability of ***H. pylori*** isolates to secrete Urease enzyme [11].

While, inhibition ***H. pylori*** Urease enzyme production are tested after growth on the Brain Heart Infusion broth containing a concentration sub-MICsof AMX, GLR and GLY separately as the results tabled in the (Table 4) Consequently, after treated samples with sub-MICs of AMX, CLR and GLY. Samples tested to screen loss ***H. pylori*** ability to Urease production by RUT and PCR test.The results found that sub-MICs of AMX wereclearly inhibiting***H. pylori*** Urease production greater than other agents with (100%) negative Urease production. However,the action of treatment with sub-MICs of CLR did not affect on the Urease enzyme and the enzyme still ongoing effectiveness to analysis urea, to give (100%) positive Urease production. But, in case of treatment with sub-MICs for GLY, this agent able to inhibit (50%) of ***H. pylori*** Urease production and (50%) unable to inhibit***H. pylori*** Urease production and the enzyme still continued its work, all these results comparing to ***H. pylori*** isolates control (no any testing agent) that have (100%) positiveUrease enzyme production.

Table 4: The effect of sub-MICs for AMX, CLR, GLYadministrated separately or in combination of AMX with CLR and AMX with GLY against Ten *H. pylori*Urease enzyme production.

Treatment with antibio and amino acid under sub-MIC	Clinical *H. pylori* isolates subjected on Ureas test									
	***A* 1**	***A* 2**	***A* 3**	***A* 4**	***A* 5**	***A* 6**	***A* 7**	***A* 8**	***A* 9**	***A*10**
AMX	-	-	-	-	-	-	-	-	-	-
CLR	+	+	+	+	+	+	+	+	+	+
GLY	-	-	+	+	+	-	-	-	+	+
AMX + CLR	-	-	-	+	+	+	-	-	-	-
AMX + GLY	-	-	-	-	-	-	-	-	-	-
Control (no drug)	+	+	+	+	+	+	+	+	+	+

- (+)Have evidence of Urease enzyme production, (-) No evidence Urease Enzyme production
- The experiment was carried out in triplicate observations.

On the basis of these results, the explanation of our data indicates before treatment with antibiotics and amino acid, ***H. pylori*** isolates tend to the production of Urease enzyme due to it is important of the survival factors, and since this enzyme consist of natural protein partials, so, once after treatment by sub-MICs of AMX the data noted the effectiveness of AMXlies in its ability to impact on the mechanisms of the possesses inhibition effect inside the cell represented by denaturated protein synthesis, causing the defect and inhibiting of ***H. pylori*** Urease production. While,the treatment with sub- MICs of CLR failed to inhibit ***H. pylori*** Urease production at all levels studied due to the antibiotic has no influence intracellular but, became inhibitor and killer extracellular.

Otherwise, in case of treatment with sub- MIC for GLY this agent may act as component of new type of therapy for resistant ***H. pylori*** isolates by its ability to reduced amount of Urease enzyme production. Few or no studies suggested that using of antibiotics in combination with amino acid may be useful in inhibition ***H. pylori*** Urease enzyme production as a complement alternative ways of controlling ***H. pylori*** infection. These combinations are able to improve reducing antibiotic-associated adverse events and increasing the eradication therapy rate with optimal synergistic efficacy and tolerability.In our experimental design ***H. pylori***Urease inhibition occur by combination sub-MICs of AMX with CLR in percentage (70) because it has been targeted the protein synthesis mechanism inside the cell and inhibition the efficiency of Urease enzyme production, nevertheless, some of ***H. pylori***isolates (30%) did not show any synergism effect when combined AMX with CLR under sub-MICs and bacteria still secret Urease enzyme even after treatment. Further,the results showed that sub-MICs of combination AMX with CLR was higher than of CLR achieved alone with highly significance deference with ($P < 0.0001$), because the combination of both these antimicrobial agents lead to enhancing the effect of CLR after mixing them togetheras illustrated in (Figure 3).

