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The Effect of Heat Stress on the Antibacterial Resistance and Plasmid Profile in *Escherichia coli* Isolates

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Abstract: In order to study the effect of heat stress on the antibacterial resistance and plasmid profile in *Escherichia coli*, thirty *E. coli* were isolated from sheep liver. Antibiotic susceptibility test were done by antibiotic disc diffusion method using filter paper disc on two 24 h cultures of each isolate which grown at 37 and 43°C simultaneously in BHI Broth (Merck VM460193 531). The isolates which grown at 43°C were under heat stress during their growth. Ten commonly used antibiotics, viz., ampicillin, erythromycin, neomycin, trimethoprim-sulfamethoxazol, lincospectine, tetracycline, gentamycin, flumequine, vancomycin and Tiamulin (Padtan Teb). The resistance level of all *E. coli* isolates against 10 antibacterial drugs compared statistically in 37 and 43°C using MINITAB Version 14 program. Plasmid DNAs were extracted from each of the *E. coli* isolates which were grown at 37 and 43°C overnight using alkali lysis method. In this study •λDNA (EcoR1+Hind III digested) was used as marker DNA. According to the results of this study, the resistance rate of *E. coli* isolates have decreased against trimethoprim-sulfamethazol, lincospectine, tiamaulin, tetracyclin and gentamycin at 43°C but only the difference between the resistance rate against gentamycin in 37°C (83.3%) and 43°C (60%) was significant Characterization of Plasmid DNAs by agarose gel electrophoresis showed that each of the thirty drug resistant *E. coli* harbored a single plasmid. There was no difference among the plasmid profiles of the thirty isolates in 37 and 43°C. As the plasmid profile did not change in 43°C (heat stress) so the resistance differences against antibacterial drugs were not significant except for gentamycine that its resistance may is chromosomal. According to the results of this study, In conclusion it can be said that heat stress could not be effective on antibacterial resistance and plasmid profile if the duration of the stress is short. The long duration of the heat stress plus other stress factors such as starvation will effect the plasmid replication and finally plasmid copy number of bacteria. Mechamism of this phenomenon remains unknown, though one might speculate that some bacterial addiction modules that are activated upon amino acid starvation, like mazEF could be involved.

Key words: *Escherichia coli*, drug resistance, plasmid profile, heat stress, plasmid replication

INTRODUCTION

Plasmids, DNA (or rarely RNA) molecules which replicate in cells autonomously (independently of chromosomes) as non- essential elements, play important roles for microbes grown under specific environmental conditions as well as in scientific laboratories and in biotechnology (Wegrzyn and Wegrrzyn, 2002).

Bacterial resistance to antimicrobial agents is a major world wide problem because of introduction a new antimicrobial agent is usually followed sooner or later by emergence of bacterial resistance to these drugs (Patway, 1994).

The drug resistance may be chromosomal DNA or plasmid DNA mediated. The plasmid mediated drug resistance is caused due to the presence of drug resistance gene(s) harboring on the plasmid DNA. These gene(s) confer the drug resistance phenomenon in the host organism (Meyer's *et al*., 1976).

Plasmids carrying drug resistance phenotype are known as R-factor which is responsible for the spread of multiple drug resistance among bacteria. R-factor consist of tow components i.e., Resistance Transfer Factor (RTF) and resistance determinant R. The complete plasmids (RTF+r) called R-factor (Patway, 1994).

Detailed mechanisms of replication initiation, which is the crucial process for efficient maintenance of plasmids in cells, have been elucidated for several plasmids.
However, to understand plasmid biology, it is necessary to understand regulation of plasmid DNA replication in response to different environmental conditions in which host cells exist (Wegrzyn and Wegrrzyn, 2002).

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Knowledge of such regulatory processes is also very important for those who use plasmids as expression vectors to produce large amounts of recombinant proteins (Wegrzyn and Wegrrzyn, 2002).

Escherichia coli is one of the serious pathogen that can cause tremendous therapeutic problem by developing resistance against antibiotics. As a result of drug resistance to several antibiotics in *E. coli* it has become a serious problem not only in the developing countries where it is endemic but also an important problem of treating drug resistant *E. coli* infection in the developed countries (Tauxe *et al*., 1990).

