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R-Factor Transfer from Salmonella Strains to *Escherichia coli*

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Abstract: *Salmonella* strains are the most common bacteria contaminating the food products, because of existing different serotypes and a wide variety of hosts and carriers. 60 *Salmonella* strains were isolated from foodstuffs suspected to contamination by *Salmonella* and were analyzed to determine their drug resistance pattern and transfer of resistance factor via conjugation. Antimicrobial resistance determining was carried out by Kirby and Bauer disk diffusion and MIC by Macrodilution method according to the NCCLS criteria. To consider the transfer of antimicrobial resistance factor, conjugation was performed by mixed culture method. The highest rate of resistance was against TE followed by SXT and NA. Multiple drug resistance was shown in 91.66% of strains. MIC was determined in order to detect exact contents of antibiotics required for making selective media. The rate of MIC was between 0.5-512 µg/ml. By using *E.coli* $DH_5\alpha F^{-}Lac^{+}Nal^{r}$ as a recipient strain, the highest rate of transfer was related to Te (44.44%) and SXT (41.17%) respectively. By using *E. coli* C_{1180} as a recipient strain, the highest rate of transfer was related to SXT (50%) and TE (45.45%) respectively. Multiple drug resistance which is related to plasmids in most of the time and is transferred among bacteria via conjugation, is a public health problem throughout the world. Limiting to apply antibiotics, performing antimicrobial susceptibility tests to select suitable antibiotic, consideration of dosage of the drug and duration of therapy can decrease developing of resistant strains.

Key words: R-factor · Antibiotic · MIC · *Salmonella* · *E. coli*

INTRODUCTION

Salmonellosis is described as an infection transmitted from animal to human. Transmitting of disease is performed within fecal-oral route, such that bowel contents of infected animal are entered in human's body along with water or nutrients. To expose foods to unsuitable temperature which allows *Salmonella* strains grow in it and insufficient thermal process or non-existence of final thermal process are different factors which participate in outhbreaking of this organism [1].

Antibiotics are used for treatment of *Salmonellosis* But the main problem in treatment of *Salmonella* infections is emergence of resistant strains of *Salmonella* against antibiotics. Uncontrolled and irregular consumption of antibiotics in human and animals, is the major cause of increasing antimicrobial resistance in bacteria. In most of the time resistance to antimicrobial agents is genetic and genes of resistance are plasmid born. One of the most important reasons of increasing antimicrobial resistance is the ability of bacteria to transfer the R-factor to the other bacteria. Intestinal bacteria possessing resistance plasmids, can enter in gastrointestinal system of human via contaminated water and foods and transfer their R-factor to the normal gastrointestinal flora. In clinical environments, in laboratory and any other environment which has suitable condition for transferring of resistance factor, R-plasmids can transfer from one bacteria to the other. Moreover many food products are exposed to contamination with *Salmonella* strains. Developing resistance among *Salmoanella* serotypes and other species of bacteria is a public health problem throughout the world [1, 2].

So in this study *Salmonella* strains were isolated from food products in order to study their ability to transfer resistance factor.

MATERIALS AND METHODS

60 *Salmonella* strains were isolated from food products. Samples of nutrients suspected to contamination by *Salmonella* were submitted to

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veterinary organization of Tehran Province. Foodstuffs were included beaf, ground meat, chicken, chicken paste and egg.

Bacteriology: Culturing and isolating of *Salmonella* strains from foodstuffs were performed according to standard methods. Final identification of *Salmonella* strains was performed through serological experiments by agglutination test using Mast Diagnostic kit (Mast group Ltd., Mersyside, UK) and serological variety and group of bacteria was clarified [1, 3].

