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Virulotyping and Antimicrobial Resistance Typing of *Salmonella enterica* Serovars Relevant to Human Health in Europe

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Abstract

The combination of virulence gene and antimicrobial resistance gene typing using DNA arrays is a recently developed genomics-based approach to bacterial molecular epidemiology. We have now applied this technology to 523 *Salmonella enterica* subsp. *enterica* strains collected from various host sources and public health and veterinary institutes across nine European countries. The strain set included the five predominant *Salmonella* serovars isolated in Europe (Enteritidis, Typhimurium, Infantis, Virchow, and Hadar). Initially, these strains were screened for 10 potential virulence factors (*avrA*, *ssaQ*, *mgtC*, *siiD*, *sopB*, *gipA*, *sodC1*, *sopE1*, *spvC*, and *bcfC*) by polymerase chain reaction. The results indicated that only 14 profiles comprising these genes (virulotypes) were observed throughout Europe. Moreover, most of these virulotypes were restricted to only one ($n = 9$) or two ($n = 4$) serovars. The data also indicated that the virulotype did not vary significantly with host source or geographical location. Subsequently, a representative subset of 77 strains was investigated using a microarray designed to detect 102 virulence and 49 resistance determinants. The results confirmed and extended the previous observations using the virulo-polymerase chain reaction screen. Strains belonging to the same serovar grouped together, indicating that the broader virulence-associated gene complement corresponded with the serovar. There were, however, some differences in the virulence gene profiles between strains belonging to an individual serovar. This variation occurred primarily within those virulence genes that were prophage encoded, in fimbrial clusters or in the virulence plasmid. It seems likely that such changes enable *Salmonella* to adapt to different environmental conditions, which might be reflected in serovar-specific ecology. In this strain subset a number of resistance genes were detected and were serovar restricted to a varying degree. Once again the profiles of those genes encoding resistance were similar or the same for each serovar in all hosts and countries investigated.

Introduction

Salmonella is a major zoonotic foodborne pathogen causing outbreaks and sporadic cases of gastroenteritis in humans worldwide (Humphrey, 2000). The primary sources of salmonellosis are food-producing animals such as poultry, pig, and cattle (Thorns, 2000). The pathogen is mainly disseminated by trade in animals and uncooked animal food products (Gillespie *et al.*, 2005). *Salmonella* is a major zoonotic pathogen in Europe, causing approximately 152,000 confirmed

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human cases in 2007 (Anonymous, 2009). The five most frequently isolated *Salmonella* serovars in humans are Enteritidis (64.5%), Typhimurium (16.5%), Infantis (1.0%), Virchow (0.8%), and Newport (0.6%) (Anonymous, 2009). However, the *Salmonella* prevalence and serovar distribution varies widely among the European Union Member States and over time.

The advent of high-throughput techniques has enabled the rapid sequencing of whole bacterial genomes. For *Salmonella*, several strains are in the process of sequencing or are already sequenced (McClelland *et al.*, 2001; Parkhill *et al.*, 2001; Cooke *et al.*, 2008; Thomson *et al.*, 2008). This information has been used to develop whole genome microarrays to study the variation in genome content within *Salmonella enterica* subspecies *enterica* (Porwollik *et al.*, 2004, 2005). Such techniques have also been used to identify conserved chromosomal genes (Anjum *et al.*, 2005) within this group of organisms. However, the wealth of data from whole genome microarrays can be difficult to handle and may be of limited use because only on the DNA microarray represented strain-specific targets are considered. Consequently, arrays with a lower number of targets, representing specific bacterial properties such as virulence genes (virulotyping), have been developed (Scaria *et al.*, 2008; Huehn *et al.*, 2009a).

The application of the virulotyping approach to *Salmonella* is beginning to inform our understanding of how various virulence gene repertoires reflect bacterial properties such as host specificity (Huehn *et al.*, 2009b). The advantages of such approaches include rapidity and cost effectiveness, which mean that many strains can be easily virulotyped.

Although some *Salmonella* serovar virulotyping studies (Prager *et al.*, 2003; Herrero *et al.*, 2006; Soto *et al.*, 2006) have been undertaken, little is known of the distribution of virulence and resistance determinants in strains across Europe. The aim of this study was to determine the distribution of virulence and resistance determinants within strains of the five most prevalent *S. enterica* serovars (Enteritidis, Typhimurium, Infantis, Virchow, and Hadar) in Europe isolated from humans and animals. Strains were collected from public health and veterinary institutes across Europe as part of the research collaboration of the European Network of Excellence Med-Vet-Net (www.medvetnet.org). They were first screened for potential virulence factors by polymerase chain reaction (virulo-PCR), and then a representative subset of strains was investigated by microarray for combinations of virulence and resistance determinants.

Materials and Methods

Participating institutes

Participating institutes provided *S. enterica* strains and performed virulo-PCR screening on their selected strains. The following institutes participated in this study: Federal Institute for Risk Assessment (BFR), Berlin, Germany; Veterinary Laboratories Agency (VLA), Surrey, United Kingdom; Health Protection Agency (HPA), London, United kingdom; Agence Française de Sécurité Sanitare des Aliments (AFSSA), Maisons-Alfort, France; National Veterinary Institute (SVA), Uppsala, Sweden; Panstwowy Zaklad Higieny (PZH), Warsaw, Poland; Statens Serum Institut (SSI), Copenhagen, Denmark; Veterinary Medical Research Institute (VMRI), Budapest, Hungary; and Instituto de Salud Carlos III (ISCIII), Madrid, Spain. All strain information and PCR data were deposited in an MS Access 2003 database (Microsoft Ltd., Reading, UK) developed and maintained by the VLA.

Bacterial strains

Participating institutes were requested to select *S. enterica* strains from their national database belonging to the serovars Enteritidis, Typhimurium, Infantis, Virchow, and Hadar on the basis of the following criteria: (i) all strains should be accurately serotyped, (ii) the year of isolation should not be before 1995, (iii) strains should be either of human or of animal (poultry, porcine, bovine, ovine, or caprine) origin and isolated from epidemiologically independent sources, (iv) any other relevant information, such as clinical symptoms or phage type, should be supplied, and (v) strains of particular interest should be included as these may be new and emerging organisms. A total of 523 strains were selected for investigation (Supplemental Table S1, available online at www.liebertonline.com). A subset of 77 strains (Table 1), selected on the basis of virulo-PCR characteristics to represent diversity and potential novel virulence combinations, was processed for DNA microarray analysis. The serovars of the strains in the subset were confirmed by slide agglutination, and the antimicrobial susceptibilities were determined to 17 antimicrobials or antimicrobial combinations (see below).

Serotyping and antimicrobial susceptibility testing

All 523 *Salmonella* strains were serotyped according to the White–Kauffmann–Le Minor scheme (Grimont *et al.*, 2007) by agglutination with O- and H-antigen-specific sera at the particular participating institute. The serovars of the subset of the 77 *Salmonella* strains were confirmed at the National *Salmonella* Reference Laboratory (Berlin, Germany) using O- and H-antigen-specific sera available from Sifin Diagnostics (Berlin, Germany).

