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Original article

Evaluation of molecular typing methods for *Salmonella enterica* serovar Typhimurium DT104 isolated in Germany from healthy pigs

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Abstract – The discriminatory power of four different DNA based typing methods was tested for the molecular subtyping of *Salmonella* Typhimurium phage type DT104 isolates. German DT104 strains (n = 133) originating from slaughter pigs were analysed by plasmid profiling, and 32 of them by pulsed-field gel electrophoresis (PFGE) using the restriction enzymes *Xba*I, *Spe*I or *Bln*I, random amplification of polymorphic DNA (RAPD) using 13 different primers and IS*200* typing. A resulting subtyping scheme was obtained which is based on the most discriminatory power of the individual methods i.e. plasmid profiling and PFGE with all three enzymes. The index of discrimination obtained by the subtyping scheme was 0.909 closely approaching the maximum value of one. Although minor differences occurred in the molecular DNA pattern of single DT104 strains, a dominating subtyping pattern was observed confirming other studies which showed, that *S.* Typhimurium DT104 isolates are highly clonal.

RAPD / IS*200* / PFGE / plasmid profiling / *Salmonella* Typhimurium DT104

Résumé – Évaluation de plusieurs méthodes de typage moléculaire de *Salmonella enterica* serovar Typhimurium DT104 isolées chez des porcs sains en Allemagne. Le pouvoir discriminant de quatre méthodes différentes basées sur le typage de l'ADN a été testé pour le sous-typage d'isolats de *Salmonella* Typhimurium DT104. Cent trente trois souches allemandes de DT104 provenant de porcs à l'abattoir ont été analysées par la méthode de profilage de plasmides, et 32 d'entre elles par électrophorèse sur gel en champ pulsé (PFGE) utilisant les enzymes de restriction *Xba*I, *Spe*I ou *Bln*I, par amplification aléatoire d'ADN polymorphe (RAPD) utilisant 13 amorces différentes, et par typage avec IS*200*. Un protocole de sous-typage, basé sur le plus fort pouvoir discriminant des méthodes individuelles, a été obtenu. Il s'agit du profilage de plasmides associé à l'électrophorèse sur gel en champ pulsé utilisant les trois enzymes de restriction. L'index de discrimination obtenu avec

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ce protocole de sous-typage était de 0,909, proche de la valeur maximale de 1. Bien que des différences mineures soient apparues dans les profils moléculaires de l'ADN des souches DT104, un profil dominant de sous-type a été observé, confirmant les résultats d'autres études ayant montré que les isolats de *Salmonella* Typhimurium étaient fortement clonaux.

amplification aléatoire d'ADN polymorphe / IS*200* / profilage de plasmide / *Salmonella* Typhimurium DT104

1. INTRODUCTION

Traditional typing methods for salmonella isolates rely on serotyping which is based on the chemical structure of the lipopolysaccharide and flagelli [24]. For subtyping within a serotype, phage typing, based on the bacterial lysis due to specific typing phages, is used [3]. For many analyses, however, these methods are not discriminative enough and modern molecular DNA-based methods had to be introduced for typing of frequently encountered serotypes. Such techniques include plasmid profiling, ribotyping, biotyping, pulsed-field gel electrophoresis (PFGE), IS*200* profiling and randomly amplified polymorphic DNA (RAPD). The high discriminatory power of DNA typing allows to differentiate related from unrelated strains isolated in a short period of time, especially strains isolated from an outbreak (for review see reference [23]).

Since 1990 increasing incidence of infections in both humans and animals caused by multiresistant *S*. Typhimurium phage type DT104 were reported in many European countries [1, 26, 29] and in the USA [8]. Molecular studies have demonstrated that the resistance to β–lactams, aminoglycosides and sulfonamides are chromosomally encoded by two different integrons [9, 27]. Resistance to tetracyclines and phenicols are in contrast located between the integrons [4]. DT104 initially emerged in cattle in 1988 in England and Wales [29]. Subsequently, the clone has been isolated from a large variety of livestock and other animals and can cause human infections via the food chain or direct animal contact [11, 21].

