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Research Article

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A molecular study of the *Salmonella enterica* serovars Abortusovis, Typhimurium, and Enteritidis

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Abstract: This study presents a molecular analysis of 3 important human and animal serovars of *Salmonella*: Typhimurium, Enteritidis, and Abortusovis. We also provide information that can be applied in the surveillance of salmonellosis. Over the course of 3 decades, 90 isolates were collected, with 30 isolates representing each type of serovar. In addition to conventional serotyping, serovars were confirmed by polymerase chain reaction (multiplex PCR and IS200 PCR). Pulsed-field gel electrophoresis (PFGE) of the serovars confirmed 12, 15, and 20 patterns, respectively. In an analysis of the results, 5 pulsotypes (80% of isolates) of *Salmonella* Typhimurium and 4 pulsotypes (64% of isolates) of *Salmonella* Enteritidis were confirmed to be prevalent, and *Salmonella* Abortusovis showed unexpectedly high diversity, with just 2 prevalent pulsotypes (40% of isolates). *S. Enteritidis* and *S. Abortusovis* were identified as polyphyletic (possibly the first report), while S. Typhimurium was identified as monophyletic. The combination of PFGE, random amplified polymorphic DNA-PCR (RAPD-PCR) with 2 primers, and antibiotic susceptibility tests showed 29, 23, and 21 distinct patterns in the serovars, respectively; for this combination of methods, the value of the discrimination index was established at more than 0/95 in each serovar. The results of this study indicate that this combination of methods can increase the discrimination index. It is therefore suitable and applicable for use in a *Salmonella* surveillance system, especially in endemic regions in which there is no active surveillance system or information.

Key words: *Salmonella*, PFGE, monophyletic, polyphyletic, discrimination index

Introduction

Salmonella serovars are one of the most frequent causes of bacterial infections in humans and other animals and are major causes of foodborne disease (1,2). The *Salmonella enterica* serovar Enteritidis is a major cause of foodborne disease and, during recent decades, it has been isolated in increasing numbers worldwide (3). The most frequently isolated serovar worldwide, however, is *S. enterica* serovar Typhimurium (4). A third serovar, *Salmonella enterica* serovar Abortusovis, is ovine-restricted and ranks among the main causes of ovine abortion in Europe and western Asia (5,6,7).

The determination and accurate identification of these bacterial isolates are essential for epidemiological surveillance and outbreak investigations. Phenotypic methods have been used to differentiate isolates for several decades. In recent

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years, however, molecular methods based on genome, protein, lipid, and lipopolysaccharide analysis have increased the sensitivity and specificity of research on *Salmonella*. These advances result from the fact that each method has specific characteristics and applications. Among the genome-based methods, different systems for analyzing chromosomal DNA, such as random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), repetitive element PCR (rep-PCR), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP), have been frequently utilized in various epidemics and studies on *Salmonella* (7-12). PFGE is probably the most commonly used molecular technique; its use worldwide has led to the detection of international outbreaks. The Centers for Disease Control and Prevention (CDC) formed an effective network of laboratories known as PulseNet, which uses standardized PFGE protocols and control strains to enable laboratories to track outbreaks (13-16). In addition to this resource, RAPD analysis provides a simple, rapid, and powerful subtyping method for *Salmonella* (3,8,12).

The present study calculates the value of the discrimination index, separately and in combination, for the evaluation of antibiotic susceptibility, RAPD-PCR, and PFGE tests (with CDC protocol) in the differentiation of isolates. These 3 human and animal serovars, *Salmonella enterica* Typhimurium, Enteritidis, and Abortusovis, were isolated over the course of more than 3 decades. We also evaluated the possible combination of these methods with the molecular analysis of serovars.

Materials and methods

Bacterial strains

The isolates examined in this study belonged to 2 groups. The first had been collected over the course of more than 3 decades, lyophilized, and stored in the microbial collection. The second groups of isolates came from clinical samples taken from different animals and poultry between 2005 and 2007, as well as over the course of this study. All of the isolates were collected at different times from various regions in Iran.

After isolation and biotyping, serotyping was administered using commercial antisera (Difco) and confirmed with multiplex PCR following the method outlined by Zahraei Salehi et al. for *S.* Typhimurium (4) and that described by Pan and Lui for *S.* Enteritidis (17). The IS200 PCR typing method was used in a previous study examining *S.* Abortusovis isolates (18).

Since *inv*A and *spv*C are the virulence genes of *S.* Typhimurium and *S.* Enteritidis, respectively (4,17), they were screened by multiplex PCR methods in the isolates. All of the *S.* Abortusovis strains used had also been isolated from abortions (18). In total, 30 isolates of each serovar were considered, with additional attention given to recently isolated strains. One strain of *S.* Typhimurium, identified with the code ATCC 14028, was added to the collection of *S.* Typhimurium for comparison with clinical isolates.

Bacterial growth

Lyophilized or recently isolated strains were subjected to overnight incubation in brain-heart infusion broth. Afterwards, they were transferred to Luria-Bertani agar (Difco, Detroit, USA) for an additional night to isolate a single colony.

