RESEARCH LETTER

Parallel evolution of multidrug-resistance in *Salmonella enterica* isolated from swine

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Abstract

The increase in frequency of *Salmonella enterica* resistant to antibiotics in food-producing animals is of great concern to public health. Determining the rate at which different resistance phenotypes are generated and maintained in the environment is thus of great importance. The distribution and evolution of antibiotic resistance and multidrug-resistance in 362 *Salmonella* stains as part of a cross-sectional study of the Canadian swine industry were investigated. The susceptibility of all isolates to 12 antimicrobial agents was tested and the statistical and phylogenetic distribution of resistance among strains characterized via multilocus sequence typing was studied to test the origin of multidrug-resistance in *Salmonella*. More than 25% of all isolates were multidrug-resistant, with predominance in serotype Typhimurium, a serotype of vital importance to public health. The strong associations between resistance phenotypes, which differ among serotypes and which is supported by the significant genetic distance between serotypes, was indicative of the independent acquisition of multidrug-resistance in at least two different serotypes, i.e. Typhimurium and Derby. The independent origin of multidrug-resistance in *Salmonella* indicates that strong selective pressures are present in the environment of the bacteria and that statistical and phylogenetic studies of antibiotic resistance are an essential part in the understanding and the control of the epidemic.

Introduction

The evolution of antibiotic resistance has been described as the most important evolutionary change in modern time, causing prolonged illness and increased cost of hospitalization for diseases that were once straightforwardly controlled (Maynard Smith *et al.*, 2000; Evans *et al.*, 2007). Resistance has dramatically increased in frequency among bacteria of clinical relevance, presumably because of selective pressures imposed by the extensive use of commercial antibiotics in human and veterinary medicine (Palumbi, 2001; Kuümmerer, 2003; Gebreyes *et al.*, 2004; Randall *et al.*, 2004; Baker-Austin *et al.*, 2006; Stepanauskas *et al.*, 2006). The use of antibiotics in combination with the horizontal transfer of resistance-conferring genes among bacteria has resulted in the emergence of multidrug-resistance, which is the resistance to two or more antibiotics, limiting greatly the therapeutic options for the treatment of disease in humans and animals (Holmberg *et al.*, 1987; Bartoloni *et al.*, 2006)

One major concern to public health has been the global dissemination of *Salmonella* Typhimurium Definitive Type 104, which commonly carries resistance to five antimicrobial agents or more (Threlfall, 2000; Poppe *et al.*, 2002a; Gebreyes *et al.*, 2004; Perron *et al.*, 2007). *Salmonella* is among the leading cause of human food-borne illnesses in developed countries and can infect people through consumption of contaminated food products (Mead *et al.*, 1999; Zhao *et al.*, 2006). The increased occurrence of multi-drug resistant *Salmonella* in food animals is thus a threat to public health as food-borne infections could bring multidrug-resistance to human population (Anderson *et al.*, 2003).

Although a large body of scientific information is available on the prevalence of antimicrobial resistance and its associated molecular mechanisms (Briggs & Fratamico, 1999; Liebert *et al.*, 1999; Poppe *et al.*, 2002b, 2005; Mulvey

et al., 2006; Chen *et al.*, 2007; Hopkins *et al.*, 2007), many aspects related to its evolution remain uncertain. In this study, the statistical and phylogenetic distribution of multidrug-resistance was analysed in a population of *Salmonella enterica* isolated from asymptomatic swine to understand the evolution of resistant strains.

Materials and methods

Bacterial strains

The methodology concerning the characterization of the sample population of asymptomatic *S. enterica* is described elsewhere (Perron *et al.*, 2007). Briefly, *Salmonella* strains were isolated from mesenteric lymph nodes taken form pig carcasses of animals with no sign of infection sampled during a cross sectional study of the Canadian swine industry. Serological identification of *Salmonella* spp. and phage-typing technique using standard methodologies were performed at the Laboratoire d'épidémiosurveillance animale du Québec in Saint-Hyacinthe and at Health Canada Laboratory for Foodborne Zoonoses, Guelph, Ontario [for details on the scheme (Poppe *et al.*, 2002b)]. In this study, *S.* Typhimurium var. Copenhagen isolates were included to *S.* Typhimurium.

