Characterization of *Salmonella* Typhimurium isolates associated with septicemia in swine

Nadia Bergeron, Jonathan Corriveau, Ann Letellier, France Daigle, Sylvain Quessy

Abstract

Salmonella Typhimurium is frequently isolated from pigs and may also cause enteric disease in humans. In this study, 33 isolates of *S*. Typhimurium associated with septicemia in swine (CS) were compared to 33 isolates recovered from healthy animals at slaughter (WCS). The isolates were characterized using phenotyping and genotyping methods. For each isolate, the phage type, antimicrobial resistance, and pulsed-field gel electrophoresis (PFGE) DNA profiles were determined. In addition, the protein profiles of each isolate grown in different conditions were studied by Coomassie Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. Various phage types were identified. The phage type PT 104 represented 36.4% of all isolates from septicemic pigs. Resistance to as many as 12 antimicrobial agents, including some natural resistances, was found in isolates from CS and WCS. Many genetic profiles were identified among the PT 104 phage types. Although it was not possible to associate one particular protein with septicemic isolates, several highly immunogenic proteins, present in all virulent isolates and in most isolates from clinically healthy animals, were identified. These results indicated that strains associated with septicemia belong to various genetic lineages that can also be recovered from asymptomatic animals at the time of slaughter.

Résumé

Salmonella *Typhimurium est souvent isolée de porcs et peut causer une gastro-entérite chez l'humain. Dans cette étude, des isolats provenant de porcs septicémiques (ASC) (*n = *33) ont été comparés à des isolats provenant de porcs sains à l'abattoir (SSC) (*n = *33). Ces isolats ont été caractérisés par des méthodes phénotypiques et génotypiques. Le type phagique, le profil d'antibiorésistance et l'analyse des profils d'ADN en gel d'électrophorèse en champ pulsé (PFGE) ont été déterminés pour chacun des isolats. Le profil des protéines pour chacun des isolats, cultivés dans différentes conditions de croissance, a aussi été déterminé par la méthode Coomassie Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) et l'immunogénicité des protéines a été évaluée par immunobuvardage. Différents types phagiques ont été identifiés parmi les isolats. Chez les souches provenant de porcs septicémiques, le type phagique PT 104 représentait 36,4 % des isolats. Les isolats pouvaient être résistants à 12 antimicrobiens, si l'on considère aussi les résistances naturelles, autant chez les souches ASC que celles appartenant au groupe SSC. Parmi les souches PT 104, plusieurs groupes génétiques ont été identifiés. Il n'a toutefois pas été possible d'identifier une protéine en particulier chez le groupe d'isolats provenant de porcs septicémiques. Il a été possible d'identifier des protéines immunogènes chez tous les isolats virulents et chez la majorité des isolats provenant de porcs sains. Ces résultats indiquent que les souches associées aux porcs septicémiques proviennent de lignées génétiques variées dont certaines peuvent être aussi retrouvées chez les porcs asymptomatiques au moment de l'abattage.*

(Traduit par les auteurs)

Introduction

Salmonella is an important problem in both humans and animals worldwide. More than 2500 serotypes have been isolated in the *Salmonella* genus: most are potential human pathogens, but only a few serotypes have been regularly associated with human infections. *Salmonella* cause diseases in humans ranging from a mild gastro-enteritis to a systemic disease that can result in death. *Salmonella* Enteritidis and Typhimurium are quantitatively the most important causative agents in human foodborne illnesses. For the serovar Typhimurium alone, more than 200 definitive phage types have been identified. The phage type PT 104 causes particular concerns because of its increasing prevalence and the presence of resistance genes to many antibiotics (1).

Although most animals may carry the bacterium without exhibiting clinical signs, in pigs, *Salmonella* are associated with diseases and economic losses. Swine may represent a reservoir for human infection (2). The infection of pigs with the serovar

Chaire de recherche en salubrité des viandes (CRSV), Groupe de recherche sur les maladies infectieuses du porc (GREMIP), Département de pathologie et microbiologie, Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec (Bergeron, Corriveau, Letellier, Quessy); Département de microbiologie et immunologie, Faculté de médecine, Université de Montréal, Montréal, Québec (Daigle).

Address all correspondence to Dr. Sylvain Quessy; telephone: (450) 773-8521 ext. 8398; fax: (450) 778-8157; e-mail: sylvain.quessy@umontreal.ca

Dr. Quessy's current address is Faculté de médecine vétérinaire, Université de Montréal, CP 5000, Saint-Hyacinthe, Québec J2S 7C6.

