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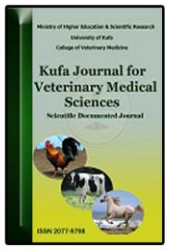

Stability of Resistance Induced by *Escherichia coli* in Comparison with That Carried by Clinical Isolates *In Vivo*

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Abstract:

This study was conducted to investigate the stability in experimentally induced resistance in sensitive *Escherichia coli* for comparison with clinical resistant strains of the same microorganism so the first step was collected of 14 strains of *E.coli* from different disease cases: diarrhea (children= 6, calve= 3, poultry= 1), UTI (urine= 2), mastitis (milk= 2). And identificated these strains by using biochemical tests.These strains were divided to sensitive and resistant strains to cefquinome (β-lactam antibacterial) according to the results of sensitivity test (Agar well diffusion method). The minimum inhibitory concentration (MIC) by tube dilution method (TDM) was estimated to 8 selected strains (4 sensitive and 4 resistance) for comparison. The MIC values for sensitive strains were 0.007, 0.003, 0.017 and 0.005 µg/ml, for resistant strains were 372, 400, 42 and 25 µg /ml for cefquinome respectively. The second step was to induce resistance to sensitive strains *in vitro* by exposing the microorganisms to sub inhibitory concentration (1/4 MIC) of antibacterial for 14 passages through which the bacteria was reidentified by using a differential media to exclude any contamination.The new MIC values were 1.25, 0.8, 2.0 and 1.5 µg/ml for cefquinome respectively. The comparison method was employed to study the degree of stability of resistance in sensitive and resistant strains against this drug are *in vivo* by multiple injections (three times) of standard suspension test microorganisms in mice followed by reisolation and reidentification from liver. The mean of drop MIC value for sensitive strainswas 10.34 folds, and for resistant strains was increase in1.40folds for cefquinome, which represent statistically significant a drop in the values of MIC for sensitive strains but in the resistant strains not significant because a slight elevation in the values of MIC.

Key words: *Escherichia coli*, sensitivity test, MIC, *in vivo*, antibacterial.

ثبات المقاومة المستحثة ومقارنتها مع العتر المقاومة المعزولة سريريا في جرثومة الاشريشيا القولونية

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الخلاصة

اجريت الدراسة الحالية لمعرفة مدى ثبات المقاومة في جراثيم الاشريشيا القولونية بالعتر الحساسة التي استحدثت بها المقاومة بالمقارنة مع العتر المقاومة سريريا وذلك من خلال جمع اربعة عشرعترة من جراثيم الاشريشيا القولونية من مختلف الامراض المسببة لها :الاسهال (اطفال=٦وعجول=٣ ودواجن=١) والتهاب المجاري البولية (٢) والتهاب الضرع

(٢). وتم تشخيص هوية الجرثومة بالأوساط الكيموحيوية والتفريقية وباستخدام عدة ال APIالخاص بها. وقسمت العتر الى مجموعتين حساسة و مقاومة للسفكوينوم (بيتا لاكتام) اعتمادا على نتائج فحص الحساسية و بعد ذلك قدرت حساسية ثماني عتر من جراثيم *E.coli* (اربعة حساسة و اربعة مقاومة) لمضاد السفكوينوم باختبار التركيز المثبط الادنى (MIC) بطريقة التخفيف بالانابيب (TDM) حيث كانت اقيام ال MIC (مكغم/مل) للعتر الحساسة هي ٠،٠٠٧ ، ٠،٠٠٣ ،٠،٠١٧ ، ٠،٠٠٥ ، ٠ للسفكوينوم وللعتر المقاومة هي ٣٧٢،٤٠٠،٤٢،٢٥ للسفكوينوم على التوالي . ولغرض إحداث المقاومة في العتر الحساسة مختبريا تم تعريض هذه العتر إلى تركيز واطئ منالسفكوينوم (ربع قيمة ال MIC) اربعة عشرتمريرة تم خلالها التاكد من هوية البكتريا بزرعها على الوسط التفريقي لاستبعاد حصول التلوث وقدرت أقيام ال MIC بعد ذلك فوجدت كالتالي (مكغم/مل) ١،٥،٢،٠،٠،٨،١،٢٥ للسفكوينوم على التوالي . و لغرض مقارنة ثبات المقاومة في العتر المقاومة طبيعيا والمستحثة استخدمت طريقة في (الحي) حيث كانت بالزرق المتكرر للجراثيم في الفئران وإعادة عزلها وتنقيتها من اكبادها وقياس معدل هبوطقيم ال MIC للعتر الحساسة هي١٠،٣٤ضعفا وللعتر المقاومةبزيادة هي ١،٤٠ضعفا للسفكوينوم . و قد لوحظ استعداد اكثر لفقدان المقاومة في العتر الحساسة بينما تظهر العتر المقاومة زيادة طفيفة في اقيام ال MIC ليس لها فرق معنوي احصائيا.