Antibiotic resistance

Antimicrobial susceptibility testing of all isolates was performed using the Kirby–Bauer disc diffusion method established by the CLSI (National Committee for Clinical Laboratory Standards NCCLS, 2002). In short, the resistance of a strain to a given antibiotic is read as the extent of growth inhibition imposed by the diffusion of the given antibiotic on a blood agar plate. The antimicrobials tested were: coamoxiclav, ampicillin, apramycin, cefoxitin, ceftiotur, cefalotin, chloramphenicol, enrofloxacin, gentamicin, neomycin, tetracycline and trimethoprim-sulfas. Results were interpreted according to the CLSI criteria following performance standards when available, or according to the manufacturer criteria. In this study, isolates with intermediate phenotype were grouped with susceptible isolates in order to not overestimate occurrence of resistance.

Multilocus sequence typing (MLST)

Bacterial isolates were genetically characterized using the MLST scheme designed for *S. enterica* (Kidgell *et al.*, 2002). Genomic DNA was prepared for a representative subsample of 282 isolates (using DNA Tissue Kit, QIAGEN, Hilden, Germany) and seven housekeeping genes were amplified and sequenced at McGill University and Genome Québec Innovation Centre, Montreal, Quebec. Known alleles and ST sequences are readily available on the central *Salmonella* MLST database (Max Planck Institute, Berlin, Germany; http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica).

Antimicrobial resistance distribution

The distribution of resistance phenotypes among serotypes, either resistance to a single antibiotic or multidrug-resistance, was tested using a contingency table analysis where rows = serotype and columns = resistance vs. susceptible. Contingency table allows to test if the proportion of resistance bacteria is the same across the different serotypes and uses the Pearson's χ^2 test to assess the statistical significance of the difference between the proportions. Only serotype with 10 strains or more (i.e. Typhimurium, Derby, Schwarzengrund, Brandenburg, London and Heidelberg) were analysed using a χ^2 distribution with five degrees of freedom. A high χ^2 value means that the resistant phenotypes are not proportionately distributed among serotypes, some serotypes having higher frequency of resistant phenotype than others.

As most serotype were associated to a single genotype, the association between serotype/genotype data and resistance data was further tested using an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN 3.1 (Excoffier *et al.*, 2005). The analysis determines how the genetic variation found among housekeeping genes is partitioned within and among populations of resistant and susceptible bacteria. Pairwise distances were computed using the Tamura and Nei distance measure with a gamma correction of 0.015.

Antimicrobial resistance association

The association between resistances to individual antimicrobial was tested using Fisher's exact tests with a Bonferroni's correction for multiple tests on 2×2 contingency tables. Positive associations were defined as the probability of finding resistance to two named antibiotics in the same isolates greater than expected by chance ($P < 0.0001$).

Results

Antimicrobial resistance distribution

Resistance to 10 of the 12 antimicrobial agents tested among the 362 asymptomatic *Salmonella* isolates was observed. Although more than 20% of all isolates showed resistance to ampicillin, chloramphenicol, neomycin, tetracycline or trimethoprim-suflas, resistance was observed predominantly in serotype Typhimurium (Table 1). Close to 90% of the Typhimurium isolates were resistant to tetracycline, an antibiotic commonly used in veterinary medicine, and more than 40% were resistant to chloramphenicol and neomycin. No resistance to enrofloxacin or gentamicin was found

Table 1. Distribution of antimicrobial resistance among serotypes

Serotype	*n*	AMC	AMP	APR	FOX	XNL	CEF	CHL	NEO	TET	SXT
Typhimurium	172	3 (1.7)	**96 (55.8)**	0 (0)	1 (<1)	1 (<1)	1 (<1)	**88 (51.2)**	**72 (41.9)**	**151 (87.8)**	**32 (18.6)**
Derby	75	1 (1.3)	4 (5.3)	2 (2.7)	1 (1.3)	3 (4.0)	3 (4.0)	2 (2.7)	6 (8.0)	54 (72.0)	3 (4.0)
Schwarzengrund	36	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	13 (36.1)	0 (0)
Brandenburg	31	0 (0)	1 (3.2)	1 (3.2)	0 (0)	1 (3.2)	0 (0)	1 (3.2)	1 (3.2)	23 (74.2)	1 (3.2)
London	16	0 (0)	1 (6.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Heidelberg	12	**4 (33.3)**	5 (41.7)	0 (0)	**2 (16.7)**	**2 (16.7)**	**5 (41.7)**	0 (0)	0 (0)	7 (58.3)	0 (0)
Infantis	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Agona	4	0 (0)	1 (25.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25.0)	1 (25.0)	1 (25.0)	1 (25.0)
Rare	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (13.3)	0 (0)
Total	367	8 (2.2)	108 (29.4)	3 (< 1)	4 (1.1)	7 (1.9)	9 (2.5)	92 (25.1)	80 (21.8)	251 (68.4)	37 (10.1)

Rare serotypes present less than two isolates. Percentages are proportion of resistance within serotypes. Bold indicates where the frequency of resistance is dependent on the serotype identity ($\chi^2 < 0.0001$). AMC, co-amoxiclav; AMP, ampicillin; APR, apramycin; FOX, cefoxitin; XNL, ceftiotur; CEF, cefalotin; CHL, chloramphenicol; NEO, neomycin; TET, tetracycline; SXT, trimethoprim-sulfas.

among the sampled population and the antibiotics in later analyses was thus ignored.

