

Original Article

Molecular Investigation of *Salmonella* Choleraesuis and *Salmonella* Hadar Strains Isolated from Humans in Turkey

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SUMMARY: Eleven *Salmonella* Choleraesuis and seven *Salmonella* Hadar strains isolated from various clinical human samples were investigated by plasmid profile analysis, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and pulsed-field gel electrophoresis (PFGE) in order to obtain information at a molecular level on the epidemiology of *S. Choleraesuis* and *S. Hadar*, which are significantly present in Turkey. Plasmid profile analysis showed that 10 (90.9%) of 11 *S. Choleraesuis* isolates harbored one to two plasmids with sizes of 2.0, 5.0 or 6.5 kb; and 5 (71.4%) of 7 *S. Hadar* isolates harbored one to three plasmids ranging from 2.5 to 70 kb. ERIC-PCR was performed using ERIC-2 primers; since isolates within each serotype showed similar band models, we concluded that ERIC-PCR is not suitable for differentiating isolates within the same serotype and for grouping into clusters. In PFGE using the *AvrII* enzyme, *S. Choleraesuis* isolates formed three clusters, and *S. Hadar* isolates formed three clusters; using the *XbaI* enzyme, *S. Choleraesuis* formed two clusters, and *S. Hadar* isolates formed four clusters. These results showed that plasmid profile analysis and PFGE are reliable and discriminative methods that would complement antibiograms, and could contribute to the investigation of outbreak epidemiology. This is the first report on *S. Choleraesuis* and *S. Hadar* isolates from Turkey investigated by plasmid profile analysis, ERIC-PCR and PFGE methods.

INTRODUCTION

Salmonellosis is still one of the most widespread foodborne bacterial illnesses in humans and can result in protean clinical manifestations, ranging from an asymptomatic state to very severe disease (1,2). Although the genus *Salmonella* comprises more than 2,000 serotypes, only a few are encountered frequently, and these few serotypes cover more than 70% of all isolates (3). Unfortunately, statistical, epidemiological and molecular data on *Salmonella* isolates in Turkey, where *Salmonella* infections constitute an important public health issue, are very limited (4). According to data obtained in Turkey, the most common serotypes isolated from clinical samples are *Salmonella* serotype Enteritidis and serotype Typhimurium, followed by *Salmonella* serotype Paratyphi B, *Salmonella* serotype Typhi, *Salmonella* serotype Choleraesuis and *Salmonella* serotype Hadar (5).

S. Choleraesuis and *S. Hadar* are rarely found in humans and are more common in extra-human hosts and the environment (6). Characteristically, *S. Choleraesuis*, a highly host-adapted pathogen, usually causes swine paratyphoid (7). This organism is notorious for its extreme invasiveness and pathogenic nature in humans, frequently causing septicemic disorders with scarce involvement of the gastrointestinal tract (primary bacteremia) (7). The incidence of *S. Choleraesuis* bacteremia has increased in the past 10 years, and this increase is associated with the upsurge of ciprofloxacin-resistant isolates (8). According to Jones et al., *S. Choleraesuis* produces a significantly higher proportion of invasive disease than *S.*

Typhimurium; cases of *S. Choleraesuis* infection were shown to be more likely to cause hospitalization than all other serotypes mentioned in their study (2). Meanwhile, *S. Hadar* causes 22.7% of the hospitalizations, 4.0% of the invasive disease, and 0.6% of the death cases, respectively (2). The increasing importance of *S. Choleraesuis* and *S. Hadar* infections has stimulated research efforts on their various aspects (7).

Serotyping was the standard for classification of *S. enterica* isolates in outbreak investigations prior to the development of molecular genotyping methods (9). More recently, several molecular methods which establish genetic relatedness among *Salmonella* strains and analyze bacterial DNA on plasmids or chromosomes have been developed and used successfully in epidemiological researches (10-12). Several authors have specifically recommended using pulsed-field gel electrophoresis (PFGE) rather than other methods for *Salmonella* genotyping (13-15).