Determining of Bacterial Resistance Against Antibiotics: Antimicrobial resistance pattern was determined by kirby and Bauer disk diffusion method. diameters of the inhibition zones were interpreted based on the NCCLS subcommittee's recommendations [3-5]. Following disks (padtan teb, Iran) were used. Ampicillin (AM, 10 µg), Cephalotin (CF, 30µg),Cephalexin (CN, 30µg), Cephotaxim (CTX, 30 µg),Cefazolin (Cz, 30 µg), Cephtriaxone (CRO, 30µg), Trimethoprim-Sulphamethoxazole (SXT, 25 µg), Streptomycin (S, 10 µg), Kanamycin (K, 30 µg), Neomycin (N, 30 µg),Gentamicin (Gm. 10 µg), Nalidixic Acid (NA, 30 µg), Ciprofloxacin (CP, 5µg), Tetracycline (TE, 30 µg) and Chloramphenicol (C, 30 µg).

Determining of MIC: MIC was carried out using macrodilution method according to NCCLS criteria [3,6]. Antimicrobial stock solutions were prepared according to potency of antibiotic powders (Iranian companies), required volume and Final concentration of solution, using suitable solvents and diluters. Range of concentrations for each antibiotic depends on it's toxicity and it's allowable content in the body. Generally the highest chosen concentration is 1024 µg/ml. The following formula is applied for preparing antimicrobial stock solutions with distinct concentration. $W = \frac{V \times C}{P}$ which W= Weight of antimicrobial agent (µg), V= Required volume of solution (ml), C= Final concentration of solution (µg/ml), P= Potency. Mueller Hinton broth was used as a medium to determine the MIC. Bacterial suspension that it's turbidity was adjusted to match a Mcfarland 0.5 barium sulfate standard was prepared. By using broth medium and antimicrobial stock solutions, serial dilutions as 0.5, 1, 2, 8, 16, 32, 64, 128, 256 were prepared. After inoculation of tubes, they incubated at 37°c overnight and then results were red. One tube served as a positive control (broth plus inoculum) and one tube which contained only broth, served as negative control. MIC of Nalidixic Acid and Ampicillin was determined for *Salmonella* strains sensitive to them and MIC of all used antibiotics was determined for recipient strains, *E. coli* $DH_5\alpha F^{-}Lac^{+}Nal^{r}$ and *E. coli* C_{1180}.

Conjugation: To consider the transfer of antibiotic resistance factor, conjugation by mixed culture method was carried out [7, 8, 9]. *E. coli* $DH_5\alpha F^{-}Lac^{+}Nal^{r}$ resistant to Nalidixic Acid but sensitive to all antibiotics and *E. coli* C_{1180} resistant to Ampicillin and Kanamycin but sensitive to all antibiotics, were used as recipient strains. Donor strains were *Salmonella* strains isolated from foodstuffs, resistant to one or more antibiotics. when using recipient strain resistant to Nolidixic Acid but sensitive to the rest of antibiotics, donor strains sensitive to Nalidixic Acid were used and when using recipient strain resistant to Ampicillin and Kanamycin, donor strains sensitive to Ampicillin were used. Donor and recipient strains were cultured on MacConkey agar at 37°C overnight, then one colony from recipient strain was inoculated in 5 ml BHI broth and one colony from donor strain was inoculated in 2 ml BHI broth and both incubated at 37°C overnight. Aliquots of overnight cultures of donor (0.1ml) and recipient (0.9ml) organisms were mixed in a final volume of 10 ml BHI broth and incubated of 37°C, 24 h and 0.1 ml was removed and plated on selective media. Also Donor and recipient strains separately were cultured on selective media as control.

Selective Medium: This medium contained two antibiotics and only transconjugant bacteria could grow on it. These bacteria were recipient strain that had acquired antimicrobial resistance from donor strains. None of donor and recipient strains could not grow on this medium. So when using *E. coli* $DH_5\alpha F^{-}Lac^{+}Nal^{r}$ as recipient, selective media contained Nalidixic Acid and one of antibiotics which donor strain was resistant to it. For donors resistant to Nalidixic Acid but sensitive to Ampicillin, *E. coli* C_{1180} was used as recipient and selective medium contained Ampicillin and one of antibiotics that donor strain was resistant to it. In this study determination of MIC was performed in order to detect exact contents of antibiotics required for making selective media. After counting the grown colonies on the surface of selective medium and confirming that these colonies were *E. coli*, the rate of transfer of resistance for each plate was calculated by the following formula [10,11].

