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Review article

Molecular characterization, spread and evolution of multidrug resistance in *Salmonella enterica* Typhimurium DT104

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Abstract – Multidrug-resistant *Salmonella enterica* serovar Typhimurium phage type DT104 has emerged during the last decade as a global health problem because of its involvement in diseases in animals and humans. Multidrug-resistant DT104 strains are mostly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (ACSSuT resistance type). The genes coding for such resistances are clustered on the chromosome. This paper reviews new developments in the characterization of *S. enterica* Typhimurium DT104, its chromosomal antibiotic resistance genes and their spread among other *S. enterica* Typhimurium phage types and other *S. enterica* serovars, the development of specific detection methods, virulence characteristics, and the evolution of multidrug-resistance with regard to the emergence of quinolone resistance.

***Salmonella* Typhimurium DT104 / multidrug resistance / *Salmonella* genomic island I**

Résumé – Caractérisation moléculaire, diffusion et évolution de la résistance multiple aux antibiotiques chez *Salmonella enterica* Typhimurium DT104. Le lysotype DT104 de *Salmonella enterica* Typhimurium présentant une résistance multiple aux antibiotiques a émergé durant la dernière décennie comme un problème mondial de santé publique qui touche à la fois humains et animaux. Les souches du lysotype DT104 sont en général résistantes à l'ampicilline, au chloramphénicol, à la streptomycine, aux sulphamides et aux tétracyclines (profil de résistance ACSSuT). Les gènes codant pour ces résistances sont groupés sur le chromosome. Cet article présente une revue des derniers développements dans la caractérisation de *S. enterica* Typhimurium DT104, ses gènes chromosomiques de résistance et leur diffusion chez d'autres lysotypes de *S. enterica* Typhimurium et d'autres serotypes de *S. enterica*, le développement de méthodes spécifiques de détection, les caractéristiques de virulence, et l'évolution de la résistance multiple au regard de l'émergence de la résistance aux quinolones.

***Salmonella* Typhimurium DT104 / résistance multiple / îlot génomique I**

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1. INTRODUCTION

Multidrug-resistant *Salmonella enterica* serovar Typhimurium phage type DT104 has emerged during the last decade as a global health problem because of its involvement in diseases in animals and humans [23, 39, 47, 48]. Multidrug-resistant strains of this phage type were first detected in the United Kingdom in cattle and humans in the late 1980s, but have become common since then in other animal species such as poultry, pigs and sheep. Human infections with multidrug-resistant DT104 isolates have been associated with the consumption of chicken, beef, pork, sausages and meat paste [52]. The DT104 epidemic is now worldwide with a considerable number of outbreaks since 1996 in the USA and Canada [7, 19, 22, 39]. The DT104 epidemic has been extensively reviewed by others [23, 39, 47, 48].

Multidrug-resistant *S. enterica* Typhimurium DT104 strains are mostly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (ACSSuT resistance type). Genes associated with these resistance properties have been found to be chromosomally encoded [49]. Additional resistance to trimethoprim, occasionally seen among DT104 strains, may be encoded by a non-conjugative but mobilizable plasmid of approximately 4.6 MDa which also encodes resistance to sulfonamides [50]. Transferable apramycin resistance has also been described in some multidrug-resistant DT104 strains [31].

This paper reviews new developments in the characterization of chromosomal DT104 antibiotic resistance genes, their spread among other *S. enterica* Typhimurium phage types and other *S. enterica* serovars, the development of specific detection methods, and the evolution in multidrug resistance with regard to the emergence of quinolone resistance.

2. CHROMOSOMAL ANTIBIOTIC RESISTANCE GENE CLUSTER

Genetic studies have demonstrated that the ACSSuT resistance profile of *S. enterica* Typhimurium DT104 is chromosomally encoded [49]. Two different integrons were identified in 1998 using PCR and sequencing [42, 43]. Each integron carried a single resistance gene cassette in addition to the *sulI* and *qacEΔ1* genes that are characteristic of class 1 integrons and specify resistance to sulfonamides and disinfectants, respectively. The first integron carried the *aadA2* gene conferring resistance to streptomycin and spectinomycin. The second integron contained the β-lactamase gene $bla_{PSE\text{-}1}$. In 1999, several research groups identified the remaining resistance genes conferring resistance to chloramphenicol and tetracyclines [4, 9, 11, 34]. These genes were located between the two previously known integrons thus rendering all antibiotic resistance genes clustered on a chromosomal locus of about 12.5 kb. The gene conferring resistance to chloramphenicol also confers resistance to its fluorinated analogue

florfenicol and has been named *cmlA*-like, *floR* or *floSt* [4, 9, 11, 34]. According to the deduced amino acid sequence homologies and the topology of the assumed protein, this gene encodes an efflux pump belonging to the 12-transmembrane segment (TMS) family export proteins of the major facilitator superfamily (MFS) reviewed by Paulsen et al. [35]. Downstream of the *floR* gene are the tetracycline resistance genes *tetR* and *tet*(G). The organization of the antibiotic resistance gene cluster is shown in Figure 1.

