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Epidemiological characteristics of *Salmonella* Typhimurium isolated from animals and feed in Poland

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SUMMARY

Fifty-seven *Salmonella* Typhimurium strains isolated from poultry, swine and animal feed in Poland during the years 1979–1998 and 2000–2002 were analysed with conventional and molecular techniques. Antimicrobial resistance as well as multiresistance was found, respectively, in 80·1% and 56·1% of the isolates and most frequently among isolates from 2000–2002. Of several phage types noted, DT104 was prevalent among poultry, swine and feed isolates. DT104, U302 and non-typable strains had a multiple resistant profile (ACSSuT) due to the presence of class I integrons. Pulse-field gel electrophoresis of *Xba*I and *Bln*I digest showed high genomic similarity between the strains and confirmed clonal spread of *S.* Typhimurium infections. Plasmid profiling allowed further differentiation of the strains. We have, therefore, confirmed the appearance of *S.* Typhimurium DT104 showing genome integrated integron-mediated antimicrobial resistance in Poland. These findings are significant for public and animal health risks and document the dissemination of DT104 epidemic strains into new geographical regions.

INTRODUCTION

Salmonellosis is considered one of the most important foodborne zoonoses contributing to public health. *Salmonella enterica* subsp. *enterica* serovar (*S.*) Typhimurium is among the most prevalent *Salmonella* serotypes worldwide and is of serious public and animal health concern. Several different *S.* Typhimurium phage types have arisen during the last decades. Most of them have been found in a wide variety of animal hosts and were characterized by particular resistance patterns [1–5]. In the early 1990s the emergence of a new clone was observed and shortly after *S.* Typhimurium DT104 gained an epidemiological importance in different animal reservoirs and subsequently in humans. Unlike the previous *S.* Typhimurium phage types with plasmid-mediated resistance, DT104 resistance genes for pentaresistant profile (ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline) were located in a genome-integrated gene cluster containing also class I integrons [1, 3, 4, 6, 7]. Recently *S.* Typhimurium DT104 have been reported from a variety of countries throughout the world [4, 5, 8, 9] and the high level of genetic homogeneity within strains of this lineage indicated a clonal spread of the pathogen [2, 3].

In Poland *Salmonella* isolates are not routinely typed and, therefore, the clonality of *S.* Typhimurium isolated from different sources remains unknown

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Table 1. *Antimicrobial break-points and the distribution of MICs in* S. *Typhimurium strains*

Antimicrobial name (abbreviation)	Break-points	MIC distribution (μg/ml*) – no. of strains														
		0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	32	64	128	256	512
Amoxicillin/clavulanic acid (Ac)	S⩽8–32⩽R							28	1	2	3	23				
Ampicillin (A)	S⩽8–32⩽R						21	6				30				
Ceftiofur	S⩽2–8⩽R					45	12									
Gentamicin	S⩽4–16⩽R						57									
Neomycin (N)	S⩽4–16⩽R							56				1				
Apramycin	S⩽8<R								57							
Spectinomycin (Sp)	S⩽64<R										3	25	2	27		
Chloramphenicol (C)	S⩽8–32⩽R							5	17	9			26			
Florfenicol (F)	S⩽8–32⩽R							11	20			17	9			
Nalidixic acid (Na)	S⩽16<R								32	6				19		
Ciprofloxacin	S⩽1–4⩽R	38		1	18											
Colistin	S⩽8<R								57							
Streptomycin (S)	S⩽8–32⩽R								1	20	1	11	24			
Tetracycline (T)	S⩽8<R							15			11	31				
Sulphamethoxazole (Su)	S⩽256<R											24	1			32
Trimethoprim/ sulphamethoxazole (Sx)	S⩽2<R						55			2						
Trimethoprim (Tm)	S⩽8<R								55			2				

* White background shows the range of antimicrobial concentrations used.

