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ORIGINAL PAPER

Molecular Characterization of Class 1 Integrons in Clinical Strains of *Salmonella* Typhimurium Isolated in Slovakia

ĽUBICA MAJTÁNOVÁ,[1] TOMÁŠ MAJTÁN[2] and VIKTOR MAJTÁN[1]*

[1] Department of Microbiology, Slovak Medical University, Bratislava, Slovak Republic
[2] Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovak Republic

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Abstract

The presence of class 1 integrons was investigated in 156 epidemiologically unrelated *Salmonella* Typhimurium isolates. Of these 156 isolates, 70 were of definitive phage type DT104 and 86 were strains of various phage type, RDNC and untypable, designated here as non-DT104 strains. Integrons were found in 47 of DT104 isolates (67.1%), while in all strains with characteristic pentaresistance (R-type ACSSuT) two integrons 1.0 kb and 1.2 kb in size were found. Among 86 non-DT104 strains, integrons with sizes of 1.6 kb and 1.9 kb in four multidrug-resistant strains DT193 and U302 were found. The integrons from selected strains were further sequenced and the *aadA1*, *aadA2*, *dhfr1*, *dhfr12* and bla_{PSE} genes were found embedded in cassettes.

Key words: *Salmonella* Typhimurium, class 1 integrons, multidrug resistance, resistance genes

Introduction

Resistance to antimicrobials is one of the best known examples for the rapid response of bacterial populations to a selective pressure. The selective advantage of resistant strains can be explained by the acquisition of resistance genes by horizontal gene transfer and/or by the accumulation of point mutations in the target genes. Especially in the case of the horizontal spread of resistance determinants between bacteria, mobile genetic elements such as plasmids, phages, transposons and integrons/gene cassettes are usually implicated (Recchia and Hall., 1995; Carattoli, 2001; Schwarz and Chaslus-Dancla, 2001; Guerra *et al*., 2004; Miko *et al.*, 2005). Integrons are genetic elements that are able to capture gene cassettes from the environment and incorporate them by using site-specific recombination (Mazel, 2006). Class 1 integrons, mostly found as part of the Tn*21* or Tn*402* transposon family are the most widely disseminated ones among the members of the *Enterobacteriaceae* family. Five classes of integrons carrying antibiotic resistance gene cassettes have been reported so far based on the homology of their integrase genes (Collis *et al.*, 2002).

Salmonella enterica is a zoonotic species that can acquire its resistance in livestock. The resulting animal food products are important vectors for the transfer of resistant bacteria from animals to humans (Threlfall, 2002; O'Hare *et al.*, 2004; Randall *et al.*, 2004). *Salmonella enterica* serovar Typhimurium (hereafter abbreviated as *S*. Typhimurium) is one of the most frequent serovars causing human salmonellosis. Many strains of *S*. Typhimurium are multidrug-resistant (MDR) and their public health importance has led to many investigations on their emergence and prevalence (Threlfall, 2002). *S.* Typhimurium isolates are usually differentiated by phage typing, denoting the phage types as definitive types (DT) (Anderson *et al.*, 1977), and by several fingerprinting methods.

The aim of this study were: 1) to identify the phage type of clinical *S*. Typhimurium strains isolated in Slovakia, 2) to determine antimicrobial susceptibility and to detect class 1 integrons in these isolates, and 3) to analyze genes cassettes content of class 1 integrons in selected MDR strains.

* Corresponding author V. Majtan, Department of Microbiology, Slovak Medical University, Limbová 12, 833 03 Bratislava, Slovak Republic. phone: + 421-2-59369445, fax: + 421-2-59369245; **e-mail: viktor.majtan@szu.sk**

Experimental

Material and Methods

Bacterial strains. One hundred and fifty six epidemiologically unrelated *S.* Typhimurium strains were analyzed. These strains were isolated from sporadic human cases with enteritis from different regions of Slovakia.

Phage typing. Phage typing was performed according to Anderson *et al*. (1977) in the National Reference Center for Phage Typing of Salmonellae at Slovak Medical University, Bratislava.

Antibiotic susceptibility testing. The strains were tested for susceptibility against ampicillin (A), ceftriaxone (CRO), chloramphenicol (C), gentamicin (G), streptomycin (S), sufisoxazole (Su), tetracycline (T), ciprofloxacin (CIP), trimethoprim (TMP) and trimethoprim-sulfamethoxazole (SxT) by the disk agar diffusion method on Mueller-Hinton agar plates according to the CLSI (formerly NCCLS) standards (1997). *Escherichia coli* ATCC 25922 was used as a reference strain. All antibiotic disks used were supplied by Oxoid (UK).