الكلمات المفتاحية: الاشريشيا القولونية،API،MIC، في الحي ،المضاد الحيوي.

Introduction:

Antibiotics constitute one of the most significant contributions of modern science. The discovery of these life-saving drugs transformed the health-care scene during the last century. Antibiotics are widely used in human and veterinary medicine to treat and prevent diseases and as growth promoters in animal intensive industries. The consequences are severe. Infections caused by resistant microbes fail to respond to treatment, resulting in prolonged illness and greater risk of death. The increasing incidence of resistance to a wide range of antibiotics by microorganisms is a major concern facing modern medicine (1). *Escherichia coli* are normal inhabitants of the gastrointestinal tract of animals and humans of which only some strains have become highly adapted to cause diarrhea and a range of extra-intestinal diseases. *Escherichia coli* is the most common cause of food and water-borne human diarrhea, urinary tract infection, meningitis, peritonitis, septicemia, and gram-negative bacterial pneumonia infection and other complications which are depending on the virulence factors *E.coli* causes (2). The development of resistance to older agents such as ampicillin and trimethoprim-sulfamethoxazole, as well as the emerging problem of fluoroquinolone resistance, may substantially limit our antibiotic choices (3). The search for more beta-lactamase-stable, broad-spectrum cephalosporins led to the development of the new class of beta-lactams: the so-called fourth generation cephalosporins 4GC such as cefquinome , an aminothiazolyl cephalosporin for exclusive use in veterinary medicine for , as well as similar cefepime and cefpirome in human medicine for injection used. It hashigher affinity to penicillin binding proteins, Lower affinity and higher stability to beta-lactamases and Improved penetration into the periplasmatic space increases the intrinsic potency. It was used to treatment of respiratory disease and mastitis (4).

Materials and Methods:

In present study 14 strains of *Escherichia coli* were collected from different disease cases. These isolated spices were identified by studying morphological examination (Gram stain, blood agar culture, MacConky agar culture, Eosin Methylene blue agar culture, motility test) and some biochemical tests (indoltest, catalase test, API 20 E).The average number of viable *E. coli* cell per ml of the stock suspension wasdetermined by taking 1 ml from overnight culture (nutrient broth) of *E. coli* suspension washing with 9 ml of Peptone water, then taking 1 ml of this suspension and making serial ten-fold dilution to comparison with Standard McFarland tube No.0.5 and

Spectrophotometer were used to measure the turbidity of *E. coli* suspension. In this study,these strains were divided to sensitive and resistant strains to cefquinome by used agar well diffusion method (sensitivity test) and broth dilution MIC methods (macrodilution). All these methods described in this protocol is in accordance with the international recommendations given by the National Committee for Clinical Laboratory Standards (NCCLS) (5). In this study the resistance in sensitive strains were induced after determining the initial MIC by exposing the test bacteria to sub minimum inhibitory concentration in Muller Hinton broth with incubation for 24 hours at 37C^{o}. Repeating this method fourteen times until induction of new resistance generations for this drug, purification of bacteria by differential media (MacConky agar) for 24 h at 37 C^{o}, and MIC values of drug was made after 14 passages and compared with initial as follow.

