# Chloramphenicol resistance genes in *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolated from human and animal sources in Hungary

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**ABSTRACT**: The presence of the chloramphenicol resistance genes *cat, cmlA, flo*, and the role of plasmids and class 1 integrons in the spread and persistence of chloramphenicol resistance were investigated on a collection of 40 *Salmonella enterica* serovar Typhimurium strains isolated from animals and humans in Hungary, by PCR and conjugation. Three groups of chloramphenicol resistant strains were identified. Eleven animal and 13 human isolates harboured the *flo* gene, encoding resistance to chloramphenicol and florfenicol, and possessed integrons of 1.0 Kb and 1.2 Kb typically found on the multidrug resistance island of *S.* Typhimurium DT104. Fifteen human strains had two different chloramphenicol resistance genes: the *catB3* gene, identified as a gene cassette within a 1.45 Kb integron, and the *catA* gene, both of which were located on and transferred by a 140 Kb plasmid from a representative strain to the *E. coli* recipient via conjugation. A single animal strain had the *catA* gene alone, which was also transferred by a 35 Kb plasmid via conjugation. These three groups of strains belonged to three distinct genetic clusters, as it was revealed by macrorestriction analysis of 18 selected strains. This study provides information on the versatile genetic background of the chloramphenicol and florfenicol resistances in *S.* Typhimurium in Hungary and points to the significance of mobile genetic elements such as conjugative R-plasmids and integrons in the spread and persistence of chloramphenicol resistance genes. The results also indicate the predominance of the *flo* gene among animal strains and its appearance among human strains in Hungary.

**Keywords**: *S.* Typhimurium; chloramphenicol resistance; *flo*; *cat*; conjugative R-plasmid

Chloramphenicol (Cm) was considered as the drug of choice to treat salmonellosis in human and veterinary medicine over a long period of time, which has led to the selection of Cm-resistant strains. The resistance to Cm is known to be mediated by the plasmid-located enzymes called chloramphenicol acetyltransferases (CAT) (Cannon et al., 1990), or by the nonenzymatic chloramphenicol resistance gene *cmlA* (Dorman and Foster, 1982), that encodes an efflux pump. The CAT enzymes encoded by the *cat* family of genes are widespread in Gram-negative bacteria. The *cat* genes are subcategorised into *catA* and *catB* groups, which are not significantly related to each other (White et al., 1999). The *catB* genes have been reported to be located on mobile genetic elements called integrons (Stokes and Hall, 1989) in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (Bunny et al., 1995; Tosini et al., 1998) and in many other bacteria such as e.g. *Enterobacter aerogenes*, *Enterobacter cloaceae* (Bunny et al., 1995) and *Pseudomonas aeruginosa* (White et al., 1999).

Since 1994 the use of Cm has been prohibited in farm animals (2701/94/EC), but in 1995 its syn-

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thetic, fluorinated analogue called florfenicol (Ffc) was approved for the treatment of bovine respiratory pathogens. Neither the *cat* nor the *cmlA* genes confers resistance to Ffc. The first gene encoding resistance to Ffc was identified from the fish pathogen *Pasteurella piscicida* (later renamed as *Photobacterium damselae* subsp. *piscicida*) and published as *pp-flo* by Kim and Aoki (1996). Only a few years later, the *flo* gene was also identified in numerous other bacterial species, in different *Salmonella* serovars such as *S.* Typhimurium (mainly of definitive phage type 104) (Bolton et al., 1999), *S.* Agona (Cloeckaert et al., 2000b), *S.* Paratyphi B (Meunier et al., 2002), *S.* Albany (Doublet et al., 2003), *S.* Newport (Meunier et al., 2003) and also in *Escherichia coli* (Cloeckaert et al., 2000a; Keyes et al., 2000) isolated from different sources. In serovars *S.* Typhimurium, *S.* Albany, *S.* Agona and *S.* Paratyphi B the *flo* gene (also called as $flo_{St}$, *floR* and *cmlA*-like) showed 97% identity to *pp-flo*, and was found within a chromosomal cluster of antibiotic resistance genes as part of a 43 Kb genomic island called SGI1 (Salmonella genomic island 1) (Boyd et al., 2001). In contrast, in serovar *S.* Newport the *flo* gene was identified on a plasmid (Meunier et al., 2003).