RAPD-PCR

In order to optimize the RAPD fingerprinting technique, method, and details of extraction (boiling and QIAGEN kit), the optimal concentrations of arbitrary oligonucleotides, DNA templates, $MgCl_2$, *Taq* DNA polymerase, and dNTPs used in PCR were first adjusted and determined. The type of primers used were selected from 9 arbitrary primers, P1254, 23L, OPA-4, OPB-6, OPB-17, OPB-15, A, Primer 1, and OPL-03, as described by Lin et al. (8), Lim et al. (10), and Tekeli et al. (19). The primers selected for this study were P1254 5′-CCGCAGCCAA-3′ and 23L 5′-CCGAAGCTGC-3′; the G+C content of both primers was 70%.

A single colony of each isolate on an agar plate was picked up and suspended in 200 µL of distilled H_2O. After vortexing, the suspension was boiled for 5 min, and 50 µL of the supernatant was collected after being spun for 10 min at 14,000 rpm in a microcentrifuge. The DNA concentration of the boiled extracts was determined with a spectrophotometer (8).

PCR was conducted in a 25-μL volume containing 40 ng of total DNA (extracted by boiling), 1.5 mM $MgCl_2$, 0.5 μM of primer, 1 U of SmarTaq DNA polymerase, and 200 mM of a dNTP mix in 1× PCR buffer. The thermal program and electrophoresis was conducted as described by Lin et al. (8).

Antibiotic susceptibility

An antibiotic susceptibility test was performed by the standard disk diffusion method in Mueller-Hinton agar; results were interpreted in accordance with the criteria of the Clinical and Laboratory Standards Institute (20). All 3 serovars were screened for resistance to the following antibiotics: cephalexin (LEX, 30 μg), oxytetracycline (T, 30 μg), trimethoprim (TMP, 5 μg), linco-spectin (LP, lincomycin and spectinomycin, 15:200), enrofloxacin (NFX, 5 μg), and trimethoprim sulfamethoxazole (SXT). Additionally, nalidixic acid (NAL, 30 μg) and nitrofurantoin (NIT, 300 μg) were administered for the samples of *S.* Typhimurium; nalidixic acid (NAL, 30 μg), furazolidone (FX, 100 μg), ampicillin (AMP, 10 μg), and neomycin (NE, 30 μg) were administered for *S.* Enteritidis; and furazolidone (FX, 100 μg), ampicillin (AMP, 10 μg), neomycin (NE, 30 μg), and chloramphenicol (CHL, 30 μg) were administered for *S.* Abortusovis. This array of antibiotics was chosen on the basis of unpublished experimental data obtained in our department on discrimination of some isolates of these serovars.

Pulsed-field gel electrophoresis

PFGE was performed according to the procedures developed by the CDC for the molecular subtyping of *Escherichia coli* O157:H7, nontyphoidal *Salmonella* serovars, and *Shigella sonnei*, as previously described (15). Briefly, agarose-embedded DNA was digested with 50 U of *Xba*I (Fermentas) overnight in a water bath at 37 °C. The restriction fragments were separated by electrophoresis in 0.5X Tris-borate-EDTA (TBE) buffer at 14 °C for 20 h at 6 V/cm using a CHEF-DR II electrophoresis system (Gene Navigator, Pharmacia, Sweden) with pulse times of 2.2-63.8 s. The gels were stained with ethidium bromide (1 μg/mL) and destained with the buffer remaining in the electrophoresis apparatus for 60-90 min.

A Gel Doc 2000 equipped with the appropriate software (Bio-Rad, Hercules, CA, USA) was used for image capture and conversion of gel images into TIFF files. Isolates presenting DNA smear patterns were retested. The size standard used for all gels was *Xba*I-digested DNA from *Salmonella braenderup* strain H9812 (American Type Culture Collection Catalog No. BAA-664), the universal size standard used by all PulseNet laboratories (21). The use of this size standard permitted normalization and comparison of DNA fingerprints from gel to gel and from lab to lab, as well as providing a type of positive control for the accuracy of the investigation. DNA fingerprint patterns were interpreted both by optical inspection and by use of Zhen Negar software, designed and optimized by the Faculty of Mathematics and Computing Sciences at Sharif University in Iran. The banding patterns were compared using Dice coefficients (22). A 5% optimization parameter and a 1% band position tolerance were used. Isolate relatedness was determined using the unweighted pair group method using arithmetic averages (UPGMA). The DNA banding patterns were interpreted as instructed by Tenover et al. (23). Simpson's index of diversity (D) was used as an indicator of the discriminatory power of each method and is calculated according to the following formula: $D = 1 - (\Sigma_n(n - 1)/N(N - 1))$, where D is the diversity, N is the total number of strains in the serovars, and n is the number of strains in each pulsotype (24).

Results

Bacterial strains

All *S.* Typhimurium and *S.* Enteritidis isolates have the virulence genes *inv*A and *spv*C (*Salmonella* plasmid virulence), respectively; *S.* Abortusovis strains were also isolated from abortions, certainly with high virulence.

RAPD profiles

RAPD analysis by primers P1254 and 23L revealed 4 and 6 polymorphic patterns of DNA in *S.* Typhimurium, 7 and 3 in *S.* Enteritidis, and 8 and 10 in *S.* Abortusovis isolates, respectively (Tables 1-3). The reproducibility of the RAPD fingerprinting technique was confirmed by comparing the fingerprint patterns obtained from duplicate runs of strains. The results obtained by using primer P1254 on some of the *S.* Abortusovis isolates are shown in Figure 1.