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Choleraesuis is usually associated with systemic disease, whereas infection with the serovar Typhimurium is associated with enteric disease. However, septicemic episodes of *S*. Typhimurium, associated with severe clinical signs and sudden death, have been observed (3,4). In these animals, the serovar Typhimurium can be isolated from multiple organs; however, at slaughter, *S*. Typhimurium is one of the most frequently isolated serovar in apparently healthy pigs (5).

Infections caused by septicemic strains of *S*. Typhimurium can be associated with significant mortality in finishing pigs. Since previous studies showed persistence of strains in various tissues for many days following infection, the presence of these strains in finishing animals also represents a food safety concern (6). Thus, it is important to better characterize these isolates in order to understand the pathogenesis of infection and develop appropriate control measures. Asymptomatic animals may, following a stress period, begin to shed the bacteria and contaminate other animals during transportation and in the lairing pen at the slaughterhouse. During evisceration procedures, direct or cross-contamination of meat may result in human foodborne infections.

Studies on the diversity of salmonellae indicate that generally *Salmonella* species have low genetic diversity. To date, various phenotypic methods have been used to characterize this bacterium, including phage typing, biotyping, and antimicrobial resistance profiling (7,8). Genotyping methods such as pulsed-field gel electrophoresis (PFGE) (8,9), amplified-fragment length polymorphism (AFLP) (10), plasmid profiling (7–9), IS200 restriction fragment length polymorphism (RFLP) (11), and ribotyping (9) were also used. The current "gold standard" method of choice for molecular typing of *Salmonella* is PFGE (12).

Some phage types such as PT 104 of *S*. Typhimurium are known to harbor genes encoding resistance to many antibiotics. Multidrug-resistant PT 104 is an important human and animal pathogen that is widespread in western and eastern Europe, North America, and the Middle East (13). For instance, PT 104 are very often found to be resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-Type AmpChlStrSulTet) (1,8,13,14). Multiple resistance to antimicrobial agents is an important concern for human health.

The aim of this study was to compare, using phenotypic and genotypic methods, isolates of *S*. Typhimurium associated with septicemia in swine and isolates recovered from clinically healthy pigs at slaughter.

Materials and methods

Bacterial isolates

Salmonella isolates (n = 33) recovered from extra-intestinal organs and/or feces of dead pigs (CS; clinical signs) (with diagnosis of salmonellosis) and submitted for necropsy were obtained from Dr. S. Messier (Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec). Isolates (n = 33) from healthy pigs (WCS; without clinical signs) were collected at slaughter from animals with no macroscopic lesions (5). Unless otherwise noted, bacterial cultures were carried out at 37°C in Luria-Bertani (LB) Muller Broth (Difco Laboratories, Detroit, Michigan, USA) or on LB containing 1.5% (w/v) agar. Isolates were stored at −70°C in LB supplemented with 35% (v/v) glycerol.

Serotyping and phage typing

Isolates were serotyped and phage typed at the Laboratoire d'Épidémiologie Animale du Québec (LEAQ) in Saint-Hyacinthe, Québec or at the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario.

Antimicrobial resistance

Resistance of all isolates to various antimicrobial agents was determined by the Kirby-Bauer disk diffusion method following criteria established by the Clinical and Laboratory Standards Institute's (CLSI) guidelines (15). The range of antimicrobial agents tested covers many classes of antibiotics: amikacin (30 μg/disk) (Amk), amoxicillin-clavulanic acid (20/10 μg/disk) (Amc), ampicillin (10 μg/disk) (Amp), bacitracin (10 IU/disk) (Bac), cefoxitin (30 μg/disk) (Fox), ceftiofur (30 μg/disk) (Ctf), ceftriaxone (30 μg/disk) (Cro), cephalothin (30 μg/disk) (Cef), chloramphenicol (30 μg/disk) (Chl), ciprofloxacin (5 μg/disk) (Cip), clindamycin (2 μg/disk) (Cli), enrofloxacin (5 μg/disk) (Enr), erythromycin (15 μg/disk) (Ery), gentamicin (10 μg/disk) (Gen), kanamycin (30 μg/disk) (Kan), nalidixic acid (30 μg/disk) (Nal), neomycin (30 μg/disk) (Neo), quinupristin/dalfopristin (4.5/10.5 μg/disk) (Q/D), streptomycin (10 μg/disk) (Str), sulfisoxazole (250 μg/disk) (Sul), tetracycline (30 μg/disk) (Tet), trimethoprim-sulfamethoxazole (1.25/23.75 μg/disk) (Sxt), and vancomycin (30 μg/disk) (Van). Results were interpreted according to the CLSI guidelines (15,16) or in accordance with the manufacturer's instructions. The diameter zone of apramycin (15 μg/disk) (Apr) was interpreted in accordance with Mathew et al (17). All the antimicrobial agents were purchased from Oxoid (Nepean, Ontario) except ceftriaxone and sulfixozazole which were purchased from Becton Dickinson (BD-Canada, Oakville, Ontario). *Escherichia coli* ATCC 25922 was used as the standard reference strain. In this study, isolates with intermediate phenotype were grouped with susceptible isolates to prevent over-estimation of occurrence of resistance.