The distribution of resistance to the different antibiotics was largely dependent on the serotype identity. More precisely, resistance to ampicillin ($\chi^2 = 105.0$; $P < 0.0001$), chloramphenicol ($\chi^2 = 107.0$; $P < 0.0001$), neomycin ($\chi^2 = 69.8$; $P < 0.0001$), tetracycline ($\chi^2 = 95.8$; $P < 0.0001$) and trimethoprim-sulfas ($\chi^2 = 246.0$; $P < 0.0001$) was significantly associated with serotype Typhimurium. On the other hand, resistance to coamoxiclav ($\chi^2 = 53.0$; $P < 0.0001$), cefoxitin ($\chi^2 = 22.5$; $P < 0.0001$), ceftiotur ($\chi^2 = 15.1$; $P < 0.01$) and cefalotin ($\chi^2 = 53.1$; $P < 0.0001$) was significantly associated to serotype Heidelberg.

Multidrug-resistance was found in serotype Derby and Heidelberg, but predominantly in serotype Typhimurium with more than 50% of the isolates bring resistant to more than one drug. For this reason, multidrug-resistance showed a significant association to the serotype ($\chi^2 = 68.777$; $P < 0.001$). In serotype Heidelberg, 41.7% of the isolates presented multidrug-resistance while 9.3% of the isolates in serotype Derby did. Interestingly, the multidrug-resistant phenotype associated with each serotypes was different (Fig. 1). The phenotypes associated with Typhimurium were generally resistant to ampicillin, chloramphenicol, neomycin, tetracycline and trimethoprim-sulfas while Heidelberg and Derby showed resistance to coamoxiclav, ampicillin, cefoxitin, ceftiotur and cefalotin.

To further investigate the distribution of antimicrobial resistance, the partitioning of variation among housekeeping genes was estimated, based on the genetic distance between the different types, within and among resistant populations using an AMOVA. This was done to test the association between the MLST data and the resistance phenotypes. Significant differences were found in the distribution of alleles between susceptible and resistant populations of bacteria for chloramphenicol, neomycin, tetracycline and trimethoprim-sulfas. AMOVA revealed that on average 17% of the variation in housekeeping genes could be explained by the susceptible vs. resistant distinction ($P < 0.0001$ in all cases). This means that genetic variation was more important between the resistant and the susceptible populations than within each population, which is indicative of a clonal (or vertical) evolution of resistance to these antibiotics. This level of partition is understandable since the major proportion of the resistant phenotypes is found in the genotypes associated with Typhimurium. A similar analysis using multidrug-resistance as distinctive trait revealed that 26% of the variation in the housekeeping genes could be explained by the presence of multidrug-resistance trait in the bacteria. This means that more variation was observed within the multidrug-resistant population than population resistant to any single antibiotic.

Antimicrobial resistance association

The strength of association between every pair of antibiotics was estimated using Fisher's exact tests. Eighteen positive associations were detected, that is the probability of finding resistance to two named antibiotics in the same isolates greater than expected by chance; all significant association, once applying the Bonferroni correction ($P < 0.0001$), are shown in Fig. 2. Ampicillin was positively associated to coamoxiclav, cefalotin, chloramphenicol, neomycin, tetracycline and trimethoprim-sulfas. Resistance to coamoxiclav was positively associated to ampicillin, cefoxitin, ceftiotur and cefalotin; cefoxitin, ceftiotur and cefalotin were also positively associated while resistance to chloramphenicol, neomycin, tetracycline and trimethoprim-sulfas was positively associated. Association in the *Salmonella* data was mainly explained by the two different patterns associated with multidrug-resistance observed in serotype Typhimurium and Derby (Fig. 2, lines patterning).