Class 1 integron content: PCR and sequence analysis. The PCR detection of class 1 integron content was performed essentially as described by Majtán *et al.* (2004). The PCR products were excised after electrophoresis on 1% agarose gel and subsequently purified using the QIAEX II kit (QIAGEN, Germany). Sequencing was performed in both directions by using BigDye method on ABI 3100-Avant Genetic Analyzer (Applied Biosystems, CA, USA). Obtained sequences were combined together using Vector NTI Advance 10 (Invitrogen, USA). The similarity search was carried out using the BLAST algorithm (McGinnis and Madden, 2004).

Results and Discussion

In contrast to the occurrence of *S*. Enteritidis strains, the percentual participation of *S*. Typhimurium strains in the etiology of salmonellosis in Slovakia is low. However, the resistance and MDR to antimicrobial agents of this serovar are relevant.

Among the 156 epidemiologically unrelated, sporadic human isolates, 17 phage types (PTs) were identified (Tables I and II). DT104 phage type included 70 (44.9%) of isolates. Other PTs, designated as non-DT104 represented the PTs, such as DT1, DT6, DT12a, DT20a, DT29, DT36, DT37, DT41, DT67, DT68, DT92, DT99, DT119, DT125, DT193, U302. Thirty two strains were untypable (UT) and 15 strains reacted with typing phages, but did not conform to designated phage types (RDNC).

Using the CLSI standard method, among the 70 DT104 isolates up to 46 (65.7%) exhibited typical pentaresistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline (ACSSuT) (Table I). Sixteen strains from 86 non-DT104 isolates were MDR, mainly the PTs such as DT193, U302, DT12a, DT68 and untypable. Resistance to sulfizoxazole and streptomycin was the most frequent among the non-DT104 strains (40.1%), while among the DT104 strains comprised only 13 strains out of 70 (18.6%). None of the 156 isolates was resistant to ceftriaxone and only one isolate was found to be resistant to ciprofloxacin. MDR in the majority of DT104 strains is due to chromosomal integration of a 43-kb structure called *Salmonella* genomic island 1 (SGI1), comprised of genes coding for resistance to ampicillin (bla_{PSE-1}), streptomycin and spectinomycin (*aad*A2), chloramphenicol and florfenicol (*flo*R), sulfonamides (*sul*1) and tetracycline (*tet*G) (Boyd *et al.*, 2001). These isolates represent a single clone that has emerged as a dominant serovar Typhimurium clone globally and has been isolated from humans, most meat-producing animals, exotic birds, and various foods (Threlfall *et al.*, 2000; Threlfall, 2002; Butaye *et al*., 2006). SGI1 was subsequently found in serovar Typhimurium strains of PTs such as DT1, DT12, DT120 and U302 (Boyd *et al.*, 2002; Carrattoli *et al.*, 2002; Lawson *et al.*, 2002). The MDR region is located at the 3´end of SGI1 in a 13-kb region corresponding to a large class 1 integron with a complex structure named In104 (Boyd *et al.*, 2001; Levings *et al.*, 2005). The resistance genes *flo*R and *tet*G are bracketed by two class 1 integrons, 1.0 kb and 1.2 kb in sizes. PCR primers int1-F and int1-B targeting conserved segments of class 1 integrons were utilized to amplify the variable region of the integrons carrying at least one resistance gene cassette. For all DT104 strains exhibiting the typical pentaresistance phenotype two PCR amplicons of 1.0 kb and 1.2 kb in length were

Table I
Incidence of antimicrobial resistance in 70 DT104 *Salmonella* strains

Antibiogram	Number of strains (%)
A C S Su SxT T	2 (2.9)
A C S Su T TMP	1 (1.4)
A C S Su T	46 (65.7)
A S Su T	1 (1.4)
G S Su T	1 (1.4)
A S Su	1 (1.4)
T Su	4 (5.7)
S T	1 (1.4)
S Su	13 (18.6)
Total	70 (100)

Table II
Incidence of antimicrobial resistance in 86 non-DT104 *Salmonella* strains