Table 2. *Antimicrobial resistance types in* S. *Typhimurium*

Code	Resistance type*	No. of strains
—	Susceptible	11
R-1	S	4
R-2	T	10
R-3	SuT	1
R-4	ASSuT	4
R-5	SSuTSxTmSp	1
R-6a	ACSSuTNaAcSpF	15
R-6b	ACSSuTNaSpF	3
R-6c	ACSSuTAcSpF	7
R-6d	ACSSuTSxTmNaAcNSpF	1

* Abbreviations for individual antimicrobials are given in Table 1.

along with the genetic background of the multi-resistance observed. In 2003, public health authorities in Poland were notified of 16 600 salmonellosis cases in humans. The incidence rate reached 43·5 cases per 100 000 inhabitants which was a decrease from 54·1 cases in the previous year [10]. Following *S*. Enteritidis (82·8%), *S*. Typhimurium was originally the second most prevalent serovar (3·8%) in Poland among both patients and asymptomatic carriers. However, within the last 2 years, *S*. Hadar has been more frequently found (4·8% and 5·0% in 2002 and 2003 respectively). This marked a significant change in epidemiology of *Salmonella* in humans compared to 1999 when *S*. Hadar represented ~1·0% of isolates [10].

Epidemiological investigations identified poultry, egg products and other food of animal origin as the most important vehicles for human salmonellosis in Poland [11]. According to National Veterinary Research Institute data, *S*. Enteritidis (57·1%), *S*. Typhimurium (11·2%) and *S*. Hadar (6·6%) were the most frequent serovars found in poultry during the years 2000–2002. *S*. Typhimurium was found in 22·8% of swine isolates. The frequency of antibiotic resistance remained low in *S*. Enteritidis but it has increased dramatically in *S*. Typhimurium in both animal and human isolates indicating the appearance of *S*. Typhimurium DT104 in Poland [11, 12].

The aim of the present study was, therefore, to characterize *S*. Typhimurium isolated from poultry, swine and animal feed and investigate the genetic background of the observed multiresistance.

METHODS

Fifty-seven *S*. Typhimurium isolates were selected for study based on the results of routine antimicrobial

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susceptibility testing performed by a standard agar diffusion method, the isolates represented the range of resistance profiles observed. The strains were isolated at veterinary laboratories in Poland during the years 1979–1998 ($n=13$) and 2000–2002 ($n=44$) from poultry ($n=23$), swine ($n=28$) and animal feed ($n=6$) and they were serotyped at the National Veterinary Research Institute.

The MIC values for the antibiotics listed in Table 1 were determined using the semi-automated, two-fold microbroth dilution method (Sensititre, Trek DS, East Grinstead, UK). The results were interpreted according to NCCLS guideline [13]. Isolates resistant to three or more antimicrobial classes were classified as multiresistant.

The presence of integrons in genome DNA was detected by a polymerase chain reaction as previously described [7]. Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, New York, NY, USA) as well as 3′-CS and att1-F primers (DNA Technology ApS, Aarhus, Denmark) were used to obtain ~1100 bp and ~1300 bp amplicons of the aminoglycoside resistance gene cassette *ant(3″)-Ia* and *pse-1* gene cassette for β-lactamase resistance respectively. The products were separated by agarose electrophoresis and compared visually against Gen Ruler 100 marker (MBI Fermentas, Vilnius, Lithuania). *S.* Typhimurium DT104 No. 9616368 was used as the positive control for the PCR set-up [7].

Phage typing was done according to the system of Anderson et al. as described previously [14].

Plasmids were extracted by alkali lysis method and separated according to molecular size by agarose electrophoresis [15]. Plasmids of *E. coli* 39R861 and V517 were used as molecular markers. Approximate molecular weights were determined using Bio-Profil software (Vilber Lourmat, Marne-La-Vallee, France).

Genotyping was performed by *Xba*I and *Bln*I macrorestriction followed by pulsed-field gel electrophoresis (PFGE). DNA samples were prepared with minor modifications according to standardized laboratory protocol for molecular subtyping of *E. coli* [16]. The gels were run at 6 V/cm and 14 °C for 20 h in CHEF Mapper (Bio-Rad, Marnes-la-Coquette, France). Pulse times were ramped from 2 s to 64 s and a 120° reorientation angle was applied. *S.* Braenderup genome DNA (strain no. H9812, CDC, Atlanta, GA, USA) was used as a molecular weight marker. The BioNumerics version 3.5 software (Applied Maths BVBA, Sint-Martens-Latem, Belgium) was applied for image analysis using UPGMA dendrogram, Dice coefficient with an optimization of 1·5% and 1·1% position tolerance. Both enzymes' DNA restriction digest profiles were used to create a composite dataset and the *Bln*I/*Xba*I internal weight profile ratio was 1:1.