Since there is no information regarding the genetic determinants responsible for the persistence of Cm resistance among *Salmonella* isolates in Hungary, the aim of this study was to investigate the genetic background of Cm resistance as well as the epidemiological relationship of the isolates in a Hungarian collection of *S.* Typhimurium strains of animal and human origin.

## MATERIAL AND METHODS

### Bacterial strains and phage-typing

In total, 40 *S.* Typhimurium strains, 12 animal and 28 human isolates were examined. The 12 animal isolates consisted of 4 turkey, 3 goose, 2 duck, 2 pig and 1 chinchilla strains isolated during the 1997 to 1999 period at different institutes and laboratories of the Hungarian Veterinary and Food Control Service. The 28 human strains were isolated at the Country Institutes of Public Health and Medical Officer Service during the same period of time. All strains were confirmed to be *Salmonella* by standard biochemical tests and serotyped according to the Kauffmann-White scheme. The strains were grown and stored on nutrient agar (Oxoid No. 2) at room temperature without antibiotic selection. The strains were phage-typed with the method of Anderson et al. (1977).

### Antibiotic susceptibility testing

Antibiotic susceptibility testing was done by disk diffusion method on Mueller-Hinton agar supplemented with sheep blood using antibiotic disks (Oxoid Ltd., Basingstoke, UK) as follows: ampicillin (A, 20 μg), chloramphenicol (C, 30 μg), streptomycin (S, 30 μg), tetracycline (T, 30 μg), gentamycin (G, 20 μg), kanamycin (K, 30 μg), sumetrolim (Sxt, 25 μg), cefoperazone (Cfp, 75 μg), cephalexin (Cfl, 30 μg), cefuroxim (Cxm, 30 μg) and nalidixic acid (N, 30 μg). MIC values for Cm resistance were determined by using *E*-test strips (Ab Biodisk, Solna, Sweden) on Mueller-Hinton agar. The test can be applied for the determination of MIC values for Cm of up to 256 μg/ml. The zones of growth inhibition were evaluated according to NCCLS standards (National Committee for Clinical Laboratory Standards, 2000).

### PCR reactions and sequencing

PCR reactions were conducted essentially as described earlier (Gado et al., 2003) using primers and PCR conditions specific for *catA* (Guerra et al., 2001), *flo* (Bolton et al., 1999), *cmlA* (Guerra et al., 2001), or class 1 integron (Levesque et al., 1995). The amplicons obtained were detected by horizontal agarose gel electrophoresis in 2% agarose gels, stained in ethidium-bromide solution, photographed under UV and evaluated using the Bio-Rad Gel-Documentation system and the Quantity One software (Bio-Rad Laboratories, CA, USA).

For partial sequencing, the amplicons were collected and purified from the 2% agarose gel using the Qiaquick Gel Extraction Kit (Qiagen Inc., Heidelberg, Germany) according to the manufacturer's instructions. Cycle sequencing was performed in the Biological Research Center of the Hungarian Academy of Sciences by applying the dye-terminator method using DNA sequencer model ABI 373. The primers used for PCR were applied as sequencing primers. In addition, internal primers were designed and applied to continue the process until the resistance genes inserted in each

amplicon were identified. The obtained sequences were analysed by the BLAST program at http://www.ncbi.nih.gov and compared to the sequences of the GenBank.

### Plasmid profile analysis

Plasmid preparation was done by the method of Kado and Liu (1981). Agarose gel electrophoresis of the preparations was performed as described by Meyers et al. (1976), using a 0.75% agarose in a vertical system. The gels were stained, photographed and evaluated as described for PCR amplicons. The approximate sizes of plasmids of the *S.* Typhimurium strains NB156 and M152 were estimated by comparison with the reference plasmids of *E. coli* V517 (2.4-54 Kb), *E. coli* RI (94 Kb) and *E. coli* R27 (170 Kb), using the Quantity One software (Bio-Rad Laboratories, CA, USA).