Antimicrobial susceptibilities of the subset of strains were tested against 17 antimicrobials or antimicrobial combinations by determining the minimum inhibitory concentration using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI, 2006) in combination with the semiautomatic Sensititre system (Trek Diagnostic Systems, Cleveland, OH). Breakpoints were applied as previously described (Schroeter *et al.*, 2004). Antibiotics tested were amoxicillin/clavulanic acid (AMC), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), florfenicol (FLO), gentamicin (GEN), kanamycin (KAN), neomycin (NEO), nalidixic acid (NAL), spectinomycin (SPE), streptomycin (STR), sulfamethoxazole (SMX), trimethoprim/sulfamethoxazole (SXT), tetracycline (TET), trimethoprim (TMP), and ceftiofur (XNL).

Virulo-PCR screening

Ten determinants with reported contributions to virulence were selected (Table 2). Five targets (*avrA*, *ssaQ*, *mgtC*, *siiD*, and *sopB*) were located on the *Salmonella* pathogenicity islands (SPIs) 1–5, three targets (*gipA*, *sodC1*, and *sopE1*) on prophages, one (*spvC*) on the *Salmonella* serovar Typhimurium virulence plasmid, and one (*bcfC*) on a fimbrial cluster. These virulence determinants represent regions known to be either highly conserved (SPIs) or variable (prophages, plasmid)

Table 1. *Salmonella* Isolates Used in This Study for Microarray Analysis and Their Phenotypic Characteristics

Institute[a]	*Country*	*Original no.*	*NRL-Salm no.*	*Serotype*	*Origin*	*Year of isolation*	*Phage type*	*Resistance*[a]
BFR	Germany	03-3402	03-3402	Typhimurium	Bovine	2003	DT104L	AMP, CHL, FFN, SMX, STR, SPE, TET
BFR	Germany	03-3875	03-3875	Typhimurium	Porcine	2003	DT104B low	AMP, CHL, GEN, FFN, KAN, NEO, SMX, STR, SPE, SXT, TET, TMP
BFR	Germany	04-2196	04-2196	Enteritidis	Poultry	2004	PT21	Susceptible
BFR	Germany	04-3244	04-3244	Virchow	Poultry	2004	PT26	Susceptible
BFR	Germany	05-0052	05-0052	Virchow	Poultry	2005	PT2	Susceptible
BFR	Germany	05-2263	05-2263	Infantis	Porcine	2005		Susceptible
BFR	Germany	04-3524	04-3524	Infantis	Poultry	2004		AMP, STR, SMX, SPE, SXT, TMP
PZH	Poland	307/05	07-3058	Typhimurium	Human	2005	DT104L	AMP, TET, STR, SMX
PZH	Poland	163/06	07-3059	Typhimurium	Human	2006	DT104L	AMP, TET, STR, SMX, NAL, CHL, FFN
PZH	Poland	425/02	07-3060	Typhimurium	Human	2002	DT104L	AMP, TET, STR, SMX, NAL, CHL, FFN
PZH	Poland	206/01	07-3054	Enteritidis	Human	2001	PT8	Susceptible
PZH	Poland	382/02	07-3055	Enteritidis	Human	2002	PT21	Susceptible
PZH	Poland	12/06	07-3056	Enteritidis	Human	2006	PT4	Susceptible
PZH	Poland	367/05	07-3057	Enteritidis	Human	2005	PT6	Susceptible
PZH	Poland	346/05	07-3062	Virchow	Human	2005		NAL
PZH	Poland	159/06	07-3063	Virchow	Human	2006		NAL
PZH	Poland	05/06	07-3064	Virchow	Human	2006		NAL
PZH	Poland	97/06	07-3061	Infantis	Human	2006		Susceptible
PZH	Poland	308/05	08-0901	Hadar	Human	2005		AMP, NAL, STR, TET
PZH	Poland	154/02	08-0902	Hadar	Human	2002		NAL, STR, TET
AFSSA	France	02EB5499SAL	07-3065	Typhimurium O5-	Porcine	2002	U302	AMP, TET, STR, SPE, CHL, FFN
AFSSA	France	04CEB8532SAL	07-3066	Typhimurium	Bovine	2004	RDNC[b]	Susceptible
AFSSA	France	04CEB10315SAL	07-3067	Enteritidis	Ovine	2004	PT4b	Susceptible
AFSSA	France	03EB4714SAL	07-3068	Enteritidis	Bovine	2003	PT4b	Susceptible
AFSSA	France	01EB288SAL	07-3071	Virchow	Poultry	2001		Susceptible
AFSSA	France	03EB3670SAL	07-3072	Virchow	Poultry	2003		AMP,TET, STR, SMX, NAL, SXT, XNL, KAN, NEO, SPE, TMP
AFSSA	France	01EB2321SAL	07-3069	Infantis	Ovine	2001		Susceptible
AFSSA	France	04CEB5290SAL	07-3070	Infantis	Poultry	2004		Susceptible
AFSSA	France	01EB165SAL	08-0424	Hadar	Bovine	2001		TET
AFSSA	France	01EB12158SAL	08-0425	Hadar	Poultry	2001		NAL, STR, TET
AFSSA	France	02EB4634SAL	08-0426	Hadar	Poultry	2002		AMP, NAL, TET
AFSSA	France	04CEB5387SAL	08-0427	Hadar	Poultry	2004		AMP, NAL, TET, STR
VMRI	Hungary	1487	07-3398	Typhimurium	Poultry	2006	RDNC	Susceptible
VMRI	Hungary	1379	07-3399	Typhimurium	Foodstuff	2006	DT12	Susceptible
VMRI	Hungary	1323	07-3400	Typhimurium	Poultry	2006	RDNC	AMP, CHL,FFN,STR,SMX, SPE, TET
VMRI	Hungary	1757	07-3405	Enteritidis	Poultry	2006	PT4	Susceptible
VMRI	Hungary	1490	07-3406	Enteritidis	Poultry	2006	RDNC	Susceptible
VMRI	Hungary	1660	07-3397	Enteritidis	Poultry	2006	PT4	Susceptible
VMRI	Hungary	584	07-3401	Infantis	Poultry	2004		NAL, STR, SMX, SPE, TET
VMRI	Hungary	588	07-3402	Infantis	Poultry	2004		NAL, STR, SMX, SPE, TET
VMRI	Hungary	605	07-3403	Infantis	Poultry	2004		NAL, STR, SMX, SPE, TET
VMRI	Hungary	597	07-3404	Infantis	Poultry	2004		NAL, STR, SMX, SPE, TET
ISCIII	Spain	7689/02	07-3085	Typhimurium	Human	2002	RDNC	Susceptible

(*continued*)