Table 1. Characteristics and results of PFGE, RAPD-PCR, and antibiotic resistance tests performed on *S.* Typhimurium isolates.

Isolate (name)	**Source**	**Year**	**R-type**[b]	**RAPD type (P125)**	**RAPD type (23L)**	**Pulsotype (*XbaI*)**	**Profiles**
S. ty 1	Pony	2003	B_1	A	A	A	1
S. ty 2	Cat	2006	C_1	A	F	A	2
S. ty 3	Chicken	1998	K_1	A	A	B	3
S. ty 4	Chicken	1998	G_1	B	E	B	4
S. ty 5	Cow	2003	M_1	C	A	F	5
S. ty 6	Cow	2003	E_1	B	A	C	6
S. ty 7	Chicken	2001	C_1	A	C	D	7
S. ty 8	Chicken	2001	C_1	A	C	D	7
S. ty 9[a]	ATCC	14028	B_1	A	A	G	8
S. ty 10	Chicken	2001	C_1	A	C	H	9
S. ty 11	Cow	2006	J_1	B	A	C	10
S. ty 12	Dove	2006	F_1	D	A	I	11
S. ty 13	Sheep	2006	C_1	A	A	A	12
S. ty 14	Dove	2005	I_1	A	A	B	13
S. ty 15	Sparrow	2005	H_1	A	A	B	14
S. ty 16	Sparrow	2005	C_1	A	F	J	15
S. ty 17	Sparrow	2005	D_1	A	F	A	16
S. ty 18	Parrot	2005	G_1	A	D	A	17
S. ty 19	Sparrow	2005	D_1	A	A	B	18
S. ty 20	Sparrow	2005	D_1	A	F	B	19
S. ty 21	Cat	1976	G_1	A	A	A	20
S. ty 22	Cat	1976	A_1	A	A	C	21
S. ty 23	Cat	1976	D_1	A	A	A	22
S. ty 24	Cat	1976	A_1	A	B	D	23
S. ty 25	Cat	1976	D_1	A	A	K	24
S. ty 26	Cow	2004	E_1	B	D	E	25
S. ty 27	Cow	2003	D_1	A	A	L	26
S. ty 28	Cow	2004	E_1	B	D	D	27
S. ty 29	Cow	2003	L_1	B	A	C	28
S. ty 30	Cow	2003	E_1	B	A	E	29
S. ty 31	Canary	2006	D_1	A	A	B	18
			Sum = 13	Sum = 4	Sum = 6	Sum = 12	Sum = 29

[a] *S.* ty 9 = *Salmonella* Typhimurium standard strain ATCC14028

[b] Letters show resistance profiles: A_1, sensitive to all antibiotics; B_1, LEX; C_1, LEX, TMP; D_1, TMP; E_1, TMP, T, LP; F_1, T, TMP; G_1, LEX, TMP, SXT; H_1, TMP, SXT; I_1, LEX, LP; J_1, LEX, T, LP; K_1, LEX, TMP, LP, SXT; L_1, T, TMP, LP, SXT; and M_1, T, NAL, TMP, LP, NIT, SXT.

Table 2. Characteristics and results of PFGE, RAPD-PCR, and antibiotic resistance tests performed on *S.* Enteritidis isolates.

Isolate (name)	Source	Year	R-type[a]	RAPD type (P1254)	RAPD type (23L)	Pulsotype (*Xba*I)	Profiles
S. e 1	Chicken	2005	A_2	A	A	C	1
S. e 2	Chicken	2005	A_2	A	C	A	2
S. e 3	Chicken	2006	A_2	A	A	C	1
S. e 4	Chicken	2005	A_2	A	C	A	2
S. e 5	Chicken	2006	D_2	A	A	E	3
S. e 6	Chicken	2005	D_2	A	A	F	5
S. e 7	Sparrow	2003	A_2	A	A	G	5
S. e 8	Sparrow	2004	A_2	A	A	H	6
S. e 9	Sheep	2006	C_2	A	C	A	7
S. e 10	Cow	2005	B_2	F	A	I	8
S. e 11	Chicken	2004	A_2	B	A	A	9
S. e 12	Cow	2003	F_2	C	C	J	10
S. e 13	Cow	2004	E_2	D	B	K	11
S. e 14	Chicken	2003	E_2	C	C	L	12
S. e 15	Cow	2005	A_2	E	B	B	13
S. e 16	Chicken	2003	A_2	A	B	M	14
S. e 17	Cow	2005	A_2	D	B	B	15
S. e 18	Cow	2004	A_2	D	B	N	16
S. e 19	Cow	2006	A_2	D	B	B	15
S. e 20	Chicken	2005	A_2	A	C	A	2
S. e 21	Chicken	2002	A_2	G	A	O	17
S. e 22	Chicken	1999	D_2	A	C	A	18
S. e 23	Chicken	2000	B_2	A	C	A	19
S. e 24	Chicken	2001	A_2	A	B	A	20
S. e 25	Chicken	2001	D_2	A	B	A	21
S. e 26	Chicken	2003	A_2	A	B	D	22
S. e 27	Chicken	2003	A_2	A	C	D	23
S. e 28	Chicken	2002	A_2	A	C	A	2
S. e 29	Chicken	2002	A_2	A	C	A	2
S. e 30	Chicken	2006	A_2	A	C	A	2
			Sum = 6	Sum = 7	Sum = 3	Sum = 15	Sum = 23

[a] Letters show resistance profiles: A_2, sensitive to all antibiotics; B_2, AMP; C_2, AMP, LEX; D_2, NAL; E_2, NAL, FX; and F_2, NAL, FX, TM, LP, T, SXT.