Proportional MIC (increase) = final MIC/initial MIC(6).

In vivo was employed to compare the stability of induced resistance with that carried by clinical isolates. Bacteria isolates (sensitive and resistance) were injected as 0.5 ml of inoculums intraperitoneally in laboratory animals (mice) using 30 mice type BALA/C, mal, range between 4-6 months age, and weighed between 18-24 g .They were divided sporadically in 3 groups:

1-Resistant bacteria group (12 mice) was divided to four sub groups, one to each strain.

2-Sensitive bacteria group (12 mice) was divided to four sub groups, one to each strain.

3-Control group (6 mice), was injected sterile media broth.

The injection was repeated three times, in each time the animals were sacrificed after three days and reisolationfrom liver on differential media for 24 hours at 37 C^{o} and purification of the bacteria was done. After that reestimated of MIC value of cefquinome in natural and induced resistance bacteria and then compared between them.

Results and Discussion:

The results of morphological and biochemical test show that the test microorganisms are motile, pink rod shape (Gram negative), pink colonies on MacConky agar because their ability to ferment lactose,Green-metallic sheen on Eosin methylene blue agar culture, positive for catalase enzyme and produce indole, this resemble the description of *Escherichia coli* mentioned by other workers (7). The API 20E test was done by incubationof strip for 24h at 37 C° and the result was readaccording to guide of manufacture company.

Antibacterial susceptibility tests: Different concentrations of cefquinome (1000, 100, 10, 1, 0.1 µg/ml) were used in agar well diffusion assay, caused different degrees of the results was seen in table (1).The organisms were selected based on their World Health Organization (WHO) classification to resistance and sensitive, when cefquinome at 10 µg/ml concentration was given the diameter of inhibition zone equal or less than19 mm is resistance while it was equal or more than23 mm is sensitive (8). In our study, the means diameter of inhibition zone to sensitive strains at 10 µg/ml are 25.67 mm while to resistant strains at the same concentration are 5.33 mm these results were close to Series of studies on the resistance of *E. coli* which were isolated from animals and humans strongly suggested that those bacteria which are resistant to antimicrobials used in animals would also be resistant to antimicrobials used in humans (9,10).

The values of MIC were estimated by **tube dilution method** are listed in table (2). These were 0.007, 0.003, 0.017, 0.005 µg/ml for sensitive strains and 372, 400, 42, 25 µg/ml for resistant strains

respectively. According to the National Committee for Clinical Laboratory Standards (NCCLS), the equivalent MIC sensitive and resistance breakpoints established are ≥4 and ≤ 8 µg/ml respectively (11). A high level of susceptibility to cefquinome has been demonstrated in sensitive group of pathogenic *E.coli*. These results are close to that limbert*et al*.,(12) when they found the MIC value ranged between 0.006-0.781 µg/ml against pathogenic *Escherichia coli*,WhileAl-Taher, (13) estimated suceptibility of *E.coli* strains isolated from diarrheic calves to cefquinome 0.06-2 µg/ml,but in our study cefquinome susceptibility reached more than these values perhaps, because it is newly used in our country, in addition to, the specific molecular structure of cefquinome provides higher affinity topenicillin binding proteins (PBPs), higher stability to AmpC-type beta-lactamase also, less likely to be hydrolyzed by extended spectrum beta-lactamases (ESBLs) and improved penetration into the periplasmatic space increases the intrinsic potency (14). The first report of resistance to cefquinome in *E. coli* of equine and cattle origin. Luhofer*et al*., (15) determined cefquinome resistance to *E.coli* to be equal or more of 8 µg/ml, but in methicillin resistant *Staphylococcus aurous* (MRSA) the MICs ranged between 1.563-50 µg/ml (12). In our study we observed that resistance to cefquinome can reach to 400 µg/ml. The predominant characteristic of beta-lactam resistance in *E. coli* and other gram-negative bacteria is the production of beta-lactamases. The relatively narrow-spectrum beta-lactamases but others have a much broader spectrum, such as extended-spectrum beta-lactamases (ESBLs), which can hydrolyze many different beta-lactams. ESBLs may be encoded by single plasmids and chromosomal independent.A change in outer membrane proteins (OMP) is a different mechanism of resistance. High-level resistance to fourth- generation of cephalosporin appears to require the synergistic activity of two mutations: enhanced beta-lactamase hyperproduction and hydrolysis, and decreased membrane permeability (16). Recently, efflux has become increasingly recognized as a major component of resistance. Some efflux pumps selectively extrude specific antibiotics such as macrolides, beta-lactames and tetracyclines, whereas others referred to as multiple drug resistance pumps. Nine proton-dependent efflux pumps have been identified in *E. coli* so far. This cause the efflux of many (two or more) antibiotics leading to miltidrug resistance MDR (17).