Molecular typing analyses indicated that DT104 isolates originating from different hosts and geographic origins are highly clonal [7, 15, 26, 30]. The determination of plasmid profiles in DT104 isolates revealed that the majority of strains possess a single 60 MDa plasmid and in some strains smaller plasmids between 5 and 1 MDa as well [26, 30]. A study in nine Danish pig herds showed, that 93 DT104 strains except one shared an identical *Xba*I-PFGE pattern [6] and *Xba*I-PFGE, IS*200* profiling with *Pst*I and ribotyping with *Pvu*II from European DT104 isolates resulted in one common clonal type [26]. A combination of randomly amplified polymorphic DNA (RAPD) using two different 10mer primers and subsequent restriction analysis indicated also a close relationship between DT104 strains [15].

Therefore, the aim of this study was to characterize the clonality of DT104 isolates originating from feces, lymphnodes and surface swabs of slaughter pig sampled in different abattoirs in Germany [18]. Possible molecular differences between single DT104 isolates will be detected by the application of several DNA based typing methods. Plasmid profiling, PFGE using different restriction enzymes, RAPD using 13 different primers and IS*200* profiling were tested for their value to discriminate between single DT104 isolates. Although the discriminative index was close to one, the study demonstrated the wide prevalence of a predominating *S.* Typhimurium DT104 clone in slaughter pigs in Germany.

2. MATERIALS AND METHODS

2.1. Bacterial strains

S. Typhimurium DT104 isolates (*n* = 403) were obtained from seven abattoirs distributed all over Germany on ten weekly sampling occasions in February through June 1996 from surface swabs, fecal or lymph node material of freshly slaughtered pigs [18].

Serotyping was performed according to the Kauffmann White scheme [24] and phage typing was performed using the Anderson scheme as applied in the public health laboratory service in Colindale [3].

2.2. Experimental procedure

The aim of the study was to evaluate established, standard phenotypic and genotypic techniques for their discriminative power to subtype *S.* Typhimurium DT104 isolates originating from slaughter pig from seven different abattoirs obtained in a study on the prevalence of *Salmonella* in German pigs [18]. Initially all 403 isolates were tested for their susceptibility against antimicrobial agents. Among those, 133 isolates belonging to representative resistance phenotypes obtained in all abattoirs and originating from fecal, swab and lymphnode material were further differentiated by plasmid profiling. Finally 32 strains representing the various plasmid profiles were further analyzed by more elaborated molecular techniques like pulsed-field gel electrophoresis (PFGE), IS*200* typing and randomly amplified polymorphic DNA-PCR (RAPD-PCR).

2.3. Antimicrobial susceptibility test

All DT104 isolates were tested for their susceptibility to 16 different antimicrobial agents by agar diffusion tests in accordance with the guidelines of the German Institute for Standards (DIN 5894 part 3) [12] with antibiotic disks (Oxoid Ltd., London, England). The antimicrobial agents used were: amikamycin (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), cefuroxim (30 μg), colistinsulphate (10 μg), enrofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (10 μg), polymyxin B (300 I.E.), streptomycin (25 μg), sulphamethoxazole-trimethoprim (25 μg), sulphonamides (300 μg) and tetracycline (30 μg).

2.4. Molecular typing techniques

2.4.1. Plasmid profile typing

Plasmid DNA was isolated by the alkaline denaturation method described previously [17] with minor modifications. Electrophoresis of plasmid DNA was performed in 1× TBE at 100 V for 2.5 h on 0.8% agarose gels, afterwards stained with ethidium bromide and photographed. The molecular weight standards were a supercoiled DNA ladder (Gibco BRL, Karlsruhe, Germany) and the plasmids R27 (112 MDa), R1 (62 MDa), RP4 (36 MDa), and ColE1 (4.2 MDa). Molecular sizes from plasmids were calculated as previously described [13].