The aim of this study was to evaluate the characteristics of *S. Choleraesuis* and *S. Hadar* isolated from humans in Turkey by plasmid profile analysis, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and PFGE.

MATERIALS AND METHODS

Eleven strains of *S. Choleraesuis* and seven strains of *S. Hadar* were selected from the culture collection of Enterobacteria Laboratory of Ankara University Medical School, Department of Microbiology and Clinical Microbiology. These strains were isolated, serotyped and tested for antimicrobial susceptibility using the agar dilution method in a previous study (16).

In the previous study (16), *S. Choleraesuis* strains were isolated from various clinical samples (blood [2], stool [9]) of sporadic cases in four Turkish provinces (Ankara [7], Bursa

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[1], Eskisehir [1], Kayseri [2]) by standard methods. Eight *S. Choleraesuis* strains were isolated from gastroenteritis patients, two *S. Choleraesuis* strains from septicemia patients and one *S. Choleraesuis* strain from a paratyphoid patient. All but two cases were male, with ages ranging from 1 to 55. *S. Hadar* strains were isolated from stool (7) samples of sporadic cases in three Turkish provinces (Ankara [3], Antalya [2], Edirne [2]) using standard procedures. *S. Hadar* strains were isolated from gastroenteritis patients (4 males, 3 females), whose ages ranged from 9 to 70.

Plasmid extraction and profile analysis: Plasmids extracted by the method of Kado and Liu (17) with modifications were separated by electrophoresis on horizontal agarose 0.7% (Serva, Heidelberg, Germany) gel containing 0.5 μ g ethidium bromide ml⁻¹ with 0.5 \times Tris-boric acid-EDTA (TBE) buffer at 100 V for 2 h. Plasmid sizes were determined by co-electrophoresis with plasmids of known sizes from *Escherichia coli* V517 (53.7, 7.2, 5.6, 5.1, 3.9, 3.0, 2.7, 2.1 kb), *S. Typhimurium* strain 020255-Ankara (90 kb) and *S. Enteritidis* strain 006956-Ankara (57, 5.8, 4.8 kb). Also, a gene ruler 1 kb DNA ladder (Fermentas Life Sciences, Vilnius, Lithuania) was used for determining plasmid sizes. DNA bands were visualized on an ultraviolet transilluminator (TFX-20M; Vilber Lourmat, Marna la Vallee, France). Control strains (*S. Typhimurium* 020255-Ankara and *S. Enteritidis* 006956-Ankara) were obtained from the Communicable Diseases Research Department, Refik Saydam National Hygiene Center, Ankara, Turkey.

ERIC-PCR: Chromosomal DNA was obtained using commercial DNA extraction kit (Nucleospin extraction kit; Macherey-Nagel, Düren, Germany) according to the manufacturer's guidelines. The PCR conditions were performed using the primer ERIC-2 (AAG TAA GTG ACT GGG GTG AGC G) as described previously (18) with minor modifications. PCR products were analyzed by electrophoresis through 1% agarose gels (Serva) containing 0.5 μ g/ml ethidium bromide with 0.5 \times TBE buffer, visualized on an ultraviolet transilluminator (TFX-20M) and then photographed. The Gene Ruler™ 100-bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA size marker.

PFGE: PFGE was performed according to WHO protocols (19). In this method, each isolate was digested with *AvrII* and *XbaI* macrorestriction enzymes (Fermentas) separately.

Briefly, all isolates were grown overnight at 37°C. Agar-

ose plugs were prepared with 1.3% low melting agarose. Genomic DNA was digested with 20 U of restriction endonucleases; *AvrII* and *XbaI* for 4 hours each according to the manufacturer's recommendations. PFGE was performed with 1.3% agarose gel using the CHEF DR II (Bio-Rad, Hercules, Calif., USA) system, at 6 V/cm, 14°C in 0.5 \times TBE for 18 h with switch times of 2.2-63.8. The gels were stained with ethidium bromide (0.2 μ g/ml) and photographed under UV light. PFGE patterns were analyzed both visually, as described previously by Tenover et al. (20), and with computer-assisted analysis using Gene Directory software (Syngene, Cambridge, UK). Similarity indexes were determined using Dice coefficients and the unweighted pair-group method with arithmetic average (UPGMA) method with 1% band tolerance.