### Genomic fingerprinting by pulsed-field gel electrophoresis (PFGE)

Eighteen strains were selected for cluster analysis. Pulsed-field gel electrophoresis was carried out according to the standardized Salmonella protocol of the CDC PulseNet (Swaminathan et al., 2001). The digestion of the plugs was done with 0.4 U/µl *XbaI* enzyme. The gel was run in CHEF DR II machine (Bio-Rad Laboratories, CA, USA) with 6 V/cm, 2 s–64 s switch time, at 14°C for 22 hours. The Lambda ladder standard (Bio-Rad Laboratories, CA, USA) was used as molecular marker. The gel was stained and photographed as described for PCR amplicons. PFGE-generated DNA profiles were entered into the Fingerprinting II Software (Bio-Rad Laboratories, CA, USA) for analysis. Cluster analysis was performed by the unweighted pair-group method (UPGMA) with arithmetic averages, and DNA relatedness was calculated on the basis of the Dice coefficient. In addition, all PFGE patterns were visually compared and assigned by letter identification according to the established guidelines of Tenover et al. (1995).

### Transfer of Cm resistance

The *catA* positive *S.* Typhimurium NB156 and the *catA* and 1.45 Kb (*catB3)* integron positive M152 strains were selected as donors and the rifampin (Rif) resistant *E. coli* K12 J-53 strain was chosen as a recipient in conjugation experiments. Overnight-grown Luria-Bertani (LB) broth cultures of the donor and the recipient cells were mixed at a ratio of 1 : 1 and plated onto the surface of LB plates for conjugation. Exconjugants were selected on LB agar containing Cm and Rif in final concentrations of 20 µg/ml and 150 µg/ml, respectively. From each conjugation experiment 3 colonies suspected to be exconjugants were tested for $MIC_{Cm}$ and for the presence of the *catA* gene, class 1 integron and transferred plasmid(s) as described above.

## RESULTS AND DISCUSSION

The origin, phage type, Cm resistance gene(s) and class 1 integron content of the 40 *S.* Typhimurium strains are summarized in Table 1. In *Salmonella*, the *cmlA* gene has been shown only recently in the emerging clone of *Salmonella enterica* serotype [4,5,12:i:–] by Guerra et al. (2001). As expected, none of our strains possessed *cmlA* type gene.

In DT104 strains, Cm resistance is encoded by the *flo* gene which confers resistance to both Cm and Ffc. It is located chromosomally, between two integrons of the salmonella genomic island 1, a 1.0 Kb that codes for *aadA1* and confers streptomycin-spectinomycin resistances and a 1.2 Kb that codes for *pse-1* and confers ampicillin resistance (Arcangioli et al., 1999; Bolton et al., 1999; Briggs and Fratamico, 1999). The DT104-like antibiotic resistance gene cluster, including the *flo* gene, has also been found in *S.* Typhimurium DT120 (Boyd et al. 2001). In the present study, the *flo* gene, as well as the 1.0 Kb and 1.2 Kb integrons characteristics of the above-mentioned genomic island, were found in fifteen DT104 and one DT120 strain, irrespective of their animal or human origin. Interestingly, *flo* was also identified in 3 animal and 3 human strains of the RDNC phage type and in 2 animal U302 strains. Since these strains had the same 1.0 Kb and 1.2 Kb integrons as the DT104 strains, we surmised that they were closely related to them and therefore examined the strains by PFGE. As suspected, all the *flo* positive strains that were selected for macrorestriction analysis had similar PFGE patterns (pattern B: lanes from 2 to 14 in Figure 1) and grouped to the same cluster (lanes from 2 to 14 in Figure 2). This finding supports the hypothesis that a single clone of DT104 may have segregated into