Using of different stress factors such as heat stress for long duration or starvation can effect the plasmid replication and so it will lead to decrease in plasmid copy numbers and finally antibacterial resistance in bacteria (Wegrzyn and Wegrzyn, 2002).

In order to study the effect of heat shock on the antibacterial resistance and plasmid profile in *Escherichia coli*, tested the antimicrobial susceptibility of thirty isolates of *E. coli* in an attempt to establish their antibiotic resistance pattern and also isolated plasmid DNAs from these isolates and characterize the plasmid DNAs in 37 and 43°C.

MATERIALS AND METHODS

Isolation and identification of the bacteria: Thirty *E. coli* were isolated from sheep liver at the Department of Microbiology, College of Veterinary Medicine, Urmia University, Iran in 2007. The isolated bacteria then sub-cultured on MacConkey agar (Biomark B238) plates. *E. coli* identified on the basis of gross morphology along with cultural characteristics and the manner in which the bacteria did response to various biochemical tests according to Quinn *et al.* (2002).

Antibiotic susceptibility tests of the bacteria: Antibiotic susceptibility tests of the isolated strains of *E. coli* were done by antibiotic disc diffusion method using filter paper discs (Bauer *et al*., 1966). Two 24 h cultures of each isolate which grown at 37 and 43°C simultaneously in BHI Broth (Merck VM460193 531) were spread on a Mueller-Hinton agar (Conda 1058) plate by using sterilized glass spreader. The isolates which grown at 43°C were under heat stress during their growth (Wegrzyn *et al*., 1996). The inoculated plates are allowed to stand for 3-5 min. The discs are placed onto the agar surface using sterile forceps. The discs should be placed no closer together than 24 mm (center-to-center). The plates are placed in a 35°C incubator within 15 min of applying the discs and incubated aerobically for 16-18 h. After incubation the plates were observed in order to calculate the diameter of clear zone produced around each disc. Such clear zone produced around each disc is the index of sensitivity to the corresponding drug. Ten commonly used antibiotics, viz., ampicillin, erythromycin, neomycin, trimethoprim-sulfamethoxazol, lincospectine, tetracycline, gentamycin, flumequine, vancomycin and Tiamulin(Padtan Teb). In order to establish antibiotic susceptibility profile of the isolated *E. coli* strains, the clear zone produce around each disc were measured in millimeter.

The resistance level of all *E. coli* isolates against 10 antibacterial drug compaired statistically in 37 and 43°C using MINITAB Version 14 program.

Extraction of plasmid DNA: Plasmid DNAs were extracted from each of the *E. coli* sisolates which were grown at 37 and 43°C overnight using alkali lysis method according to Sambrook *et al*. (1989).

Agarose gel electrophoresis of the extracted DNA: Plasmid DNA extracted from each of the *E. coli* isolate was subject to gel electrophoresis with 0.8% agarose gel according to Meyer's *et al*. (1976). In this study •λDNA (EcoR1+Hind III digested) was used as marker DNA (Rezina *et al*., 2001).

RESULTS

All the *E. coli* isolates were resistant to ampicillin, erythromycin, neomycin, trimethoprim-sulfamethoxazol, lincospectine, tetracycline, gentamycin, flumequine, vancomycin and Tiamulin at 100, 100,100,10, 56.6, 20, 83.3, 100, 100 and 93.3% at 37°C, respectively and also all *E. coli* isolates were resistant to ampicillin, erythromycin, neomycin, trimethoprim-sulfamethoxazol, lincospectine, tetracycline, gentamycin, flumequine, vancomycin and Tiamulin at 100, 100, 100, 3.3, 36.6, 16.6, 60,100, 100 and 83.3% at 43°C (heat stress), respectively (Table 1). The drug resistance pattern of 30 *E. coli* isolates against 10 antibacterial agents at 37 and 43°C (heat stress) are showed in Table 2 and 3.