2.4.2. Randomly amplified polymorphic DNA (RAPD)

It is well known, that RAPD patterns depend on many parameters like $MgCl_2$ and KCl concentrations, template concentration and the thermal cycling protocol [14]. We got the most reliable and well-separated fragments with a two step thermal cycling protocol including 20 °C and 30 °C annealing temperature as well as a 3 mM $MgCl_2$ and 50 mM KCl concentration for each reaction. The inclusion of 1 $\mu g \cdot \mu L^{-1}$ BSA in the RAPD reaction strengthened weaker bands but did not influence the overall pattern.

Thirteen 10-mer primers were chosen for typing *S*. Typhimurium DT104 strains.

Twelve primers were previously described: 23L, OPB17, OPA4, OPB6 and OPB15 [19], OPG08, OPH13, OPG04, OPG10 and OPH04 [20], 1283 and 1254 [2]. Primer OPB15/B was designed on the basis of the sequence of OPB15 containing at the 5' and 3' end a variable nucleotide A, C, G or T. The genomic DNA as template for RAPD-PCR was prepared as follows: the cells of 1 mL overnight broth culture were collected by centrifugation (15 000 × *g*, 10 min), resuspended in 300 μL of sterile distilled water and boiled (10 min) in a water bath. After centrifugation (15 000 × *g*, 10 min) 5 μL of supernatant containing DNA was used as a template in the RAPD-PCR reaction. Amplification reactions were performed in volumes of 25 μL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.001% gelatin, 200 μM (each) dATP, dCTP, dGTP and dTTP, 25 pmol primer, 25 μg BSA, 50 ng genomic DNA and 1.5 units Taq DNA polymerase (Perkin Elmer Biosystems, Weiterstadt, Germany). A Perkin Elmer GenAmp 2400 thermal cycler was used for amplification. The cycling program was 1 min at 94 °C, six cycles at 94 °C for 30 s, 20 °C for 30 s, 72 °C for 1 min and 35 cycles at 94 °C for 30 s, 30 °C for 30 s, 72 °C for 1 min. Finally an incubation at 72 °C for 4 min was performed. After PCR 5 μL amplification products were analyzed by electrophoresis on 2% agarose gels (Gibco BRL) and detected by staining with ethidium bromide. Faint amplicons occurring in RAPD patterns were excluded from the analysis.

2.4.3. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis after digestion of genomic DNA with *Spe*I, *Xba*I and *Bln*I from *Salmonella* strains resulted in suitable numbers of well-separated fragments [25, 28, 31]. These restriction enzymes were therefore chosen for further subtyping of the DT104 isolates.

Cells grown on Luria-Bertani agar plates for 16 h were suspended in phosphate buffered saline (10 mL, 5×10^8 cells·mL^{-1}), centrifuged and resuspended in 1 mL of 1% agarose (ImBed LMP agarose, New England Biolabs, Schwalbach, Germany). 0.1 mL plugs were chilled in plastic moulds (BioRad, München, Germany) for 20 min. The plugs were incubated overnight at 37 °C in 50 mL lysis buffer (1 μg·mL^{-1} RNAse, 1% Sarkosyl, 6 mM Tris-HCl, 100 mM EDTA, pH 8.0), and overnight at 50° C in 2.5 mL of ESP solution (1% Sarkosyl, 1 mg·mL^{-1} proteinase K, 500 mM EDTA, pH 8.0). The plugs were washed four times in 50 mL of TE buffer pH 8.0 at 37 °C for 30 min and could be stored at 4 °C up to 3 months. Quartered plugs were equilibrated with 0.3 mL of digestion buffer for 15 min and after replacing the buffer incubated overnight at 37 °C with 10 units of *Xba*I (New England Biolabs), 5 units of *Spe*I (New England Biolabs) or 5 units of *Bln*I (Roche Diagnostics, Mannheim, Germany). The plugs were melted at 65 °C and loaded on 1% agarose gels (Seakem GTG agarose, FMC BioProducts, Maine, USA). Electrophoresis (BioRad, CHEF-DRII) was performed in 0.5 × TBE at 10 °C, at 200 V with pulse ramping from 2 to 40 s over 24 h (*Spe*I), 5 to 50 s over 24 h (*Xba*I) or 8 to 80 s over 25 h (*Bln*I). The gels were stained with ethidium bromide and photographed under UV light. Molecular weight standards were lambda concatemers Mid Range II Markers (New England Biolabs). For analysis a fragment size range from 750 kb to 30 kb was considered.