Table 1. (Continued)

Institute[a]	*Country*	*Original no.*	*NRL-Salm no.*	*Serotype*	*Origin*	*Year of isolation*	*Phage type*	*Resistance*[a]
ISCIII	Spain	761/06	07-3086	Typhimurium O5-	Human	2005	U302	AMP, TET, STR, SMX
ISCIII	Spain	4116/03	07-3087	Typhimurium	Human	2003	DT195	Susceptible
ISCIII	Spain	3582/04	07-3088	Typhimurium O5-	Human	2004	U302	AMP, CHL, FFN, SMX, STR, SPE, TET
ISCIII	Spain	6670/05	07-3083	Enteritidis	Human	2005	PT1	AMP, TET, STR, NAL, CHL, SPE, SMX, XNL
ISCIII	Spain	5968/02	07-3084	Enteritidis	Human	2002	PT8	Susceptible
ISCIII	Spain	37/03	08-2104	Hadar	Human	2002		AMP, NAL, STR, TET
ISCIII	Spain	6596/04	08-2105	Hadar	Human	2004		AMP, NAL, TET
ISCIII	Spain	3222/05	08-2106	Hadar	Human	2005		AMP, KAN, NAL
SVA	Sweden	109-04	07-3667	Typhimurium	Feline	2004	DT40	Susceptible
SVA	Sweden	482-04	07-3668	Typhimurium	Bovine	2004	DT104L	AMP, CHL, FFN, SMX, STR, SPE, TET
SVA	Sweden	189-00	07-3665	Enteritidis	Pork	2000	PT4	Susceptible
SVA	Sweden	487-05	07-3666	Enteritidis	Poultry	2005	PT1b	Susceptible
SVA	Sweden	167-99	07-3669	Virchow	Feline	1999		Susceptible
SVA	Sweden	287-00	07-3670	Virchow	Canine	2000		Susceptible
SVA	Sweden	S 1098-06	07-3671	Infantis	Human	2006		Susceptible
SVA	Sweden	438-04	08-2005	Hadar	Poultry	2004		Susceptible
SVA	Sweden	S 332-06	08-2006	Hadar	Human	2006		NAL
SVA	Sweden	S 882-06	08-2007	Hadar	Human	2006		AMP, KAN, NAL, STR, TET
SSI	Denmark	0511R7057	07-3658	Enteritidis	Human	2005	PT4	Susceptible
SSI	Denmark	0604H19005	07-3660	Virchow	Human	2006		AMP, GEN, NAL, TET
SSI	Denmark	0505T38978	07-3659	Infantis	Human	2005		Susceptible
SSI	Denmark	0602F18345	08-0377	Hadar	Human	2006		NAL, STR, TET
SSI	Denmark	0603M39016	08-0378	Hadar	Human	2006		TMP, SMX
HPA	England	H0 7032 0132	07-3662	Typhimurium	Human	2007	RDNC	STR, SMX
HPA	England	P575439/0	07-3661	Enteritidis	Human	2003	PT14b	Susceptible
HPA	England	H0 7018 0329	07-3664	Virchow	Human	2006	PT8	NAL, SMX, SXT, TET, TMP
VLA	England	25/P4632080	07-3675	Typhimurium O5-	Human	nk	U302	AMP, CHL, FFN, SMX, STR, SPE, TET
VLA	England	27/5949/98	07-3676	Typhimurium	Poultry	1998	DT104L	AMP, CHL, FFN, SMX, STR, SPE, TET
VLA	England	26/P4987540	07-3677	Typhimurium	Human	nk	DT104L	AMP, CHL, FFN, SMX, STR, SPE, TET
VLA	England	23/6878/00	07-3678	Typhimurium O5-	Bovine	2005	DT135	Susceptible
VLA	England	22/1759/99	07-3679	Typhimurium O5-	Bovine	1995	DT135	Susceptible
VLA	England	02/4400/98	07-3672	Enteritidis	Poultry	1998	RDNC	Susceptible
VLA	England	12/4662/00	07-3673	Enteritidis	Poultry	2000	PT6	Susceptible
VLA	England	10/P5283131	07-3674	Enteritidis	Human	2000	PT11	Susceptible

[a]Abbreviations: see Materials and Methods section.
[b]React with phages but does not conform with definite or provisorial types.
nk, not known.

Table 2. Target Genes for Virulo-Polymerase Chain Reaction Screening

Gene designation	*Function*	*Location*	*Oligonucleotide sequences (5′-3′)*	*PCR product size (in bp)*	*Reference*
avrA	Inhibits the key proinflammatory, antiapoptotic NF-kappa B pathway	SPI-1, Centisome 63	F: cct gta ttg ttg agc gtc tgg B: aga aga gct tcg ttg aat gtc c	422	Collier-Hyams *et al.* (2002), Streckel *et al.* (2004)
ssaQ	Secretion system apparatus protein, component of second T3SS	SPI-2, Centisome 31-30,5	F: gaa tag cga atg aag agc gtc c B: cat cgt gtt atc ctc tgt cag c	455	Hensel (2004), Soto *et al.* (2006)
mgtC	Intramacrophage survival protein	SPI-3, Centisome 82	F: tga cta tca atg ctc cag tga at B: att tac tgg ccg cta tgc tgt tg	677	Blanc-Potard *et al.* (1999), Soto *et al.* (2006)
siiD (*spi4_D*)	HLYD family secretion protein	SPI-4, Centisome 92	F: gaa tag aag aca aag cga tca tc B: gct ttg tcc acg cct ttc atc	655	McClelland *et al.* (2001), Soto *et al.* (2006)
sopB	Translocated effector protein (phosphoinositide phosphatase) via T3SS	SPI-5, Centisome 20	F: tca gaa gRc gtc taa cca ctc B: tac cgt cct cat gca cac tc	1231	Rahman (2006), Wood *et al.* (1998)
gipA	Peyer's patch-specific virulence factor	Gifsy-1 bacteriophage	F: acg act gag cag gct gag B: ttg gaa atg gtg acg gta gac	518	Stanley *et al.* (2000)
sodC1	Periplasmic Cu, Zn-superoxide dismutases	Gifsy-2 bacteriophage	F: cca gtg gag cag gtt tat cg B: ggt gcg ctc atc agt tgt tc	424	Fang *et al.* (1999), Herrero *et al.* (2006)
sopE1	Translocated T3SS effector protein	Cryptic bacteriophage	F: cgg gca gtg ttg aca aat aaa g B: tgt tgg aat tgc tgt gga gtc	422	Ehrbar and Hardt (2005), Streckel *et al.* (2004)
spvC	Spv region promotes rapid growth and survival within the host	pSLT plasmid	F: act cct tgc aca acc aaa tgc gga B: tgt ctt ctg cat ttc gcc acc	467	Libby *et al.* (2000), Guerra *et al.* (2000a)
bcfC	Bovine colonization factor, fimbrial usher	Chromosome	F: acc aga gac att gcc ttc c B: ttc tga tcg ccg cta ttc g	467	Weening *et al.* (2005)