Table 1. Categories of 40 Cm-resistant *S.* Typhimurium strains according to their Cm resistance gene(s), class 1 integron content, origin and phage type

| Cm resistance gene(s) and class 1 integrons | Origin and phage type* | |
|---|---|---|
| | human ($n$ = 28) | animal ($n$ = 12) |
| *cmlA* | – | – |
| *flo*, 1.0 kb and 1.2 kb | 13[a] | 11[b] |
| *catA* | – | 1[c] |
| *catA*, *catB3*, 1.45 kb and 2.05 kb | 15[d] | – |

*the distribution of the different phage types among the strains within a group was as follows
[a]DT104: 10; RDNC (reacts but does not conform): 3
[b]DT104: 5; U302: 2; DT120: 1; RDNC: 3
[c]DT8: 1
[d]UNTY: untypable (refers to strains that do not react with any of the phages): 10; DT193: 5

different subclones with altered phage types (Ridley and Threlfall, 1998; Sandvang et al., 1998). Since the data suggested a chromosomal location of the *flo* gene, we did not examine the transferability of the gene in these strains.

Only one animal isolate did not possess the *flo* gene. The strain had been isolated from a goose, had no integron and the *catA* gene encoded its Cm resistance. The strain contained two large plasmids of 100 Kb and 35 Kb, and the latter transferred by conjugation to the *E. coli* recipient strain (Table 2). The transfer of this large conjugative R-plasmid resulted in the appearance of the *catA* gene in the exconjugants and the same level of resistance as that of the donor (Table 2). Based on its PFGE pattern (pattern A, lane 1 in Figure 1), the strain represented a distinct genetic cluster (lane 1 in Figure 2).

Fifteen human strains produced the expected amplicon with the *catA* primers and had the same patterns consisting of 1.45 Kb and 2.05 Kb integrons (Table 1), but none of the animal strains showed this pattern. The integrons of strain M152 were isolated, purified and sequenced by two independent PCR reactions. Sequencing revealed the presence of *aadB* (aminoglycoside adenyltransferase) and *catB3* (chloramphenicol acetyltransferase) genes in the 1.45 Kb integron, and the presence of *oxa1* (beta-lactamase) and *aadA1* (aminoglycoside adenyltransferase) genes in the 2.05 Kb integron. The sequences showed a 99-100% identity with that of the integron type 1 (In-t1) (Ac. No.: AJ 009818) and integron type 2 (In-t2) (Ac. No.: AJ009819) of *S.* Typhimurium, respectively (Tosini et al., 1998). Conjugation experiments carried out using the M152 strain as a donor resulted in exconjugants having the 140 Kb plasmid of the donor strain only. The integrons and the *catA* gene was also detected in the exconjugants (Table 2), indicating that these resistance elements are located on the 140 Kb conjugative R-plasmid as observed by Tosini et al. (1998). Four of the 15 strains were selected for macrorestriction analysis. The PFGE pattern of these four strains (pattern C, lanes from 15 to 18 in Figure 1) was similar to each other but distinct from that of the other two groups of strains

Table 2. $MIC_{Cm}$ values, Cm resistance genes, class 1 integron(s) and plasmid(s) of the M152 and NB156 donor strains and of their *E. coli* K12 J-53 exconjugants

| Strain | MIC for Cm (μg/ml) | *catA* | 1.45 kb (*catB3*) | Plasmid(s) (kilobase-pair) |
|---|---|---|---|---|
| M152 donor | 256 | + | + | 140, 45, 7 |
| M152 × *E. coli* K12 J53-exconjugants | 256 | + | + | 140 |
| NB156 donor | 256 | + | – | 100, 35 |
| NB156 ×*E.coli* K12 J53-exconjugants | 256 | + | – | 35 |