Table 3. Characteristics and results of PFGE, RAPD-PCR, and antibiotic resistance tests performed on *S.* Abortusovis isolates.

Isolate (name)	Province	Year	R-type[a]	RAPD type (P1254)	RAPD type (23L)	Pulsotype (*Xba*I)	Profiles
S. a.o 1	Tehran	1970	B_3	A	A	C	1
S. a.o 2	Tehran	1970	C_3	C	B	D	2
S. a.o 3	Tehran	1970	K_3	D	C	E	3
S. a.o 4	Tehran	1970	B_3	A	D	F	4
S. a.o 5	Tehran	1970	J_3	C	E	G	5
S. a.o 6	Tehran	1970	J_3	H	E	B	6
S. a.o 7	Tehran	1970	A_3	E	C	H	7
S. a.o 8	Tehran	1970	J_3	C	F	I	8
S. a.o 9	Tehran	1970	A_3	B	D	B	9
S. a.o 10	Khorasan	1970	A_3	B	D	B	9
S. a.o 11	Esfehan	1970	J_3	C	E	J	10
S. a.o 12	Esfehan	1970	H_3	B	C	K	11
S. a.o 13	Esfehan	1970	I_3	B	C	L	12
S. a.o 14	Tehran	1970	A_3	D	B	M	13
S. a.o 15	Khorasan	1970	J_3	C	G	N	14
S. a.o 16	Khorasan	1970	G_3	F	F	O	15
S. a.o 17	Khorasan	1970	E_3	D	G	P	16
S. a.o 18	Khorasan	1970	B_3	D	D	q	17
S. a.o 19	Khorasan	1970	B_3	H	H	R	18
S. a.o 20	Khorasan	1970	F_3	D	C	S	19
S. a.o 21	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 22	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 23	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 24	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 25	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 26	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 27	Chaharmahal-Bakhtiari	2000	D_3	D	J	T	21
S. a.o 28	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 29	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
S. a.o 30	Chaharmahal-Bakhtiari	2000	A_3	G	I	A	20
			Sum = 11	Sum = 8	Sum = 10	Sum = 20	Sum = 21

[a] Letters show resistance profiles: A_3, sensitive to all antibiotics; B_3, T; C_3, T, LP; D_3, T, LP, FX; E_3, T, LP, FX, AMP; F_3, T, FX, AMP; G_3, T, TM; H_3, LP, CHL; I_3, T, LP, CHL, FX, AMP; J_3, SXT, T, LP, TM, FX; and K_3, SXT, T, LP, TM, LEX, FX, AMP.

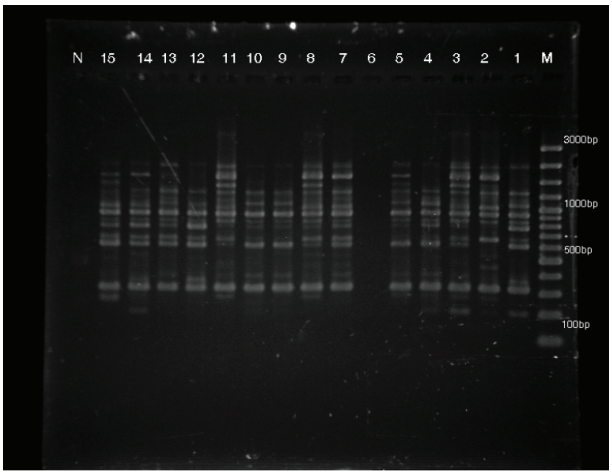

Figure 1. RAPD fingerprints of some *S.* Abortusovis (*S.* ao) isolates using primer P1254 (M: 100 bp marker, N: negative control, 1-15: *S.* ao 1 through *S.* ao 15).

Antibiotic susceptibility test

With the application of 8 (*S.* Typhimurium), 10 (*S.* Enteritidis), and 10 (*S.* Abortusovis) antibiotics, 30 isolates of the bacteria from each of the serovars could be divided into 13, 6, and 11 patterns of resistance type (R-type) in *S.* Typhimurium, *S.* Enteritidis, and *S.* Abortusovis, respectively (Tables 1-3).

It was seen that 2 isolates of *S.* Typhimurium were susceptible to all of the antimicrobials tested, compared to 20 isolates of *S.* Enteritidis and 13 isolates of *S.* Abortusovis.

In *S.* Typhimurium, 9 isolates were found to be resistant to 1 antimicrobial, 9 isolates were resistant to 2, 8 isolates were resistant to 3, 2 isolates were resistant to 4, and only 1 isolate was resistant to 6 antimicrobials. Further results indicated that in *S.* Enteritidis, 6 isolates were resistant to 1 antimicrobial, 3 isolates were resistant to 2, and only 1 isolate was determined to be resistant to 6 antimicrobials. Finally, in the tested samples of *S.* Abortusovis, 4 isolates were shown to be resistant to 1 antimicrobial, 3 isolates were resistant to 2, 2 isolates were resistant to 3 antimicrobials, 1 isolate was resistant to 4, 6 isolates were resistant to 5, and only 1 isolate was resistant to 7 antimicrobials.