Growth conditions for protein production

The growth conditions known to encourage the *Salmonella* invasion process include anaerobiosis, high osmolarity, late-log-phase growth, and neutral pH (18,19). Aerobic growth conditions for bacterial cultures were induced by vigorous agitation (200 rpm) of tubes. Anaerobic conditions were induced by static incubation in an anaerobe jar equipped with a pressure gauge (Oxoid) and supplied with a gas generator envelope and a resazurin strip (Oxoid); the gauge and strip served to confirm the establishment of an anaerobic environment. The effect of high osmolarity (0.3 M NaCl) was assessed by the use of LB or nutrient broth (NB) (Difco Laboratories) supplemented with NaCl. The effect of iron was tested by supplementing NB with 40 μM $FeSO_4$ (Sigma-Aldrich Canada, Oakville, Ontario) and depleting iron with 100 μM 2,2′-dipyridyl (Sigma). To analyze the effect of pH, strains were grown in NB buffered with 0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma) at pH of 5.0 or 6.5.

SDS-PAGE

LB agar plates were first inoculated with bacteria stored in frozen glycerol and incubated at 37°C overnight. A 50-mL sample of one of the media described herein was inoculated with single colonies and incubated for 20 h at 37°C. Optical density (OD) of ~0.6 at 600 nm was obtained. The bacterial suspensions were centrifuged and resuspended in PBS and sonicated twice, for 2 min each time, on ice (Sonics Material, Danbury, Connecticut, USA). The suspension was centrifuged again for 20 min at 4°C. Proteins present in the supernatant were harvested, mixed with an equal volume of buffer to insure solubility, boiled 5 min and separated by SDS-PAGE standard technique in 12.5% polyacrylamide vertical slab gel with 4.5% stacking gel. Gels were stained with Coomassie brillant blue stain (Bio-Rad Laboratories, Mississauga, Ontario).

Production of antisera

Pigs (n = 5) were injected intramuscularly once a week for 6 wk with 1 mL of a formalin-killed (0.5% v/v, 18 h) suspension of 10^9 CFU/mL of a field isolate serovar Typhimurium PT 104 (obtained from a septicemic pig) and grown overnight in NB. Pigs were euthanized 1 wk later and blood was collected. All procedures using animals were done in accordance with guidelines of the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Western blotting

Following SDS-PAGE, proteins were transferred to the nitrocellulose membrane (Bio-Rad) by electroblotting in a transblot apparatus (Bio-Rad) with methanol-Tris-glycine buffer according to the standard technique. Casein (2%, w/v) in Tris-buffered saline was used to block unreacted sites and the nitrocellulose membrane was incubated overnight with 1:400 (v/v) dilutions of the pig antisera raised against whole cell antigen (see above). After washing in Tris-NaCl, the membrane was incubated with a peroxidase-conjugated goat anti-swine IgG (heavy + light chains) (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for 60 min at a dilution of 1:2000 in 2% (w/v) casein in Tris-NaCl. After repeated washings, the presence of bound antigens was visualized by reacting the nitrocellulose membrane with 0.06% 4-chloro-1-naphthol (Sigma) in cold (−20°C) methanol mixed to 0.02% H_2O_2 in Tris-NaCl. Apparent molecular weights were calculated by comparison with standards of known molecular weight (Bio-Rad).

Pulsed-field gel electrophoresis (PFGE)

Genomic DNA was prepared in agarose gel plugs from an overnight bacterial culture on LB agar using a modified protocol (20). Briefly, bacterial cultures were resuspended to obtain an OD of 1.4 at 610 nm. Bacterial culture and Seakem Gold agarose 1.5% (Cambrex Corporate, East Rutherford, New Jersey, USA) were mixed in a mold to form plugs. The agarose plugs were incubated 2 h at 54°C in lysis solution and proteinase K (Quiagen, Mississauga, Ontario). Cell debris and any excess proteinase K were removed by washing twice with millipore water and 4 times with Tris-EDTA buffer. Genomic DNA was digested with restriction endonucleases, *Spe*I (recognition sequence ACTAGT) and *Xba*I (recognition sequence TCTAGA) (Invitrogen Canada, Burlington, Ontario).

