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Drug resistance, plasmid profile and random amplified polymorphic DNA analysis of Iranian isolates of *Salmonella* Enteritidis

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SUMMARY

The aim of this study was to characterize 49 *Salmonella* Enteritidis (SE) isolates from different sources (poultry, human, cow, poultry house environment) in Iran with respect to drug resistance, plasmid profile, and random amplified polymorphic DNA (RAPD) analysis. Antimicrobial susceptibility test to 29 agents found 33 resistance patterns among the isolates. No resistance was observed to danofloxacin, levofloxacin, norfloxacin, ceftriaxone, imipenem, and amikacin. The highest resistance (38.8%) was observed to flumequine. Thirty (61.2%) isolates were multidrug-resistant. Six plasmid profiles were detected and a 68-kb plasmid was found in 98% of isolates. Two different primers, MK22 and P1254, were used for RAPD analysis which each produced six profiles. For MK22 and P1254 primers, 83 and 86% of the isolates, respectively, belonged to one profile only and the rest distributed among other 5 patterns. The findings of the present study showed that SE isolates from poultry-related sources were closely related to human SE isolates. This study confirmed previous evidence that molecular techniques such as RAPD-PCR or plasmid profile alone do not demonstrate sufficient discriminatory power in epidemiological studies and a combination of patterns obtained by several techniques will provide more discriminatory power.

KEY WORDS: Salmonella Enteritidis, RAPD-PCR, Plasmid profile, Antimicrobial resistance, Poultry, Iran

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INTRODUCTION

Non-typhoidal salmonellosis is an important food-borne infection with worldwide distribution **(**Velge *et al.*, 2005). In 2005, more than 45000 human cases of non-typhoidal salmonellosis were reported to the Centers for Disease Control and Prevention (CDC) (Anonymous, 2007). *Salmonella enterica* serotype Enteritidis (*Salmonella* Enteritidis) has been one of the most common causes of food-borne infections in last three decades (Velge *et al.*, 2005). This serotype ranked among the top two most frequently isolated serotypes from human sources reported to CDC in 2006 (Anonymous, 2006). Poultry and poultry products are considered major sources of *Salmonella* Enteritidis (SE) infections for humans (Velge *et al.*, 2005). Recent studies in US have found 4.3% *Salmonella* positive samples from meat, poultry, and egg products in which 1.3% of *Salmonella* isolates were identified as SE (White *et al.*, 2007). From 2000 to 2005, among 51327 broiler rinses sampled in US boiler abattoirs, 12.4% were positive for *Salmonella,* in which 280 (4.4%) isolates were found to be SE (Altekruse *et al.*, 2006). Recent surveys of *Salmonella* infection in the UK detected a prevalence of 11.7% (54 out of 454) and 10.7% (41 out of 382) in commercial layer and broiler flock holdings respectively (Snow *et al.*, 2007; Snow *et al.*, 2008). The most common serotype in both types of flocks was SE. In The Netherlands' surveillance program of *Salmonella* spp. in laying hens flocks, from 1999 to 2002, a prevalence of 21.1% in 1999 to 13.4% in 2002 was recorded. *Salmonella* Enteritidis was

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the predominant serotype and accounted for one third of the positive flocks (van de Giessen *et al.*, 2006). In our recent investigation in Iran, a total of 3202 pooled samples were obtained from 142 poultry flocks, cultured, and 123 (3.8%) *Salmonella* isolates were recovered (unpublished data). Of the 123 *Salmonella* isolates, 70 (57%) were identified as SE. In the same study, serologic examination of 171 flocks by ELISA revealed anti-SE antibody in 65.5% of flocks tested. Because of the importance of *Salmonella* as the cause of a food-borne disease, many typing methods including phenotypic and genotypic methods have been used to trace the outbreak to the contaminated source and to elucidate the epidemiology of infection (Lukinmaa *et al.*, 2004). Using DNA-related techniques, researchers now are able to better differentiate *Salmonella* isolates below the level of serotypes. These techniques include plasmid profile, ribotyping, pulsed-field gel electrophoresis (PFGE), IS200 fingerprinting, PCR-ribotyping, ribosomal DNA intergenic spacer amplification and heteroduplex analysis, amplified fragment length polymorphism, automated 5' nuclease PCR assay, and random amplified polymorphic DNA (RAPD) analysis. RAPD analysis is the most general procedure for the comparison of several isolated genomes over the course of a few days. RAPD analysis produces reproducible, and often distinctive, sets of DNA fragments with a single primer selected from an arbitrary nucleotide sequence (Lukinmaa *et al.*, 2004). This method is faster, relatively simple and more economical than other genomic typing methods.
This study compared 49 SE isolates from human, avian, and bovine sources by antibacterial resistance, plasmid profile, and RAPD analysis to assess genetic diversity and relationship between isolates of SE from different sources.