PFGE results

S. Typhimurium

A total of 12 distinct patterns were generated by PFGE with PulseNet protocol and *Xba*I enzyme digestion among the 30 isolates of *S.* Typhimurium. Some 10 to 12 bands were identified in different pulsotypes and 7 bands were common: 40, 70, 90, 230, 260, 380, and 670 kb. It was also revealed that 80% of the isolates belonged to 5 pulsotypes (A, B, C, D, and E), with the largest group of isolates (47%) representing A and B. Results indicated that 7 and 4 isolates belonged to the A and D pulsotypes, respectively, and were determined to be common to other animals and poultry. A further 7 isolates of the B pulsotype and 4 isolates of the C pulsotype were identified as being specific to poultry and other animals. Finally, 2 isolates of pulsotype E were identified as specific to bovines (Table 1, Figures 2 and 3).

Figure 2 shows the results obtained by PFGE for some *S.* Typhimurium isolates. General PFGE profiles of each serovar can be seen in Figure 4.

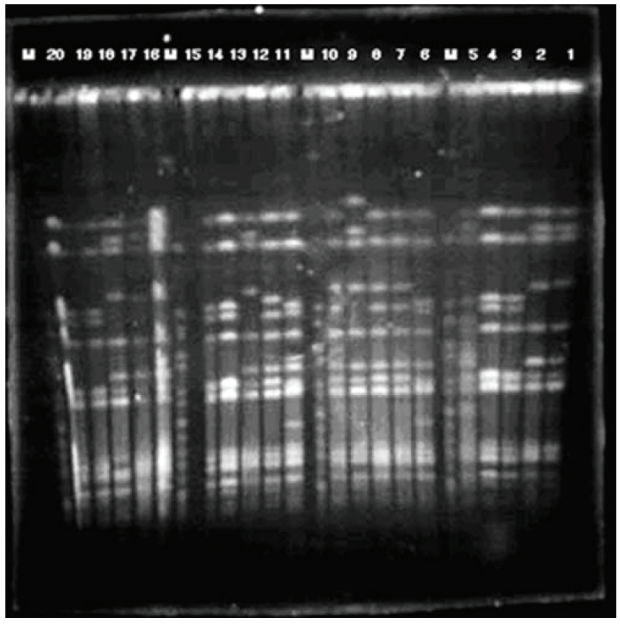

Figure 2. PFGE with *Xba*I enzyme digestion of some *S.* Typhimurium (*S.* ty) isolates (M: *S.* Braenderup H9812 marker, 1-20: *S.* ty 1 through *S.* ty 20).

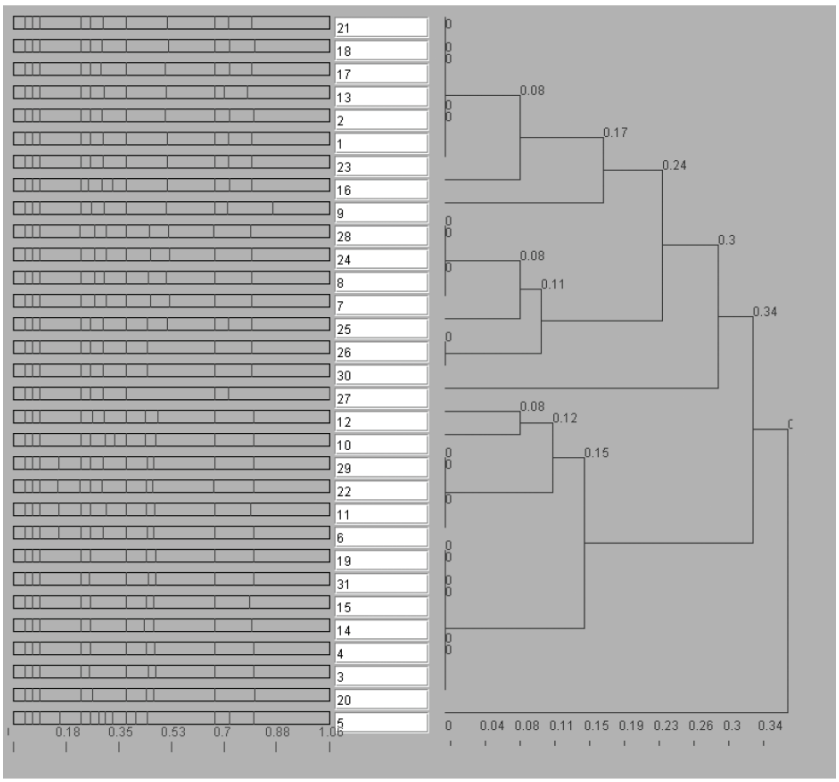

Figure 3. Patterns and phylogenetic tree of *S.* Typhimurium isolates. Numbers in the center indicate patterns of *S.* ty 1 through *S.* ty 31; unit of measurement = Mb.

In addition, the strain of *S.* Typhimurium identified as ATCC 14028 showed a unique pulsotype in PFGE, the most prevalent RAPD type, and an R-type similar to another isolate (related to ponies). Overall, it presented a specific, combined pattern (Table 1, Figure 3). For this reason, the comparison of clinical isolates with the standard strain did not provide any new information and was not repeated in the other serovars.