PCR, polymerase chain reaction; SPI, *Salmonella* pathogenicity islands.

within the *Salmonella* genome. The oligonucleotide primer sequences for each PCR target gene are shown in Table 2. Primers used for the target genes *ssaQ*, *mgtC*, *siiD* (formerly designated *spi4_D*), *sodC1*, and *spvC* have been previously published (Guerra *et al.*, 2000a; Herrero *et al.*, 2006; Soto *et al.*, 2006). Primers for the remaining target genes were designed in this study and based on a multiple alignment of the specific gene to avoid polymorphisms within the amplified sequences. DNA for PCRs was prepared by thermal cell lysis. Briefly, a 1-mL aliquot of an overnight culture of *Salmonella* cultured in Luria–Bertani broth at 37°C was centrifuged for 5 min at 10,000 *g* and 4°C. The supernatant was discarded and the cell pellet resuspended in 300 μL TE buffer (10 mM Tris [pH 8] and 0.1 mM ethylenediaminetetraacetic acid). The resuspension was heated for 10 min at 95–100°C, and then immediately cooled and again centrifuged at 14,000 *g* for 10 min. As template in PCR, a 5-μL aliquot (approximately DNA of 10^6 bacterial cells) of a 1:10 dilution in sterile deionized H_2O was used. A typical 25 μL PCR contained 0.4 μM of each primer, 200 μM of each dNTP, 1×PCR buffer (20 mM Tris-HCl [pH 8.4] and 50 mM KCl), 1.5 mM $MgCl_2$, 0.75 U Platinum *Taq* polymerase (Invitrogen GmbH, Karlsruhe, Germany), and 5 μL DNA template. Participating laboratories were allowed to use other DNA polymerases and reaction buffers commercially available. The incubation conditions for all targets, except *bcfC*, were 95°C for 1 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, and a final step of 72°C for 4 min. For the *bcfC* PCR the annealing temperature was 53°C. A 10-μL aliquot of a PCR product was loaded on a 1.5% agarose gel and electrophoresed at 6 V/cm for 90 min. The presence of a clear fragment with the correct amplification size, after staining the gel in ethidium bromide, was assessed as a positive signal indicating the presence of the gene. For all targets, except *sopE1*, a positive control DNA from *Salmonella enterica* serovar Typhimurium strain LT2 (McClelland *et al.*, 2001) was used. For *sopE1* DNA from *Salmonella enterica* serovar Hadar strain 99-0601 was used as the positive control.

DNA microarray analysis

The DNA microarray probes, microarray production, whole genome DNA labeling, hybridization, analysis, and validation were all as previously described (Huehn *et al.*, 2009a). Briefly, the array comprises 275 60mer oligonucleotide probes derived from *Salmonella* sequences deposited at GenBank at NCBI (www.ncbi.nlm.nih.gov/). The probes were assigned to seven different marker groups depending on the functionality of the corresponding gene sequence (number of probes): pathogenicity (80), resistance (49), serotyping (33), fimbriae (22), DNA mobility (57), metabolism (21), and prophages (13). In addition, three 60mer oligonucleotides derived from the *Arabidopsis thaliana* genes RCA (M86720), RCP1 (NM_12175), and PRKASE (X58149) were designed as negative control probes on the microarray.

Virulence determinants for each strain analyzed were categorized according to their location on the *Salmonella* genome: SPIs, prophages, plasmid, islets, and fimbrial clusters. Microarray signals, which were assigned as uncertain by microarray analysis, were re-analyzed by PCR using primers as previously described (Huehn *et al.*, 2009a). After PCR testing an individual decision was made for the presence or absence of this target.

Data analysis

Normalized presence/absence data for each strain was imported into BioNumerics (version 5.1; Applied Maths, Sint-Martens-Latem, Belgium) as character values. A cluster calculation analysis was performed with the simple matching binary coefficient using the unweighted-pair group method with arithmetic averages (UPGMA dendrogram type).

Results

Virulo-PCR screening

The presence or absence of 10 selected virulence determinants in the panel of 523 *S. enterica* strains, including 146 serovar Typhimurium, 151 serovar Enteritidis, 82 serovar Infantis, 66 serovar Virchow, and 78 serovar Hadar strains, was investigated in multiple laboratories using a standardized PCR (Table 3). Little or no variation was observed for genes located in SPIs or for the fimbrial marker; that is, only one Enteritidis, one Virchow, and two Infantis strains were negative for gene *avrA*, and all the strains tested were positive for *ssaQ*, *mgtC*, *siiD*, and *bcfC*. The highest variation was observed in the gene targets located in a prophage or on a plasmid. The virulence plasmid-encoded gene *spvC* was only identified in serovars Enteritidis and Typhimurium, but even then not in all strains tested. Similarly, the *sodC1* gene was always present in serovars Enteritidis and Typhimurium but

Table 3. Results of Virulo-Polymerase Chain Reaction Screening

	Number of strains positive by PCR				
Target	*Enteritidis*	*Typhimurium*	*Infantis*	*Virchow*	*Hadar*
avrA	145	151	80	65	78
ssaQ	146	151	82	66	78
mgtC	146	151	82	66	78
siiD (spi4_D)	146	151	82	66	78
sopB	146	151	81	66	78
gipA	13	75	0	58	0
sodC1	146	151	0	0	0
sopE1	146	7	4	59	57
spvC	124	102	0	0	0
bcfC	146	151	82	66	78
Total no. of strains	**146**	**151**	**82**	**66**	**78**

not in serovars Infantis, Virchow, and Hadar. All strains of serovar Enteritidis and most strains of Virchow (59/66) and Hadar (57/78) but few strains of serovars Typhimurium (7/151) and Infantis (4/82) tested were positive for the *sopE1* gene. Interestingly, all seven *sopE1*-negative serovar Virchow strains were isolated in Spain or Germany. Altogether, 14 different virulence gene combinations have been detected in the five serovars (Table 4). Most diversity in virulence gene combinations was observed for serovar Typhimurium (six combinations), followed by Enteritidis, Virchow (five combinations), Enteritidis (four combinations), Infantis (three combinations), and Hadar (two combinations). Some, but not all, of the virulence gene combinations observed were clearly serovar specific.

Virulence determinants characterization by microarray analysis

A subset of 77 *Salmonella* strains (Table 1) were selected for microarray analysis using an array of 275 60mer oligonucleotide probes to detect 102 previously identified *Salmonella* virulence- and 49 resistance-associated determinants. Twenty-one strains belonged to serovar Typhimurium, 19 to serovar Enteritidis, 14 to serovar Hadar, and 11 to each of serovars Infantis and Virchow.

