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Genotypic diversity and antimicrobial resistance in asymptomatic *Salmonella enterica* serotype Typhimurium DT104

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Abstract

The recent emergence of multidrug-resistant *Salmonella enterica* subspecies enterica serotype Typhimurium phage-type DT104 represents a challenge to health workers from both human and veterinary medicine. In an effort to develop effective control strategies, it is vital to characterize accurately the extent of genetic and phenotypic variation present in the pathogen population. While previous studies have focused on disease-associated populations, we report the genetic diversity and the diversity in antimicrobial resistance associated with asymptomatic *Salmonella* Typhimurium DT104 isolates found in pigs. Using pulsed-field gel electrophoresis (*Xba*I and *Spe*I), we identified 62 different genotypes associated to 13 different antimicrobial resistant phenotypes among 80 asymptomatic DT104 isolates collected from the Canadian swine industry. When rarefaction curves of a subsample the asymptomatic isolates were compared to that of disease-associated isolates from a similar spatial and temporal scale, we identified significantly more genotypes among the asymptomatic populations with 27 PFGE patterns against 23.0 ± 1.4 (95% confidence intervals) in the latter. Also, using Simpson's diversity index, we observed considerably higher genetic and phenotypic diversity in the asymptomatic isolates within herds and detected possible patterns of periodic selection within the disease-associated population. We thus concluded that an important aspect of *Salmonella* population structure and ecology is overlooked in investigations of disease-associated isolates.

Keywords: *Salmonella* spp.; Typhimurium DT104; Pathogenic bacteria; Ecology; Genetic diversity; Antimicrobial resistance; Public health; Population structure; Infectious disease

1. Introduction

Non-typhoidal salmonellosis is an important food-borne problem throughout the world. Even though more than 2500 serovars of *Salmonella enterica* have thus far been recognized (Farmer, 1999), few appear to cause foodborne illnesses (Gebreyes et al., 2006). One non-host-adapted serovar common to animals and humans is Typhimurium, which has been reported as one of the top two food-borne infections in developed countries (Poppe et al., 2002). More importantly, the emergence of multidrug-resistance in Typhimurium as been described internationally and mostly associated to isolates belonging to phage type or definitive type (DT) 104 (Threlfall et al., 1994; Helms et al., 2005).

Salmonella spp. can infect people through consumption of contaminated food products. Thus, the increased occurrence of multidrug-resistant DT104 in animals and in food of animal origin poses a threat to public health (Fone and Barker, 1994; Wall et al., 1995). Until recently, DT104 was primarily associated with cattle and human (Gresham, 1996; Threlfall, 2000). Although high levels of virulence and mortality have been associated with DT104 in cattle and humans, an unexpected large number of seemingly asymptomatic isolates have been observed in swine, suggesting pigs as another potential significant reservoir of DT104 (Davies and Wray, 1997; Côté et al., 2004; Gebreyes et al., 2006).

Previous studies on *Salmonella* diversity have focused on disease-associated isolates or disease outbreaks (Hilton and Penn, 1998; Casin et al., 1999; Prager et al., 1999; Baggesen et al., 2000; Ebner and Mathew, 2001; Murphy et al., 2001; Liebana et al., 2002; Heir et al., 2002; Kim et al., 2004). Since very little genetic variation has been observed among DT104

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isolates differing in host (Murphy et al., 2001), location (Baggesen et al., 2000; Kim et al., 2004) or time (Ribot et al., 2002; Lawson et al., 2004), it has been concluded that DT104 spread clonally throughout the world (Threlfall, 2000). However, isolates from epidemiological outbreaks are expected to be closely related, after descent from a common ancestor, and, consequently, to show nearly identical genotype (Achtman, 2002). Collections of strains that concentrate on the most virulent isolates, e.g. disease outbreaks, may thus introduce a selection bias (Gupta and Maiden, 2001).

The lack of study of the relationship between pathogenic strains of bacteria and their abundant counterpart causing no symptoms in humans or animals has been pointed out by Maynard Smith et al. (2002). Like *Salmonella* Typhimurium, many pathogenic bacteria do not rely on the generation of pathological conditions for their long-term evolutionary survival, but because they can cause disease opportunistically, their population structure is mainly studied from disease-associated populations. In this study, we focused on the asymptomatic population of *S. enterica* Typhimurium DT104 found in healthy pig carriers in order to establish the genetic and phenotypic diversity associated with the broader ecology of pathogenic bacteria.