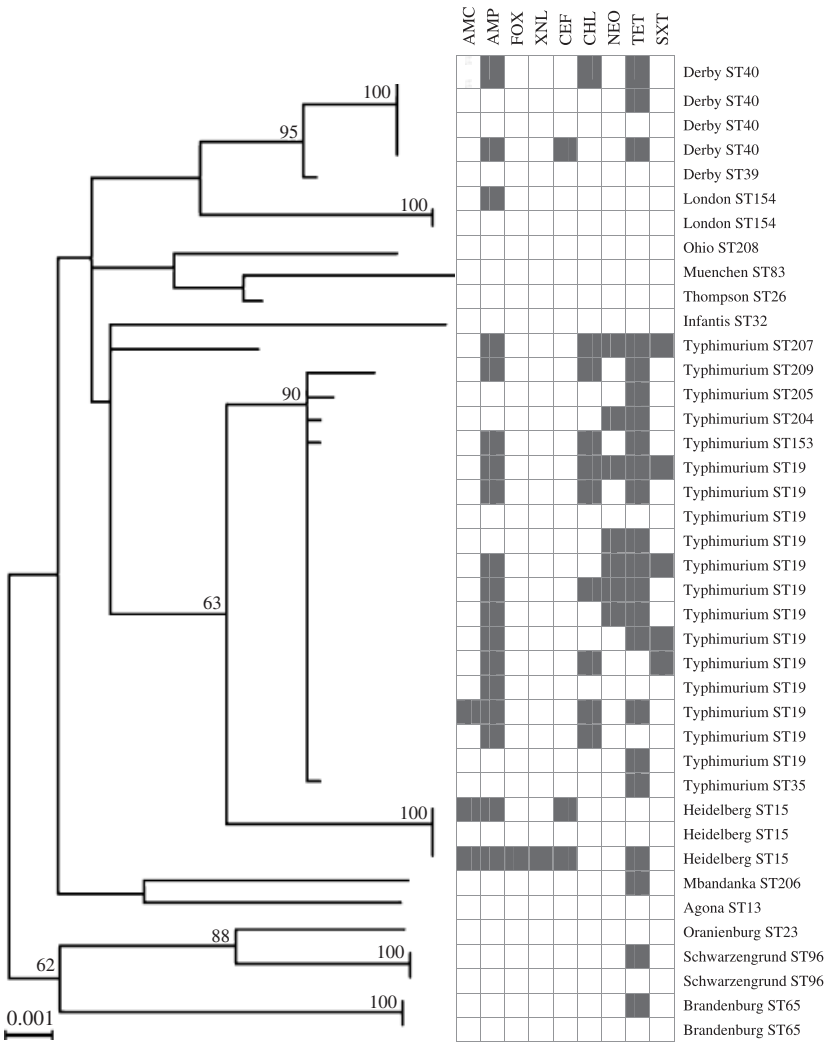

Fig. 1. Phylogenetic tree and distribution of antimicrobial resistance phenotypes among *Salmonella* isolates represented by a neighbor-joining MLST tree from concatenated sequences. Number above the nodes are bootstrap scores based on 1000 pseudoreplicates. The tree is identical in its gross topology to one produced by maximum likelihood. Representation of a subsample of genotypes identified with different genotypes only. The black and white grid represents the presence or absence, respectively, of the assayed resistance phenotype. AMC, coamoxiclav; AMP, ampicillin; FOX, cefoxitin; XNL, ceftiotur; CEF, cefalotin; CHL, chloramphenicol; NEO, neomycin; TET, tetracycline; SXT, trimethoprim-sulfas.

The association between each antibiotic and multidrug-resistance as a phenotype was estimated. Of all resistance phenotypes, only resistance to apramycin did not show positive association to multidrug-resistance. Although cefoxitin only showed marginally significant association to multidrug-resistance ($P < 0.01$), it was found to be significantly associated to cefalotin ($P < 0.0001$) and ceftiotur ($P<0.0001$). This is indicative that at least one type II error has been committed; most likely, the association between cefoxitin and multidrug-resistance was only marginally detected because of the conservative statistical power imposed by the Bonferroni correction.

Discussion

Two main conclusions emerge from this study on antimicrobial resistance. First, antimicrobial resistance is widespread among asymptomatic *Salmonella* with more than 70% of all isolates being resistant to at least one antibiotic. Of the 12 commercial antimicrobial agents tested, only one proved to be totally effective against all isolates. Furthermore, resistance to antibiotics rarely used in veterinary medicine or agriculture was also found to be frequent, most likely because of its association with other resistance factor or cross-resistance. For example, resistance to chloramphenicol was present in more than 50% of Typhimurium isolates although it has been illegal to use in food-producing animals in Canada since 1985.