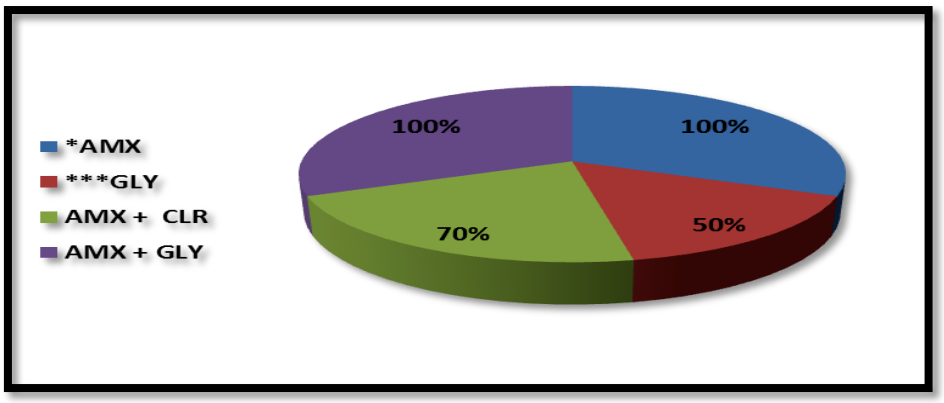

Figure 3: Percentage of synergistic effect on *H. pylori* Urease inhibition occurs by combination of AMX with CLR and combination AMX with GLY.

Besides that, antimicrobial synergy has traditionally been showed through the AMX and CLR mixing that allows for different mode of mechanisms action for bacterial Urease inhibition cell walls in order to facilitates uptake and passage of CLR into the periplasmic space [55], beside the ability of AMX to enhance the uptake of CLR then finally this pathway act on inhibition Urease from its work inside bacterial cells, and some isolates still give positive Urease, even after the addition AMX with CLR may be related to the antagonism effect occurrence. The results obtained by researcher [56] have observed very little effected on Urease activity in ***H. pylori*** isolates after the combination between AMX andMacrolidesand their mixing together led to appeared antagonistic activity because AMX does not support the uptake ofantibiotic.These findings were in a concordance with the results citedby [57] who found the activity combination of AMX with CLR was probably has inhibitory effect on the bacterial Urease system in many bacterial species.

Table 5: The number of *H. pylori*Urease enzyme production under separately of sub-MICs of AMX, CLR, GLY or in combination AMX with CLR and AMXwith GLY.

Treatment with antibiotics and amino acid under sub-MICs	Number of *H. pylori*Urease enzyme inhibition	Rate of inhibition %
AMX	10	100

CLR	0	0
GLY	5	50
AMX + CLR	7	*70
AMX + GLY	10	*100
Control (no drug)	0	0
Statistical Analysis		
Cell: AMX + CLR Effect : row 5 on row 1 Significance level: 0.05	$P < 0.0001$	
Cell: AMX + GLY Effect : row 6 on row 2 Significance level: 0.05		

* There is highly significance difference ($P < 0.0001$).

Furthermore the researchers [58, 59] suggested the importance of mixing AMX with CLR in order to inhibition Urease enzyme production inmost Gram negative bacteria especially Enterobacteriaceae. From the other hand,the results observed the combination AMX with GLY under sub-MICs led completely inhibition Urease enzyme in all ***H. pylori*** isolates to give negative results (100%) as presented in the (Table 5) after tested by RUT and PCR.Accordingly, our finding the interpretations of these results could be attributed to the many mechanism action in which GLY inhibits Urease activity produced by ***H. pylori*** remains elusive. Whereas the possible roles in the research of [60] suggested that GLY antimicrobial activity such synergistic effect appeared due to attachment to cell receptors and inhibition of important metabolic enzymes resulting in disruption of microbial cell reproduction and respiration.Moreover, [61] showed the likely mode of GLY actionmay be through disruption of energy production by inhibitiona number of available active enzymes involved in cell wall synthesis, such as DD- and DL- carboxypeptidases, which are important in the formation of cell wall-bound peptidoglycan (PG), and because GLY mainly inhibits LD-carboxypeptidases, the modifying effect of GLY on cell wall synthesis was explained to be largely due to this mechanism exactly. While,β- Lactamantibiotics as AMX are involve capability of inhibiting not only DL-carboxypeptidases but, also DD-carboxypeptidases [62], which are required for the synthesis of cross-linked (PG) in ***H. pylori***, and this refers to the

strong synergistic activity between AMX and GLY, so more importantly, it could be said that mixing both Tow these agents can enhance the effect of one another.