A UPGMA cluster analysis based on the presence or absence of the 102 virulence determinants (pathogenicity and fimbrial markers) present on the microarray and previously described (Huehn *et al.*, 2009a) grouped all strains belonging to the same serovar in a distinct clade (Fig. 1). The diversity of virulence determinant combinations (virulotypes) varied between serovars, with Typhimurium being the most diverse and Infantis, the least. Serovar Typhimurium strains belonging to phage types DT104L and U302 clustered closely together independent of their country of origin or source. In general, country-specific associations were not recognized in these 77 strains. However, four serovar Enteritidis strains from humans in Poland had identical virulotypes. In addition, three serovar Infantis strains isolated from poultry in Hungary also had identical virulotypes. Obviously, those isolates may have an epidemiological association.

The microarray data indicated that virulence determinants located in the SPIs 1–5 were highly conserved among the five serovars (Fig. 1). However, some variation occurred in SPI-3 at the 3′-region, such that in most of the strains belonging to serovars Hadar, Infantis, or Virchow, *sugR* and *rhuM* were absent. Genes located in SPI-7 were uniformly absent in all strains excepting in one serovar Infantis (*tviB* present, previous designation *wcdA*), one serovar 4,12:-:1,2 (phenotypic variant of serovar Typhimurium, *pilV* present), and one serovar Enteritidis strain (*pilV* present).

Some of the virulence-associated genes were serovar specific (Table 5). Others, such as those located on islets of *Salmonella*, were invariably present in all serovars (Fig. 1). This latter group of highly conserved genes included those encoding regulators (*barA*, *oxyR*, *phoP*, *entF*, *leuO*, and *slyA*) or effector proteins (*pipB2*, *sifA*, *sopA*, *sopD*, *sopE2*, *sseK2*, and *sspH2*) secreted by type III or II secretion systems and encoded by SPI-1 or SPI-2, respectively. Other genes varied in prevalence, including *trhH* (encoding pilus assembly protein), *sirA* (two-component system with *barA*), *pagK* (PhoPQ-activated protein), and *sseK1* (encoding putative-secreted effector protein). The *msgA* gene, which is essential to *Salmonella* mouse virulence (Gunn *et al.*, 1995), was consistently found in serovar Typhimurium strains but not observed in other serovars (Table 5).

The highest variation was observed in genes located on prophages. The Gifsy-1-associated gene *gipA* was detected in 9 out of 11 serovar Virchow strains, 9 out of 21 Typhimurium, and 4 out of 19 serovar Enteritidis strains. The other Gifsy-1-encoded gene, *gogB*, was present in 20 out of 21 Typhimurium strains and 4 out of 19 Enteritidis strains. Gifsy-2 encodes the virulence-associated genes *gtgA*, *sodC1*, and *sseI*, and these three genes were present in all serovar

Table 4. Virulence Gene Combinations and Frequency Identified by Virulo-Polymerase Chain Reaction Screening (10 Targets)

Gene combination[a]	*Number (percent) of strains*				
avrA-ssaQ-mgtC-siiD-sopB-gipA-sodC1-sopE1-spvC-bcfC	*Enteritidis*	*Typhimurium*	*Infantis*	*Virchow*	*Hadar*
1-1-1-1-1-0-0-0-0-1	0	0	76 (93)	2 (3)	21 (27)
1-1-1-1-1-0-0-0-1-1	0	0	0	0	57 (73)
1-1-1-1-1-0-0-1-0-1	0	0	4 (5)	5 (8)	0
0-1-1-1-1-0-0-0-0-1	0	0	2 (2)	1 (2)	0
1-1-1-1-1-1-0-1-0-1	0	0	0	54 (82)	0
1-1-1-1-1-1-0-0-0-1	0	0	0	4 (6)	0
1-1-1-1-1-0-1-0-1-1	0	67 (44)	0	0	0
1-1-1-1-1-1-1-0-1-1	0	32 (21)	0	0	0
1-1-1-1-1-0-1-0-0-1	0	9 (6)	0	0	0
1-1-1-1-1-1-1-0-0-1	0	32 (24)	0	0	0
1-1-1-1-1-1-1-1-0-1	3 (3)	5 (3)	0	0	0
1-1-1-1-1-1-1-1-1-1	10 (7)	2 (1)	0	0	0
1-1-1-1-1-0-1-1-1-1	114 (78)	0	0	0	0
1-1-1-1-1-0-1-1-0-1	19 (13)	0	0	0	0
Total no. of strains	**146**	**151**	**82**	**66**	**78**
Virulence gene combinations	**4**	**6**	**3**	**5**	**2**

[a]The presence (1) or the absence (0) of the specific PCR product for the respective target gene.