$$\text{The rate of transfer} = \frac{\textit{The number of transconjugant cells in } 1\ ml}{\textit{The number of donor cells in } 1\ ml}$$

After inoculating the donor bacteria in 2 ml of BHI broth and incubating at 37°C overnight, OD was red. If OD in 550 nanometer was 0.8-1, the number of donor cells was considered 9×10^9 cell per millilitre.

RESULTS AND DISCUSSION

By using bacteriological and biochemical experiments 60 *Salmonella* strains were identified and by serological experiments, group and serotype of *Salmonella* strains were determined.

Determining of Bacterial Resistance Against Antibiotics: The highest rate of resistance was against tetracycline (86.66%) followed by trimethoprim – sulphamethoxazole (73.33%), Nalidixic Acid (66.66%) and Streptomycin (61.66%) (Table 1). All the strains were resistant to at least one antibiotic. Five strains(8.33%) were resistant only to Nalidixic Acid. Fifty five strains (94.66%) were resistant to two or more antibiotics. Sixty *Salmonella* strains showed twenty two different patterns of resistance to the antimicrobial agents used in this study. The most common antimicrobial resistance pattern of these isolates was NA/SXT/TE/N/K/S (13.33%).

Determining of MIC: The rates of MIC of Nalidixic Acid for twenty *Salmonella* strains sensitive to it and the rates of MIC of Ampicillin for twelve *Salmonella* strains sensitive to it, has been shown in Table 2 and Table 3 respectively. MIC of antibiotics used in this study for recipient strain *E. coli* $DH_5\alpha F^- Lac^+ Nal^r$ and *E. coli* C_{1180} has been shown in Table 4 and Table 5 respectively.

Conjugation: E. coli $DH_5\alpha F^- Lac^+ Nal^r$ was used as a recipient for twenty *Salmonella* strain sensitive to Nalidixic Acid. The highest rate of transfer was for TE(44.44%) followed by SXT (41.17%), C(37.5%), AM (25%), CN (14.28%) and CTX (10%) (Figure1).

E. coli C_{1180} used as a recipient for twelve *Salmonella* strains sensitive to Ampicillin. The highest rate of transfer was for SXT (50%) followed by TE (45.45%), C(42.85%), CTX (33.33%) and CN (28.57%) (Figure 2). The rate of transfer was between 1.6×10^{-8} -7.77×10^{-7}.

Salmonella is one of intestinal gram negative bacteria that is a main cause of gastroenteritis due to foodstuffs. Major habitat of *Salmonella* is intestine of animals such ad birds, reptiles, farm animals, human and probably insects. Development of antibiotic resistance in

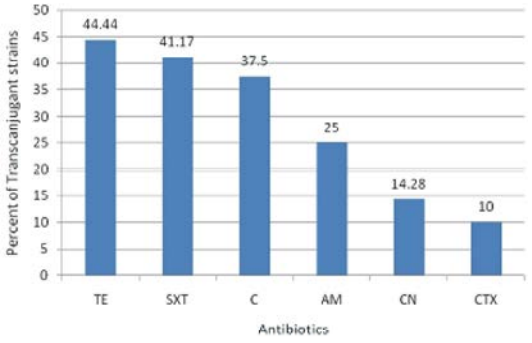

Fig. 1: Transfer of antibiotic resistance factor in Salmonella strains sensitive to NA

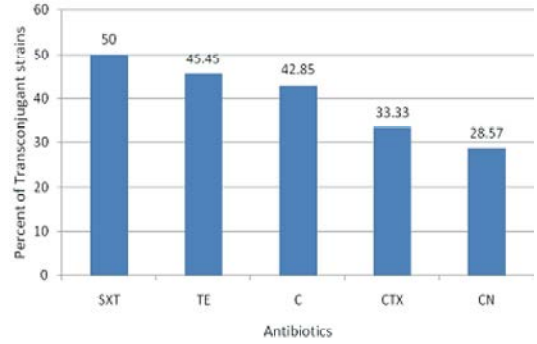

Fig. 2: Percent of antibiotic resistance factor in Salmonella strains sensitive to AM