MATERIALS AND METHODS

Bacterial isolates

Forty-nine *Salmonella* Enteritidis (SE) isolates were used in this study. Thirty-five isolates (29 from chickens and 6 from poultry environment) were from our collection of SE isolates from poultry sources (unpublished data). Nine human isolates, recovered from the stools of patients, were obtained from a children's medical center in Tehran. Five fecal bovine SE isolates were kindly provided by Dr. A. Barin (Faculty of Veterinary Medicine, University of Tehran). All specimens were collected during 2005-2007. For comparison, a known strain of *Salmonella* Enteritidis PT21 (Dr. Misaghi, Faculty of Veterinary Medicine, University of Tehran) was included in DNA-based techniques of this study.

Drug susceptibility test

The susceptibility of the SE isolates to a panel of antimicrobial agents was determined by the agar disk diffusion method and the interpretation of results was carried out according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2000). The antimicrobial agents tested and their concentrations (µg) were: ciprofloxacin (5), danofloxacin (10), ofloxacin (5), norfloxacin (10), enrofloxacin (5), levofloxacin (5), nalidixic acid (30), flumequine (30), cephalothin (30), ceftazidime (30), ceftriaxone (30), cefixime (5), ampicillin (10), amoxi-clav (30), carbenicillin (100), piperacillin (100), imipenem (10), kanamycin (30), neomycin (30), streptomycin (10), amikacin (30), gentamicin (10), tobramycin (10), lincospectin (15/200), chloramphenicol (30), florfenicol (30), furazolidone (100), tetracycline (30), and trimethoprim-sulfamethoxazole (1.25/23.75). All antibacterial disks were provided from Padtan Teb Co (Tehran, Iran). The ATCC reference strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, ATCC 27853, and *E. coli* ATCC 35218 were used for quality control purposes. In this study, the SE isolates with intermediate susceptibility classification were considered not to be resistant to that drug and multi-resistance was defined as resistance to more than one drug.

Plasmid profile analysis

A high pure plasmid isolation kit (Roche Applied Science, Mannheim, Germany) was used to extract and purify plasmid DNA from the bacterial isolates. Plasmids were separated by gel electrophoresis (Apelex, France) in 0.7% agarose gel in 1 x TAE buffer (Sambrook and Russell, 2001). The gels were run for 10 minutes at 100 volts and then approximately 2 hours at 70 volts, stained with ethidium bromide, exposed to ultraviolet light and photographed (Visi-Doc-It system, UVP, UK). Commercial DNA ladders (Fermentas,

Germany) and an *E. coli* strain, AC11, containing three plasmids of 68 kb, 2.7 kb, and 1.7 kb (Peighambari *et al.*, 1994) were used as molecular-weight markers in each gel running.