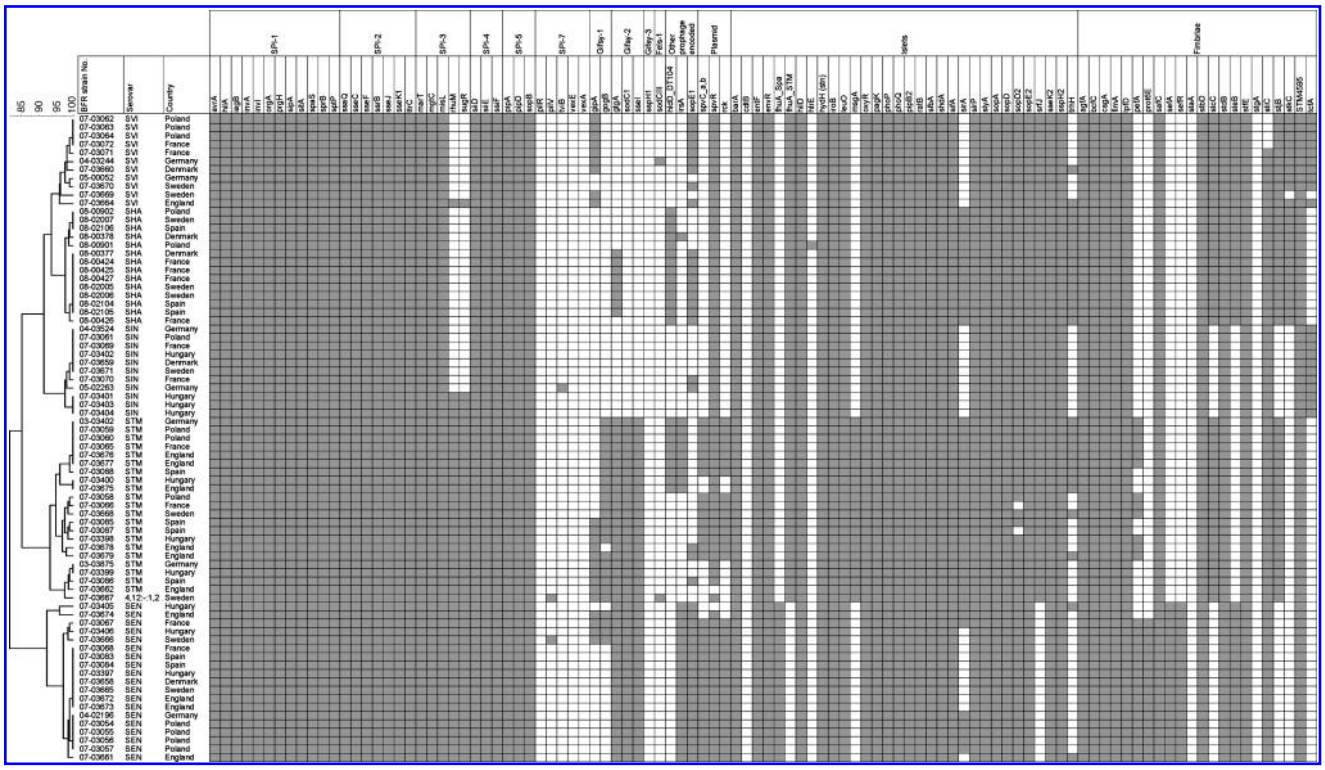

FIG. 1. Virulence determinants microarray data for 77 *Salmonella* strains analyzed. At the top, the analyzed genes are grouped according to their particular genomic location (SPI-1 to SPI-7; Prophages Gifsy-1, Gifsy-2, Gifsy-3, and Fels-1; plasmids; and islets) or function (fimbrial). On the left, a UPGMA dendrogram followed by the BFR strain number, serovar (SVI, Virchow; SHA, Hadar; SIN, Infantis; STM, Typhimurium; SEN, Enteritidis), and origin (country of isolation) is indicated. The order of strains represents their relatedness according to the UPGMA dendrogram type performed in BioNumerics 5.1. The hybridization result of a distinct strain is shown by row. A white box indicates the absence and a gray box indicates the presence of the target sequence in the strain. SPI, *Salmonella* pathogenicity islands; UPGMA, unweighted-pair group method with arithmetic averages.

Table 5. Pathogenicity and Fimbrial Genes Exclusively Present in Certain Serovars (in Percent)

	Serovar				
Target	*Enteritidis* (n = *19*)[a]	*Typhimurium* (n = *21*)[a]	*Infantis* (n = *11*)[a]	*Virchow* (n = *11*)[a]	*Hadar* (n = *14*)[a]
sodC1	100	100	0	0	0
stcC	0	100	0	100	100
steB	100	0	0	100	100
stjB	0	100	0	100	100
stkC	0	0	0	91	100
sefA/*sefR*	100	0	0	0	0
prot6e	89	0	0	0	0
srfJ	0	100	100	100	100
tcfA	0	0	100	91	0
sseI	100	100	0	0	0
sirA	74	100	0	0	100
msgA	0	100	0	0	0
*fhuA*_Spa	100	0	0	0	0
*fhuA*_STM	0	100	100	100	100
*hldD*_DT104	0	43[b]	0	0	100
irsA	100	43[b]	0	0	9
sugR/*rhuM*	100	100	27	9	0

[a]*n*, number of strains tested.
[b]All strains belong to *Salmonella enterica* serovar Typhimurium phage types DT104L or U302.

Typhimurium and Enteritidis strains. Most strains (8 out of 14) belonging to serovar Hadar harbored a *gtgA* gene (*pipA* gene homolog) but lacked *sodC1* and *sseI* from Gifsy-2. The Gifsy-3-located gene *sspH1* was not found in any strain tested. The Fels-1 prophage encodes *sodCIII* (a Cu/Zn superoxide dismutase precursor). This gene was only present in one serovar Virchow strain and one serovar Typhimurium strain. Genes *rck* and *spvC*, located on the *Salmonella* virulence plasmid, were exclusively present in strains of serovars Typhimurium and Enteritidis and seemed to be associated with each other, which was consistent with the presence or absence of the plasmid. However, the other plasmid located gene, *spvR*, also apparently located on this plasmid, could be detected in nearly all serovars, suggesting that this gene, or a highly similar sequence detected by the probe, might be located elsewhere on the chromosome in serovars Hadar, Infantis, and Virchow.

Twenty-two probes on the microarray represent 21 different fimbrial clusters (there are two probes for SEF-14 fimbrial cluster) collected from genome sequences of serovars Typhimurium, Typhi, and Enteritidis. Each serovar demonstrated its own specific fimbrial profile (Fig. 1). In all strains the fimbrial markers *bfcC*, *agfA*, *csgA*, *fimA*, *lpfD*, *safC*, *stbD*, *stfE*, and STM4595 were present. The *staA* gene, originally identified in serovar Typhi, was found to be absent from all strains. The *prot6E* (located on the 60-kb virulence plasmid) and *sefA*/*sefR* genes were exclusively present in serovar Enteritidis. Two serovar Enteritidis strains (07-3405 and 07-3674) lacked the typical 60-kb virulence plasmid because all plasmid-located targets (*rck*, *pefA*, *spvC*, and *prot6E*) were absent. The *steB* gene was exclusively present in strains of serovars Virchow, Hadar, and Enteritidis and *stkC* exclusively in strains of serovars Virchow (excepting one, 07-3669) and Hadar (Table 5). The fimbrial cluster represented by the *tcfA* gene was present exclusively in serovar Infantis and Virchow, whereas the *stcC* and *stjB* genes were absent in all serovar Infantis and Enteritidis strains (Table 5).

Antimicrobial resistance characterization

Thirty-nine out of the 77 strains analyzed by microarray were resistant to one or more antimicrobials (Fig. 2) by phenotypic methods. All 39 strains, except 3 serovar Virchow strains, were resistant to two or more antimicrobial compounds tested (multiresistant). Multiresistance was frequently observed in strains belonging to serovars Hadar (93%) and Typhimurium (68%).

Serovar Enteritidis strains were all susceptible, except one highly multiresistant strain (07-3083) isolated in Spain from a human case of salmonellosis. This strain, and a serovar Virchow strain (07-3072) from France were phenotypically resistant to the β-lactam ceftiofur. However, since those microarray probes corresponding to genes encoding extended spectrum β-lactamases or AmpC enzymes (conferring a resistance to third-/fourth-generation cephalosporins) were all negative, PCRs for several other genes potentially responsible for this resistance were performed. These PCRs indicated that the ceftiofur resistance was encoded by a $bla_{\text{SHV-1-like}}$ gene in the serovar Enteritidis strain and a $bla_{\text{CTX-M-9}}$ gene in the serovar Virchow strain.

The majority of serovar Virchow, Hadar, and 4 out of 11 Infantis strains were resistant to nalidixic acid and ciprofloxacin. A plasmid harboring the *qnrS* gene (Robicsek *et al.*, 2006), conferring quinolone resistance, was detected in one serovar Hadar strain (08-2007). The remaining phenotypic antibiotic resistances correlated perfectly with the presence of a distinct gene responsible for the respective antimicrobial phenotype (Fig. 2).