RESULTS

Plasmid profile analysis: Plasmid DNAs of each *Salmonella* isolate were identified by their molecular weights in kilobase pairs (kb). Plasmid profile analysis showed that 10 (90.9%) of 11 *S. Choleraesuis* isolates harbored one to two plasmids with sizes of 2.0, 5.0 or 6.5 kb; all plasmid-harboring strains possessed the 5.0-kb plasmid (Table 1).

Five (71.4%) of seven *S. Hadar* isolates harbored one to three plasmids ranging from 2.5 to 70 kb. All plasmid-harboring strains possessed the 7.0-kb plasmid; two solely and three in combination with other plasmids (Table 2).

ERIC-PCR: *S. Choleraesuis* strains isolated using ERIC-2 primers produced 4-8 bands ranging from 100-1,100 bp. The band patterns were found inadequate for separating the isolates into different clusters.

S. Hadar strains isolated using ERIC-2 primers produced 7-10 bands ranging from 100-5,000 bp. These band patterns were also found inadequate for separating the isolates into different clusters. We concluded that genotyping procedures involving ERIC-PCR using ERIC-2 primers were unable to detect genetic differences among *S. Choleraesuis* and *S. Hadar* isolates from various clinical samples from sporadic human cases.

PFGE: Analysis of 11 *S. Choleraesuis* strains by PFGE after digestion with macrorestriction enzymes *AvrII* and *XbaI* generated 7-8 and 11-13 restriction fragments with molecular sizes ranging from 48.5 to 921 kb and from 48.5 to 727 kb, respectively. Dendrograms of PFGE patterns yielded after digestion with macrorestriction enzymes *AvrII* and *XbaI*

Table 1. PFGE patterns after restriction with *AvrII* and *XbaI* enzymes and plasmid profiles of *S. Choleraesuis* isolates

Strain no.	Isolation date (D/M/Y)	Province	Sample	Resistance ¹⁾ pattern	Plasmid profile (kb)	PFGE pattern ²⁾	
						<i>AvrII</i>	<i>XbaI</i>
1	23/07/2000	Ankara	Blood	C	5.0	AC3	XC2
2	10/08/2000	Bursa	Stool	C	5.0	AC3	XC1
3	02/09/2000	Eskisehir	Stool	C	5.0	AC2	XC1
4	07/02/2001	Ankara	Stool	S	5.0	AC3	XC1
5	08/02/2001	Ankara	Stool	S	6.5 5.0	AC3	XC1
6	10/03/2001	Ankara	Stool	S	5.0	AC1	XC1
7	08/01/2001	Ankara	Stool	S	-	AC1	XC1
8	28/04/2001	Ankara	Stool	S	5.0 2.0	AC1	XC1
9	07/08/2001	Kayseri	Blood	S	5.0	AC2	XC1
10	16/09/2001	Kayseri	Stool	S	5.0	AC1	XC2
11	29/08/2001	Ankara	Stool	A	5.0	AC1	XC2

¹⁾ S, susceptible to all antimicrobials; A, resistant to ampicillin; C, resistant to chloramphenicol.

²⁾ Clusters, AC3-XC2; AC3-XC1; AC2-XC1; AC1-XC1; AC1-XC2.

Table 2. PFGE patterns after restriction with *AvrII* and *XbaI* enzymes and plasmid profiles of *S. Hadar* isolates

Strain no.	Isolation date (D/M/Y)	Province	Sample	Resistance pattern ¹⁾	Plasmid profile (kb)	PFGE pattern ²⁾	
						<i>AvrII</i>	<i>XbaI</i>
12	23/01/2001	Ankara	Stool	A	–	AH1	XH3
13	21/02/2001	Ankara	Stool	AA/CCTT/S	7.0 2.5	AH1	XH1
14	14/08/2001	Antalya	Stool	AA/CT	70 7.0 2.5	AH1	XH1
15	21/07/2001	Antalya	Stool	AA/CT	7.0 2.5	AH1	XH1
16	12/07/2001	Edirne	Stool	AA/CT	7.0	AH1	XH2
17	07/08/2001	Edirne	Stool	AA/CT	7.0	AH2	XH2
18	07/11/2001	Ankara	Stool	AA/CCT	–	AH3	XH4