The PFGE was performed using a CHEF DR II system (Bio-Rad) on a horizontal agarose gel electrophoresis in a 1% (w/v) SeaKem Gold agarose gel at 6 V/cm with 0.5× Tris-Borate electrophoresis buffer. Gels were stained with SYBR Safe DNA Gel Stain (Invitrogen) and photographed on a UV transilluminator. *Salmonella* Braenderup "Universal Marker" was used as the reference marker (21). The PFGE patterns obtained were analyzed by the Bionumerics Software (Applied Maths, Kortrijik, Belgium) using algorithm for clustering and the unweighted pair group method with arithmetic mean (UPGMA) tree-building approach with optimization of 2.0% and 4.0% and 3.5% for position tolerance for *Xba*I and *Spe*I, respectively. Visual inspection of the patterns was performed as a final validation step.

Statistical analysis

Computer software (SAS version 9.1; SAS Institute, Cary, North Carolina, USA) was used to analyze the data. Univariate analysis using the exact chi-squared test examined the relationship between various categorical variables. The comparison was made between the isolates from septicemic pigs and isolates from healthy pigs. Another comparison was made between the isolates from phage type PT 104 and isolates from the group of other phage types. The statistical significance was set at $P < 0.05$.

Results

Typing of isolates

Salmonella Typhimurium was the only serovar identified in this study out of the 33 isolates from CS. When isolates were phage typed, it was found that 36.4% (12/33) belonged to PT 104 while the others belonged to 11 different phage types. For the 33 isolates from WCS, the prevalence of strains belonging to PT 104 was 51.5% (17/33) while the second most prevalent type was PT 193 (8/33). No association was found between the origin of the isolates and the phage type PT 104 (P = 0.32). Only 3 isolates could not be phage typed.

Salmonella isolates were found in extra-intestinal organs or feces from pigs. Animals came from a total of 55 farms, 7 farms were sampled more than once. Among these 7 farms, isolates from both CS and WCS were found in 3 farms.

Antimicrobial susceptibility testing

In this study, 24 antimicrobial agents (AMA) were tested. Among those showing resistance, 5 AMA were considered as natural resistance (Bac, Cli, Ery, Q/D, Van). All isolates were resistant to up to 7 AMA among the 19 remaining AMA tested. Six isolates in both groups showed no resistance, but none of these were PT 104. Among all isolates, 19 different antimicrobial resistance profiles were identified. Twelve different profiles were observed in isolates from CS, 13 different profiles were found in isolates from WCS while 6 profiles were common to both CS and WCS isolates. The most prevalent resistance profile (Amp, Chl, Neo, Tet, Kan, Bac, Cli, Ery, Q/D, Van, Str, Sul) was found in both CS and WCS.

Among the *S*. Typhimurium PT 104 isolates from CS, 3 different profiles were observed, whereas among PT 104 isolates from WCS, 4 antimicrobial resistance profiles were found. Two profiles were common in PT 104 isolates from both CS and WCS. Among the 3 non-typable isolates 2 showed the same antimicrobial resistance profiles.

PT 104 isolates from CS were significantly more resistant to some AMA compared with other phage types from CS: Amp ($P = 0.0002$), Chl ($P < 0.0001$), Kan ($P = 0.009$), Neo ($P = 0.009$), Str ($P = 0.0008$), and Sul ($P = 0.01$). PT 104 isolates from WCS were also more resistant to some AMA versus other phage types: Amp ($P = 0.0003$), Chl ($P < 0.0001$), Kan ($P = 0.02$), and Str ($P = 0.002$). Other phage types were more resistant than PT 104 in WCS: Gen ($P = 0.02$).

Protein profiles by SDS-PAGE and Western blotting

In order to reflect the various environmental conditions encountered by *Salmonella* during colonization and invasion of the host, different growth conditions were used to study the protein profiles of the 2 groups of isolates. For a given environmental growth condition, protein profiles of isolates associated with septicemia and those isolated from clinically healthy pigs were similar (data not shown). It was not possible to relate any proteins to septicemic isolates; however, several different patterns were expressed when a given isolate was grown under different environmental conditions (data not shown). For instance, when grown in iron-restricted media, expression of a ~33 kDa protein was observed on Western blots for all isolates in both groups of pigs. In addition, few immunogenic proteins were found to be expressed in all growth conditions.

Pulsed-field gel electrophoresis (PFGE)

A total of 17 different PFGE profiles were found when *Xba*I was used. Twelve different profiles were observed for isolates from CS compared with 11 profiles for isolates from WCS. Six profiles were common among isolates from CS and WCS. Profile 1 was significantly more prevalent in isolates from CS compared with WCS ($P = 0.03$). A total of 19 different profiles were found with *Spe*I. Using *Spe*I, 11 different profiles were identified in isolates from CS, while 13 profiles were identified from isolates from WCS. Five profiles were common to isolates of both CS and WCS. Profile 20 was significantly more prevalent among isolates from WCS compared with CS ($P = 0.02$).