Random-amplified polymorphic DNA (RAPD) analysis

To extract bacterial DNA, 1 ml pure overnight culture of each SE isolate grown overnight at 37° C for 16 h was transferred to a clean 1.5 ml microtube and centrifuged for five min at 10000 x *g*. The supernatants were carefully removed and discarded. The pellet was re-suspended in 300 µl sterile double distilled water by vortexing, incubated for 15 min at 100° C, chilled on ice immediately, and centrifuged again for five min at 14000 x *g* in 4° C. The supernatant was removed and used as template DNA. The concentration of DNA was determined by Biophotometer (Eppendorff, Germany) and adjusted to approximately 200 ng for each PCR reaction. The supernatant was stored at -20° C for further use. Two primers, MK22 (TGA GCA TAG ACC TCA) (Nguyen *et al.*, 1994) and P1254 (CCG CAG CCA A) (Lin *et al.*, 1996) were used. The primers and other materials used in PCR reaction were provided by Cinnagen (Tehran, Iran). Amplification reactions for MK22 primer were carried out in a 50 µl reaction volume containing 5 µl 10 x PCR buffer, 200 µM (each) dATP, dCTP, dGTP, and dTTP, 108 pmol of primer, 1.25 U of *Taq* polymerase DNA, 2.5 mM $MgCl_2$, and dH_2O. Approximately 200 ng of template DNA (5 µl) were added to the mixture. Negative controls (dH_2O instead of template DNA) were included in all PCR reaction sets. Amplification was programmed in a thermocycler (Gradient Mastercycler, Eppendorff, Germany) as follows: 94° C for 7 min followed by 45 cycles of 94° C for 1 min, 40° C for 1 min, 72° C for 2 min, and a final extension at 72° C for 5 min (Fadl *et al.*, 1995). The amplification products were detected by gel electrophoresis in 1% agarose gel at 70 V for 80 min in 1 x TAE buffer. The reaction mixture for P1254 primer was prepared as described for MK22 primer, only the concentration of primer (123.75 pmol) and $MgCl_2$ (3.5 mM) differed. For P1254 primer, the amplification program was as follows: 4 cycles of 94° C for 4 min, 35° C for 4 min, and 72° C for 4 min followed by 30 cycles of 94° C for 30s, 35° C for 1 min, 72° C for 2 min, and a final extension at 72°C for 5 min (Lin *et al.*, 1996). Amplified products were electrophoresed on 1% gels at 70 V for 80 min. Reproducibility of the RAPD patterns was confirmed using duplicate runs by two operators on separate occasions but on the same thermocycler.

RESULTS

Drug susceptibility test

A low resistance was observed to most of quinolones tested except for flumequine to which 34.5, 60, and 66.7% of poultry, bovine, and human isolates were resistant, respectively (Table 1). Resistance to cephalosporins varied among the isolates from different sources. Twenty percent of bovine isolates showed resistance to cephalothin and 24.2% of poultry isolates demonstrated resistance to cefixime. Among penicillins, the highest resistance was observed to ampicillin among all SE isolates, ranging from 17 to 40%. High resistance to other classes of drugs such as kanamycin, lincospectin, chloramphenicol, furazolidone and tetracycline were notable among SE isolates from different sources (Table 1). No resistance was observed to danofloxacin, norfloxacin, levofloxacin, ceftriaxone, imipenem and amikacin. Thirty-three resistance patterns were found among 49 isolates, in which 22 isolates (45%) belonged to 6 patterns and the remaining 27 isolates (55%) each belonged to a single pattern (Table 2). Nine (18.4%) isolates were sensitive to all drugs, 10 (20.4%) were single resistant, and the other 30 (61.2%) isolates were multidrug-resistant (MDR) type. The pattern of MDR varied from 2 to 13 antibacterial agents.

Plasmid profile

Of the 49 isolates, 48 (98%) carried one to six plasmids with molecular sizes ranging from 1.5 to 68 kb. All of 48 isolates harbored the 68 kb plasmid of which 20 (41%) isolates harbored this single plasmid. The other plasmids were small and below 10 kb. Six different plasmid profiles (A to F) were demonstrated (Figure 1). The distribution of profiles among 49 isolates was as follows: profile A in 17 isolates, B in two isolates, C in eight isolates, D in one isolate, E in 20 isolates, and F in one isolate (Table 3). Two patterns (A and E) were more frequent and included 75% of