Table 1: The drug resistance rate of 30 *E. coli* isolates against 10 antibacterial agents at 37 and 43°C

	(%) of resistant isolates	
Antibacterial agent	37°C	43°C
Ampicillin	100.0	100.0
Erythromycin	100.0	100.0
Neomycin	100.0	100.0
Trimethoprim-sulfamethoxazol	10.0	3.3
Lincospectine	56.6	36.6
Tetracycline	20.0	16.6
Gentamycin	83.3	60.0
Flumequine	100.0	100.0
Vancomycin	100.0	100.0
Tiamulin	93.3	83.3

Table 2: The Drug resistance pattern of 30 *E. coli* isolates against 10 antibacterial agents at 37°C

No. of isolates	Resistance pattern at 37°C
1	Am, Van, Tm, Gm, E, LP, Fm, Neo
2	Am, Van, Tm, E, LP, Fm, Neo
3	Am, Van, Tm, Gm, E, LP, Fm, Neo
4	Am, Van, Tm, Gm, E, LP, Fm, Neo
5	Am, Van, Tm, Gm, E, Fm. Neo
6	Am, Van, Tm, Gm, E, Fm, Neo
7	Am, Van, Tm, Gm, E, LP, Fm, Neo
8	Am, Van, Tm, Gm, E, LP, Fm, Neo
9	Am, Van, Tm, Gm, E, Fm, Neo
10	Am, SXT, Van, Tm, Gm, E, LP, Te, Fm, Neo
11	Am, Van, Tm, E, LP, Fm, Neo
12	Am, Van, Tm, E, LP, Fm, Neo
13	Am, Van, Tm, Gm, E, LP, Te, Fm, Neo
14	Am, Van, Tm, Gm, E, LP, Fm, Neo
15	Am, Van, Tm, Gm, E, LP, Fm, Neo
16	Am, Van, Tm, Gm, E, Fm, Neo
17	Am, Van, Gm, E, LP, Fm, Neo
18	Am, Van, Tm, Gm, E, LP, Fm, Neo
19	Am, Van, Tm, Gm, E, Te, Fm, Neo
20	Am, Van, Tm, Gm, E, Fm, Neo
21	Am, Van, Tm, Gm, E, LP, Te, Fm, Neo
22	Am, Van, Tm, Gm, E, Fm, Neo
23	Am, Van, Tm, Gm, E, Te, Fm, Neo
24	Am, Van, Tm, Gm, E, Fm, Neo
25	Am, Van, Tm, Gm, E, LP, Fm, Neo
26	Am, Van, Tm, Gm, E, LP, Fm, Neo
27	Am, Van, Tm, Gm, E, Fm, Neo
28	Am, Van, Tm, Gm, E, Te, Fm, Neo
29	Am, SXT, Van, E, Fm, Neo
30	Am, SXT, Van, Tm, E, Fm, Neo

Am = Ampicillin, SXT = Trimethoprim-Sulfamethazol, Van = Vancomycin, Tm = Tiamulin, Gm = Gentamycin, E = Erythromycin, LP = Lincospectine, Te = Tetracyclin, Fm = Flumequine, Neo = Neomycin

Table 3: The Drug resistance pattern of 30 *E. coli* isolates against 10 antibacterial agents at 43°C