The results of exposure of susceptible microorganisms to sub inhibitory concentration (1/4 of MIC value) of cefquinome used for seven and fourteen passages are listed in table (3). After 7 passages the mean MIC values was 0.386 µg/ml for cefquinome, which represent an increase of 57.29 folds. After 14 passages the MIC values was 1.39 µg/ml , which represent an increase of 205.35 folds. Although, the elevation in cefquinome resistance did not pass the breakpoint resistance because it is highly sensitive and need more passages, nevertheless we called resistance metaphorically. Exposure of *E.coli* to different levels of antibacterial drug may result in increase in degree of resistance as reported before by many workers (18, 19, 20).

InVivo: The stability of antibacterial resistance when bacteria were injected and reisolated for three times in mice. The results of this experiment represent all sensitive strains showed dramatic drop in the values of MIC. The greatest drop was seen (26.66 folds) however, the values did not return to the value seen before exposure to sub inhibitory concentration. All drops in resistance was statistical significant. In contrast resistant strains showed insignificant increase, the mean of elevation folds were (1.28, folds), see the

table (4,5). The difference in rate of resistance lose can be explained on basis of type of resistance (plasmid or chromosomal) and whether it is stable or unstable (21).The resistance tended to be lost after passage this strain in vivowas nonspecific and unstable because it found onsmall plasmids bands (22). The interaction between *E.coli* and the host immune system is complex. The outcome of an infection is the result of a balance between the *in vivo* environment where the bacteria survive, grow and the regulation of fitness genes at a level sufficient for the bacteria to retain their characteristic rate of growth in a given host. This adaptation does not confer increased resistance but can be detected as an enhancement in the bacterial net growth rate later in the infection. The enhanced growth rate is lost upon a single passage *in vitro*, and it is therefore transient and not due to selection of mutants (23). This study was supported the insignificant resistance increase which occurred resistant clinical isolates. Enterobacteriaceae are capable of exchanging resistance genes under intestinal conditions in animals. It has been shown that genetic transfer of determinants for drug resistance can occur rapidly in vitro, but frequency of transfer in vivo is lower (10).

Conclusion:

Induced resistance to cefquinome by exposure to subinhibitoryconcentration was unstable when the microorganisms were passed for three times in mice while no change in degree of resistance in resistant clinical isolates when these were passed for three times in mice.

Table (1): Mean diameter of inhibition zone (mm) ± SE of cefquinome at different concentrations against different resistant and sensitive strains of *E.coli*.

Conc. (µg/ml)					
Mean± SE of sensitive strain	**1000**	**100**	**10**	**1**	**0. 1**
	30.50 ± 0.41	**28.42 ± 0.59**	**25.67 ± 0.92**	**20.75 ± 0.74**	**16.75 ± 0.56**
Mean± SE of resistant strain	**19.83 ± 0.86**	**14.50 ± 1.05**	**5.33 ± 0.42**	**0.00 ± 0.00**	**0.00 ± 0.00**
T- test Value	**6.54 ***	**5.89 ***	**8.05 ***	**6.33 ***	**4.17 ***
(P<0.05)*					

T-test value comparison between mean of sensitive and resistant strains for each concentration.