TABLE 1 - *Resistance of 49 Salmonella Enteritidis isolates to 29 antimicrobial drugs.*

	% Resistant isolates from				
Antimicrobial drugs	*Poultry*	*Environment*	*Cow*	*Human*	*Total*
1 Ciprofloxacin (CF)	3.4	0	0	0	2
2 Danofloxacin (DF)	0	0	0	0	0
3 Enrofloxacin (EF)	6.9	0	0	11.1	6.1
4 Levofloxacin (LF)	0	0	0	0	0
5 Norfloxacin (NF)	0	0	0	0	0
6 Ofloxacin (OF)	3.4	0	0	0	2
7 Nalidixic acid (NA)	24.1	0	80	77.8	36.7
8 Flumequine (FL)	34.5	0	60	66.7	38.8
9 Cephalothin (CP)	10.3	0	20	11.1	10.2
10 Ceftazidime (CZ)	6.9	16.7	0	0	6.1
11 Ceftriaxone (CT)	0	0	0	0	0
12 Cefixime (CX)	24.1	16.7	0	0	16.3
13 Ampicllin (AP)	17.2	33.3	40	33.3	24.5
14 Amoxi-Clav (AC)	6.9	16.7	0	0	6.1
15 Carbenicillin (CB)	3.4	0	20	22.2	10.2
16 Piperacillin (PP)	10.3	16.7	0	0	8.1
17 Imipenem (IP)	0	0	0	0	0
18 Amikacin (AK)	0	0	0	0	0
19 Gentamicin (GE)	3.4	16.7	0	0	4.1
20 Kanamycin (KA)	3.4	0	0	22.2	6.1
21 Streptomycin (ST)	10.3	16.7	20	3.3	16.3
22 Tobramycin (TB)	3.4	0	0	0	2
23 Neomycin (NE)	0	16.7	20	11.1	8.2
24 Furazolidone (FU)	34.5	33.3	0	55.6	34.7
25 Chloramphenicol (CL)	10.3	0	20	11.1	10.2
26 Florfenicol (FF)	3.4	0	0	0	2
27 Linco-spectin (LS)	20.7	16.7	40	11.1	20.4
28 Tetracycline (TE)	24.1	66.7	40	33.3	32.6
29 Trimethoprim-Sulfamethoxazole (TS)	17.2	0	0	11.1	12.2

TABLE 2 - *Drug resistance patterns among 49 Salmonella Enteritidis isolates.*

Pattern[#]	*Resistant to*[a]	*No. of isolate (%)*
1	None of drugs tested	9 (18.4)
2	FL	4 (8.2)
3	FU	3 (6.1)
4	TE	2 (4.1)
5	FU, TE	2 (4.1)
6	AP, CX, CZ, FU, GE, LS, TE	2 (4.1)
7	NA	Each pattern included only one isolate (2%)
8	FL, NA	
9	AC, NE	
10	LS, ST	
11	FU, LS, TE	
12	CP, CX, LS	
13	CX, FU, TS	
14	FL, NA, TE	
15	CB, LS, NA	
16	NA, ST, TE	
17	FL, FU, NA	
18	CB, FL, FU, NA	
19	AC, AP, CX, TS	
20	AP, PP, ST, TE	
21	AP, FL, NA, NE	
22	AP, CB, FL, NA	
23	AP, EF, FU, KA, NA	
24	CB, FL, NA, ST, TE	
25	AP, CP, FL, FU, NA	
26	AP, FL, FU, KA, NA	
27	CL, FL, LS, NA, ST, TE	
28	AP, CB, CL, CP, FL, NA, TE	
29	AP, CP, CX, FL, FU, PP, TE, TS	
30	AC, AP, CX, NA, OF, PP, TE, TS	
31	CF, CL, EF, FL, FU, LS, NA, ST, TE	
32	CL, EF, FL, FU, KA, LS, NA, NE, ST, TE, TS	
33	FF, CL, CP, CX, CZ, FL, FU, LS, PP, ST, TB, TE, TS	

[a]For abbreviations of drugs refer to Table 1.

isolates. The frequency of plasmid profiles among isolates from different sources varied and was as follows: bovine (A, B), environmental (A, C, E), human (A, C, E) and poultry (A, C, D, E, F). The SE PT21 strain showed a different pattern with two plasmids of 4.5 and 2.4 kb.