2. Materials and methods

2.1. Bacterial isolates

2.1.1. Asymptomatic isolates

Asymptomatic *Salmonella* Typhimurium DT104 isolates were sampled during a cross-sectional study of the Canadian swine industry and collected from August to December 2003. Bacteria were isolated from 1 g samples of mesenteric lymph nodes taken from pig carcasses showing no apparent macroscopical lesions. A total of 7441 carcasses from 312 different herds were randomly sampled (first animal randomly selected and every fourth one thereafter for a total 20–25 animals per herd) in ten Canadian abattoirs receiving animals from either Quebec, Ontario, Manitoba, Saskatchewan or British Columbia. Bacterial cultures were isolated from collected samples in the laboratory of the Research Chair in Meat Safety of the Université de Montreal using methods described elsewhere (Côté et al., 2004). In order to establish the asymptomatic status of each animal at farm, we estimated the level of antibodies against *Salmonella* present in the blood of the animal using indirect enzyme-linked immunosorbent assays (Côté et al., 2004). We also excluded bacterial isolates collected from animals issued from farm with a history of clinical infection.

2.1.2. Disease-associated isolates

Clinical isolates of *Salmonella* Typhimurium DT104 ($n = 44$) represent all isolates collected from sick pigs with salmonellosis episode in the province of Quebec between the month of August 2003 and January 2004. Isolates were obtained from the Laboratoire d'épidémiosurveillance animale du Québec (LEAQ) in St-Hyacinthe, Quebec, and were collected from 23 different farms. Since *S.* Typhimurium DT104 and *S.* Typhimurium var. Copenhagen DT104 are both highly relevant to public health and genetically closely related, isolates from both types were combined in asymptomatic and disease-associated collections.

2.2. Phenotypic characterization

2.2.1. Serotyping and phage-typing

Serological identification of *Salmonella* spp. and phage-typing technique using standard methodologies were performed at the LEAQ laboratories in Saint-Hyacinthe and at Health Canada Laboratory for Foodborne Zoonoses, Guelph, Ontario (details in Poppe et al., 2002).

2.2.2. Antimicrobial resistance (AMR)

Antimicrobial susceptibility testing of all isolates was performed using the Kirby–Bauer disc diffusion method following criteria established by the NCCLS (2006) (performance standards for antimicrobial disc and dilution susceptibility tests for bacteria isolated from animals, 2004). The range of antimicrobials tested covers four classes of antibiotics broadly used: co-amoxiclav (Ax), ampicillin (Am), apramycin (Ap), cefoxitin (Cfx), ceftiotur (Ct), cephalothin (Ce), chloramphenicol (Cm), enrofloxacin (En), gentamycin (Gm), neomycin (Ne), tetracycline (Te) and trimethoprime-sulfas (T/S). Results were interpreted according to the NCCLS criteria following performance standards when available, or accordingly to the manufacturer. In this study, isolates with intermediate phenotype were grouped with susceptible isolates in order to not over-estimate occurrence of resistance.

2.3. Genotypic characterization

2.3.1. Pulse-field gel electrophoresis (PFGE)

Genomic DNA preparation and digestion with restriction endonucleases, *Xba*I and *Spe*I (Invotrogen, CA USA), were done in accordance with the Center for Disease Control and Prevention standard protocol (1-day (24–28 h) standardized laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by pulse field gel electrophoresis (PFGE), 2004). The resulting DNA fragments were separated by agarose gel electrophoresis in a 1% (w/v) SeaKem Gold agarose (Cambrex, USA) gel with a CHEF DR II system (BioRad, USA) at 6 V/cm with 0.5× Tris–Borate electrophoresis (TBE) buffer. *Salmonella* Braenderburg 'Universal Marker' was restricted with *Xba*I and was used as the reference marker while certain test isolates were included in more than one gel as an internal control. Analysis of PFGE data was performed using Bionumerics (Applied Maths, Kortrijik, Belgium) using 'different bands' algorithm for clustering and the unweighted pair group for arithmetic means (or UPGMA) tree-building approach with optimization of 0.5% and 2.5% position tolerance. Visual inspection of the patterns and internal controls matching was performed as a final step for analysis. Individual genotypes within each restriction enzyme analyses

are referred to as pulse-type while the genotype formed by the combined pulse-type of both analyses is referred to as PFGE pattern.