Table 1: Resistance of *Salmonella* Serotypes against antibiotics

Antibiotic	N. and % of resistant strains
AM	14 (23.33%)
CZ	15 (25%)
CTX	16(26.66%)
CRO	-
CN	18 (30%)
CF	16(26.66%)
C	22(36.66%)
CP	17(28.33%)
SXT	44(73.33%)
GM	4(6.66%)
K	29(48.33%)
NA	40(66.66%)
N	33(55%)
S	37(61.66%)
TE	52(86.66%)

Table 2: MIC of NA for 20 *Salmonella* strains sensitive to it

Concentration of NA (µg/ml)	4	8	16	32	64	128	256
N. and % of Salmonella strains	2 (10%)	1 (5%)	1 (5%)	2 (10%)	8 (40%)	2 (10%)	4 (20%)

Table 3: MIC of used antibiotics for *E. coli* $DH_5\acute{a}F^{-}Lac^{+}$ Nal^{r}

Antibiotic	MIC (µ g/ml)
Cephtriaxone	2
Ciprofloxacin	2
Gentamicin	4
Cephotaxim	4
Cefazolin	16
Chloramphenicol	32
Tetracycline	32
Trimethoprim	64
Sulphamethoxazole	128
Cephalexin	128
Ampicilling	256

Table 4: MIC of AM for 12 *Salmonella* strains sensitive to it

Concentration of AM (µg/ml)	16	32	64	256	512
N. and % of Salmonella strains	2 (%16.6)	1 (%8.3)	4 (%33.3)	3 (%25)	2 (%16.6)

Table 5: MIC of used antibiotics for *E. coli* C_{11800}

Antibiotic	MIC (µg/ml)
Cephtriaxone	0.5
Ciprofloxacin	0.5
Gentamicin	0.5
Cephotaxim	2
Tetracycline	8
Cefazolin	16
Chloramphenicol	64
Trimethoprim	64
Cephalexin	128
Sulphamethoxazole	256
Nalidixic Acid	512

Salmonella serotypes has been considered as a public health problem throughout the world. This resistance is transferable among *Salmonella* serotypes. and other species of bacteria [1,2]. In this study *Salmonella* strains showed high percentage of resistance against antibiotics which were used.

In Hidetake *et al.* investigation in Japan (2004), antimicrobial resistance of *Salmonella* strains isolated from cow, pig and birds was determined. All the serotypes showed resistance to Ampicillin, Dihydrostreptomycin, Kanamycin and Oxytetracycline [12].In Altier study (2004) on *Salmonella* strains isolated from pig, two resistance pattern (AM/K/S/SXT/TE and AM/K/S/SXT/TE) were identified [13]. In Poppe *et al.* investigation in Canada (2006), *Salmonella* entrica serotype Newport isolated from animals and nutritional products showed resistance to at least eleven antibiotic specially Cephalosporins[14]. Ray *et al.* (2006) showed that *Salmonella* strains isolated form farms and milch cows were resistant to the most of antibiotics [15]. Stevenson *et al.* in USA (2007), announced increasing of resistance to Nalidixic Acid in *Salmonella entrica* strains. The common resistance pattern among those isolates was NA/AM/C/S/SXT/TE [16]. In Van et al study in Vietnam (2007), antimicrobial resistance in *Salmonella* strains isolated from chicken, cow and pig was determined and all of the isolates showed high rate of resistance to a variety of antibiotics. Furthermore the highest rate of resistance was against Tetracycline [17]. In Vojdanifar *et al.* study in Tehran (2005), antimicrobial resistance of *Salmonella* strains isolated from chicken was determined and all the strains were resistant to Streptomycin, Penicillin and Erythromycin [18]. Obviously in our study and other studies throughout the world *Salmonella* strains show high rate multi drug resistance.