No. of isolates	Resistance pattern at 43°C
1	Am, Van, Tm, Gm, E, LP, Fm, Neo
2	Am, Van, Tm, E, LP, Fm, Neo
3	Am, Van, Tm, Gm, E, LP, Fm, Neo
4	Am, Van, Tm, Gm, E, LP, Fm, Neo
5	Am, Van, Tm, Gm, E, Fm. Neo
6	Am, Van, Tm, E, Lp, Fm, Neo
7	Am, Van, Tm, E, Fm, Neo
8	Am, Van, Tm, E, LP, Fm, Neo
9	Am, Van, Tm, LP, E, Fm, Neo
10	Am, Van, Tm, Gm, E, Te, Fm, Neo
11	Am, Van, Gm, E, LP, Fm, Neo
12	Am, Van, Tm, Gm, E, LP, Fm, Neo
13	Am, Van, Tm, Gm, E, Te, Fm, Neo
14	Am, Van, Tm, Gm, E, Fm, Neo
15	Am, Van, Tm, Gm, E, Fm, Neo
16	Am, Van, Tm, Gm, E, Fm, Neo
17	Am, Van, Tm, E, Fm, Neo
18	Am, Van, Tm, Gm, E, Fm, Neo
19	Am, Van, Tm, E, Te, Fm, Neo
20	Am, Van, Tm, E, Fm, Neo
21	Am, Van, Tm, Gm, E, Te, Fm, Neo
22	Am, Van, Tm, E, Fm, Neo
23	Am, Van, Gm, E, Te, Fm, Neo
24	Am, Van, Tm, Gm, E, Fm, Neo
25	Am, Van, Tm, E, LP, Fm, Neo
26	Am, Van, Tm, Gm, E, LP, Fm, Neo
27	Am, Van, Gm, E, Fm, Neo
28	Am, Van, Gm, E, Fm, Neo
29	Am, Van, E, Fm, Neo
30	Am, SXT, Van, Tm, E, Fm, Neo

Am = Ampicillin, SXT = Trimethoprim-Sulfamethazol, Van = Vancomycin, Tm = Tiamulin, Gm = Gentamycin, E = Erythromycin, LP = Lincospectine, Te = Tetracyclin, Fm = Flumequine, Neo = Neomycin

According to the results of this study, the resistance rate of *E. coli* isolates have decreased against trimethoprim-sulfamethazol, lincospectine, tiamaulin, tetracyclin and gentamycin. The resistance level of all *E. coli* isolates against antibacterial drugs in 37 and 43°C compaired statistically using MINITAB Version 14 program. Only the difference between the resistance rate against gentamycin in 37 (83.3) and 43°C (60) was significant ($p<0.05$).

Actually in this study the heat stress (43°C for 24 h) has not any effect on antibacterial drug resistance except the gentamycine.

Characterisation of Plasmid DNAs by agarose gel electrophoresis showed that each of the thirty drug resistant *E. coli* harbored single plasmid.

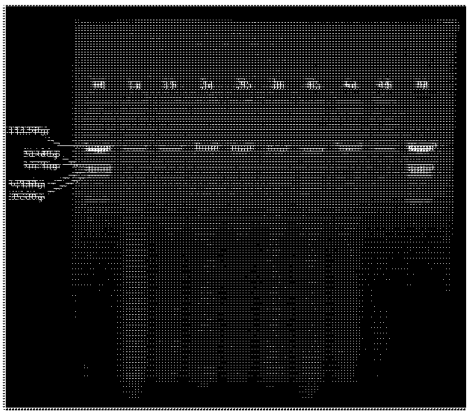

Fig. 1: The plasmid profile of *Escherichia coli* isolates (sample No. 1-4) in 37 and 43°C Lane M represents DNA marker ladder, Lane 1a-4a plasmid profile at 37°C and Lane 1b-4b Plasmid profile in 43°C (heat stress)

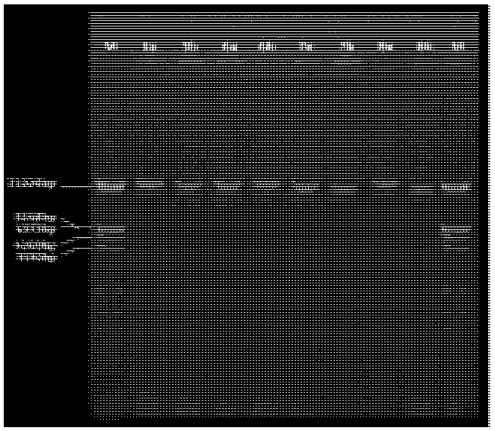

Fig. 2: The plasmid profile of *Escherichia coli* isolates (sample No 5-8) in 37 and 43°C, Lane M represents DNA marker ladder, Lane 1a-4a plasmid profile at 37°C and Lane 1b-4b Plasmid profile in 43°C (heat stress)

From the pattern of bands observed in the gel, the molecular size of the plasmid DNAs were calculated. There was no difference among the plasmid profiles of the thirty isolates in 37 and 43°C (Fig. 1,2). As the figures show the heat stress which have been used in this study had no effect on plasmid profile in different isolates of *E. coli*.