2.4. Diversity analyses

Fist, we compared the genotypic and phenotypic diversity of Quebec asymptomatic and disease-associated isolated using computed rarefaction curves with 95% confidence intervals (PAST: Paleontological statistics, Version 1.40, program available from the University of Oslo website). Rarefaction is an ecological tool to standardize the calculation of taxa richness for a given number of sampled isolates and rarefaction curves is a plot of the number of taxa, e.g. genotype, as a function of the number of isolates sampled (Gotelli and Colwell, 2001). Second, we used the Simpson's diversity index $(1 - \sum(n/N)^2$; where n is the total number of isolates of a particular genotype and N the total number of isolates) to compare the genotypic and phenotypic diversity of asymptomatic and disease-associated isolates partitioned: (1) among herds; (2) within herds over time; (3) within herds at one time. The value can range from zero, where all isolates in the sampled population share the same genotype, or can get close to one, indicating a population where each isolates has a unique genotype.

3. Results

3.1. Diversity in asymptomatic DT104

A total of 85 asymptomatic *S.* Typhimurium DT104 isolates were collected out of a total of 391 *S. enterica* strains identified among healthy swine carriers. DT104 was the most frequent phage type observed, with a proportion of approximately 50% of the Typhimurium isolates, and around 22% of the entire *Salmonella* population (Table 1). Using PFGE, we identified a total of 62 different genotypes among the DT104 isolates. More precisely, 25 and 27 pulse-types were identified using *Xba*I and *Spe*I respectively for PFGE, with a total of 50 isolates (62.5%) showing a unique PFGE pattern.

Using antimicrobial resistance, a total of ten resistance patterns were observed. Within the resistant phenotypes, 73 isolates (91.3%) harbored resistance to at least one antimicrobial and one isolate was resistant to eight antimicrobials (AxAmCfxCftCeCmTeT/S). The most common patterns were AmCmTe (32.5%), AmCmNeTe (26.3%) and AmCmNeTeT/S (21.3%). The remaining phenotypes were present at different frequencies: AmNeTeT/S (3.8%), AmCm (2.5%) and AxAmCmTe (1.3%). Seven isolates (8.8%) were totally susceptible to the antimicrobial tested.

Table 1
Proportion of asymptomatic *Salmonella* Typhimurium DT104 in Canada

Serovar	Location			
	Quebec[a]	Ontario[a]	Others[a]	Total[a]
Typhimurium DT104	31 (21.1)	52 (34.7)	2 (2.1)	85 (21.7)
Typhimurium others	47 (32.0)	30 (20.0)	10 (10.6)	87 (22.3)
Others *Salmonella* spp.	69 (46.9)	68 (45.3)	82 (87.2)	219 (56.0)
Total[b]	147 (37.6)	150 (38.4)	94 (24.0)	391

[a] Data is presented as presence and percent within location.
[b] Data is presented as total presence and percent from total population.

3.2. Comparison of diversity of asymptomatic and disease-associated isolates

Based on PFGE genotyping of Quebec isolates, a total of 27 PFGE patterns were observed among 31 asymptomatic isolates against 30 patterns among the 44 disease-associated isolates. Using rarefaction curves to compare both populations with samples of equal size and estimate the 95% confidence intervals, we observed significantly more genotypes in the asymptomatic collection (Fig. 1a). Standardized to a sample size of 31, we would expect to identify 23.0 ± 1.4 (95% CI) genotypes in the disease-associated population, which is significantly lower that the 27 observed genotypes among the asymptomatic isolates. Similar results are obtained when using rarefaction curves on *Xba*I or *Spe*I analyses separately (Fig. 1b and c).