More recently this chromosomal antibiotic resistance gene cluster has been shown to be part of a 43-kb genomic island termed *Salmonella* genomic island I (SgiI) [10]. By comparison with the genome sequence of *S. enterica* Typhimurium LT2, SgiI has been found in the genome between the *thdf* gene, coding for a thiophene and furan oxidation protein, and a prophage CP-4-like integrase gene (*int2*), and is flanked by an imperfect 18-bp direct repeat [10]. Downstream of SgiI, a 1.9 kb retron sequence was located between genes *int2* and *yidY* – coding for a putative drug translocase – of the *S. enterica* Typhimurium genome. This retron sequence may be unique to *S. enterica* serovar Typhimurium since it has not been detected in other *S. enterica* serovars [10]. The SgiI comprises a number of genes – with no known functions and no homologies with other proteins in databases – which might contribute to virulence as seen with the *Salmonella* pathogenicity islands (M. Mulvey, personal communication). If this is confirmed, the particular situation of the DT104 antibiotic resistance gene cluster associated with a pathogenicity island might explain the current worldwide epidemic of this pathogen. A fact that may support this hypothesis is that DT104 was an uncommon phage type before acquiring the multidrug-resistance phenotype [47].

Several authors have speculated on the origin of the DT104 antibiotic resistance gene cluster and consecutive spread of multidrug-resistant DT104 strains [3, 19, 20]. The use of antimicrobial agents in agriculture might have contributed to the emergence of multidrug-resistant DT104 strains [3]. Since the genes included in the multidrug-resistance gene cluster of DT104 strains confer resistances to drugs of four of the five classes of antimicrobials (tetracyclines, β-lactams, aminoglycosides and sulfonamides) most frequently used in veterinary medicine, co-selection of the entire cluster may result from the use of any of these drugs. While some genes in the cluster, such as *aadA2*, bla_{PSE-1}, or *sul*I, are widely distributed among *Enterobacteriaceae*, the remaining two genes, *floR* and *tet*(G), are most probably not of enterobacterial origin. Florfenicol is a veterinary antimicrobial agent that has been used in aquaculture in Asia since the early 1980s. A *floR* homolog was first identified on a plasmid in *Pasteurella piscicida* recently renamed *Photobacterium damselae*, a fish pathogen [28]. In addition, the class G tetracycline resistance gene associated with the *floR* gene in the DT104 antibiotic resistance gene cluster was first identified in *Vibrio anguillarum*, also a fish pathogen [56]. The *tet*(G) gene has also been detected on plasmids of *Photobacterium damselae* [27]. The *floR* and *tet*(G) genes in *S. enterica* Typhimurium DT104 have a similar G + C content (58%) and could thus have the same origin. Based on these data, Angulo and Griffin [3] suggested that the resistance determinants of multidrug-resistant DT104 strains may have emerged among bacteria in aquaculture and subsequently been horizontally transferred to *S. enterica* Typhimurium DT104. Once multidrug-resistant *S. enterica* Typhimurium DT104 is introduced into food animals in a region, the use of antimicrobial agents in animals will favour the dissemination of the multidrug-resistant strains [3]. However, another hypothesis by Davis et al. [20] is the possible *Pseudomonas* sp. origin. Indeed, *tet*(G) also occurs in bacteria of this genus [45], and similarly, *floR* is closely related to the *P. aeruginosa* chloramphenicol-resistance

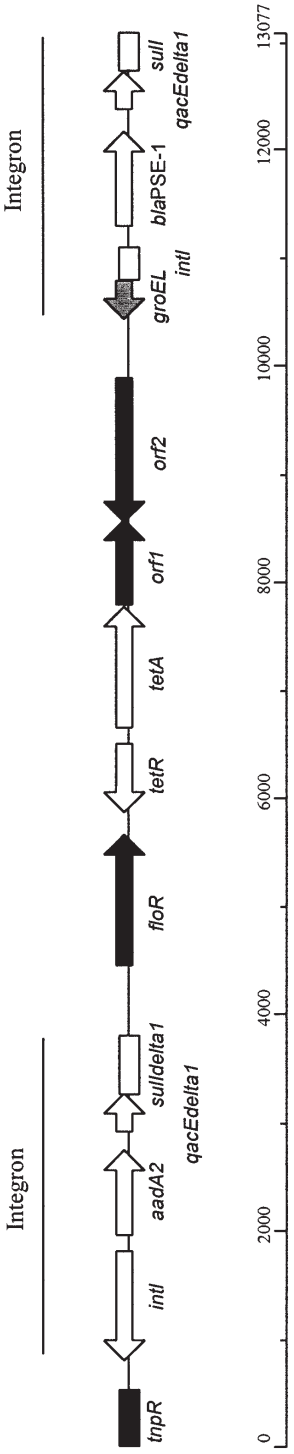

Figure 1. Gene organization of the chromosomal antibiotic resistance gene cluster of *Salmonella enterica* Typhimurium DT104 according to Arcangioli et al. [4], Briggs and Fratamico [11], Cloeckaert et al. [18] and Ng et al. [34].