More importantly for public health considerations, resistance was mostly concentrated in serotypes frequently isolated from food producing pigs or associated with disease in human, such as Typhimurium, Derby or Heidelberg (Poppe *et al.*, 2001; Gebreyes *et al.*, 2004, 2006; Randall *et al.*, 2004; Zhao *et al.*, 2006). The combined resistances of the three serotypes covered all classes of antibiotics tested and showed

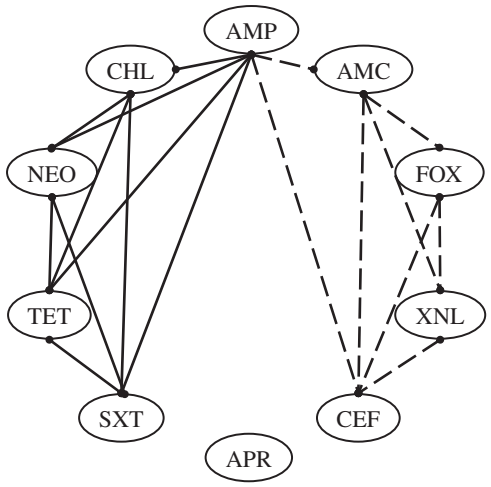

Fig. 2. Positive associations of antibiotic resistance in *Salmonella enterica*. Full lines represent significant positive associations found in serotype Typhimurium while dashed line represent significant positive associations found in serotype Derby. A positive association between two antibiotics represents the probability of finding resistance to the two named antibiotics in the same isolates greater than expected by chance. All positive associations were estimated using a Fisher's exact test and applying a Bonferroni's correction ($P < 0.0001$ in every case). AMP, ampicillin; AMC, coamoxiclav; APR, apramycin; FOX, cefoxitin; XNL, cefalotin; CEF, ceftiotur; CHL, chloramphenicol; NEO, neomycin; TET, tetracycline; SXT, sulfa-trimethoprim.

two significant different patterns of multidrug-resistance determined by association tests. Hopefully, the significant association of certain resistance phenotypes can aid health practitioners and veterinarians by providing clear predictions on how to choose new antibiotics when confronted to treatment complications caused by resistance bacteria.

The second conclusion is that the evolution of multidrug-resistance may have at least two independent origins in *Salmonella*. The strong associations between resistance phenotypes, which differ among serotypes and which is supported by the significant genetic distance between serotypes, is indicative of the independent acquisition of multidrug-resistance in serotype Typhimurium/Heidelberg and Derby. Also, the association between resistant phenotypes of Heidelberg differs from that of Typhimurium, which suggest a third origin of multidrug-resistance. However, bootstrap values for branches differentiating serotypes Typhimurium and Heidelberg are poorly supported and the phenotype could be due to either the loss of a resistance trait in the Heidelberg branch or a gain or resistance traits in Typhimurium. In any case, the gross topology of the tree was consistent across a variety of phylogenetic methods and, although more genetic details will be required to refine the tree, the authors are confident with the marginally significant topology of the tree to assert that the evolution of multidrug-resistance is a dynamic process.

The evolution and spread of antibiotic resistance is known to be promoted by horizontal transfer and can, outside a phylogenomic framework, be independent of the core genome of bacteria. This study shows, however, that there is a significant difference between multidrug-resistant populations and that although variable, multidrug-resistance is clustered in certain groups or lineages of bacteria. Although all serotypes were isolated from a similar environment, only three were found to be multidrug-resistant. Perhaps certain serotypes appear to have the unique ability to acquire antimicrobial resistance horizontally through conjugation or transformation, which in itself represent an important adaptation leading to multidrug-resistance (Randall *et al.*, 2004; Poppe *et al.*, 2005; Mulvey *et al.*, 2006; Hopkins *et al.*, 2007).

Although research on resistance in *Salmonella* is diverse, few studies of antimicrobial resistance in *S. enterica* have used a precise phylogenetic framework. An MLST approach was used to characterize the *S. enterica* core genome. By mapping the distribution of antimicrobial resistance onto the MLST phylogeny, clear hypotheses can be drawn on the evolutionary origin of such phenotypes (Hwang *et al.*, 2005). The understanding of resistance mechanisms is important for the development of new drugs, but should not be relied upon as the sole solution to fight resistant infections. History has provided a case for the evolution of resistance to every new antibiotic within years of its introduction (Palumbi, 2001), even to promising new drugs (Perron *et al.*, 2006). To control the spread of resistance and to preserve the efficacy of antibiotics, it is vital to understand the rate at which resistance is generated and how it is maintained in the environment, whether in hospitals, animals or people.

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