The antimicrobial resistance phenotype diversity is greater in disease-associated isolates than in asymptomatic DT104 isolates from Quebec, with 13 patterns observed in the disease-associated isolates and five patterns within the asymptomatic isolates. Using rarefaction curves, we observed significantly more phenotype in the disease-associated isolates, with 10.9 ± 1.1 (95% CI) phenotypes (Fig. 1d). The most common resistance patterns among the disease-associated isolates were AmCmNeTe (36.4%), AmCmNeTeT/S (18.2%) and AmCmTe (13.6%). The remaining isolates also showed various resistance patterns at low frequencies: AmNeTeT/S (6.8%), AmCmNe and AmTeT/S (4.5% each), AxAmCmNe, AmCmT/S, AmCmNeT/S, AmCmGmNeTe, AmApCmNeTeT/S, AmApCmGmNeTe and CmTe were all found in 2.3% of the isolates. When considering intermediate resistance phenotype as well, similar patterns are observed with a steady increase in the number of resistance profile in both asymptomatic and disease-associated isolates (unpublished).

We also used Simpson's diversity index to compare the genotypic and phenotypic diversity of the two populations observed among and within herds (Table 2). In accordance with the rarefaction analysis, the genotypic diversity observed among herds is slightly greater in asymptomatic isolates while the phenotypic diversity is larger in the disease-associated isolates. Yet, when considering the diversity observed within a single herd over time, the genotypic as well as the phenotypic diversity becomes larger. Finally, when one considers the diversity observed within herd at one given time for the disease-associated isolates (i.e. similar conditions to that of the asymptomatic isolates), the genotypic and the phenotypic diversity significantly decreases below the diversity observed in the asymptomatic isolates. For example, the diversity in resistant phenotypes drops down to zero among disease-associated isolates indicating that only a single phenotype is ever observed within each herd at any given time.