gene *cmlA* [4, 8, 9, 11]. Moreover, the bla_{PSE-1} encoded β-lactamase is a common feature of hospital *P. aeruginosa* isolates [36]. Thus the hypothesis that multidrug resistant *S. enterica* Typhimurium DT104 acquired resistance genes horizontally from nosocomial pseudomonads might also be worth considering [20]. The association of *floR* with the *tetR* and *tet*(G) genes has nevertheless not yet been described in bacteria other than *S. enterica* Typhimurium DT104 and *S. enterica* serovar Agona (see below).

Whatever the origin of multidrug-resistant *S. enterica* Typhimurium DT104, animal- or human-related or not related to antibiotic use, we must now consider the importance of the inclusion of the DT104 antibiotic resistance gene cluster within SgiI. The antibiotic resistance cluster might only represent the visible part of an iceberg and the spread of multidrug-resistant *S. enterica* Typhimurium DT104 might occur even without the selective pressure imposed by the use of antibiotics.

3. CLONAL SPREAD AND HORIZONTAL TRANSFER OF THE ANTIBIOTIC RESISTANCE GENE CLUSTER

Using pulsed-field gel electrophoresis (PFGE), several studies have concluded that multidrug-resistant *S. enterica* Typhimurium DT104 has probably been spread clonally in European countries and the United States [6, 16, 30, 37, 40, 48]. However, a recent study by Markogiannakis et al. [32] also using PFGE showed that six distinct clones were present among Greek multidrug-resistant *S. enterica* Typhimurium DT104 isolates. In a recent review, Tauxe [47] also stated that an important epidemiological feature of the *S. enterica* Typhimurium DT104 phenomenon is that it is not a monoclonal strain and that multidrug-resistant DT104 represents a cluster of strains with related lysotyping patterns, including DT104, DT104a, DT104b, and U302. Thus the epidemic would be more accurately described as being due to a cluster of related strains [47]. It is also interesting to note that strains showing the same macrorestriction pattern nevertheless exhibit genetic diversity when using other methods such as infrequent restriction site PCR (IRS-PCR) and amplified fragment length polymorphism (AFLP) [46]. The latter methods may be more discriminatory than PFGE and the usefulness and limits of each method are described by Aarts et al. [1] in this special issue. The occurrence of the DT104 antibiotic resistance gene cluster in several clones would suggest horizontal transfer of the cluster and possibly of the entire SgiI among *S. enterica* Typhimurium strains.

The best evidence for horizontal transfer of the antibiotic resistance gene cluster is the recent discovery of this cluster in another *S. enterica* serovar, namely serovar Agona [18]. These multidrug-resistant serovar Agona strains were isolated from poultry in Belgium during the 1992–1997 period [24], but have not yet been reported in other countries. Besides this observation, the DT104 antibiotic resistance gene cluster has also been shown to occur in phage type DT120 of serovar Typhimurium [18]. A possible mechanism by which this antibiotic resistance gene cluster may be horizontally transferred is phage-mediated transfer. Recently, it has been shown that the DT104 antibiotic resistance genes can be efficiently transduced by P22-like phage ES18 and by phage PDT17, which are released by all DT104 isolates analyzed so far [44].

4. MOLECULAR IDENTIFICATION AND DETECTION METHODS

Florfenicol resistance and detection of the *floR* gene by PCR-based methods have been proposed as means of rapidly identifying multidrug-resistant *S. enterica* Typhimurium DT104 strains [9] as phage typing remains a laborious task only available in specialized laboratories. Multiplex

PCR based on the surrounding genes of the antibiotic resistance gene cluster has also been proposed for the identification of *S. enterica* Typhimurium DT104 strains [5, 12, 26]. In the light of evidence of horizontal transfer of the DT104 antibiotic resistance gene cluster to other Typhimurium phage types or other *S. enterica* serovars [18], these methods appear to be no longer suitable for the specific detection of multidrug-resistant *S. enterica* Typhimurium DT104. Let us also mention the recently described occurrence of florfenicol resistance encoded by the *floR* gene on conjugative plasmids or on the chromosome of *Escherichia coli* isolated from cattle and poultry [17, 25, 53].