Table 3. *Plasmid profiles observed in* S. *Typhimurium*

Code	Plasmid profile	No. of strains
P-1	98·0 kb	17
P-2	98·0, 2·7 kb	8
P-3	98·0, 6·1 kb	8
P-4	83·0, 3·9, 3·0 kb	3
P-5	98·0, 3·9 kb	3
P-6	98·0, 2·4 kb	2
P-7	98·0, 6·1, 3·9 kb	2
P-8	98·0, 6·9 kb	2
P-9	6·9 kb	1
P-10	114·0, 83·0, 57·0, 2·4 kb	1
P-11	98·0, 3·0, 2·4 kb	1
P-12	98·0, 3·0, 2·7 kb	1
P-13	98·0, 3·4 kb	1
P-14	98·0, 3·9, 2·9 kb	1
P-15	98·0, 3·9, 3·0 kb	1
P-16	98·0, 3·9, 3·4 kb	1
P-17	98·0, 64·0 kb	1
P-18	98·0, 7·2, 2·4 kb	1
P-19	98·0, 7·4, 6·9 kb	1
P-20	98·0, 74·0 kb	1

RESULTS

MIC distribution is shown in Table 1 and the characteristics of the tested *S.* Typhimurium strains are shown in the Figure. Eleven strains were susceptible to all antimicrobials, 15 were resistant to one or two antimicrobials and the remaining 31 strains were multiresistant (Table 2). Both resistance and multiresistance were more frequently observed in strains isolated during 2000–2002 than in those from 1979–1998 (9 and 29, and 5 and 2 strains respectively). Although resistant strains were identified from different sources, most of the multiresistant isolates (48·4%) originated from poultry. A total of nine resistance profiles was noted and the pentaresistant profile (ACSSuT) was found in 26 strains. Twenty-four of these strains belonged to the predominating phage type DT104, and the remaining two were U302 or non-typable (NT).

Class 1 integrons were identified in all pentaresistant *S.* Typhimurium strains. The first integron-positive

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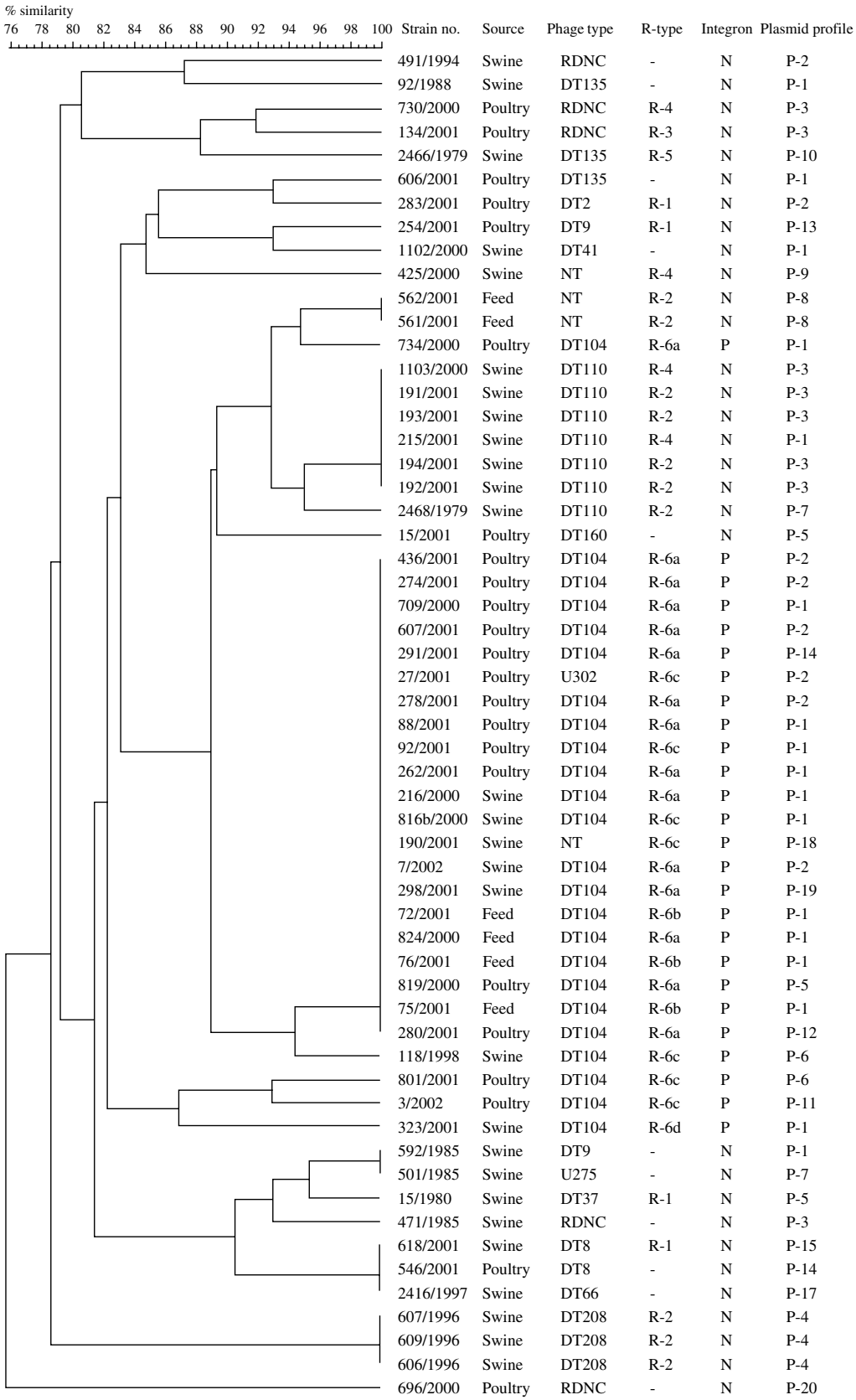