For *Xba*I, among the *S*. Typhimurium PT 104 isolates from CS, 7 different genotypes were observed, profile 2 being significantly more prevalent in PT 104 isolates compared with other phage types ($P < 0.0001$). Profile 1 was significantly more prevalent in isolates from other phage types compared with PT 104 ($P = 0.03$). Profiles 2, 7, 16, and 17 for isolates from CS and profiles 2, 5, and 11 for isolates from WCS were found in PT 104 isolates only. The 3 non-typable isolates harboured different profiles for *Xba*I.

For *Spe*I, among the *S*. Typhimurium PT 104 isolates from CS, 8 different profiles were found; none more frequently than another. It was not possible to identify a major profile with *Spe*I in the group of PT 104 isolates and in the group of other phage types. Profiles 30 and 35 for isolates from CS and profiles 29, 30, and 34 for isolates from WCS were found in PT 104 isolates. Only 2 of the 3 isolates, which could not be typed, had the same *Spe*I profile. Summaries of the results are presented in Tables I and II.

Discussion

In this study, PT 104 isolates were compared with all other phage types because of their antimicrobial resistance, recognition as food-borne pathogens, greater virulence, suggested clonale origin, and zoonotic potential (9,13,22). Phage type PT 104 was found to be the most prevalent phage type in characterized isolates. This agrees with other studies that reported similar results in Ontario, the United States, and France in isolates from apparently healthy animals (23–25). However, previous studies from Quebec and Spain indicated that PT 104 was the second phage in importance after PT 108 and PT 193, respectively (4,26).

Phage type PT 104 was found in 31.4% (11/35) of *S*. Typhimurium isolates from apparently healthy food-producing animals in Japan, as determined by the Japanese Veterinary Antimicrobial Resistance Monitoring Program (1999–2001) (27). It was found that the percentage of PT 104 isolates was lower in animals from WCS, as opposed to what was observed in our study. On the other hand, another similar study completed by the same group between 2002 and 2005 showed a significant decrease ($P < 0.01$) to 4.1% (2/48) of the PT 104 *S*. Typhimurium isolates (28).

In the current study, PT 104 isolates were generally found to be more resistant to antimicrobial agents than other phage types but it was not possible to associate this phage type to pigs with CS. Many authors from various countries reported that most isolates of *S*. Typhimurium PT 104 are resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (1,8,13,14,24,29,30) and that some isolates are also kanamycin resistant. In our study, 89.7% (26/29) of PT 104 isolates possess the complete pentaresistance profile; among these isolates, 69.0% (20/29) showed additionnal resistence to kanamycin and neomycin. It has been described that the pentaresistance genes in PT 104 isolates are situated on the chromosome and located on integrons, and thus rarely horizontally transmissible (13,31–33). The origin of DNA containing the pentaresistance genes is unknown, previous studies proposing a possible clonal dissemination of the *S*. Typhimurium PT 104 in the population (9,22,30,31). Our antimicrobial resistance results suggested that other genetic lineages can be found in PT 104 isolates from swine in Canada, particularly in healthy animals.

Bacteria obtained from infected animals are routinely examined by SDS-PAGE and immunoblotting to search for the presence of putative virulence determinants. In our study, we had the opportunity to compare isolates from diseased animals to isolates from healthy animals. Although we made the assumption that isolates from healthy animals were less likely to express some virulence factors, we cannot rule out the possibility that some of the isolates from healthy animals were in fact isolates from animals that had recovered from disease. However, since the occurrence of outbreaks associated with *S*. Typhimurium is quite low in Quebec, it seemed reasonable to expect that isolates from healthy animals were less likely to be virulent. Nevertheless, although differences in protein profiles were observed when strains were grown in different environmental growth conditions, it was not possible to associate a particular protein to septicemic strains. On the other hand, we observed the expression of a ~33 kDa protein when isolates were grown under iron-limiting conditions. Expression of a protein

Table I. Antimicrobial resistance and pulsed-field gel electrophoresis (PFGE) profiles of *S.* Typhimurium PT 104 isolates from diseased and healthy pigs