RAPD analysis

Using primer MK22, six different RAPD types (G to L) were observed among the 49 isolates (Figure 2a). The RAPD profiles differed in the number of fragments (1-6) which ranged from 0.2 to 2 kb in molecular weight. Forty-one (84%) isolates had pattern G, and the other 8 (16%) isolates were distributed in 5 patterns (Table 3). Pattern G was prevalent among 5 (83%), 27 (93%), and 9 (100%) of environmental, poultry, and human isolates, respectively. All five bovine isolates belonged to two patterns of K (3 isolates) and L (2 isolates). The SE PT21 strain showed pattern G. With primer P1254, we observed six RAPD patterns which were assigned M to R (Figure 2b). These profiles had 3 to 9 fragments ranging from 0.3 to 2.6 kb in molecular weight. Forty-two (86%) isolates had pattern M, and the other 7 (14%) isolates were distributed in 5 patterns (Table 3). Profile M was found among 2 (40%), 5 (83%), 26 (90%), and 9 (100%) bovine, environmental, poultry, and human isolates, re-

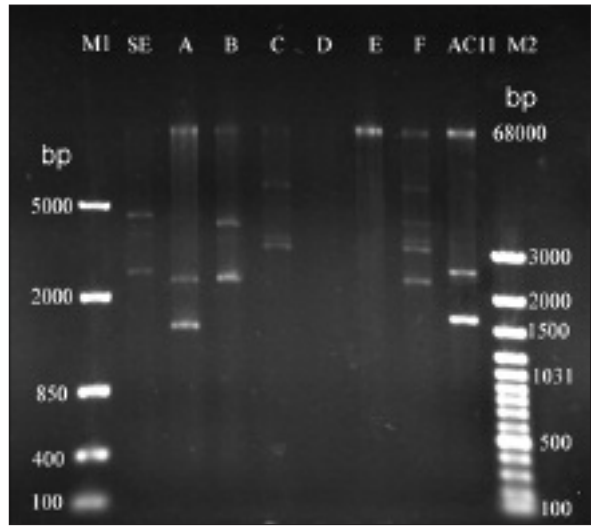

FIGURE 1 - *Plasmid profiles of Salmonella* Enteritidis *isolates. M1, 100 bp ladder; M2, 100 plus bp ladder; SE, Salmonella* Enteritidis *PT21 strain; AC11, an Escherichia coli strain; Lanes A-F, plasmid profiles.*