DISCUSSION

Antimicrobial susceptibility testing of intestinal micro-organisms like *E. coli* is important consideration because the administration of antimicrobial substances can alter the intestinal microbial balance and resulted in the suppression of certain beneficial bacterial disorders (Rezina *et al*., 2001).

The results of this study revealed that all isolates of *E. coli* were resistant to different antimicrobial drugs and their efficiency varied from antibiotics to antibiotics in both 37 and 43°C. These findings were in accord with the Azad and Shahjahan (1999). They have documented reports of isolation of multi drug resistant *E. coli* which were resistant to at least eight commonly used antibiotics including ampicillin, tetracycline and chloramphenicol. The Resistance factor may transfer among the gram negative bacteria specially in the family of enterobacteriaceae (Ahmadi, 2005; Anderson and Dalta, 1965; Kayvanfar and Firouzi, 1998).

Heat shock proteins are absolutely necessary for initiation of DNA replication from Ori λ (Taylor and Wegrzyn, 1995; Taylor and Wegrzyn, 1998; Wegrzyn *et al*., 1996). On the basis of the presented fact, one could predict that heat stress might effect on the replication of the plasmids. It has demonstrated that λ plasmid copy number is decreased at 42°C relative to lower temperatures (e.g., 30 or 37°C) (Wegrzyn, 1995).

More detailed studies revealed that the replication pathway dependent on the function of the heritable replication complex is impaired by heat shock. Namely, the heritable replication complex, which under standard laboratory conditions is a stable structure able to function for many cell generations, is disassembled relatively shortly after transfer of bacteria from 30 to 43°C. This disassembly was found to be dependent on GroEL and GroES heat shock proteins (Wegrzyn *et al*., 1996). In fact, this was the first demonstration, supported by subsequent studies that the GroEL/GroES molecular chaperons system is engaged in an *in vivo* disassembly of a highly organized protein structure (Chatellier *et al*., 1998).

Combination of prolonged (several hours) cultivation of bacteria at increased temperature (42-43°C) and amino acid starvation has deleterious effects on plasmids. Namely, plasmid DNA degradation was observed under these conditions (Neubauer *et al*., 1996).

The effects of heat stress on the antimicrobial drug resistance of *E. coli* of the intestinal tract of swine were studied in animals from a farm that had not been supplementing antimicrobials in feed for the past 10 years. Antomicrobial resistance levels after stress were significantly higher compared with pre stress levels for amikacin. Ampicillin, cephalothin, neomycin and tetracycline from fecal samples (Moro *et al*., 2000).

The effects of heat stress on the antimicrobial drug resistance and plasmid profile were studied in 30 *Escherichia coli* isolates. Antimicrobial resistance levels after stress were lower compared with pre-stress levels for gentamycin ($p<0/05$). There was no significant difference in the plasmid profile after the establishment of heat stress. In this case we have used •λDNA (EcoR1+Hindø digested) as marker DNA. The molecular size of the plasmid DNAs isolated from thirty *E. coli* isolates were about 21 kbp. In this study it was revealed that each of thirty drug resistant *E. coli* harbored single plasmid in both 37 and 43°C.

As the plasmid profile did not change in 43°C so the resistance against gentamycine may is chromosomal.

According to the results of this study and other researches, In conclusion it can be said that heat stress could be effective on antibacterial resistance and plasmid profile, of course this effects correlated with the origin of the isolated bacteria and the duration of the stress (Moro *et al*., 2000; Wegrzyn and Wegrrzyne, 2002; Giraldo-Suarez *et al*., 1993). The duration of the heat stress will effect the plasmid replication and finally plasmid copy number of bacteria. Mechanism of this phenomenon remains unknown, though one might speculate that some bacterial addiction modules that are activated upon amino acid starvation, like mazEF could be involved (Aizenman *et al*., 1996).

Finally, we suggest studying the effect of combination of stress factors on bacteria in order to decrease the plasmid copy in bacteria which they carry out the resistance in bacteria.

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