Antimicrobial profile	PFGE (*Xba*I)[ab]	PFGE (*Spe*I)[ab]
Amp, Chl, Neo, Tet, Kan, Bac, Cli, Ery, Q/D, Van, Str, Sul	5^{aa} 17^{a} 1^{ab} $2^{aaaabbbb}$ 3^{bbb} 6^{bb} 7^{b} 9^{b}	23^{a} 24^{aa} 25^{a} 35^{a} 18^{ab} 19^{abb} 30^{ab} 20^{bb} 22^{b} 26^{b} 27^{b} 29^{bb} 34^{b}
Amp, Chl, Tet, Bac, Cli, Ery, Q/D, Van, Str, Sul	7^{a} 16^{a} 2^{ab} 1^{b} 5^{b}	19^{a} 21^{a} 23^{a} 18^{b} 20^{b} 27^{b}
Amp, Neo, Tet, Sxt, Kan, Bac, Cli, Ery, Q/D, Van, Sul	8^{a}	23^{a}
Tet, Bac, Cli, Ery, Q/D, Van, Sul	1^{b}	18^{b}
Neo, Kan, Bac, Cli, Ery, Q/D, Van, Str	11^{b}	19^{b}

[a] Isolates with clinical signs (CS).

[b] Isolates without clinical signs (WCS).

Table II. Antimicrobial resistance and pulsed-field gel electrophoresis (PFGE) profiles of *S.* Typhimurium isolates belonging to phage types other than PT 104 from diseased and healthy pigs

Antimicrobial profile	PFGE (*Xba*I)[ab]	PFGE (*Spe*I)[ab]
Amp, Chl, Neo, Tet, Kan, Bac, Cli, Ery, Q/D, Van, Str, Sul	1^{a} 5^{a} 8^{a} 14^{a} 3^{b} 13^{b}	18^{a} 25^{a} 28^{a} 24^{ab} 27^{b}
Bac, Cli, Ery, Q/D, Van	1^{aaaa} 4^{b} 6^{b}	18^{a} 21^{aa} 31^{a} 20^{b} 26^{b}
Apr, Gen, Tet, Sxt, Bac, Cli, Ery, Q/D, Van, Sul	3^{a} 10^{a} 1^{aab}	21^{aa} 28^{a} 32^{a} 36^{b}
Tet, Bac, Cli, Ery, Q/D, Van	1^{aa} 3^{b} 10^{b}	18^{aab} 19^{b}
Neo, Tet, Kan, Bac, Cli, Ery, Q/D, Van, Sul	15^{a} 4^{b}	19^{a} 20^{b}
Amp, Tet, Bac, Cli, Ery, Q/D, Van	1^{a} 12^{a}	18^{aa}
Apr, Gen, Tet, Sxt, Bac, Cli, Ery, Q/D, Van, Str, Sul	1^{a}	23^{a}
Amp, Tet, Sxt, Bac, Cli, Ery, Q/D, Van, Sul	1^{a}	18^{a}
Tet, Bac, Cli, Ery, Q/D, Van, Str	1^{a}	18^{a}
Bac, Cli, Ery, Q/D, Van, Sul	1^{a}	18^{a}
Gen, Tet, Bac, Cli, Ery, Q/D, Van, Str, Sul	3^{b} 4^{bb}	20^{b} 22^{b} 33^{b}
Apr, Gen, Neo, Tet, Sxt, Bac, Cli, Ery, Q/D, Van, Sul	1^{bb}	18^{b} 25^{b}
Amp, Neo, Tet, Kan, Bac, Cli, Ery, Q/D, Van, Str, Sul	4^{b}	22^{b}
Tet, Bac, Cli, Ery, Q/D, Van, Str, Sul	9^{b}	26^{b}
Amp, Neo, Tet, Kan, Bac, Cli, Ery, Q/D, Van	7^{b}	22^{b}

[a] Isolates with clinical signs (CS).

[b] Isolates without clinical signs (WCS).

of a similar molecular weight, named SitA, under iron-limiting conditions was also reported by Zhou et al (34). These authors identified an iron transport system, encoded within the *Salmonella* pathogenicity island 1 (SPI1) of *S.* Typhimurium. As observed in this study, the induction of *sit* gene expression was prevented by the addition of Fe^{2+} to the growth medium. Furthermore, Janakiraman and Slauch (35) demonstrated that the putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *S.* Typhimurium. The fact that this protein was found in both types of isolates suggests that it may also be needed for colonization of pigs in sub-clinical infections.

Although no protein was associated with septicemic isolates, many proteins that are strongly recognized by swine antisera were found in both type of isolates in all growth conditions. In particular, a ~37 kDa protein was found to be present in all septicemic isolates and in most isolates from clinically healthy animals and might be considered for vaccine production given its high immunogenicity.