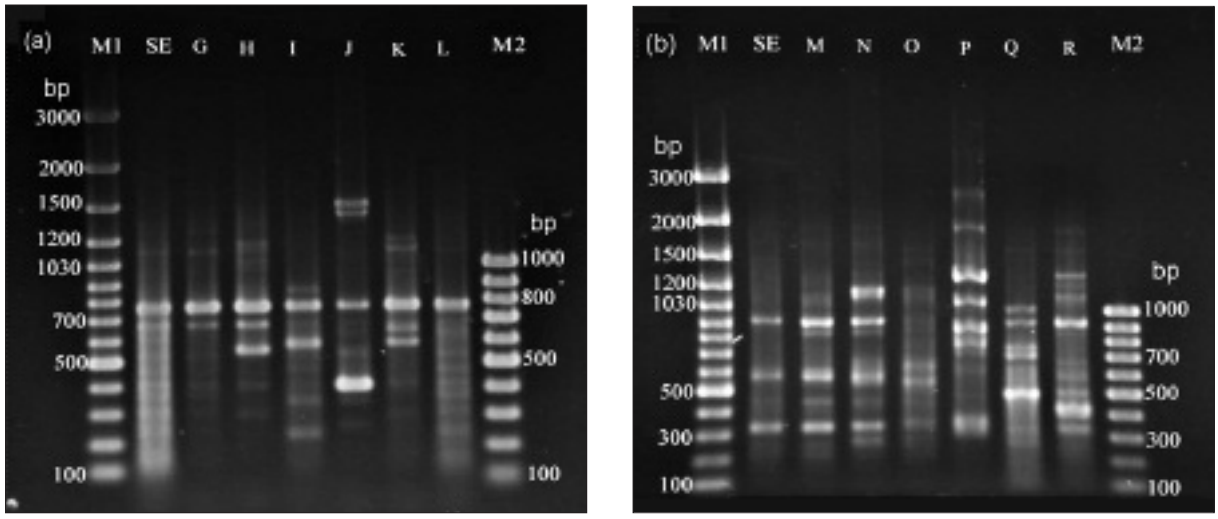

FIGURE 2 - *DNA fingerprints patterns of Salmonella* Enteritidis *isolates by RAPD-PCR with (a) MK22 primer and (b) P1254 primer. M1, 100 plus bp ladder; M2, 50 bp ladder; SE, Salmonella* Enteritidis *PT21 strain; Lanes G-L and M-R, RAPD patterns.*

TABLE 3 - *Results of plasmid profiling and RAPD fingerprinting of Salmonella Enteritidis isolates using two arbitrary primers.*

Source	*No. of isolates (%)*	*Plasmid profile*	*RAPD profile with primer*	
			MK22	*P1254*
Poultry	12 (24.5)	E	G	M
Poultry	9 (18.4)	A	G	M
Poultry	4 (8.2)	C	G	M
Poultry	1 (2)	A	H	N
Poultry	1 (2)	C	G	O
Poultry	1 (2)	F	G	M
Poultry	1 (2)	D	J	P
Poultry environment	4 (8.2)	E	G	M
Poultry environment	1 (2)	C	G	M
Poultry environment	1 (2)	A	I	Q
Cow - feces	3 (6.1)	A	K	R
Cow - feces	2 (4)	B	L	M
Human - stool	4 (8.2)	E	G	M
Human - stool	3 (6.1)	A	G	M
Human - stool	2 (4)	C	G	M

spectively. The remaining 3 (60%) bovine isolates belonged exclusively to profile R. The SE PT21 strain belonged to a unique pattern different from profile M to R. Combination of RAPD patterns obtained using two different primers, provided 7 profiles (Table 3). Profile GM was found in 40 (82%) isolates of human, poultry, and environmental sources. The remaining four isolates from these sources each belonged to one profile. Five bovine isolates were differentiated from other isolates and distributed in two profiles KR (3 isolates) and LM (2 isolates).

When the results of plasmid profiles and RAPD analysis were combined, 10 groups were found among the 49 SE isolates of this study (Table 3). Twenty (41%) isolates belonged to group EGM, 12 (24.5%) isolates belonged to group AGM, and 7 (14.2%) isolates belonged to group CGM which were all from human, poultry and environmental sources. The remaining five isolates from these sources each belonged to one profile. Bovine isolates again were distributed in two separate profiles of AKR (3 isolates) and BLM (2 isolates).

DISCUSSION

The emergence of *S.* Enteritidis (SE) as one of the major food-borne pathogens and a cause of acute gastroenteritis worldwide (Velge *et al.*, 2005) has prompted researchers and health authorities to carefully monitor the epidemic spread of this pathogen. This report describes the antibiotic susceptibility, plasmid profiles and RAPD types of SE isolates from human, poultry and bovine sources. The methods applied in our study are among the methods used for epidemiologic analysis of important zoonotic bacterial pathogens. The choice of a suitable typing technique depends on a number of factors such as ease of operation, speed, cost and the discriminatory power and reproducibility of the method (Lukinmaa *et al.*, 2004).