2.4.4. IS200 typing

Digoxigenin (DIG)-labeled IS*200* probes (557 bp) were synthesized by PCR amplification (30 cycles of 1 min at 94 °C, 1 min at 63 °C, 1 min at 72 °C; final incubation at 72 °C for 4 min) with genomic DNA of *S.* Typhimurium strain LT2 as template. The primers for amplification IS*200*-L2 and IS*200*-R2 were described previously [10].

Amplification reactions were performed in volumes of 50 µL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM (each) dATP, dCTP, dGTP and dTTP, 10 pmol of each primer, 50 µg BSA, 50 ng genomic DNA and 1.5 units Taq DNA polymerase (Perkin Elmer Biosystems). The PCR product was DIG labeled with the random primed labeling hexanucleotide mix (Roche Diagnostics) according to the manufacturers protocol.

Genomic DNA isolation for IS*200* typing was performed as previously described [5]. One microgram of genomic DNA was digested with *Pst*I according to the manufacturers' instructions and separated on 1% agarose gels in 1 × TBE at 45 V for 19 h. The gels were stained with ethidium bromide and photographed. DNA was transferred from agarose gels to Hybond N nylon membranes (Amersham Pharmacia, Freiburg, Germany) in 10 × SSC using a vacuum blotter (model 785, BioRad) and hybridized to the Digoxigenin-labeled IS*200* fragment at 42 °C overnight in a solution containing 50% formamide. Unbound IS*200* probe was removed by washing the membranes twice with 2 × SSC and 0.1% SDS at room temperature and twice with 0.1 × SSC and 0.1% SDS at 68 °C. The bound probe was recognized by a colorimetric assay as described in the DIG DNA Detection Kit (Roche Diagnostics).

2.5. Calculation of discriminatory power

The discriminatory power of the different molecular typing methods was calculated by determining the indices of discrimination (D values) according to Hunter and Gaston [16]. These values calculate the probability that two strains from the test population will be classified into different typing groups. The lower the index value is, the less discriminative is the typing method.

3. RESULTS

3.1. Susceptibility to antimicrobial agents

Among the 403 DT104 strains originating from slaughter pigs 94% were identified as multiresistant. Among them 367 (91%) of the strains exhibited only the typical pentaresistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamides (Su) and tetracycline (T) (= R-type ACSSuT) described for DT104 [29, 30]. Twenty-four (6%) of the strains investigated were sensitive to all sixteen antimicrobial agents tested.

3.2. Molecular typing of pig isolates

Of the 403 isolates, 133 were initially characterized for their plasmid profile (Tab. I). The isolates selected represented arbitrary samples distributed evenly over the seven abattoirs and samplings [18]. Thirty-two representative strains were selected from the 133 plasmid typed DT104 isolates for the application of other molecular typing techniques. Table II describes the genotypes obtained.

3.2.1. Plasmid profile patterns

All 133 strains possessed at least one to four plasmids which resulted in eight different plasmid profile patterns (A to H) (Fig. 1, Tab. I). A 60 MDa plasmid was observed in seven plasmid profile patterns which represents the typical serovar specific virulence plasmid (pRQ28) [13] of *S.* Typhimurium. Plasmid pattern A (24%), B (20%) and C (47%) predominated and represented 92% of the 133 isolates. The ten strains belonging to the pattern D, E, F and G were always sensitive to antimicrobial agents (Tab. I).

The results showed that some of the DT104 isolates of the slaughter pigs could already be discriminated by their plasmid profiles.

Table I. Plasmid profile patterns and resistance or sensitivity to antimicrobial agents of 133 DT104 isolates originating from slaughter pigs.