S. Enteritidis

In this serovar, 15 distinct patterns were identified among 30 isolates. Of the 7-13 bands of pulsotypes, 6 were common to all of the profiles: 110, 180, 250, 300, 330, and 670 kb. A majority of 64% of isolates belonged to 4 pulsotypes (A, B, C, and D), with the largest group (40% of the total) made up of pulsotype A. This pulsotype was identified in 12 isolates and, with the exception of a single isolate, was found to be specific to poultry. Pulsotype B, with 3 isolates, was specific for other animals, and the C and D pulsotypes, each with 2 isolates, were shown to be specific to poultry (Table 2, Figure 5).

S. Abortusovis

In *S. Abortusovis*, 20 distinct pulsotypes were identified among the 30 isolates. Of the 8-15 bands of pulsotypes, only 2 were common to all of the profiles: 70 and 100 kb. Except for 12 isolates (40% of total) representing the A and B pulsotypes, other isolates revealed nonidentical PFGE patterns in *Xba*I enzyme digestion.

In addition, all 9 isolates of the A pulsotype were isolated from different places and cities of 1 province at same time (Chaharmahal and Bakhtiari Province) (Table 3, Figure 6).

Correlation between pulsotypes and serotypes

Because the sizes of common bands of pulsotypes of serovars were clearly different from each other, the pulsotypes identified as specific for serotypes and the correlation between pulsotypes and serotypes were completely identified in this study. The reproducibility of the PFGE was confirmed by comparing the fingerprint patterns obtained from duplicate runs of strains.

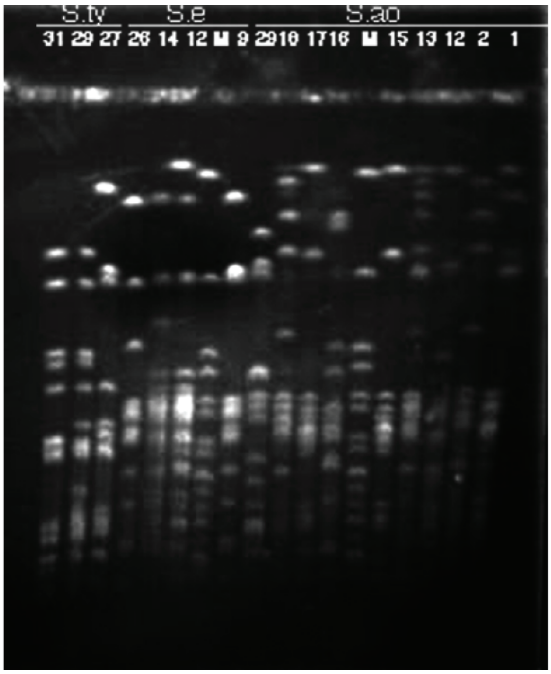

Figure 4. PFGE with *Xba*I enzyme digestion of some isolates of this study in retesting the 3 serovars (*S.* ty = *S.* Typhimurium; *S.* e = *S.* Enteritidis; *S.* ao = *S.* Abortusovis; M: *S.* Braenderup H9812 marker).

Data analysis

In total, 29, 23, and 21 compound profiles were identified in *S.* Typhimurium, *S.* Enteritidis, and *S.* Abortusovis, respectively, by a combination of profiles of PFGE with *Xba*I, RAPD-PCR with 2 primers (P1254 and 23L), and R-typing (Tables 1-3).

In each serovar, the discriminatory power of each method was calculated with Simpson's diversity index; calculations were performed on each method separately and in combination with the others. For the combination of methods, the final value of the discrimination index obtained was more than 0/95 in each serovar. Results are shown in Table 4.

Discussion

In recent years, phenotypic typing methods such as biotyping, serotyping, phage typing, and antibiotic resistance testing have been found to lack discriminatory power due to the expanded diversity of isolates. This may be a result of selective pressures, such as widespread illegal and irregular antibiotic