Antibiogram	Number of strains (%)	Phage type
A CIP G C S Su SxT T TMP	1 (1.2)	DT193 (1)
A S Su T SxT TMP	4 (4.7)	DT193 (1), NT (2),U302(1)
A S Su T TMP	1 (1.2)	NT (1)
A G S Su T	1 (1.2)	NT (1)
A C S Su T	1 (1.2)	NT (1)
A S Su T SxT	1 (1.2)	NT (1)
S T Su SxT	2 (2.3)	DT12a (1), DT68 (1)
C S Su T	1 (1.2)	NT (1)
A S Su T	4 (4.7)	DT193 (2), NT (2)
S Su T	9 (10.5)	DT12a(1),DT68(1),DT6(1),DT92(1),RDNC(3) NT(2)
A S Su	6 (6.9)	NT (6)
A S T	1 (1.2)	NT (1)
Su T	1 (1.2)	NT (1)
Su S	35 (40.1)	DT37(1),DT41(5),DT6(2),DT99(1),DT68(1)DT67(2),DT20a(4),DT1(2),
		DT119(2),RDNC (8), NT(7)
Su A	1 (1.2)	DT68 (1)
A	2 (2.3)	NT (1), DT36 (1)
S	4 (4.7)	DT125 (1), NT (2), RDNC (1)
Su	10 (11.6)	DT41(3),DT1(1),DT29(1),RDNC(2),NT(3)
susceptible	1 (1.2)	RDNC(1)
Total	86 (100)	

NT = non typable; RDNC= reacted with typing phages but did not confirm to designated phage types

obtained. DT104 strain possessing resistance to ampicillin, sulfisoxazole and tetracycline contained only the 1.2 kb PCR amplicon (Table III). The integrons with PCR amplicons of 1.6 kb and 1.9 kb in size were observed in four non-DT104 MDR strains of DT193 and U302 PTs, respectively (Table III).

In the following step, two multidrug resistant DT104 and two non-DT104 strains were tested to unfold integron content (Table IV, Figure I). The first selected DT104 strain (30/04) with the typical pentaresistance phenotype yielded two different PCR amplicons: 1.0 kb and 1.2 kb. Sequence analysis of these amplicons revealed that each carried a single resistance gene cassette, *aad*A2 and bla_{PSE}, respectively. These two amplicons as well as gene cassettes are typical for the DT104 type (Poirel 1999; Ahmed *et al.*, 2005; Miko *et al.*, 2005). For the second selected DT104 strain (29/03) with resistance to ampicillin, streptomycin, sulfisoxazole and tetracycline, PCR and DNA sequencing analysis identified only one 1.2 kb PCR amplicon carrying a single resistance gene cassette

Table III
Integron patterns of *S.* Typhimurium strains

IP	Amplicons (bp)	No of strains	
		DT104	Non-DT104
1	1200	1	
2	1600		3
3	1900		1
4	1000, 1200	46	
		47	4

Table IV
Characteristics of the chosen *S.* Typhimurium isolates containing integrons

Isolate	Phage type	Resistance profile	Integron length	Resistance genes	GenBank Acc. No
10/02	DT193	A Cip G C S Su T SxT TMP	1891	*dhfr*12-ORFF-*aad*A2	EF204550
8/03	U302	A S Su T SxT TMP	1586	*dhfr*1-*aad*A1	EF204551
29/03	DT104	A S Su T	1134	bla_{PSE}	EF204552
30/04	DT104	A C S Su T	958	*aad*A2	EF204553
			1156	bla_{PSE}	EF204554