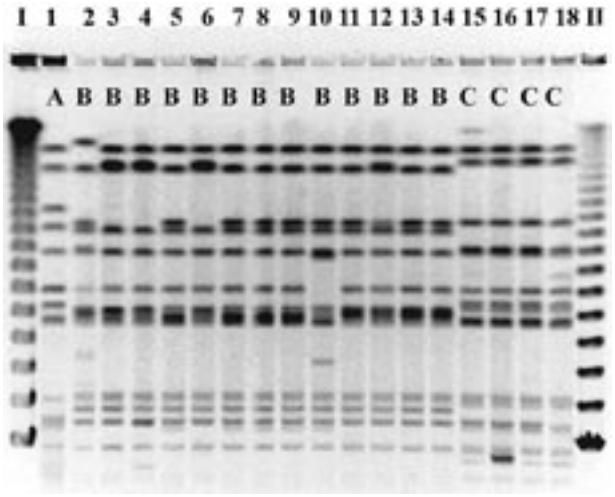


Figure 1. Pulsed-field gel electrophoresis profiles of 18 Cm resistant *S.* Typhimurium strains selected for cluster analysis

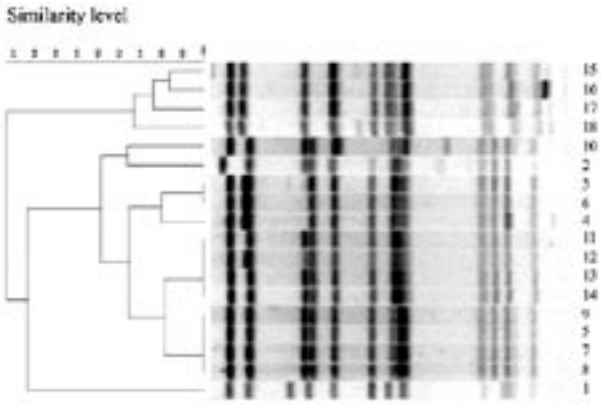


Figure 2. Dendrogram generated by Fingerprinting II software showing the results of cluster analysis on the basis of PFGE fingerprinting

The designations, origin, definitive phage type (DT) and Cm resistance gene(s) of 18 *S.* Typhimurium strains that were selected for PFGE analysis and grouped to patterns A, B and C are as follows:
I and II: Lambda ladder standard
pattern A: lane 1 = NB 156, goose, DT8, *catA*
pattern B: lane 2 = NB215, turkey, DT120, *flo*; lane 3 = NB137, turkey, RDNC, *flo*; lane 4 = NB 216, turkey, RDNC, *flo*; lane 5 = NB 289, chinchilla, RDNC, *flo*; lane 6 = M519, human, RDNC, *flo*; lane 7 = NB129, pig, DT104L, *flo*; lane 8 = NB270, duck, DT104L, *flo*; lane 9 = NB285, duck, DT104L, *flo*; lane 10 = NB128, pig, DT104L, *flo*; lane 11 = NB188, goose, DT104L, *flo*; lane 12 = M117, human, DT104L, *flo*; lane 13 = NB140, goose, U302, *flo*; lane 14 = NB204, turkey, U302, *flo*
pattern C: lane 15 = M98, human, DT193, *catA*, *catB3*; lane 16 = M630, human, DT193, *catA*, *catB3*; lane 17 = M152, human, UNTY, *catA*, *catB3*; lane 18 = M182, human, UNTY, *catA*, *catB3*
Lane serial numbers in Figure 2 correspond to lane numbers in Figure 1

mentioned above. The strains represented another genetic cluster (lanes 15–18 in Figure 2).

In summary, the *S.* Typhimurium strains described here can be categorized into three groups on the basis of the genes encoding their Cm resistance. The first group includes all the DT104 and some non-DT104 strains of both animal and human origin that have the *flo* gene and clustered to the same genetic clone as the DT104 strains. In contrast, in most of the non-DT104 strains the A and/or B subtypes of the *cat* gene was identified. In one group the *catA* gene alone was identified and located outside the integron. In the other group, in addition to the non-integron located *catA* gene, a *catB3* gene was also identified as part of a 1.45 Kb class 1 integron. In both cases the *cat* genes were located on large, transferable, conjugative plasmids, which could facilitate the spread, acquisition and persistence of Cm resistance, even a decade after the prohibition of Cm as a therapeutic agent.

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