¹⁾ A, resistant to ampicillin; A/C, resistant to amoxicillin/clavulanic acid; C, resistant to chloramphenicol; T/S, resistant to trimethoprim/sulfamethoxazole; T, resistant to tetracycline.

²⁾ Clusters, AH1-XH3; AH1-XH1; AH1-XH2; AH2-XH2; AH3-XH4.

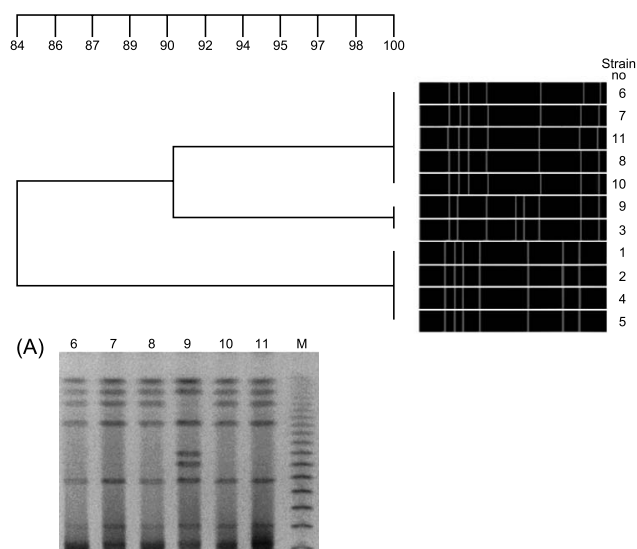


Fig. 1. Dendrogram of PFGE *AvrII* band patterns of *S. Choleraesuis* isolates generated by Gene Directory software using the UPGMA method. (A) Examples of representative PFGE fingerprint patterns of *S. Choleraesuis* strains after restriction with *AvrII* enzymes. 6-11, strain no.; M: lambda ladder PFGE marker (N0340S, New England Biolabs, Hertfordshire, UK. Band size 48.5-1018.5 kb).

using Dice coefficients and UPGMA method with a 1.0% tolerance limit are shown in Figs. 1 and 2, respectively.

Examples of representative PFGE fingerprint patterns of *S. Choleraesuis* strains after restriction with *AvrII* and *XbaI* enzymes are shown in Figs. 1A and 2A, respectively.

S. Choleraesuis strains after digestion with macrorestriction enzyme *AvrII* generated three different PFGE clusters (AC1, AC2 and AC3) consisting of 7-8 restriction fragments. Five isolates with genetic similarity showed PFGE pattern AC1; two isolates belonged to PFGE pattern AC2; and four isolates that were 100% similar displayed PFGE pattern AC3. The genetic similarity between clusters AC1 and AC2 was 91%, and the genetic similarity between these two clusters and AC3 was 84% (Fig. 1).

S. Choleraesuis strains after digestion with macrorestriction enzyme *XbaI* generated two different PFGE patterns (XC1 and XC2) consisting of 11-13 restriction fragments. Eight isolates with genetic similarity showed PFGE pattern XC1, and three isolates that were 100% similar belonged to PFGE pattern XC2. The genetic similarity between these two clusters was 91% (Fig. 2).