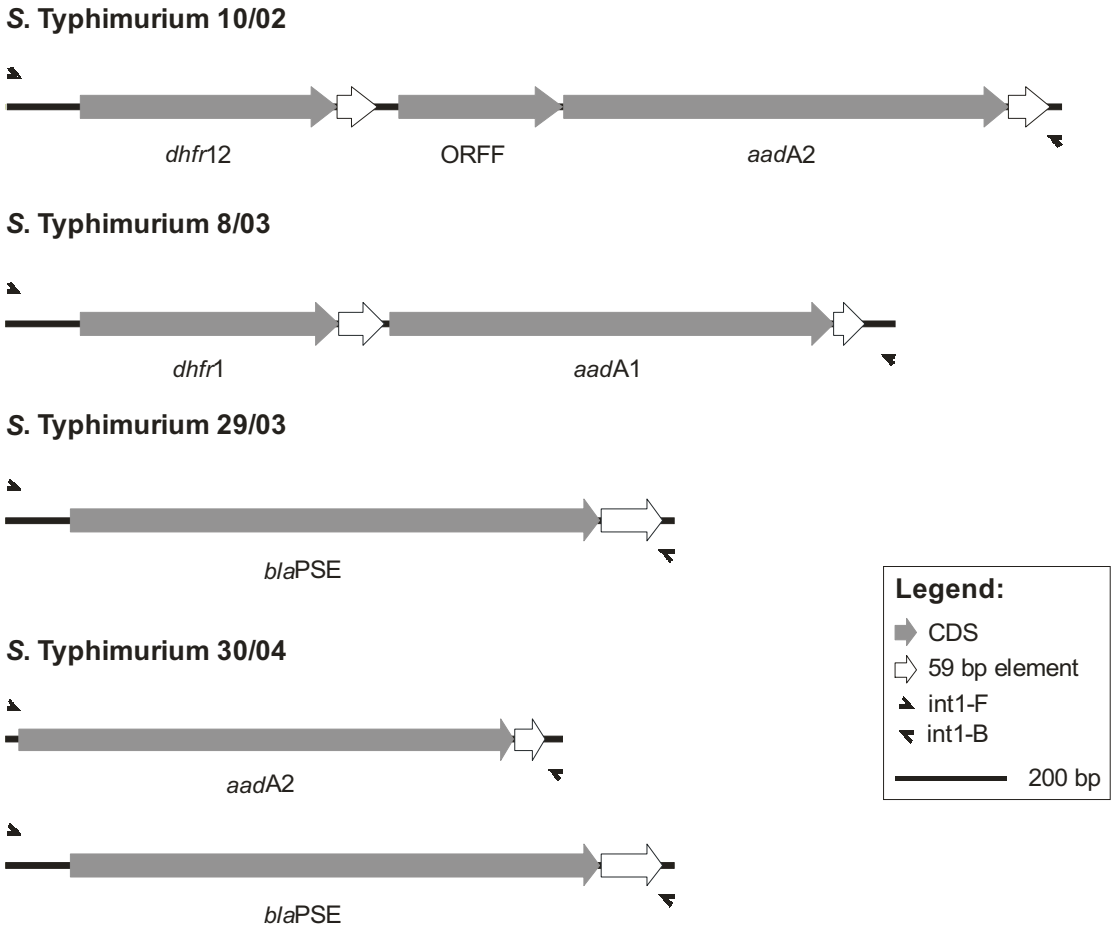

Fig. 1. Genetic organization of sequenced variable regions of class 1 integrons. The regions were amplified by using int1-F and int1-B primers targeting 3'- and 5'- conserved segments of class 1 integrons

of $bla_{PSE.}$ PCR screening of MDR *S.* Typhimurium U302 and DT193 strains detected amplicons of 1.6 kb and 1.9 kb in size, respectively. Two gene cassettes, *dhfr*1 and *aad*A1 were found in MDR U302 strain (8/03) and similarly two gene cassettes *dhfr*12 and *aad*A2 in MDR DT193 strain were found. The nucleotide sequences of variable regions of class 1 integrons were deposited in GenBank database under accession numbers EF204550 – EF204554.

Integrons possessing these arrays of gene cassettes have been already described in several studies on animal, clinical and foodstuffs isolates (Guerra *et al.*, 2000; Carattoli, 2001; White *et al.*, 2001; Lindstedt *et al.*, 2003; Gebreyes *et al.*, 2004; Randall *et al.*, 2004). The 1.6 kb amplicon contained gene cassettes with the *dhfr*A1 and *aad*A1 genes in *S.* Typhimurium isolate from Belgium was found. This *dhfr*A1-*aad*A1 – containing integron was confirmed to be plasmid located (Tosini *et al.*, 1998). The same PCR amplicon with *dhfr*A1-*aad*A1 gene cassettes has been described in Irish *S.* Typhimurium animal and food isolates recently (Daly and Fanning, 2000). The *aad*A1 and *aad*A2 genes coding for aminoglycoside adenylyltransferase confer resistance to streptomycin and spectinomycin. The *dhfr*1 and *dhfr*12 encode dihydrofolate reductase, which confers resistance to trimethoprim and bla_{PSE} gene product confers resistance to ampicillin. Gene bla_{PSE} was found in MDR DT104-like isolates exclusively, while *aad*A2 gene was found, in addition, in serovars Agona, Derby, Heidelberg, and non-DT104 Typhimurium (Miko *et al.*, 2005).

Of the MDR *S.* Typhimurium DT104 as well as non-DT104 isolates investigated, 76.1% were shown to carry class 1 integrons suggesting the important role of these genetic elements in the complexicity of antimicrobial resistance dynamics. Mainly MDR DT104 strains can be considered as the most important carrier of class 1 integrons. Because class 1 integrons have become integrated into the chromosome in MDR DT104 and its relatives, they are able to persist even in the absence of antimicrobial selective pressure.

Surveillance and monitoring of antimicrobial-drug resistance, including screening for class 1 integrons as likely indicators of evolution of drug resistance mechanisms and acquisition of new resistance traits, are necessary steps in planning effective strategies for containing this phenomenon within foodborne infections organisms.

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