The susceptibility of SE isolates to a panel of antimicrobial agents common in human and veterinary medicine provided a wide range of patterns, with 81.6% of isolates showing resistance to at least one agent. Less than 35% of poultry isolates showed resistance to all drugs tested. A much higher frequency of resistance was observed to some drugs among human and bovine isolates. Probably both human and veterinary uses of some classes of drugs have significantly contributed to the emergence of resistant strains of *Salmonella*. Horizontal transfer and clonal spread of resistance genes among food producing animals and humans may occur (Hawkey, 2008).

Quinolones are currently used against invasive and systemic infections caused by *Salmonella* spp. that occur in humans and animals (Giraud *et al.*, 2006). In our study, a much higher resistance to nalidixic acid and flumequine was observed among isolates compared with that of other agents of this class. Resistance to these two agents ranged 60%-80% among human and bovine isolates which was much higher than those of poultry isolates. The frequency of resistance to flumequine among poultry isolates, considering the fact that flumequine is an extensively used drug in the Iranian poultry industry against *Escherichia coli* infections, was much lower compared to that of *E. coli*. Our previous study on 150 *E. coli* isolates from avian colibacillosis cases showed 92% resistance to flumequine (Khoshkhoo and Peighambari, 2005). In the present study, resistance to other agents of quinolones class was relatively uncommon or very low among SE isolates from different sources. The frequency of resistance to ampicillin was also notable. Isolates from poultry origin were much less resistant to ampicillin than isolates from other origins. Ampicillin is commonly used in human medicine but is uncommon in the Iranian poultry industry. An increased frequency of ampicillin-resistant SE has been reported in other countries and associated with SE strains belonging to phage type 6a (Vatopoulos *et al.*, 1994). Our study found resistance to furazolidone in 55.6% and 34.5 % of human and poultry isolates, respectively. This drug is used in human medicine against intestinal infections but its use is banned in food animals.

Increased MDR has been reported in *Salmonella* isolates in many countries including Iran (Butaye *et al.*, 2006, Madadgar *et al.*, 2008). In our study, 61.2% of SE isolates demonstrated the MDR pattern and the number of antibacterial agents varied from 2 to 13 among MDR types. MDR bacterial isolates of animal origin may spread into human population by direct contact and through animal-origin foods (Soulsby, 2008). These resistant bacteria may be colonized in the human

intestinal tract and the genes coding for antibiotic resistance can be transferred to the bacteria of natural microflora or pathogenic bacteria. The resistance bacteria that are shed in the environment may infect animals and then through the food chain back to humans. Examples of the transfer of antibiotic resistance genes from humans to animals or vice versa have been reviewed (Hawkey, 2008). The development of resistance to antimicrobial agents among bacterial strains should be carefully monitored throughout the world. Plans for controlled antimicrobial therapy should be implemented and continuous efforts should be made to develop effective new agents to combat resistant microbes.

The value of plasmid profiling as an epidemiological tool in analyzing SE is limited by the prevalence of such elements in the isolates being investigated (Lukinmaa *et al.*, 2004; Chu and Chiu, 2006). Plasmids are known to carry the genes that confer antibiotic resistance. In one study, plasmid analysis of SE isolates had limited potential because the frequency of occurrence of the plasmid identified was relatively low (Martinetti and Altwegg, 1990). The number of plasmids and the related profiles vary among SE isolates (Brown *et al.*, 1994; Vatopoulos *et al.*, 1994; Fernandes *et al.*, 2003; Bakeri *et al.*, 2004; Liebana *et al.*, 2004). One study found 7 plasmid patterns among 105 SE isolates from human and non-human sources (Fernandes *et al.*, 2003). In other studies, Bakeri *et al.* (2004) and Liebana *et al.* (2004) reported 9 and 17 plasmid profiles among 65 and 250 SE isolates, respectively, from human and animal sources. In our study, six different plasmid patterns were detected among 49 isolates. Two patterns (A and E) were more frequent and included 75% of isolates.