Analysis of seven *S. Hadar* strains by PFGE after digestion with macrorestriction enzymes *AvrII* and *XbaI* generated 7-

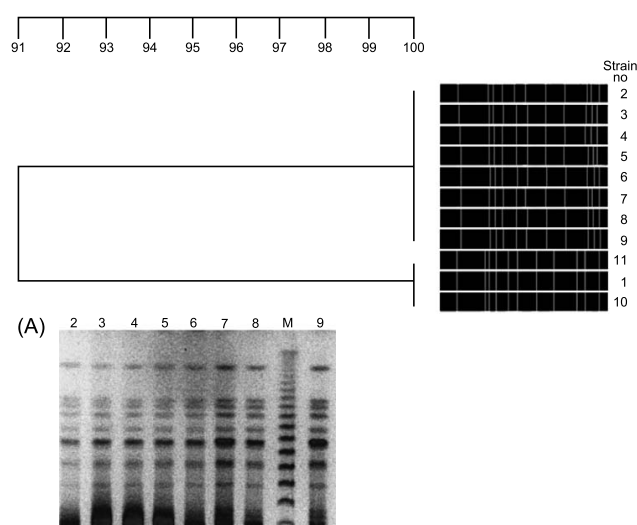


Fig. 2. Dendrogram of PFGE *XbaI* band patterns of *S. Choleraesuis* isolates generated by Gene Directory software using the UPGMA method. (A) Examples of representative PFGE fingerprint patterns of *S. Choleraesuis* strains after restriction with *XbaI* enzymes. 2-9, strain no.; M, lambda ladder PFGE marker (N0340S. Band size 48.5-1018.5 kb).

8 and 11-13 restriction fragments with molecular sizes ranging from 48.5 to 1,018 kb and 48.5 to 873 kb, respectively. Dendrograms of PFGE patterns yielded after digestion with macrorestriction enzymes *AvrII* and *XbaI* using Dice coefficients and UPGMA method with a 1.0% tolerance limit are shown in Figs. 3 and 4, respectively. PFGE fingerprint patterns of *S. Hadar* strains after restriction with *AvrII* and *XbaI* enzymes are shown in Figs. 3A and 4A, respectively.

S. Hadar strains after digestion with macrorestriction enzyme *AvrII* generated three different PFGE clusters (AH1, AH2 and AH3) consisting of 7-10 restriction fragments. Five isolates with PFGE pattern AH1 showed 100% genetic similarity, and two independent strains that produced two different PFGE patterns (AH2 and AH3) showed only 60% genetic similarity with PFGE pattern AH1 (Fig. 3).

S. Hadar strains after digestion with macrorestriction enzyme *XbaI* produced four different PFGE patterns (XH1, XH2, XH3 and XH4) consisting of 10-13 restriction fragments. Three isolates showing PFGE pattern XH1 and two isolates showing PFGE pattern XH2 constituted a major cluster demonstrating 86% genetic similarity; there was 53% genetic similarity between this major cluster and the two isolates which produced PFGE patterns XH3 and XH4, respectively (Fig. 4).

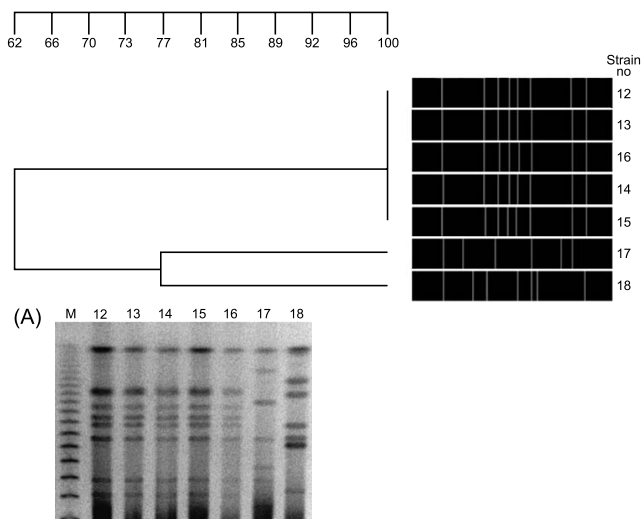


Fig. 3. Dendrogram of PFGE *AvrII* band patterns of *S. Hadar* isolates generated by Gene Directory software using the UPGMA method. (A) PFGE fingerprint patterns of *S. Hadar* strains after restriction with *AvrII* enzymes. 12-18, strain no.; M, lambda ladder PFGE marker (N0340S. Band size 48.5-1018.5 kb).