Among genotyping methods, PFGE is considered the reference method for DNA fingerprinting in *Salmonella* and other foodborne pathogens (12), and has been proposed as a system for differentiating epidemic strains from endemic ones (36). The use of PFGE (*Xba*I) is considered a good tool for the epidemiological typing of *S.* Typhimurium (37). If genetic variation does not significantly impact the size or electrophoretic mobility of a restriction fragment, then the change may not be identified as a separate pulsotype (8). By the use of 2 or more enzymes for PFGE analysis, the discriminatory power of the method may be enhanced for differentiating *Salmonella* isolates (38). Some authors, however, have emphasized that *S.* Typhimurium has often been considered very clonal and PFGE may not have sufficient discriminatory power to differentiate the various phage types (39). In this study, PFGE was able to demonstrate genetic variability among *S.* Typhimurium isolates in general, while it was also instrumental in demonstrating that some genetic clusters were associated with isolates from diseased animals.

In this study, different procedures were used in order to differentiate *S.* Typhimurium isolates recovered from septicemic animals from those of healthy pigs. Overall, a poor correlation was observed between the various typing methods, as previously observed in other studies (8,9). Usually a multiple typing approach is used to increase the ability to differentiate strains, especially when trying to separate isolates that appear to have clonal distribution (8,9).

However, we demonstrated, using genetic typing methods, a very high genetic diversity in isolates from sick animals, suggesting that multiple genetic lineages might be responsible for clinical outbreaks

in swine herds. Some genetic profiles, however, were found almost exclusively in diseased animals. In a recent study that compared genetic variability of both groups of isolates within the herds (40), a significantly higher difference of genetic diversity in strains from asymptomatic animals was also observed, suggesting that once a virulent strain is established within a herd, this genetic lineage may persist for a prolonged period. In addition, the fact that some genetic clusters can be found in both types of isolates can be explained by transport up to the slaughter period and septicemic isolates following recovery from the disease.

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References

1. Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N Engl J Med 1998;338:1333–1338.
2. Berends BR, Van Knapen F, Snijders JMA, Mossel DAA. Identification and quantification of risk factors regarding *Salmonella* spp. on pork carcasses. Int J Food Microbiol 1997;36:199–206.
3. Desrosiers R. Les maladies en émergence chez le porc. Méd Vét Québec 1999;29:185–188.
4. Letellier A, Messier S, Paré J, Ménard J, Quessy S. Distribution of *Salmonella* in swine herds in Québec. Vet Microbiol 1999;67:299–306.
5. Letellier A, Messier S, Quessy S. Prevalence of *Salmonella* spp. and *Yersinia enterocolitica* in finishing swine at Canadian abattoirs. J Food Prot 1999;62:22–25.
6. Côté S, Letellier A, Lessard L, Quessy S. Distribution of *Salmonella* in tissues following natural and experimental infection in pigs. Can J Vet Res 2004;68:241–248.
7. Threlfall EJ, Frost JA. The identification, typing and fingerprinting of *Salmonella:* Laboratory aspects and epidemiological applications. J Appl Bacteriol 1990;68:5–16.
8. Foley SL, White DG, McDermott PF, et al. Comparison of subtyping methods for differentiating *Salmonella enterica* serovar Typhimurium isolates obtained from food animal sources. J Clin Microbiol 2006;44:3569–3577.
9. Liebana E, Garcia-Migura L, Clouting C, et al. 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 2002;40:4450–4456.
10. Lindstedt BA, Heir E, Vardund T, Kapperud G. A variation of the amplified-fragment length polymorphism (AFLP) technique using three restriction endonucleases, and assessment of the enzyme combination *Bgl*II-*Mfe*I for AFLP analysis of *Salmonella enterica* subsp. *enterica* isolates. FEMS Microbiol Lett 2000;189:19–24.
11. Baquar N, Threlfall EJ, Rowe B, Stanley J. Phage type 193 of *Salmonella* Typhimurium contains different chromosomal genotypes and multiple IS200 profiles. FEMS Microbiol Lett 1994;115:291–295.
12. Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV. PulseNet: The molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis 2001;7:382–389.
13. Humphrey T. *Salmonella* Typhimurium definitive type 104. A multi-resistant *Salmonella*. Int J Food Microbiol 2001;67:173–186.
14. Gebreyes WA, Altier C. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolates from swine. J Clin Microbiol 2002;40:2813–2822.
15. Clinical and Laboratory Standards Institue (CLSI). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard. 2nd ed. [CLSI Document M31-A2] Wayne, Pennsylvania: CLSI, 2002.
16. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Thirteenth Informational Supplement. [CLSI Document M100-S13] Wayne, Pennsylvania: CLSI, 2003.
17. Mathew AG, Saxton AM, Upchurch WG, Chattin SE. Multiple antibiotic resistance patterns of *Escherichia coli* isolates from swine farms. Appl Environ Microbiol 1999;65:2770–2772.
18. Galan JE, Curtiss R, 3rd. Expression of *Salmonella* Typhimurium genes required for invasion is regulated by changes in DNA supercoiling. Infect Immun 1990;58:1879–1885.
19. Lee CA, Falkow S. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc Natl Acad Sci U S A 1990;87:4304–4308.
20. Michaud S, Arbeit RD, Gaudreau C. Molecular strain typing of *Campylobacter jejuni* by pulsed-field gel electrophoresis in a single day. Can J Microbiol 2001;47:667–669.
21. Hunter SB, Vauterin P, Lambert-Fair MA, et al. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: Converting the national databases to the new size standard. J Clin Microbiol 2005;43:1045–1050.
22. Baggesen DL, Sandvang D, Aarestrup FM. Characterization of *Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. J Clin Microbiol 2000;38:1581–1586.
23. Farzan A, Friendship RM, Dewey CE, Muckle AC, Gray JT, Funk J. Distribution of *Salmonella* serovars and phage types on 80 Ontario swine farms in 2004. Can J Vet Res 2008;72:1–6.
24. Gebreyes WA, Altier C, Thakur S. Molecular epidemiology and diversity of *Salmonella* serovar Typhimurium in pigs using phenotypic and genotypic approaches. Epidemiol Infect 2006;134:187–198.
25. Lailler R, Grimont F, Jones Y, Sanders P, Brisabois A. Subtyping of *Salmonella* Typhimurium by pulsed-field gel electrophoresis and comparisons with phage types and resistance types. Pathol Biol (Paris) 2002;50:361–368.