A serotype specific virulence plasmid has been reported among several *Salmonella* serotypes including Enteritidis (Chu and Chiu, 2006, Rychlik *et al.*, 2006). Serotype specific virulence plasmids vary in size (50-100 kb) but all carry the *spv* operon that is known to be involved in the virulence expression of the serotypes in their specific hosts. These virulence plasmids share many properties but each appears to be specific to its host. A 36 MDa serotype specific virulence plasmid was found in 22 (%100) (An -Küçüker *et al.*, 2000) and 96% of SE isolates studied (Fernandes *et al.*, 2003). Our findings were in comparison with previous findings in that 98% of our isolates were shown to harbor a large plasmid (68 kb) that appears to be a serotype specific virulence plasmid. RAPD fingerprinting is one of powerful DNA-based typing methods for the comparison of genomes (Lukinmaa *et al.*, 2004). RAPD analysis has been useful for studying the epidemiology of SE (Fadl *et al.*, 1995; Lin *et al.*, 1996). Using MK22 primer, Fadl and coworkers (1995) found seven RAPD patterns among 33 SE isolates from human and avian sources. In another study, when patterns obtained by six primers were combined, 14 RAPD patterns were observed among 29 SE isolates, and a higher discriminatory power was established for RAPD-PCR when it was compared to other subtyping methods such as phage typing, ribotyping and PFGE (Lin *et al.*, 1996). RAPD analysis of 275 human SE isolates in Hong Kong, using MK22 primer, revealed six different RAPD types in which 95% of isolates belonged to one pattern only (Ling *et al.*, 1998). Other researchers were able to differentiate 47 SE isolates from different geographic locations and even within a specific geographic locale, using RAPD analysis (Hudson *et al.*, 2001). The primers that they used (1247, 1283, Opa4, Opb17) were different from the primers used in our study. In the same study, PFGE produced only one pattern and failed to disclose the genetic differences among the SE isolates. In the present study, we used two primers to enhance the discriminatory power of the RAPD method. RAPD with MK22 primer showed a high similarity between human, poultry and environmental isolates. One pattern, G, was prevalent in 83, 93 and 100% of environmental, poultry and human isolates, respectively. However, bovine isolates were distributed into two patterns, K and L, and differentiated from isolates obtained from other sources. Results with P1254 primer were nearly the same. The pattern M was found among 83, 90 and 100% of environmental, poultry and human isolates, respectively, as well as among 40% of bovine isolates. The remaining 60% of bovine isolates exclusively belonged to pattern R. The discriminatory power was slightly increased when the patterns obtained by two primers were combined. Seven types were observed in which 82% of isolates from human, poultry, and environment belonged to one type; and as for MK22 primer, the bovine isolates were differentiated from isolates of other sources. Much higher dis-

criminatory power among SE isolates was reported when the combination approach was used. Hudson *et al.* (2001) were able to differentiate among specific SE phage types when the results of RAPD with two or more primers were combined. These researchers even subtyped SE phage type 4 isolates, showing identical PFGE patterns, using a combination of patterns obtained by different primers.

Our results showed that the discriminatory power of two methods used in this study, plasmid profile and RAPD analysis, did not differ and both methods were similarly able to differentiate the SE isolates into 6 subtypes. A single method may not produce sufficient differentiating power for subtyping SE strains (Ling *et al.*, 1998). To increase the discriminatory power for subtyping of SE, researchers combined the results of several molecular methods to achieve this goal (Ling *et al.*, 1998; Liebana *et al.*, 2004). In the present study, upon combining the results from plasmid profile and RAPD analysis by two different primers, the collection of 49 SE isolates was differentiated into 10 subtypes in which three subtypes included about 80% of the isolates. All human isolates and more than 83% of poultry and environmental isolates belonged to two of these three subtypes. This highly shared RAPD and plasmid patterns among human and poultry isolates might indicate that the infection in humans could have originated from poultry products. Two subtypes were exclusively found among bovine isolates and this dissimilarity of bovine subtypes with those from other sources was notable. However, more bovine origin SE isolated from different places should be analyzed for a better clarification of this matter.

Further comprehensive studies are required to elucidate whether the patterns observed in this study are predominant clones of human and poultry *Salmonella* Enteritidis circulating in Iran.

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