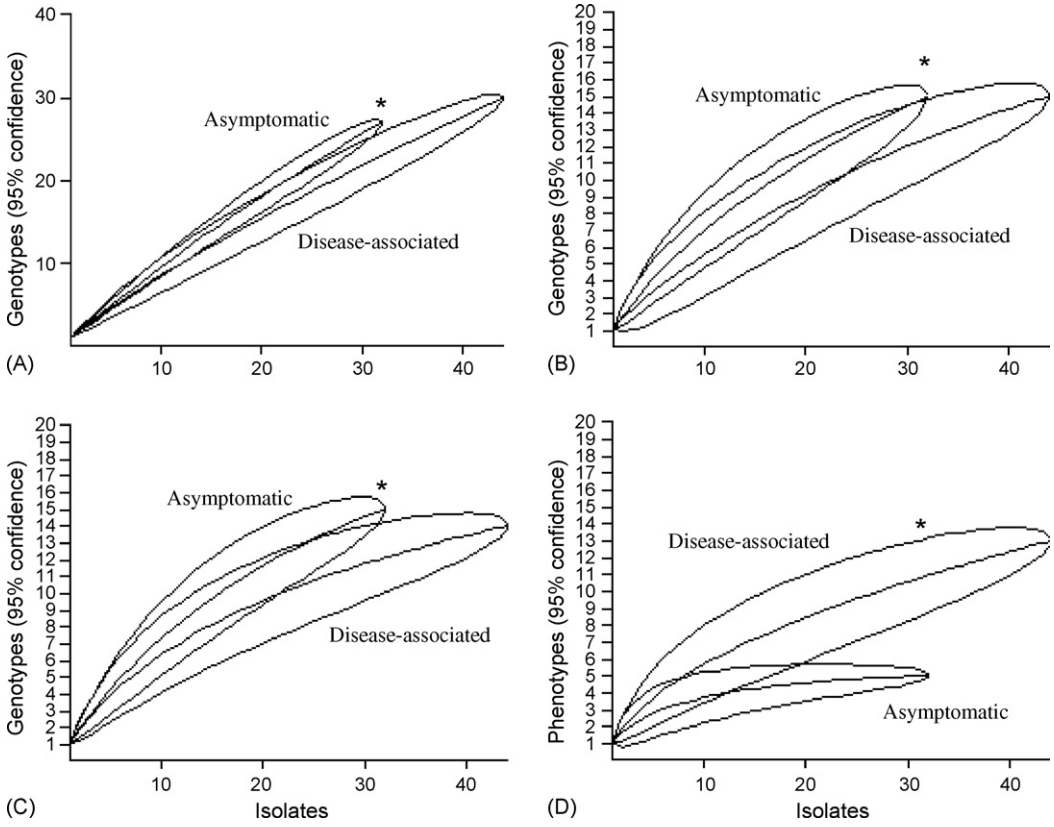

Fig. 1. Rarefaction curves are presented with 95% confidence intervals comparing the number of genotypes observed within asymptomatic and disease-associated *Salmonella* Typhimurium DT104 isolates for (A) PFGE patterns; (B) *Xba*I pulse-types; (C) *Spe*I pulse-types; (D) the number of phenotypes observed in the same two populations. Significant differences at comparable sample size are denoted by*.

Table 2
Diversity of *Salmonella* Typhimurium DT104 within and among herds

Population	Partition	Diversity			
		PFGE patterns	*Xba*I	*Spe*I	AMR
Asymptomatic	Among herds[a]	0.96	0.87	0.89	0.77
	Within herds[b]	0.64 ± 0.06	0.51 ± 0.10	0.56 ± 0.05	0.27 ± 0.07
Disease-associated	Among herds[a]	0.93	0.72	0.85	0.80
	W/over time[b]	0.48 ± 0.08	0.38 ± 0.09	0.46 ± 0.07	0.23 ± 0.11
	W/one time[b]	0.25 ± 0.11	0.25 ± 0.11	0.25 ± 0.11	0.00 ± 0.00

[a] Data are presented as Simpson's diversity index ($1 - S$).
[b] Data are presented as mean Simpson's diversity index ($1 - S$) with standard error.

4. Discussion

To our knowledge, this is the first study to asses the genotypic and phenotypic diversity of *Salmonella* Typhimurium DT104 issued from an asymptomatic animal population. Although asymptomatic and disease-associated isolates show similar diversity among all herds over time, asymptomatic isolates show greater genotypic and phenotypic diversity within individual herds. Numerous studies also using PFGE as a genotyping method and focusing on disease-associated isolates have previously reported low genetic diversity in DT104 (Hilton and Penn, 1998; Casin et al., 1999; Prager et al., 1999; Baggesen et al., 2000; Ebner and Mathew, 2001; Murphy et al., 2001; Liebana et al., 2002; Heir et al., 2002; Kim et al., 2004). Poppe et al. (2002) were the first to observe considerable genetic diversity within DT104 isolates of different origins, although, a single variant represented more than 70% of all isolates. Even so, longitudinal studies of human cases made in the USA and UK reported indistinguishable DT104 isolates over a 15 years period (Ribot et al., 2002; Lawson et al., 2004). It thus becomes clear that an important aspect of *Salmonella* Typhimurium DT104 population structure and ecology is neglected when considering disease or outbreaks isolates only.

The lower diversity in the disease-associated bacterial population was probably caused by a selection bias for the most ''virulent'' bacterial strains following a selective sweep of a highly adaptive genotype within individual herds. The low diversity observed within herds at any given time contrasting

with the high diversity observed over time and among herds suggests the sequential pattern of fixation predicted by models of periodic selection (Atwood et al., 1951). Conversely, asymptomatic isolates may be less prone to strong periodic selection induced by the host immune response or antimicrobial treatments administered to sick animals. Moreover, the diversity observed in asymptomatic isolates could be explained by the recent diversification of DT104 strains that would have successfully colonized asymptomatic pigs as a new ecological niche (Threlfall et al., 2005). The extent of diversity observed over time in disease-associated isolates suggests that migration of new DT104 genotypes and/or phenotypes is frequent in pig herds, and is most likely a significant source of new types in this animal. Movement of DT104 strains between herds could result from animal trade or most likely from passive and active transport due to human, insect or rodent's activity.

Resistance genes are often located on transferable elements, like conjugative plasmids (Poppe et al., 1996, 2001), and may thus affect the genotypic diversity of bacteria. However, the lack of relationship observed between genotypes and resistance profiles in disease associated isolates is most likely due to our consistent exclusion of unreliable small bands (which could have been associated with plasmids) from PFGE analyses, and is thus suggestive of the active role plasmid-like structures may play in virulence and its associated traits.