Notably, thedemonstration of week synergistic effect for combination AMX with CLRcomparing to the highest synergistic effect from combination AMX with GLY on***H. pylori***Urease enzyme inhibition as showed in (Table 5).

Clearly, our data obtained in this study hypothesize the confirming synergism Glycine's activity an increasing the antimicrobial efficacies of AMX antibiotic is mainly due to its inhibition of Urease enzyme function that is an important part of the defence system in the bacteria themselves against any antimicrobial agents by influenced the synergistic effect level comparing to separately used of AMX or GLY and this may showed further useful utilization method in reduction the virulent bacteria through inhibiting Urease enzyme production and decrease the chances of infection with bacterium complications and treatment P.U.D. caused by ***H. pylori*** pathogen.

Subsequent studies on Urease inhibitors by amino acids have been recently attracted much attention as potential new anti-urease drugs.[63]. Additionally, the results in the study of [64] revealed that the effect of some amino acid on nickel cofactor of Urease enzyme, cause decreasing the expression of Urease and hence prevent the ***H. pylori*** colonization in stomach.It is of great importance; the results presented here open new avenues toward more effective eradication therapy for ***H. pylori***and suggest that the administration of a combination of a proton pump inhibitor, antibiotics and amino acid deserves further evaluation. Interestingly,from these findings the direct comparisons of our work with other study impossible due to different experimental designs used and because this data appeared for the first time so, we could not find corresponding literatures agree or disagree in their results supporting our data and the lack of research on an effective therapeutic dose that showed there was a significant reduction in the Urease activity produced by ***H. pylori*** isolates, therefore, it considers the designer of our current experiment being the first time report in Iraq.

Genotypic detection of *UreA* gene in *H. pylori* using (PCR) test:

Molecular genotypic analysis further were done to make sure screening the losses or the presence ***H. pylori****UreA* genethat responsible for encoding Urease enzyme production in ***H. pylori*** isolates before and after treatment. The data showed that all Ten ***H. pylori*** isolates

found positive Urease producers depending on PCR test and they were harbouring *UreA*gene before treated with amino acid or antibiotics to yield amplification fragments products with *UreA*-PCR specific primersin all the tracks at the same level. Whereas, the absence of such amplicon in DNA extracted from treated ***H. pylori*** isolates with sub-MICs of AMX alone to give (100%) negative results. While, in case of DNA extracted from treated ***H. pylori*** isolates with sub-MICs of CLR alone the data give (100%) positive results, and when treated isolates with sub-MICs of GLY alone the date reveal that (50%) of extracted DNA from treated ***H. pylori*** isolates givepositive results and (50%) of extracted DNA from treated ***H. pylori*** isolates negative results as show in (Figure 5) thatseen the presence of PCR products running in 1.5% Agarose gel electrophoresis for amplification*UreA*gene extracted from genomic DNA of gastric Six ***H. pylori*** isolates number (*A*1, *A*2, *A*3, *A*4, *A*5, *A*6) used as template for PCR of *Ure A*: in line 1 positive control (template DNA extracted from isolate number *A*1 pre-exposure to separately sub-MICs GLY to gain a clear PCR product; line 2 negative control (without template DNA); lines 3, 4, and 5 are multiple positive PCR results treated with sub-MICs GLY showing shiny bands and amplified molecularsize ~ 340 bpfragments which represent a part of the gene was detected in the lanes containing genomic DNA extracted from isolates number *A*3, *A*4, *A*5, compared with 100 bp DNA ladder which represents the whole size of *UreA* gene in line (M) molecular weight as marker; lines 6, 7, and 8 are multiple negative PCR products (that inhibited *UreA* gene after treated by GLY under sub-MICs, was not detected in the lanes that containing genomic DNA extracted from isolates number *(A*1, *A*2, *A*6). the results described above indicate there is evidence to thatUrease activity was strongly inhibited in the samplenumber *(A*1, *A*2, *A*6) isolate after treated with sub-MICs GLY, although no inhibition of Urease activity the in same***H. pylori*** isolate number *A*1 pre-exposure was observed and ***H. pylori***cells were still production Urease enzyme, even of trying repeated and changing different optimizing conditions of PCR experiment for several times.