Fig. Dendrogram of *S*. Typhimurium genome macrorestriction profile similarity and other epidemiological characteristics. Vertical lines at 100% similarity indicated homologous PFGE pattern. The abbreviations for resistance types and plasmid profiles are given in Tables 2 and 3.

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strain was isolated from swine in 1998, and thereafter others were found in poultry, swine and animal feed. Besides DT104, U302 and NT, nine other phage types were distinguished (Fig.) with differences in the distribution of phage types evident among poultry, swine and feed isolates, i.e. DT37, DT41, DT66, DT110, and DT208 in swine, and DT160, DT2 and U302 exclusively in poultry. Nine strains were either non-typable (NT, $n=4$) or showed different reactions with no confluent lysis (RDNC, $n=5$).

None of the tested strains was plasmid-free. A plasmid of $\leqslant 98$ kb was found in 52 isolates and 15 additional plasmids ranging from 114 kb to 57 kb and 7·2–2·2 kb were noted (Table 3).

PFGE typing revealed 17 *Xba*I band patterns consisting of 13–21 DNA fragments and 18 *Bln*I profiles of 8–11 bands ranging in size from $\leqslant 20{\cdot}5$ kb to 1135 kb. Most of the strains that were indistinguishable with one enzyme were also clustered together with the other enzyme. The epidemiological concordance (E) of both enzyme digests was 0·79. Some discrepancies, however, were noted. Strains showing the most prevalent *Xba*I profile ($n=21$) were divided into two *Bln*I profiles comprising 19 and two isolates and likewise the predominant *Bln*I profile ($n=23$) was subdivided further by *Xba*I of 20 isolates and three single distinct strains. A total of 26 different profiles were found on combination of both enzyme macrorestriction profiles. As shown in the Figure the profile similarity of the investigated *S.* Typhimurium strains reached 76%. Twenty PFGE profiles were unique for single strains and the remaining strains were gathered in six genomic clusters. The predominant cluster comprised 21 multiresistant and integron-positive strains belonging to phage type DT104, U302 or NT whereas the other five clusters consisted of 6, 3, 3, 2, and 2 isolates.

DISCUSSION

Antimicrobial resistance in bacteria is nowadays one of the major threats to public and animal health and much attention has been given to multiresistant *S.* Typhimurium DT104 which is one of the most prevalent clones in different countries [4, 5, 17, 18]. An increasing number of antimicrobial-resistant *Salmonella* isolates have also been observed in Poland with the greatest prevalence in *S.* Typhimurium strains originating from animals [12]. A clinically significant feature of multiresistant *S.* Typhimurium infection elsewhere is the reduced antimicrobial efficacy of fluoroquinolones for treatment [8, 12, 19, 20] and this has also been seen in our study (Table 1). Earlier Szych et al. [11] reported up to 50% multiresistance in humans isolates with pentaresistance being predominant amongst 48 resistance profiles noted in Poland.