From a public health perspective, it thus becomes essential to integrate the ecology of pathogenic bacteria into models of evolution and population structure. Such models are necessary for epidemiological investigations and to predict the response of pathogen populations to selective pressures imposed by host immunity (Levin et al., 1999). The extent and structure of genetic diversity in microbial populations is also of great importance for the effective management of antimicrobial drugs (Levin et al., 1999; Palumbi, 2001; Witte, 2004).

Because the high presence and diversity of asymptomatic DT104 in pigs can have dramatic effects on public health and the swine industry (Baggesen and Aarestrup, 1998; Molbak et al., 1999), it is essential that the present asymptomatic collection truly reflects the biology of asymptomatic population of pathogenic bacteria. Because the risk of cross-contamination during transport or in holding pens is high, we collected samples from the mesenteric lymph node of animals with high levels of antibody against *Salmonella* antigen present in their serum, which is a method recognized as a better indicator of the animal's status at farm (Fravalo et al., 1999; Carlson and Blaha, 2001; Côté et al., 2004). To include additional precautions (Gebreyes et al., 2004), we also excluded bacterial isolates collected from animals or farms with a history of clinical infection. Although it is possible that a number of strains studied in this work expressed some level of pathogenocity, the most stringent conditions were hold to define asymptomatic DT104 isolates which were made to ensure inclusion of only true asymptomatic strains in this group.

Some limitations are inherent to the use of PFGE in ecological or evolutionary studies of bacteria. Although this technique has been the standard for *Salmonella* epidemiological surveys because of its high discriminatory power, PFGE does not accurately depict the process by which bacterial clones diversify (Spratt, 2002; Spratt et al., 2004; Feil et al., 2004). PFGE integrates information from total genomic DNA, while population structure of bacterial species should be assessed by examining genes for which the genetic variation is likely to be selectively neutral (Selander et al., 1990; Achtman, 2002).

In this study, we identified considerable genetic and phenotypic diversity among asymptomatic *S.* Typhimurium DT104 isolates and we observed a high proportion of multidrug-resistant DT104 among apparently healthy pigs, indicating the importance of pigs, in addition to cattle and humans, as reservoirs of *Salmonella* serovars and vectors of antimicrobial resistance. We thus conclude that an important aspect of *Salmonella* population structure and ecology was previously overlooked in investigations of disease-associated isolates. Although conventional clinical studies of pathogenic bacteria are still desirable, it is essential to perform this type of studies to increase our understanding of pathogens populations and to improve public health policies on the control of bacterial infections and drug resistance.

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References

Achtman, M., 2002. A phylogenetic perspective on molecular epidemiology. In: Sussman, M. (Ed.), In Molecular Medical Microbiology, vol. 1. Academic Press, London, pp. 485–509.

Atwood, K.C., Schneider, L.K., Ryan, F.J., 1951. Periodic selection in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 37, 146–155.

Baggesen, D.L., Aarestrup, F.M., 1998. Characterization of recently emerged multiple antibiotic-resistant *Salmonella enterica* serovar Typhimurium DT104 and other multiresistant phage types from Danish pig herds. Vet. Rec. 143, 95–97.

Baggesen, D.L., Sandvang, D., Aarestrup, F.M., 2000. Characterization of *Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. J. Clin. Microbiol. 38, 1581–1586.

Carlson, A.R., Blaha, T., 2001. In-herd prevalence of *Salmonella* in 25 selected Minnesota swine farms. J. Swine Health Prod. 9, 7–10.

Casin, I., Breuil, J., Brisabois, A., Moury, F., Grimont, F., Collatz, E., 1999. Multidrug-resistant human and animal *Salmonella typhimurium* isolates in France belong predominantly to a DT104 clone with the chromosome- and integron-encoded β-lactamase PSE-1. J. Infect. Dis. 179, 1173–1182.

Center for disease control and prevention, 2004. One-day (24 h–28 h) standardized laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal Salmonella serotypes, and Shigella sonnei by pulse field gel electrophoresis (PFGE). CDC, Atlanta, Georgia, USA.

Côté, S., Letellier, A., Lessard, L., Quessy, S., 2004. Distribution of *Salmonella* in tissues following natural and experimental infection in pigs. Can. J. Vet. Res. 68, 241–248.