Presence of Salmonella genomic island 1

In 10 strains belonging to serovar Typhimurium, targets were detected that indicated the presence of the *Salmonella* genomic island 1 (i.e., int_SG1 [S001], *rep* [S003], *trhH* [S012], *int1* [S028], *tnpR* [S027], *aadA2* [S029], *qacE*Δ1 [S030/S039], *sul1* [S031/S040], *floR* [S032], *tet*[G] [S034], ISCR3 [S036], $bla_{\text{PSE-1}}$, and IS*6100* [S043]) (Boyd *et al.*, 2001). Six of these 10 strains were assigned to phage type DT104L, 3 to U302, and 1 to RDNC (reacts with phages but does not conform with definite or provisionial types) (07-3400). One other DT104L strain (07-3058) and a U302 strain (07-3086) exhibited an AMP, STR, SMX, and TET (ASSuT) phenotype, encoded by bla_{TEM}, *strA*/*B*, *sul2*, as well as *tet*(A) or *tet*(B). One serovar Typhimurium strain (03-3875), belonging to phage type DT104Blow, showed a phenotypic resistance pattern including resistance against AMP, CHL/FFN, STR/SPE, SMX, and TET (ACSSuT), indicating the presence of an SGI-1 variant, in which several molecular targets characteristic for SGI-1 (including antibiotic resistance determinants) could not be detected.

The resolvase-encoding gene *tnpR* (S027) was exclusively present in SGI-1 harboring strains. Other targets present in SGI-1 were also either sporadically or consistently detected in other serovars or strains. In one serovar Virchow strain (07-3660), the SGI-1-associated integrase gene (int_SGI-1, S001) was detected. The *trhH* gene, putatively encoding a pilus assembly protein, was consistently detected in serovar Hadar strains but was only sporadically detected in strains of serovars Enteritidis and Virchow. The replication gene *rep* (S003) was present in 87%, and the transposase gene IS*6100* in 42%, of all the 77 strains tested.

Discussion

The five *Salmonella* serovars, Enteritidis, Typhimurium, Infantis, Virchow, and Hadar, are consistently in the top 10 most frequently reported serovars in Europe. All are known to induce gastroenteritis in a broad range of unrelated host species (Anonymous, 2009). The serovars Enteritidis and Typhimurium are the most important ones causing approximately 80% of all human cases of salmonellosis. The reason for the successful spread of these serovars remains to be elucidated. The nature and severity of *Salmonella* infections in different animal species and in humans vary enormously and reflect both bacterial and host factors. It is striking that the prevalence of these five serovars in food-producing animals mirrors their impact in terms of human salmonellosis. In general, serovar Enteritidis is the most frequently isolated serovar from poultry and its products (poultry meat and table eggs), whereas serovar Typhimurium is predominant in pigs and cattle and their products. In contrast, serovars Infantis, Hadar, and Virchow occur less frequently in both poultry and pigs (Anonymous, 2009).

The aim of the study reported here was to investigate the distribution of virulence and resistance determinants in these five serovars. A unique feature of this study was the use of a panel of 523 *Salmonella* strains, distributed among the

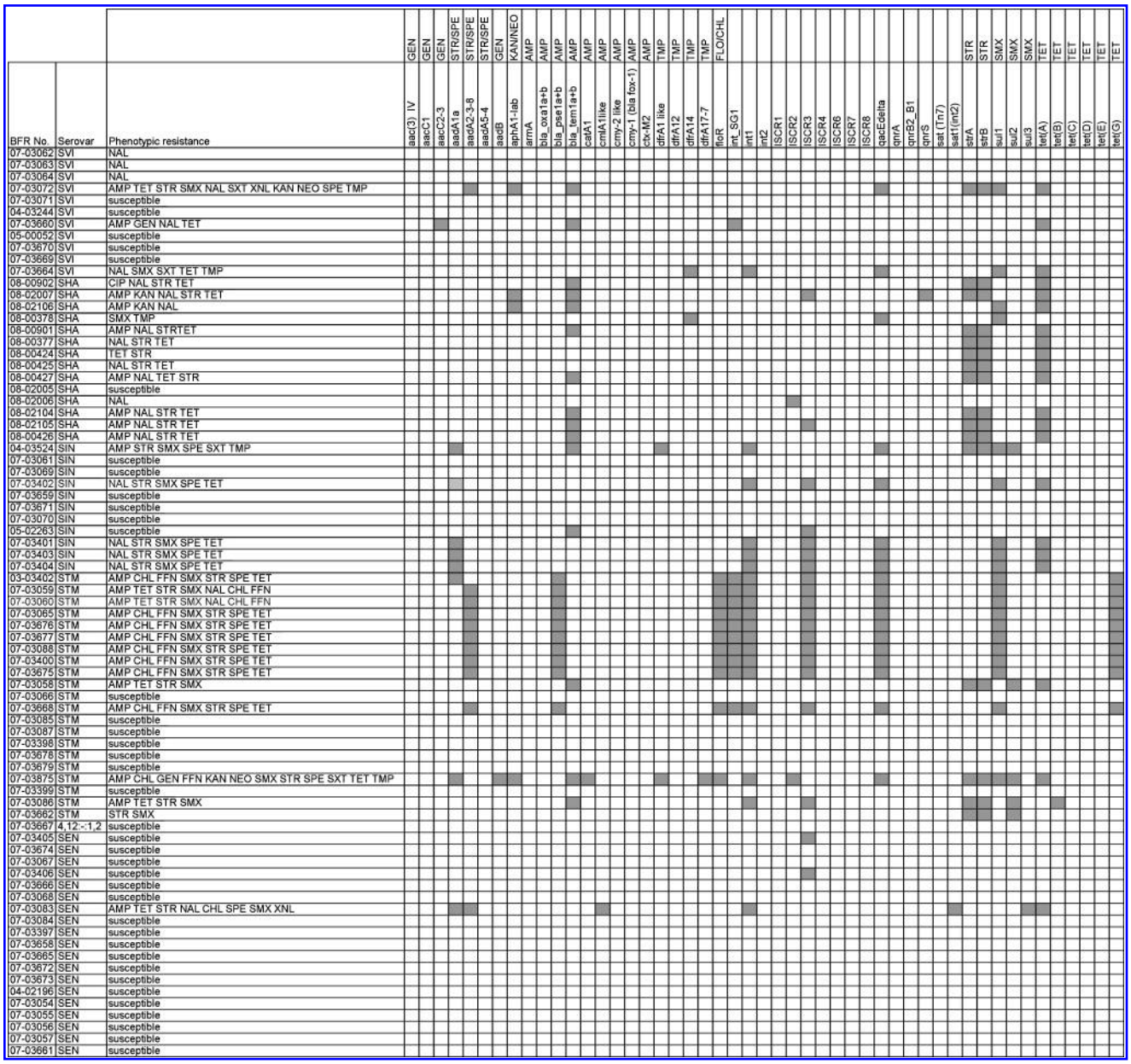

FIG. 2. Resistance determinants microarray data for the 77 strains analyzed. On the top, the analyzed genes are listed in alphabetical order, resulting in resistance to several antibiotics shown in abbreviations (see Materials and Methods for definitions) or genetic resistance elements like integron-associated integrases and transferases. On the left, the strain numbers, corresponding serovar (SVI, Virchow; SHA, Hadar; SIN, Infantis; STM, Typhimurium; SEN, Enteritidis), and antimicrobial resistance phenotype are indicated. The row right to the phenotype shows the hybridization result of a particular strain. A gray box indicates the presence of the target sequence in the strain, and a white box indicates the absence.