Plasmid size (MDa)	Plasmid profile pattern	Number of strains	
		Resistant	Sensitive
60	A	24	8
60, 1.4	B	25	2
60, 2.2, 1.4	C	63	0
60, 4, 2.4, 1	D	0	5
2.8	E	0	1
68, 60, 55, 1.4	F	0	2
60, 4.6	G	0	2
60, 20	H	1	0
Total number	8	113	20

Note: Some of the patterns correlate to the ones described by Threlfall et al. [30] namely (our nomenclature - Threlfall's nomenclature) A = A, B = C, D = G, G = E.

3.2.2. IS200 typing

Four distinct patterns could be detected within the 32 selected DT104 strains (Fig.1, Tab. II). Pattern 1 occurred in 27 isolates whereas pattern 2 and 4 were observed in two strains each (strains: 62L129, 62L142, genotype 15 and 310K21, 310L23, genotype 2) and pattern 3 in one strain only (strain 67L10, genotype 16). All strains belonging to the IS*200* patterns 2 to 4 were sensitive to antimicrobial agents. The number of IS*200* elements varied between eleven in pattern 1 and six in pattern 2 (Fig. 1).

3.2.3. PFGE typing

Seven distinct pulsed-field gel electrophoresis patterns were observed with *Spe*I (designated S1 to S7) and *Bln*I (B1 to B7) (Tab. II). *Xba*I digestions resulted in four different patterns (X1 to X4) among the 32 isolates originating from pig. The patterns S1, X1 and B1 were most predominantly observed in 69% (*Spe*I), 81% (*Xba*I) or 63% (*Bln*I) of the cases and selected as reference patterns. Pattern S1 (*Spe*I) contained 21 fragments ranging in sizes from 20 to 440 kb (Fig. 1). *Xba*I produced 15 fragments (pattern X1) ranging in sizes from 20 to 700 kb and *Bln*I 10 fragments (pattern B1) ranging in sizes from 20 to 750 kb (data not shown). In most of the cases pulsed-field gel patterns of one restriction enzyme differed by one to three fragments only, indicating a close relationship of the strains. In some cases (five strains, genotype 2, 15 and 16) the PFGE pattern differed for each restriction enzyme used (Tab. II) indicating a different common ancestor. Thirteen strains showed PFGE patterns, where only one out of the three restriction enzymes caused a negligible different PFGE pattern indicating a common ancestor.

3.2.4. RAPD typing

The reproducibility of RAPD was tested with 10 different genomic DNA preparations from one strain of *S.* Typhimurium DT104 (51K61) and one primer (23L). In addition, 10 different strains isolated in one abattoir containing identical plasmid profiles and resistance patterns were tested in RAPD with primer 23 L and OPB15/B. The resulting patterns were identical (data not shown). This showed the high reproducibility of RAPD under the conditions used.

RAPD was carried out with the 32 representative DT104 strains. The patterns except one (OPG08) consisted of 6 to

Table II. Genotypes of 32 representative DT104 isolates originating from slaughter pigs.

Genotype	No. of strains[a]	Resistance[b]	Plasmid profile	PFGE pattern			IS*200* pattern (no. of bands)	RAPD pattern[c]
				*Spe*I	*Xba*I	*Bln*I		
1	8	ACSSuT	A	1	1	1	1 (11)	111111111111
2	2	sensitive	A	5*	2*	4*	4 (10)	354112623322
3	2	ACSSuT	B	1	1	2	1 (11)	211111112121
4	2	ACSSuT	B	1	1	2	1 (11)	211211112121
5	1	ACSSuT	B	7	1	1	1 (11)	111111111111
6	2	ACSSuT	B	1	1	1	1 (11)	111111111111
7	1	CSSu	B	1	1	7	1 (11)	141111111111
8	1	ACSSuT	B	1	1	7	1 (11)	141111111111
9	1	ACSSuT	B	1	1	3	1 (11)	111111111111
10	1	ACSSuT	B	4	1	1	1 (11)	116111111111
11	1	sensitive	B	4	1	1	1 (11)	116111511111
12	2	ACSSuT	C	1	1	1	1 (11)	111111111111
13	1	ACSSuT	C	1	1	1	1 (11)	131111111111
14	1	ACSSuT	C	1	1	1	1 (11)	111111311111
15	2	sensitive	D	5*	2*	5*	2 (6)	322111222222
16	1	sensitive	E	3	3*	6	3 (10)	373111134233
17	1	sensitive	F	6	1	1	1 (11)	165111411111
18	1	sensitive	G	2	4	1	1 (11)	111111111111
19	1	ACSSuT	H	1	1	1	1 (11)	411111112121