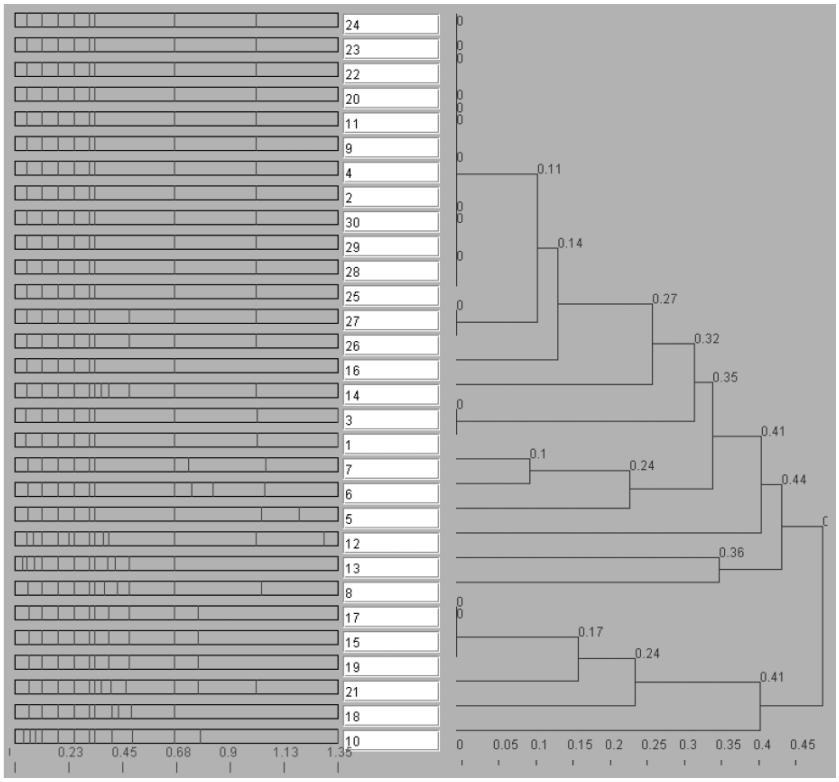

Figure 5. Patterns and phylogenetic tree of *S.* Enteritidis (*S.* e) isolates. Numbers in the center indicate patterns of *S.* e 1 through *S.* e 31; unit of measurement = Mb.

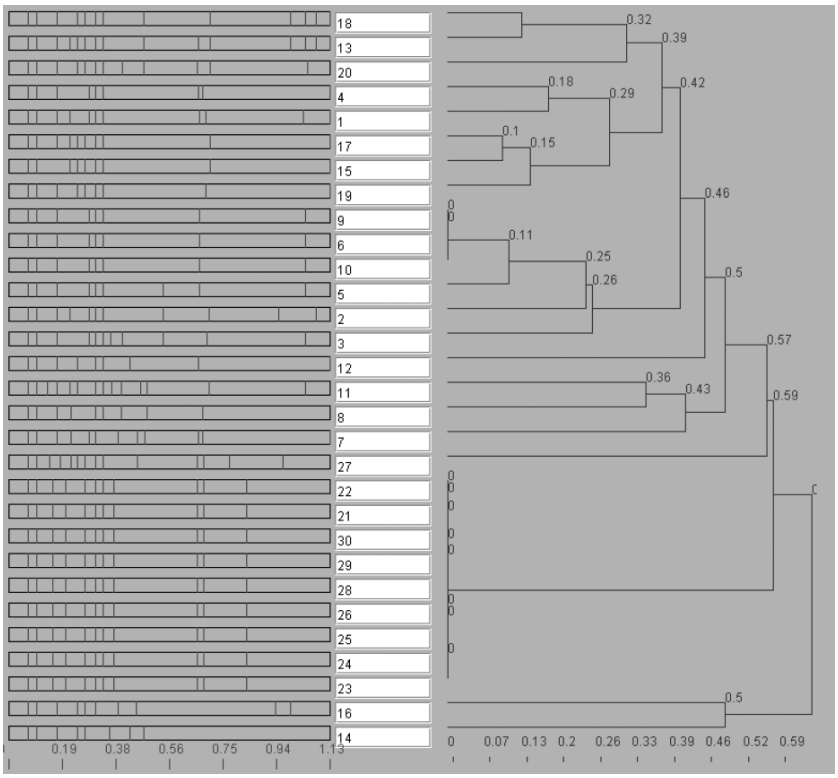

Figure 6. Patterns and phylogenetic tree of *S.* Abortusovis (*S.* ao) isolates. Numbers in the center indicate patterns of *S.* ao 1 through *S.* ao 31; unit of measurement = Mb.

Table 4. The discrimination index of every method in each serovar using Simpson's index (*S.* ty = *S.* Typhimurium; *S.* e = *S.* Enteritidis; *S.* ao = *S.* Abortusovis).

Methods / Serovars	R-type	RAPD-PCR (P1254)	RAPD-PCR (23L)	PFGE (*Xba*I)	RAPD-PCR (P1254+23L)	RAPD-PCR (P1254+23L) + R-type	All methods
***S.* ty**	**0/89**	**0/45**	**0/60**	**0/88**	**0/79**	**0/96**	**0/995**
***S.* e**	**0/54**	**0/54**	**0/68**	**0/83**	**0/83**	**0/92**	**0/96**
***S.* a.o**	**0/78**	**0/84**	**0/86**	**0/91**	**0/90**	**0/91**	**0/91** **0/995**[a]

[a] With the exception of 9 isolates of a probable epidemic.

consumption, crowded husbandry systems, and expanded transportation of humans and animals. For this reason, molecular typing methods including RAPD-PCR, IS200 typing, and protein profiles have been used for the differentiation of isolates in the serovars of *Salmonella* (3,7,9,18,25). In parallel with global surveillance systems of *Salmonella*, the present study combined PFGE, the "gold standard" typing method for *Salmonella* as approved by the CDC, with the PulseNet protocol for RAPD-PCR, a highly sensitive molecular typing method. As a phenotypic approach, antibiotic susceptibility tests were selected and the combination of these techniques was evaluated. Our aim was to increase the discrimination index of isolates in precise and important epidemiologic studies in endemic regions without any active surveillance system, like Iran, and to investigate the possible clonality of each important serovar.

The finding of virulence genes *inv*A and *spv*C in all of the *S.* Typhimurium and *S.* Enteritidis isolates of this study, respectively, and the isolation of all *S.* Abortusovis isolates from abortion samples increases the value of our research because these strains have virulence properties and clinical importance in *Salmonella*.