In the present investigation we have characterized *S.* Typhimurium isolated from poultry, swine and animal feed and investigated the genetic basis of the observed multiresistance. A major cluster of indistinguishable strains, by means of PFGE, comprised 21 strains of the 'DT104 family' (DT104 and U302) and these shared a common feature of a gene cluster linked to chromosome-mediated multiresistance. Although integron-mediated multiresistance has been found in a single extended-spectrum β-lactamase-producing *S.* Mbandaka human isolate [11], these elements have not been reported in *S.* Typhimurium in Poland so far. We can, therefore, firmly state that integron-positive *S.* Typhimurium DT104 appeared in Polish swine as far back as 1998 and we have noted the presence of the multiresistant clone in poultry and animal feed in Poland since 2000. As the PFGE profile and the other epidemiological markers of the single DT104 strain (816b/2000) isolated from imported animals were indistinguishable from several other isolates, we conclude that the possible source of introduction of the phage type to Poland was through imported, chronically infected animals. The example of *S.* Typhimurium DT208 strains showing unique characteristics found in animals imported from a Mediterranean country and different from *S.* Typhimurium clones originating from Poland also gives support to this conclusion.

In our study DT104 strains were also found in feed which with other environmental factors might also serve as a transmission vector for the animal population [1, 2, 4, 9]. This is in agreement with previous observations of multidrug-resistant *S.* Typhimurium DT104 spreading clonally within and among different geographical sites and with similar or highly homogenous PFGE profiles [3, 5, 6]. However, some minor variations in clonal populations have been observed in plasmid and genome profiling [1, 4, 8, 21].

The largest *S.* Typhimurium cluster found in the present study comprised several multiresistant and integron-positive isolates belonging mostly to DT104 or the related U302 phage type. Although these strains shared the same PFGE profile, they displayed a variety of plasmid and resistance profiles. This may

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be due to different sources and routes of infection that favour genetic rearrangements occurring during the transmission of bacterial populations [1, 4, 6, 7, 22]. Changes in bacteriophage receptors can also result in the appearance of different phage types showing a high level of genome homogeneity [4, 5, 23].

Since the strains in the present study were not selected at random it is not possible to show the exact estimates for the prevalence of *S*. Typhimurium phage types in animals and feed. Most of the phage types other than DT104 were found in a few or single isolates and the observed differences in phage type distribution might be explained by different management practices of poultry and swine [9] or animal host specificity of particular phage types [2]. The epidemiologically relevant observation, however, is that the frequency of *S*. Typhimurium DT104 is increasing in Poland. A broader investigation may help to elucidate the spread and the prevalence of *S*. Typhimurium DT104 in this country and its relationship to isolates from different parts of the world [1, 17], although the DT104 strains recovered here appear to be highly homogeneous.

It is worth noting that most of the tested strains carried extrachromosomal DNA elements of molecular weight similar to the *Salmonella*-specific virulence plasmid observed in *S*. Typhimurium [1, 17, 22, 24] which might have implications for the course and severity of the infection in humans [1, 25].

Molecular typing has been used for characterization of a variety of *Salmonella* serovars. The efficacy of typing methods depends on the polymorphism of the analysed feature and usually several methods are needed for precise differentiation of *S*. Typhimurium strains [1, 3, 4, 8, 15, 26]. In our study combined enzyme macrorestriction achieved a discrimination index of 0·85 which was slightly less than that shown by plasmid profiling (0·88) although the limitations of the latter resulting from inherent mobility of plasmid elements should be borne in mind [3, 4, 22, 23, 26]. The discriminatory power of antimicrobial resistance typing and phage typing was lower (0·82 and 0·80 respectively).

We have identified some characteristics of *S*. Typhimurium isolated from animals in Poland but the public health consequences of our findings remain unknown. Although *S*. Hadar has recently become the second most frequent *Salmonella* serovar found in humans in this country the prevalence of *S*. Typhimurium has been stable in recent years [10]. The question remains as to what are the possible routes for *S*. Typhimurium to reach the human population. The Dutch study found a different phage type distribution in human and animal strains which argues against direct transmission of the pathogen [9]. Danish reports, however, clearly demonstrate that domestic as well as imported food of animal origin must be considered as an undisputed vector [27]. Imported food does not constitute a major part of the Polish market and unlike Denmark and some other countries infections acquired abroad are rare [10, 18, 27]. Thus, *Salmonella* infections most probably originate from national livestock [1, 3, 5, 8, 9, 15, 17] but further studies are necessary to detail the routes of infection from animal to man. Although in some countries the *S*. Typhimurium epidemic is in decline [3] we foresee, based on our limited results, a continuous rise in *S*. Typhimurium infections as clones move to other geographic sites to pose new public and animal health risks.

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