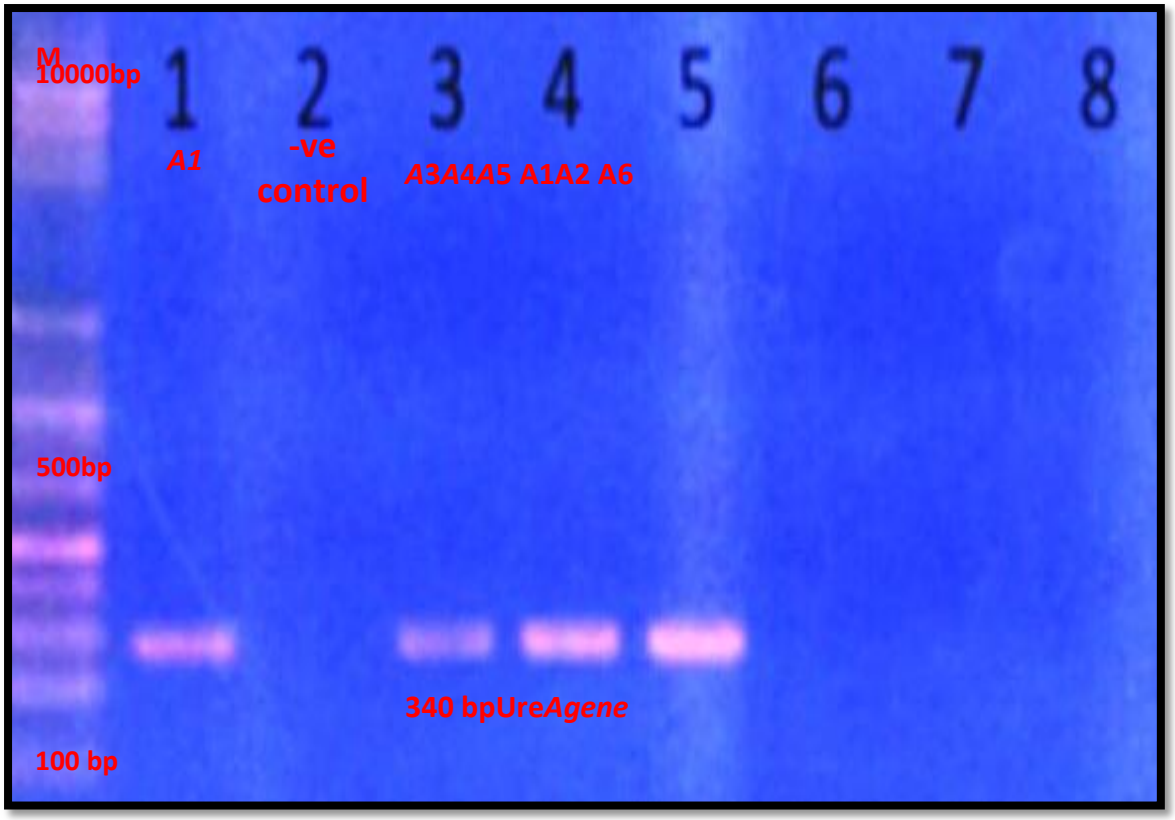

Figure 5: PCR analysis subjecting in 1.5%Agarose gel electrophoresis (70 Voltage and 100 ampir for 60 min) supplied with Ethedium bromide staining to detect PCR amplification of *UreA* gene from DNA of Six *H. pylori* isolates number (*A*1, *A*2, *A*3, *A*4, *A*5, *A*6) used as template. The photocomposition of the figure was obtained with Gel documentary camera.

But, in case of exposure ***H. pylori*** isolatesto the under sub-MICs of combination AMX with GLY the negative result of PCR product showed the absence of the amplified *UreA* gene to give (100%) negative results due to the lack bands of such amplicon indicating that non of***H. pylori*** isolates were produced the Urease enzyme after treatment. High sensitivity of PCR assay for detection of ***H. pylori***Urease enzyme production from pure culture was in agreement with observation of [65] whoindicated that PCR quite effective in detection of ***H. pylori***Urease enzyme productionin broth cutler.

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التأثير التأزري للمضادات الحياتية والأحماض الامينية على أنتاج أنزيم اليوريز

لبكتريا المعدة الحلزونية *Helicobacter pylori*.

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