Thus the development of new molecular methods not based on the occurrence of the antibiotic resistance gene cluster is required for the specific detection of *S. enterica* Typhimurium DT104. By PCR amplification of the 16S-to-23S spacer region of bacterial rRNA genes, bovine isolates of *S. enterica* Typhimurium DT104 demonstrated a unique band as compared to other phage types [41]. The nucleotide sequence of this band was determined and a search of the nucleotide sequence databases did not produce any matches to known ribosomal sequences [41]. A PCR test based on this sequence was thus further developed and was shown to be specific for phage type DT104 and related phage type U302 among serovar Typhimurium isolates. However, this sequence was also detected in other *S. enterica* serovars such as Brandenburg, Lille, Muenchen, Dublin, and Enteritidis [41]. Another rapid identification approach worth mentioning is the development and use of specific monoclonal antibodies to the lipopolysaccharide of *S. enterica* Typhimurium DT104 [55].

5. QUINOLONE RESISTANCE

The emergence of decreased susceptibility to fluoroquinolones in multidrug-resistant *S. enterica* Typhimurium DT104 is causing particular concern [15, 33, 48, 51]. The emergence and spread of such DT104 strains followed the licensing for veterinary use of enrofloxacin in 1993 [48]. This fluoroquinolone antibiotic has subsequently been used in cattle and poultry for treatment resulting in the selection of quinolone-resistant strains [48]. During a *S. enterica* Typhimurium DT104 Danish outbreak in 1998, some hospitalized patients did not respond to treatment with ciprofloxacin, a fluoroquinolone used exclusively for human therapy, and two died [33]. However, these were obviously immunocompromised patients and the strains isolated from these patients, although quinolone-resistant, did not show clinical resistance to fluoroquinolones [15].

The mechanisms of decreased susceptibility to fluoroquinolones in DT104 isolates involve point mutations in the quinolone resistance determining region (QRDR) of the target gene *gyrA* [42, 48, 51]. Another important mechanism may be active efflux due to overproduction of the AcrAB efflux pump [21]. Overproduction of this efflux pump probably involves mutations in the regulatory genes such as those of the *marRAB* operon [38] but not yet explored in *Salmonella*. An important feature of this overproduction is the contribution to multidrug-resistance of DT104 strains to other unrelated antibiotics. For example such DT104 strains showed an eight-fold increase in resistance levels to chloramphenicol and florfenicol when compared to quinolone-susceptible DT104 strains (unpublished result).

6. CHANGE IN VIRULENCE?

The hypothesis of hypervirulence of multidrug-resistant *S. enterica* Typhimurium DT104 has been investigated by testing invasiveness in cell lines and virulence in the mouse model of systemic salmonellosis [2, 13, 14]. However, the strains tested did

not appear more invasive than non-resistant cohorts and some DT104 isolates appeared even less invasive than non-resistant relatives [13]. With regard to invasion capacity, however, it is interesting to note that *S. enterica* Typhimurium DT104 infection resulted in a higher egg contamination rate than *S. enterica* Enteritidis PT4 [54]. In the mouse model of infection, multidrug-resistant DT104 strains did not reveal evidence of enhanced nor reduced virulence when compared with ATCC *S. enterica* Typhimurium strain 14028s [2].

Other animal models using primary hosts are required to precisely determine the role of SgiI in virulence. Preliminary results using the experimental poultry model of infection showed that a multidrug-resistant DT104 strain was more virulent than a susceptible strain lacking SgiI, suggesting a potential role for SgiI in virulence (unpublished result). This result needs to be confirmed by testing a DT104 strain experimentally deleted in SgiI.

Another questionable aspect is the evolution of virulence in multidrug-resistant DT104 strains showing decreased susceptibility to fluoroquinolones in particular with regard to their ability to overproduce the AcrAB efflux pump. The AcrAB efflux pump, besides conferring multiple antibiotic resistance, has been shown in *S. enterica* Typhimurium to play an important role in resistance to biliary salts and detergents and in murine intestinal colonization [29].

7. FUTURE DIRECTIONS

In the light of evidence of horizontal transfer of the DT104 antibiotic resistance gene cluster or of the entire SgiI, future directions for research should involve the identification of specific stable markers of *S. enterica* Typhimurium DT104 by genomic or proteomic approaches and consecutive development of specific identification tests. The potential role of SgiI and particular new genes of SgiI in virulence should be carefully investigated in primary animal host models of infection. These approaches may yield a possible explanation of the *S. enterica* Typhimurium DT104 world-wide epidemic. The development of fluoroquinolone resistance in *S. enterica* Typhimurium DT104 strains with a concomitent increase in multidrug resistance should be carefully monitored.

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