* Difference in more than three bands in respect to reference pattern.

[a] The following strain designations belong to the genotypes (genotype-strain designations): 1-51K61, 51O20, 22O42, 24K8, 65K163, 16O3, 26K121, 610O12; 2-310K21, 310L23; 3-32K112, 24K200; 4-32L126, 32O151; 5-13K195; 6-24O137, 110L54; 7-55L34; 8-55O93; 9-26K117; 10-110K11;11-110O138; 12-12O194, 16K51;13-54K91; 14-17O134; 15-62L129, 62L142; 16-67L10; 17-110L44; 18-310K100; 19-51O47. The first number encodes the abattoir, the second number the week of isolation, the letter at the third position indicates the material of isolation from the slaughtered pigs, K (fecal), O (surface swab), L (lymphnode). The following number designates each individual slaughter pig.

[b] A (ampicillin), C (chloramphenicol), S (streptomycin), Su (sulphonamides) and T (tetracycline).

[c] Each number defines a certain RAPD pattern in the order of the following primers: 23L-OPB15/B-OPB15-1283-OPG10-OPH04-OPH13-OPB17-OPA4-OPG04-OPB6-1254.

12 bands ranging from about 3 kb to 200 bp. The primer OPG08, which is applicable for *S.* Enteritidis subtyping [20] generated only a 2 kb fragment and was therefore excluded from further studies. The number of patterns of each primer ranged between one and seven (OPG10: one pattern; 1283, OPH04: two; OPB17, OPG04, OPB6, 1254: three; 23L, OPA4: four; OPB15, OPH13: six; OPB15/B: seven) and patterns generated by one primer differed mostly in one or two bands. The 12 primers differentiated the 32 strains in 13 typing patterns. Because of their low or identical discriminatory power, the primers OPG10, 1254, OPG04, OPA4, OPH04 could be excluded from the analysis without influencing the number of patterns.

RAPD results correlated with the results of the other molecular typing methods described above. In addition, the patterns of some primers correlated with the patterns derived from PFGE. For example four strains (Tab. II: strains 32K112, 32L126, 32O151 and 24K200, genotypes 3 and 4) possessed corresponding patterns generated by primer 23L and by *Bln*I-PFGE (pattern B2). Two other strains shared unique patterns of primer OPB15/B and *Bln*I-PFGE B7.