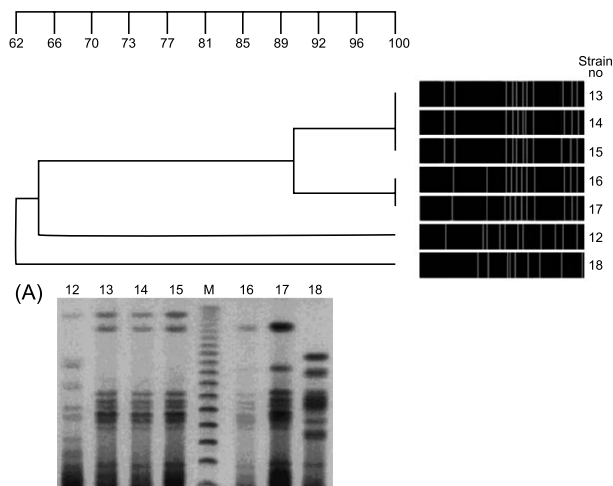


Fig. 4. Dendrogram of PFGE *XbaI* band patterns *S. Hadar* isolates generated by Gene Directory software using the UPGMA method. (A) PFGE fingerprint patterns of *S. Hadar* strains after restriction with *XbaI* enzymes. 12-18, strain no.; M, lambda ladder PFGE marker (N0340S. Band size 48.5-1018.5 kb).

DISCUSSION

In Turkey, *S. Choleraesuis* and *S. Hadar* are the prevalent serotypes of group C salmonellae in humans (16). Few epidemiological typing methods have been established to identify the sources of the bacteria and the transmission chains. The strains are usually defined phenotypically by serotyping and antimicrobial resistance tests in microbiology laboratories of Turkey. Since phenotyping data suggest the clonal diffusion of a limited number of local strains, there is a need for practical techniques to discriminate *Salmonella* isolates and to evaluate the association of *Salmonella* strains in epidemiological studies (21).

Here, we present the results of a molecular study of *S. Choleraesuis* and *S. Hadar* isolates in Turkey conducted to investigate the genotypic characterization of strains circulating since the year 2000. Plasmid profile analysis, ERIC-PCR and PFGE techniques were used as molecular subtyping

methods for sporadic human isolates.

Plasmid profile analysis is based on the numbers and molecular weight of plasmids after extraction of plasmid DNA. Several *Salmonella* epidemics have been elucidated with plasmid profile analysis (22,23), but this method is limited to strains possessing plasmids (24). Plasmid profiles are not very useful for subtyping *Salmonella* serotypes when strains do not carry plasmids, when they possess only serotype-specific plasmids, or when only a few strains harbor plasmids (3,22).

In this study plasmid analysis of *S. Choleraesuis* revealed that 10 (90.9%) of 11 *S. Choleraesuis* isolates carried one to two plasmids with sizes of 2.0, 5.0 or 6.5 kb; five (71.4%) of seven *S. Hadar* isolates harbored one to three plasmids ranging from 2.5 to 70 kb.

Plasmids of *S. Choleraesuis* strains yielded three distinct plasmid profiles. Strains revealing different antibiotic resistance patterns carried similar plasmid profiles, and strains showing similar antibiotic resistance patterns possessed different plasmids. *S. Hadar* strains showed three different plasmid profiles, and strains revealing similar antibiotic resistance patterns carried different plasmid profiles. These data suggest that there is not a close relation between antibiotic resistance and plasmids. Even though 15 out of 18 *Salmonella* isolates analyzed carried plasmids (83.3%), strains which revealed similar antibiotic resistance patterns carried different plasmid profiles, indicating that plasmid profile analysis is only useful for typing of *S. Choleraesuis* and *S. Hadar* if the results are combined with antibiotic resistance patterns.

PCR-based typing methods were performed to determine the diversity among bacteria. ERIC-PCR and repetitive element (REP)-PCR, using BOX, ERIC and REP primers, were performed to determine the molecular nature of the isolates; since these methods use stable primers they are considered to be reproducible genotyping methods for the genetic typing of *S. enterica* (25).