26. García-Feliz C, Collazos JA, Carvajal A, et al. *Salmonella enterica* infections in Spanish swine fattening units. Zoonoses Public Health 2007;54:294–300.
27. Esaki H, Morioka A, Kojima A, et al. Epidemiological characterization of *Salmonella* Typhimurium DT104 prevalent among food-producing animals in the Japanese Veterinary Antimicrobial Resistance Monitoring Program (1999–2001). Microbiol Immunol 2004;48:553–556.
28. Kawagoe K, Mine H, Asai T, et al. Changes of multi-drug resistance pattern in *Salmonella enterica* subspecies *enterica* serovar Typhimurium isolates from food-producing animals in Japan. J Vet Med Sci 2007;69:1211–1213.
29. Daly M, Buckley J, Power E, et al. Molecular characterization of Irish *Salmonella enterica* serotype Typhimurium: Detection of class I integrons and assessment of genetic relationships by DNA amplification fingerprinting. Appl Environ Microbiol 2000;66:614–619.
30. Ng LK, Mulvey MR, Martin I, Peters GA, Johnson W. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob Agents Chemother 1999;43:3018–3021.
31. Ridley A, Threlfall EJ. Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella* Typhimurium DT 104. Microb Drug Resist 1998;4:113–118.
32. Schmieger H, Schicklmaier P. Transduction of multiple drug resistance of *Salmonella enterica* serovar Typhimurium DT104. FEMS Microbiol Lett 1999;170:251–256.
33. Threlfall EJ, Frost JA, Ward LR, Rowe B. Epidemic in cattle and humans of *Salmonella* Typhimurium DT 104 with chromosomally integrated multiple drug resistance. Vet Rec 1994;134:577.
34. Zhou D, Hardt WD, Galan JE. *Salmonella* Typhimurium encodes a putative iron transport system within the centisome 63 pathogenicity island. Infect Immun 1999;67:1974–1981.
35. Janakiraman A, Slauch JM. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella* Typhimurium. Mol Microbiol 2000;35:1146–1155.
36. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–2239.
37. Doran G, Morris D, O'Hare C, et al. Cost-effective application of pulsed-field gel electrophoresis to typing of *Salmonella enterica* serovar Typhimurium. Appl Environ Microbiol 2005;71:8236–8240.
38. Ridley AM, Threlfall EJ, Rowe B. Genotypic characterization of *Salmonella* Enteritidis phage types by plasmid analysis, ribotyping, and pulsed-field gel electrophoresis. J Clin Microbiol 1998;36:2314–2321.
39. Weill FX, Guesnier F, Guibert V, et al. Multidrug resistance in *Salmonella enterica* serotype Typhimurium from humans in France (1993 to 2003). J Clin Microbiol 2006;44:700–708.
40. Perron GG, Quessy S, Letellier A, Bell G. Genotypic diversity and antimicrobial resistance in asymptomatic *Salmonella enterica* serotype Typhimurium DT104. Infect Genet Evol 2007;7:223–228.