five predominant serovars, and collected from veterinary and public health laboratories in nine European countries. It was anticipated that this panel would enable the detection of any host-related and/or geographic specificity in virulence or resistance determinants. Screening by PCR based on 10 well-recognized virulence genes was applied to the panel. The results from this approach indicated that a limited number ($n = 14$) of virulotypes are observed throughout Europe. Moreover, most of these virulotypes were restricted to only one ($n = 10$) or two ($n = 3$) serovars (Table 4). The data also indicated that the virulotype did not vary significantly with host source or geographical location.

An extended virulence and resistance gene typing was then performed, on a subset of 77 strains, using a DNA array. The results, collated on the basis of 102 virulence genes, confirmed and extended the previous observations using the PCR screen. In particular, it was striking that all strains belonging to each serovar grouped together in the dendrogram (Fig. 1), indicating that the virulence-associated gene complement corresponded with serovar. There were, however, some differences in the virulence gene complement between strains belonging to an individual serovar. This variation occurred primarily within those virulence genes that were prophage encoded, in fimbrial clusters or in the virulence plasmid (Table 5). For

prophages this is not surprising as the acquisition and loss of such material in bacterial genomes has been explained as a fast mode of evolution (Brüssow *et al.*, 2004). Prophage genomes can contain additional genes, which might play a role in bacterial virulence, as in, for example, the prophages Gifsy-1, 2, and 3, Fels-1 and 2, and SopEΦ (Ehrbar and Hardt, 2005). The inclusion and reassortment of such prophage-associated virulence genes could enable *Salmonella* to adapt to different environmental conditions and to conquer new niches that might be reflected in serovar-specific ecology.

Similarly, fimbriae are considered to contribute to the diversity and host adaptation of *Salmonella* serovars. Fimbriae are responsible for the initial adhesion of the bacterium to the eukaryotic cells. They are frequently highly host specific and, therefore, an obvious factor that potentially influences host range/specificity. A number of different fimbrial clusters are encoded in a single *Salmonella* genome. For both serovars Typhimurium and Enteritidis, 13 fimbrial clusters have been identified (McClelland *et al.*, 2001; Thomson *et al.*, 2008). A core set of fimbrial determinants (including *bcf*, *agf*, *csg*, *fim*, *lpf*, *saf*, *stb*, *stf*, and STM4595) is common between the five serovars investigated. Such a common set of adherence determinants would contribute to the colonization of a broad range of animal species and human. Conversely, the absence of some fimbrial determinants is expected to contribute to host adaptation and, possibly, lower outbreak potential. For example, the serovar 4,12:d:- is highly adapted to poultry, like serovar Enteritidis, but seldom causes illness in humans (Huehn *et al.*, 2009a). Previous studies have shown that serovar 4,12:d lacks the *lpfD* gene of the Long polar fimbrial cluster (LPF) (Huehn *et al.*, 2009a). LPF of serovar Typhimurium mediates adhesion to murine Peyer's patches and are required for full virulence (Bäumler *et al.*, 1996), but such strains exhibit no significant defect in colonization of chickens (Allen-Vercoe *et al.*, 1999).

Horizontal gene transfer in combination with selective pressure from antimicrobial substances is largely responsible for the dissemination of resistance genes via mobile elements, such as transposons and integrons (Schwarz and Chaslus-Dancla, 2001). A number of resistance gene cassettes resulting in multiresistant strains have been described in various *Salmonella* serovars (Guerra *et al.*, 2000b; Miko *et al.*, 2003). The resistance gene profiles of the subset of 77 strains revealed that serovar Typhimurium strains belonging to phage types DT104 and U302 possessed the typical penta-resistance ACSSuT, encoded by the SGI-1 region (Boyd *et al.*, 2001). Whereas tetracycline resistance is encoded by *tet*(G) in these strains, in strains belonging to serovars Virchow, Hadar, and Infantis, this resistance is exclusively encoded by *tet*(A), often in combination with *strA/B* (STR resistance) and/or bla_{TEM} (AMP resistance).

Interestingly, resistance was detected in only one serovar Enteritidis strain, and this was multiresistant (isolated from a human). This confirms previous observations (Soto *et al.*, 2003) that, until recently, antimicrobial resistance was rarely described in this serovar. Since serovar Enteritidis, like serovars Hadar and Infantis, is frequently isolated from poultry in Europe, it is to be expected that selective pressure through the treatment of poultry with antibiotics would influence the uptake of resistance genes in this genome. However, it would seem that the uptake of such resistance genes by genome of the serovar Enteritidis is hampered by as yet unknown factors.

The microarray data indicated that the SPI-located genes were highly conserved. The only variation detected occurred in SPI-3. In particular, the genes *sugR* and *rhuM* were absent in all strains belonging to serovars Virchow (except one strain), Hadar and Infantis. Such variation in SPI-3 has previously been reported for serovars Virchow and Infantis (Amavisit *et al.*, 2003), and these studies concluded that the acquisition of the *sugR/rhuM* region was likely to be a relatively recent event. As a consequence, serovars Enteritidis and Typhimurium are likely to be common ancestors of Virchow, Hadar, and Infantis.

Conclusions

Virulotyping and antimicrobial resistance typing of strains from the five most important serovars of human salmonellosis within the European Union, Enteritidis, Typhimurium, Infantis, Virchow, and Hadar, has demonstrated serotype-specific repertoires for genes associated with virulence and resistance. Moreover, identical or similar virulotypes for each serovar were distributed across Europe, indicating the successful spread of those virulotypes across borders, presumably enabled by trade in food and food-producing animals. Those differences observed in genotype within a serovar were mainly based on the distribution of prophage-encoded virulence genes. A number of different resistance genes have been identified that were responsible for the expression of antimicrobial resistance belonging to various antibiotic classes. They were serovar restricted to a varying degree. In addition, the profiles of those genes encoding resistance were similar or the same for each serovar in all hosts and countries investigated. The dataset results support evidence of the role of mobile elements, such as phages, plasmids, and integrons, in the rapid evolution of *Salmonella* in adaptation to environmental stresses enabling the survival and widespread distribution of these *Salmonella* serovars throughout the food chain.

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Disclosure Statement

No competing financial interests exist.

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