Although it has been claimed that a combination of REP-PCR with the ERIC primer pair and phage typing was useful in discriminating between the epidemic isolates and epidemiologically unrelated isolates in some *Salmonella* serotypes (26), in the present study we performed ERIC-PCR using ERIC-2 primers and obtained similar band patterns for all *S. Choleraesuis* and *S. Hadar* strains isolated from sporadic cases in Turkey; hence, this method proved unsuitable to determine the diversity among the isolates of these serotypes.

PFGE has provided molecular fingerprints in *Salmonella* genotyping. Use of PFGE with endonuclease *XbaI* has been widely recognized as a sensitive means of fingerprinting *Salmonella* serotypes (27). Murase et al. also demonstrated that macrorestriction analysis with two enzymes was a more discriminatory method for subtyping *Salmonella* (13).

In our study we analyzed a total of 18 isolates of *S. Choleraesuis* and *S. Hadar* obtained from various clinical samples of sporadic cases with PFGE using *AvrII* and *XbaI* macrorestriction enzymes. *S. Choleraesuis* strains after digestion with macrorestriction enzyme *AvrII* generated three different PFGE patterns, and *S. Hadar* strains also generated three different PFGE patterns; *S. Choleraesuis* strains after digestion with macrorestriction enzyme *XbaI* generated two different PFGE patterns, while *S. Hadar* strains generated four different PFGE patterns. Using both enzymes, *S. Choleraesuis* strains and *S. Hadar* strains divided into five distinct PFGE cluster profiles each (e.g., AC3-XC2 and AH1-XH3) as shown in Tables 1 and 2.

In the present study, 15 (83.3%) of 18 *Salmonella* isolates

carried plasmids, and all plasmid-carrying strains gave six distinct plasmid profiles; the PFGE of *AvrII*- and *XbaI*-digested chromosomal DNA revealed five distinct cluster profiles each for *S. Choleraesuis* and *S. Hadar* isolates. *Salmonella* serotypes showing similar resistance profiles could be distinguished easily by plasmid profiling and PFGE after digestion with two macrorestriction enzymes (Tables 1 and 2). The three strains (no. 6-no. 8) of *S. Choleraesuis* from cluster AC1-XC1 were sensitive to antimicrobial agents and possessed various plasmid profiles (without plasmid; 5.0, and 5.0, 2.0). *S. Choleraesuis* strains (no. 4 and no. 5) belonging to cluster AC3-XC1 diverged, with distinct antibiotic resistance patterns and plasmid profiles (Table 1). These *S. Choleraesuis* strains (no. 4-no. 8) were isolated from stool cultures of gastroenteritis patients from various hospitals in different districts of Ankara. Although these five strains seemed to be genetically related according to PFGE using the *XbaI* enzyme (clusters AC1-XC1 and AC3-XC1), the patients from whom the strains were obtained were sporadic gastroenteritis cases; there was absolutely no epidemiological relation among them.

Three strains (no. 13-no. 15) of *S. Hadar* from cluster AH1-XH1 were different in terms of their antibiotic resistance patterns and plasmid profiles (Table 2). These three *S. Hadar* strains were obtained from sporadic gastroenteritis cases in Ankara and Antalya. There was no indication of an epidemiological relation among the cases.

This study has demonstrated that PFGE using *AvrII* and *XbaI* combined with resistance patterns and plasmid profiles could constitute a useful tool allowing detection of genetic relationships between isolates with different resistance patterns and also differentiation of isolates with the same resistance phenotype.

To conclude, analysis of small numbers of isolates using PFGE with *AvrII* and *XbaI* is a reliable discriminative method complementing antibiogram and plasmid profiles and may contribute to investigations into the *S. Choleraesuis* and *S. Hadar* strains, which cause a significant number of human infections in Turkey.

The study presented here is the first report on *S. Choleraesuis* and *S. Hadar* isolates from Turkey investigated by plasmid profile analysis, ERIC-PCR and PFGE methods.

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