Davies, R., Wray, C., 1997. Study of multi-resistant *Salmonella typhimurium* infection in pig herds: preliminary findings. Pig J. 40, 80–88.

Ebner, P.D., Mathew, A.G., 2001. Three molecular methods to identify *Salmonella enterica* serotype Typhimurium DT104: PCR fingerprinting, multiplex PCR and rapid PFGE. FEMS Microbiol. Lett. 205, 25–29.

Farmer, J.J., 1999. Enterobacteriaceae. Introduction and identification. Manual of clinical microbiology. American Society of Microbiology, Washington, DC.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., Spratt, B.G., 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bact. 186, 1518–1530.

Fone, D.L., Barker, R.M., 1994. Association between human and animal infections with *Salmonella typhimurium* DT104 in Herefordshire. Commun. Dis. Rep. CDR Rev. 4, R136–R140.

Fravalo, P., Rose, V., Eveno, E., Salvat, G., Madec, F., 1999. Bacteriological assessment of the Salmonella status of market-aged pigs: Evolution from rearing to slaughter. Journées de la Recherche Porcine en France (in French) 31, 383–389.

Gebreyes, W.A., Davies, P.R., Turkson, P.K., Morrow, W.E., Funk, J.A., Altier, C., 2004. *Salmonella enterica* serovars from pigs on farms and after slaughter and validity of using bacteriologic data to define herd *Salmonella* status. J. Food Prot. 67, 691–697.

Gebreyes, W.A., Altier, C., Thakur, S., 2006. Molecular epidemiology and diversity of *Salmonella* serovar Typhimurium in pigs using phenotypic and genotypic approaches. Epidemiol. Infect. 134, 187–198.

Gotelli, N.J., Colwell, R.K., 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecol. Lett. 4, 379–391.

Gresham, A.C.J., 1996. An old disease of increasing significance–*Salmonella* infection in pigs in Great Britain. Pig J. 37, 59–64.

Gupta, S., Maiden, M.C., 2001. Exploring the evolution of diversity in pathogen populations. Trends Microbiol. 9, 181–185.

Heir, E., Lindstedt, B.A., Nygard, I., Vardund, T., Hasseltvedt, V., Kapperud, G., 2002. Molecular epidemiology of *Salmonella typhimurium* isolates from human sporadic and outbreak cases. Epidemiol. Infect. 128, 373–382.

Helms M, Ethelberg S, Mølbak K, DT104 study group, 2005. International Salmonella Typhimurium DT104 infections, 1992–2001. Emerg. Infect. Dis. 11, 859–867.

Hilton, A.C., Penn, C.W., 1998. Restriction enzyme analysis of randomly amplified polymorphic DNA amplicons of *Salmonella enterica* ser. Enteritidis PT4 and Typhimurium DT104. Lett. Appl. Microbiol. 27, 158–162.

Kim, S., Chun, S.G., Lim, O.Y., Park, M.S., Kang, Y.H., Park, Y.H., Lee, B.K., 2004. Genomic relationship of *Salmonella enterica* serovar typhimurium DT104 isolates from Korea and the United States. J. Microbiol. 42, 14–19.

Lawson, A.J., Desai, M., O'Brien, S.J., Davies, R.H., Ward, L.R., Threlfall, E.J., 2004. Molecular characterization of an outbreak strain of multiresistant *Salmonella enterica* serovar Typhimurium DT104 in the UK. Clin. Microbiol. Infect. 10, 143–147.

Levin, B.R., Lipsitch, M., Bonhoeffer, S., 1999. Population biology, evolution, and infectious disease: convergence and synthesis. Science 283, 806–809.

Liebana, E., Garcia-Migura, L., Clouting, C., Clifton-Hadley, F.A., Lindsay, E., Threlfall, E.J., McDowell, S.W., Davies, R.H., 2002. Multiple genetic typing of *Salmonella enterica* serotype typhimurium isolates of different phage types (DT104, U302, DT204b, and DT49) from animals and humans in England, Wales, and Northern Ireland. J. Clin. Microbiol. 40, 4450–4456.

Maynard Smith, J., Feil, E.J., Smith, N.H., 2002. Population structure and evolutionary dynamics of pathogenic bacteria. BioEssays 22, 1115–1122.