In *S.* Typhimurium, results showed the spread of this serovar in other animals and poultry, but its specificity to other animals seems to include more than poultry. From samples collected in 1976, 5 isolates showed 1 of the 5 dominant pulsotypes, indicating that these profiles might have been in existence for more than 30 years. The 13 antibiotic resistance profiles in this serovar may be due to the irregular and wide-ranging consumption of antibiotics in different regions and times; it could also be attributed to the illegal transporting of animals and food from neighboring countries without optimum surveillance. Resistance is usually common in serovars such as *S.* Typhimurium that are associated with bovine animals, because of the concentration of resistance genes in phage types associated with bovine animals, but it is relatively uncommon in serovars associated with poultry, such as *S.* Enteritidis (26). The antibiotic resistance test can therefore be considered a powerful phenotypic method for the differentiation of *S.* Typhimurium isolates in contaminated endemic regions. The sensitivity of all isolates of *S.* Typhimurium to a new type of antibiotic (enrofloxacin) showed the importance of establishing legal protocols to monitor the consumption of this antibiotic. Finally, *S.* Typhimurium is probably monophyletic, since it has relatively few pulsotypes (12 pulsotypes), few differences in band numbers between pulsotypes (10-12 bands), and a relatively large number of common bands (7 bands) in the pulsotypes of this serovar.

In *S.* Enteritidis, results showed specificity of this serovar to poultry rather than to other animals. Pulsotype A, with 12 isolates (40% of all isolates), was shown to be the dominant pulsotype in our isolates. This may be attributed to the selective environmental pressures on this clone in different regions (26). The fact that only 6 profiles of antibiotic resistance were found and the sensitivity of 20 isolates (67%) to all of the antibiotics tested indicated that the overall level of antimicrobial resistance in *S.* Enteritidis is lower than that of *S.* Typhimurium and further implied that multiple-drug resistance is rare in isolates from other animals. Recently, an alarming increase in multidrug-resistant *S.* Enteritidis strains has been reported in many countries (27-29). The present study shows that the sensitivity rate (67%) of *S.* Enteritidis isolates to all antibiotics is high, and it is probably due either to the relatedness of the isolates to at least 5 years ago when selective antibiotic pressures making multidrug resistant strains were lower than today, or to lower overall selective antibiotic pressures on *S.* Enteritidis in Iran. Finally, *S.* Enteritidis appears to be polyphyletic due to the relatively large number of pulsotypes (15) and the great difference of band numbers between pulsotypes (7-13 bands) in the short space of 10 years.

The results of our examination of *S.* Abortusovis were unexpected. The relatively large number of 20 pulsotypes, the great difference in band numbers between pulsotypes (8-15 bands), and the relatively small number of common bands between pulsotypes (2 bands) all point to the polyphyletic identity of this serovar. In the absence of any documentation on this subject, this may be the first report of this finding for the world. The following hypotheses may offer a possible explanation for this polymorphism: large variations in the reservoirs of this serovar in animals

and the environment; lower selective pressure, such as lower antibiotic consumption, in nomadic types of sheep and goat husbandry; or a probable high rate of gene transfer (plasmid, phage, etc.). These hypotheses require further study.

From Chaharmahal and Bakhtiari Province, 9 out of 10 recent isolates showed the same pulsotype, RAPD type, and R-type (resistance type) (Table 3, Figures 1 and 6). According to Tenover et al. (23), there was an epidemic and an identical clone in that place and year. This highlights the specificity, reproducibility, and identical diagnostic ability of these 3 methods, together with the accuracy of the tests performed in this study. Ultimately, it seems likely that the lower consumption of antimicrobials in nomadic sheep and goat husbandry systems was to some degree responsible for the sensitivity of 13 of the tested isolates to all antibiotics.

Lower genetic distances of *S.* Typhimurium isolates in comparison with *S.* Enteritidis and *S.* Abortusovis in their phylogenetic trees confirm the close relationship and monophyletic identity of serovar Typhimurium in comparison with Enteritidis and Abortusovis, which seem to be polyphyletic (Figures 3, 5, and 6).

Differences between RAPD types or R-types of isolates that have the same pulsotype in this study may be due to the high sensitivity of RAPD-PCR to very small mutations or differences. Another factor may be the plasmid and phage transfer of antibiotic resistance, which does not cause remarkable differences in pulsotypes because PFGE is not sensitive to differences at up to 50-100 kb (30,31). It is therefore possible that these isolates are related to the same clone, as indicated by the fact that each isolate was limited to 1 epidemic and 1 year; they may have acquired small differences over the years.

Data were analyzed by visual inspection and by using the Zhen Negar software examination of pulsotypes, separately. Zhen Negar confirmed all of the results obtained by visual examination.

In the PFGE of each serovar, a discriminatory index value greater than 0/80 was obtained. This figure increased to more than 0/95 in the evaluation of the combination of the 4 methods. In comparison to the results of other studies, such as that of Fernandez et al. (25) or Nikbakht et al. (18), the latter of which yielded a DI of 0/52 using the IS200 typing method on *S.* Abortusovis, the method presented here is optimum and shows the high discriminatory power of the combination of PFGE (with CDC protocol for PulseNet), RAPD-PCR (with the protocol of this research), and the antibiotic resistance test, which is applicable both in surveillance systems and in endemic regions in which there is no active surveillance system. If this method cannot differentiate isolates, RAPD-PCR and PFGE with additional primers and enzymes are recommended.

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