Significant increase in the incidence of resistance is due to over the counter availability and uncontrolled use of antimicrobial drugs in human and animals which result in disappearing of sensitive bacteria and choosing resistant strains. Antibiotics have a variety of applications. They are prescribed for controlling and treatment of bacterial diseases in human and animals in most cases. Moreover antibiotics are applied in some animals such as chicken and turkey as feed additives for growth promotion and prevention of disease. Therefore, selective pressure due to use of antibiotics is too high in poultry. So there is high rate of resistant bacteria in their fecal flora. These resistant strains transfer from gastrointestinal system to the carrion during slaughter and cause contamination of chicken meat with multi drug resistant strains. They also transmit to human body directly or via meal and colonize in digestive system and may transfer resistance genes to human flora. Antibiotic resistance genes are plasmid born in most of the time and one of the most important reason in incidence of antimicrobial resistance in bacteria is the ability of them to transfer these plasmids to the other bacteria. Enteric bacteria containing resistance plasmids, can enter to human system via water and contaminated nutrients and transfer their R-factor to natural flora of enteric system. In clinical environments, laboratories and any other environment which has suitable condition for transferring resistance factor, R-plasmids can transfer from a bacterium to another [2]. In our study transfer of resistance factor by conjugation in *Salmonella* strains isolated from food products was analyzed.

Many resistant strains contained R-factors. The most common transferred antimicrobial resistance pattern was TE/SXT/C/AM/CN/CTX. Farhoudi moghaddam *et al.* in Iran(1990),studied transfer of resistance factor via conjugation in children under 5 years old. They isolated *Salmonella* from their feces specimens. 71.9% of resistant strains contained transferable resistance factor. The highest rate of transfer belonged to Chloramphenical (77.6%) and the lowest to Streptomycin (20%)[7]. Lazaro *et al.* (2004) studied R-factors in *Salmonella* entrica isolated from pig. They showed transfer of resistance against Sulphamethoxazole, Tetracycline and Sulphonamide[19]. Nogrady et al in Poland (2005), showed transfer of resistance to Choramphenicol via conjugation in *Salmonella* strains [8]. Poppe *et al.* in Canada (2006), used *Salmonella newport* strains as donors and *E.coli* C_{600} Nal^r as recipient in conjugation process. Transferable plasmids in their study, transferred, resistance to the most antibiotics [14]. Romani et al (2008) used *E. coli* K_{12} J_5 as recipient for *Salmonella* strains as donors. They showed transfer of resistance against Ampicillin and Tetracycline [20]. In lindsey et al study (2009) R-plasmids were known as important agents in emergence of antimicrobial resistance [21]. Khan et al (2009) showed a large plasmid which contained resistance genes to Trimethoprim-Sulphamethoxazole and Streptomucin. They gained this plasmid from *Salmonella* strains isolated from sea foods [22]. A wide variety of studies were performed on transfer of antibiotic resistance at aqueous environments such as rivers and wastes and isolated bacteria showed multi drug resistances. [10,23,24]. Results of transfer of antimicrobial resistance in our study is compatible with current investigations. It should be mentioned that some factors prevent form this type of transfer. A successful transfer of resistance depends on the existence of transferable plasmids and suitable conditions for conjugation. Inability to transfer antimicrobial resistance may be related to the presence of nontransferable plasmids or resistance genes may be chromosomal. Nowadays emergence of multi drug resistant pathogenic bacteria, is one of the most important problem in treatment of infectious diseases throughout the world. Significant increase in the incidence of resistance in *Salmonella* strains is due to increased use of antimicrobial agents and high rate transfer of R-factor. Restricting use of antibiotics in human and animals, performing antimicrobial susceptibility tests to select suitable antimicrobial agent, application of recommended dosage of antibiotic and following duration of therapy can decrease developing of resistant strains.

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