Molbak, K., Baggesen, D.L., Aarestrup, F.M., Ebbesen, J.M., Engberg, J., Frydendahl, K., Gerner-Smidt, P., Petersen, A.M., Wegener, H.C., 1999. An outbreak of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104. N. Engl. J. Med. 341, 1420–1425.

Murphy, T.M., McNamara, E., Hill, M., Rooney, N., Barry, J., Egan, J., O'Connell, A., O'Loughlin, J., McFaddyen, S., 2001. Epidemiological studies of human and animal *Salmonella typhimurium* DT104 and DT104b isolates in Ireland. Epidemiol. Infect. 126, 3–9.

National Committee for Clinical Laboratory Standards, Performance Standards for Antimicrobial Disk Susceptibility Tests, Ninth Edition*: Approved Standard M02-A9*. NCCLS, Villanova, PA, USA, 2006.

Palumbi, S.R., 2001. Human as the World's greatest evolutionary force. Science 293, 1786–1790.

Poppe, C., McFadden, K.A., Demczuk, W.H.B., 1996. Drug resistance, plasmids, biotypes and susceptibility to bacteriophages of *Salmonella* isolated from poultry in Canada. Int. J. Food Microbiol. 30, 325–344.

Poppe, C., Ayroud, M., Ollis, G., Chirino-Trejo, M., Smart, N., Quessy, S., Michel, P., 2001. Trends in antimicrobial resistance of Salmonella isolated from animals, foods of animal origin, and the environment of animal production in Canada, 1994–1997. Microbial. Drug Resist. 7, 197–212.

Poppe, C., Ziebell, K., Martin, L., Allen, K., 2002. Diversity in antimicrobial resistance and other characteristics among *Salmonella* Typhimurium DT104 isolates. Microb. Drug Resist. 8, 107–122.

Prager, R., Liesegang, A., Rabsch, W., Gericke, B., Thiel, W., Voigt, W., Helmuth, R., Ward, L., Tschape, H., 1999. Clonal relationship of *Salmonella enterica* serovar typhimurium phage type DT104 in Germany and Austria. Zentralbl. Bakteriol. 289, 399–414.

Ribot, E.M., Wierzba, R.K., Angulo, F.J., Barrett, T.J., 2002. *Salmonella enterica* serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. Emerg. Infect. Dis. 8, 387–391.

Selander, R.K., Beltran, P., Smith, N.H., Helmuth, R., Rubin, F.A., Kopecko, D.J., 1990. Evolutionary genetics relationships of clones in *Salmonella* serovars that cause human typhoid and other enteric fevers. Infect. Immunol. 58, 2262–2275.

Spratt, B.G., 2002. Exploring the concept of clonality in bacteria. From methods in molecular biology. In: Woodford, N., Jonhson, A. (Eds.), Genomics, Proteomics, and Clinical Bacteriology: Methods and Reviews, vol. 266. Humana Press, Totowa, New Jersay, pp. 323–352.

Spratt, B.G., Hanage, W.P., Li, B., Aanensen, D.M., Feil, E.J., 2004. Displaying the relatedness among isolates of bacterial species—the eBURST approach. FEMS Microb. Lett. 24, 129–134.

Threlfall, E.J., 2000. Epidemic *Salmonella typhimurium* DT104–a truly international multiresistant clone. J. Antimicrob. Chem. 46, 7–10.

Threlfall, E.J., Frost, J.A., Ward, L.R., Rowe, B., 1994. Epidemic in cattle and humans of *Salmonella typhimurium* DT 104 with chromosomally integrated multiple drug resistance. Vet. Rec. 134, 577.

Threlfall, E.J., Hopkins, K.L., Ward, L.R., 2005. Diversification in *Salmonella* Typhimurium DT104. Emerg. Inf. Dis. 11, 980–981.

Wall, P.G., Morgan, D., Lamden, K., Griffin, M., Threfall, E.J., Ward, L.R., Rowe, B., 1995. Transmission of multi-resistant *Salmonella typhimurium* from cattle to man. Vet. Rec. 136, 591–592.

Witte, W., 2004. International dissemination of antibiotic resistant strains of bacterial pathogens. Inf. Gen. Evol. 4, 187–191.