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

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## Abstract

**Background:** The increasing incidence of resistance to a wide range of antibiotics by microorganisms is a major concern facing modern medicine because these 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.

**Objectives:** The aims of this study are: 1-Comparing the changes of induced resistance in *E.coli* with that present in clinical isolates of the same microorganism. 2-Determine the properties of cefquinome especially the development of resistance and the stability of it

**Methods:** fourteen strains of *E.coli* were collected from different disease cases: diarrhea (children= 6, calve= 3, poultry= 1), UTI (urine= 2), mastitis (milk= 2) from Al-Yarmook hospital and farms of veterinary college (Baghdad). The MIC was estimated eight selected strains (4 sensitive and 4 resistances) before and after passages these strains *in vivo* for carried out the comparison.

**Results:** A significant drop in induced resistance for sensitive strains as compared with clinical isolates which are not significant elevate in resistance. Most *E.coli* strains showed highly susceptible to cefquinome but some of them were appeared resistance although this antibacterial was newly used in this country.

**Conclusion:** loss of induced resistance when passing *in vivo* because the resistant microorganisms actually were unstable group when comparison with stable resistance in clinical isolates was not changed when exposed to the same condition.

**Keywords**: *E. coli*, MIC, *in vivo*, antibacterial.

**الخلاصة**

**الاهداف:** الاهداف من هذه الدراسة

١- المقارنة بين المقاومة المستحثة و مقاومة العتر المعزولة سريريا في جرثومة الاشريشيا القولونية

٢- معرفة خصائص السيفكوينوم للمقاومة و ثباتيتها

**طرق العمل:** جمعت اربعة عشرعزلة من جراثيم الاشريشيا القولونية من مختلف الامراض المسببة لها:اسهال (اطفال=٦وعجول=٣ ودواجن=١) والتهاب المجاري البولية (٢) والتهاب الضرع (٢) من مستشفى اليرموك ومن حقول كلية الطب البيطري (بغداد .اختيرت ثماني عزلات لقياس اقيام التركيز المثبط الادنى قبل وبعد عدة تمريرات في الحي لغرض اجراء المقارنة بينهما.

**النتائج:** انخفاض معنوي في المقاومة المستحثة للعتر الحساسة عند مقارنتها مع ارتفاع غيرمعنوي في العتر المقاومة سريريا.

معظم عزلات الاشريشيا القولونية اظهرت حساسية عالية السفكوينوم لكن القليل منها اظهر مقاومة عالية بالرغم من انه حديث الاستخدام بالقطر.

**الاستنتاج:** فقدان المقاومة المستحثة بتمريرها في الحي لانها تعد من النوع الغير ثابت بعكس المقاومة المكتسبة طبيعيا تكون ثابتة ولا تتغير تحت نفس الظروف.

## Introduction

*Escherichia coli* is normal inhabitant 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 [(1)]. *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 the 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 has higher 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 used to treatment of respiratory disease and mastitis [(4)].

## Materials and Methods

In present study 14 strains of *E.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 (indol test, catalase test, API 20 E). The average number of viable *E. coli* cell per ml of the stock suspension was determined by taking 1 ml from overnight culture (nutrient broth) of *E. coli* suspension washed with 9 ml of Peptone water, then taking 1 ml of this suspension to make 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 was 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 against 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. Bacterial isolates (sensitive and resistant) were injected (0.5 ml of inoculums intraperi-toneally 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 bacterial group: 12 mice were divided to 4 sub groups, one to each strain.

2-Sensitive bacterial group: 12 mice were divided to 4 sub groups, one to each strain.

3-Control group: 6 mice were injected sterile media broth.

The injection was repeated three times, each time the mice were sacrificed after

three days and reisolation from liver on differential media for 24 hours at 37 $C^o$ and purification of the bacteria was performed. Then 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 showed that the test microorganisms are motile, Gram negative rod shape, 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 [7]. The API 20E test was carried out by incubation of strip for 24h at 37 C° and the result was read according 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 are listed in table 1. The test strains were selected based on their world health organization (WHO) classification for resistance and sensitive, when cefquinome at 10 µg/ml concentration gave the diameter of inhibition zone equal or less than 19 mm is resistance and when it was equal or more than 23 mm is sensitive [8]. The results of this study showed that, the means diameter of inhibition zone for sensitive strains at 10 µg/ml are 25.67 mm and the 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 [9,10].

The values of MIC were estimated by **tube dilution method** and 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] who found the MIC value ranged between 0.006-0.781 µg/ml against pathogenic *E. coli*, while Al-Taher, [13] estimeted suceptibility of *E.coli* strains isolated from diarrheic calves to cefquinome 0.06-2 µg/ml, but in this study the cefquinome susceptibility reached more than these studies above, because it is newly used in this country, in addition to, the specific molecular structure of cefquinome provides higher affinity to penicillin 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] was estimated cefquinome resistance to *E.coli* to be equal or more of 8 µg/ml, but in Methicillin resistant *Staphylococcus aurous* (MRSA) the MICs were ranged between 1.563-50 µg/ml [12]. This study was observed the resistance to cefquinome can reach to 400 µg/ml.

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 elevated in cefquinome resistance not passed the breakpoint resistance because it is highly sensitive and need to 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 [16, 17, 18].

***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, table 4. The greatest drop was seen (26.66 folds) however, the values not returned 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 elevated folds were (1.28, folds), show the table 5. The difference in rate of resistance loss can be explained on basis of type of resistance (plasmid or chromosomal) and whether it is stable or unstable [19]. The resistance tended to be lost after passage these strains *in vivo* was nonspecific and unstable because it found on small plasmids bands [20]. 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*, [21]. This study was supported the insignificant resistance increase which occurred resistant clinical isolates.

## Conclusions

1-The strains of *E.coli* used in this study was more susceptible to cefquinome.

2- loss of induced resistance when passing *in vivo* because the resistant microorganisms actually were unstable group when comparison with stable resistance in clinical isolates was not changed when exposed to the same condition.

Table 1: Mean± SE of diameter of inhibition zone (mm) 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-test value | 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 | 420.00± 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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