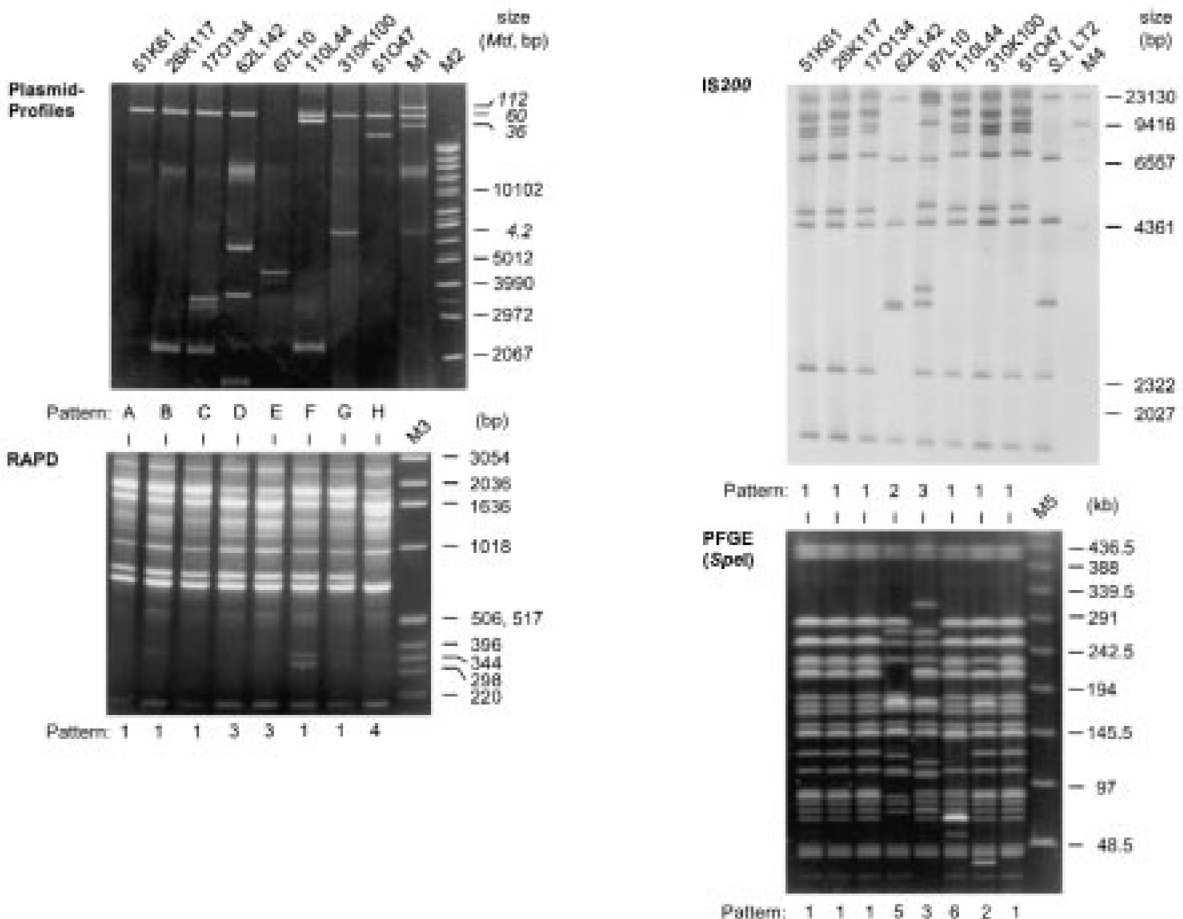

Figure 1. Molecular typing patterns of *S.* Typhimurium DT104. Eight plasmid profile patterns (A to H) are presented and correlated to RAPD with primer 23L, IS*200* with *Pst*I digested genomic DNA and *Spe*I-PFGE. Each lane contains the sample of an identical strain. The designated molecular typing pattern of each sample and method is shown below the gels. Molecular sizes at the right correspond to bands of the size markers. The reference strain designation is shown above the plasmid profile and IS*200* gels. Molecular size marker on individual gels: lane M1, size marker (italic type) containing R27 (112 MDa), R1 (60 MDa), RP4 (36 MDa) and ColE1 (4.2 MDa); lane M2, size marker (GibcoBRL, supercoiled DNA ladder; lane M3, size marker (BRL, 1-kb molecular size standard); lane M4, size marker (Roche Diagnostics, DIG labeled molecular size standard MII); M5, size marker (New England Biolabs, lambda ladder PFG marker).

Table III. Discrimination indices of molecular typing methods applied to 32 DT104 isolates.