Table (2): Initial MIC value of sensitive and resistant strains of *E.coli* to cefquinome.

Strains	Initial MIC (µg/ml) for sensitive strains	Strain	Initial MIC (µg/ml) for resistant strains
S1	**0.007 ± 0.002**	**R5**	**372.00 ±1.20**
S2	**0.003 ± 0.001**	**R6**	**400.00 ±5.78**
S4	**0.017 ± 0.006**	**R8**	**42.00 ± 1.15**
S5	**0.005 ± 0.002**	**R10**	**25.00 ± 2.89**
Mean± SE	**0.008 ± 0.002**	**Mean ± SE**	**209.75 ± 6.21**
T-testValue	**28.05 ***		
(P<0.05)*			

T-test value comparison between mean ofinitial MIC for sensitive and resistant strains.
R: Resistance strain. S: Sensitive strain.

Table (3): The initial and final MIC values of sensitive strains of *E.coli* after seven and fourteen passages in vitro in media contains sub inhibitory concentration (1/4 MIC) of cefquinome and the folds of elevation.

Strains	Initial MIC (µg/ml)	MIC after 7 times of passages	Fold of elevation	MIC after 14 times of passages	Folds of elevation
S1	**0.007 ± 0.002**	**0.500 ± 0.057**	**71.42**	**1.25 ± 0.144**	**178.50**
S2	**0.003 ± 0.001**	**0.145 ± 0.058**	**48.33**	**0.80 ± 0.057**	**266.66**
S3	**0.017 ± 0.006**	**0.500 ± 0.057**	**29.41**	**2.00 ± 0.289**	**117.64**
S5	**0.005 ± 0.002**	**0.400 ± 0.057**	**80.00**	**1.50 ± 0.288**	**258.60**
LSD Value	**12.96 ***				
*** (P<0.05)**					

Table (4): Stability of resistance of cefquinome which was measured after bacteria were injected for 3 times in laboratory animals (mice).

Strains	Initial MIC (µg/ml)	Final MIC (µg/ml)	Change in folds of resistance
S1	1.25 ± 0.14	0.08 ± 0.02	15.62 ↓
S2	0.80 ± 0.05	0.03 ± 0.01	26.66 ↓
S4	2.00 ± 0.28	0.26 ± 0.06	7.69 ↓
S5	1.25 ± 0.14	0.08 ± 0.02	10.34 ↓
Mean± SE	1.33 ± 0.27	0.11 ± 0.03	15.08 ± 2.16
R5	372.00 ±1.20	400.00± 15.3	1.12 ↑
R6	400.00 ±5.78	428.33± 6.00	1.07 ↑
R8	42.00 ± 1.15	65.00± 7.64	1.54 ↑
R10	25.00 ± 2.89	35.00± 2.89	1.40 ↑
Mean± SE	209.75 ± 6.21	237.08 ± 6.33	1.28 ± 0.06
T-test Value	44.32 *	41.93 *	6.33 *
(P<0.05)*			

T-test value comparison between mean of initial and final MIC for sensitive and resistant strains.

R: Resistance strain. S: Sensitive strain. ↓ = **Decrease.** ↑ = **Increase.**

Table (5): Change of resistance (folds) to cefquinome was resulted after three injections in laboratory animals.

Strain	Cefquinome	Strain	Cefquinome
R5	1.12 ↑	S1	15.62 ↓
R6	1.07 ↑	S2	26.66 ↓
R8	1.54 ↑	S4	7.69 ↓
R10	1.40 ↑	S5	10.34 ↓
L.S.D Value	0.846 NS	L.S.D Value	6.31 *
(P<0.05)*			

R: Resistance strain. S: Sensitive strain. ↓ = **Decrease.** ↑ = **Increase.**

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