Method	Number of types	Discrimination index
Plasmid profiles	8	0.762
PFGE (*Spe*I)	7	0.520
PFGE (*Xba*I)	4	0.332
PFGE (*Bln*I)	7	0.599
PFGE (*Spe*I, *Xba*I, *Bln*I)	12	0.796
IS*200* (*Pst*I)	4	0.288
RAPD (23L/OPB15/B)	9	0.673
RAPD (12 primers)	13	0.778
Plasmid profiles/PFGE (*Spe*I)	11	0.853
Plasmid profiles/PFGE (*Xba*I)	9	0.794
Plasmid profiles/PFGE (*Bln*I)	12	0.893
Plasmid profiles/PFGE (*Spe*I, *Xba*I, *Bln*I)	14	0.909
Plasmid profiles/RAPD (23L)	10	0.859
Plasmid profiles/RAPD (OPB15/B)	11	0.841
Plasmid profiles/PFGE (*Bln*I)/RAPD (OPB15)	13	0.905

3.3. Discriminatory power and application of a subtyping scheme

The calculated indices for each individual typing method and the method combinations are given in Table III. Plasmid profiles possessed the highest discriminatory power among the 32 isolates followed by RAPD in combination with the two most discriminatory primers 23L and OPB15/B (Tab. III) as well as PFGE with the restriction enzyme *Bln*I. The very low index of IS*200* typing indicates that this typing method is not a suitable tool for subtyping DT104 isolates. Nevertheless, each molecular typing method per se seems to be not discriminative enough. The combination of the methods with the highest D values resulted in better discrimination. Acceptable levels of discrimination (> 0.9) were obtained by the combination of plasmid profiling, PFGE with *Bln*I and RAPD with primer OPB15. Alternatively, the combination of plasmid profiles and PFGE patterns from all three restriction enzymes resulted in an index of 0.909.

4. DISCUSSION

In this study, the efficiencies of four different DNA based typing methods were evaluated for subtyping German *S.* Typhimurium phage type DT104 isolates. DT104 strains (n = 133) originating from slaughtered pigs were subject to plasmid profiling, and 32 to PFGE, RAPD and IS*200* typing.

Plasmid profiling of the 32 pig isolates resulted in an index of discrimination of 0.762. Sixty to 70% of the isolates had identical *Xba*I, *Spe*I or *Bln*I PFGE-patterns with an index of discrimination of 0.332 for *Xba*I, 0.520 for *Spe*I and 0.599 for *Bln*I. RAPD using 12 different primers in combination detected 13 different genotypes resulting in an index of discrimination of 0.778. Individual primers showed only a moderate discriminatory power. IS*200* typing of the 32 isolates revealed only four different patterns (D = 0.288). The D values confirm that the molecular typing methods tested have different discriminatory power. A theoretical maximum value of one indicates a 100% discrimination probability for each strain tested. According to our results, plas-

mid profiling alone is the most discriminatory method. In addition it is fast, easy to perform and reproducible and should consequently be the first method of choice for subtyping DT104 isolates.

The combination of different typing methods increased the index of discrimination and resulted in a useful subtyping scheme for DT104 isolates. The combination of plasmid profiling with PFGE patterns from *Xba*I, *Spe*I and *Bln*I digestions resulted in a maximum of discrimination. Therefore, we recommend these methods for subtyping of DT104 in the future. Similar results were reported for the serotype *S.* Hadar [31]. Alternatively, RAPD can be applied for the discrimination of DT104 isolates using at least the primers 23L and OPB15/B in combination. The disadvantage of RAPD is its sensitivity to several parameters and lack of comparability of data obtained in different laboratories. The discriminatory power of RAPD depends mainly on the specificity of the primer. Among the 32 isolates from pig origin OPB15/B, 23L, OPB15 and OPH13 were the most discriminative primers. It is necessary that the proposed subtyping scheme will be applied to further DT104 isolates obtained from other livestock species in order to confirm the observed discrimination indices.

The study has shown that in general only minor differences occur in the DNA pattern of single DT104 strains isolated from slaughter pigs. These clonal subtypes most likely represented variants of the predominating clone. Such variants did not establish successfully and did not lead to a clonal replacement within the ten weeks and among the abattoirs. Clonal replacement within a short period of time is possible as described for an epidemic clonal group of *Neisseria meningitidis* [22]. The data presented here in contrast show that the spread of DT104 from German pigs is highly clonal and stable.

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