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Genetic relatedness of a rarely isolated *Salmonella*: *Salmonella enterica* serotype Niakhar from NARMS animal isolates

J. D. Tankson[1], P. J. Fedorka-Cray[1]*, C. R. Jackson[1] and M. Headrick[2]

[1]*USDA/Agricultural Research Services, Bacterial and Epidemiology Antimicrobial Resistance Research Unit, 950 College Station Road, Athens, GA 30605, USA;* [2]*US Food and Drug Administration, Center for Veterinary Medicine, 7500 Standish Place HFV-200, Rockville, MD 20855, USA*

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***Background*: In the United States, *Salmonella enterica* serotype Niakhar is infrequently isolated. Between 1997 and 2000, the animal arm of the National Antimicrobial Resistance Monitoring System—Enteric Bacteria (NARMS) assayed a total of 22 383 *Salmonella* isolates from various animal sources (swine, cattle, chickens, turkeys, cats, horses, exotics and dogs) for antimicrobial susceptibility. Isolates originated from diagnostic and non-diagnostic submissions.**

***Objectives*: To study the phenotypic and genotypic characteristics of *Salmonella* Niakhar.**

***Methods and results*: Only five (0.02%) of the 22 383 isolates were identified as *Salmonella* Niakhar. Antimicrobial resistance testing indicated that three isolates were pan-susceptible, one isolate was resistant to ampicillin and one isolate was resistant to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole. RAPD–PCR analysis, PFGE and ribotyping indicated that two pan-susceptible isolates were genetically similar, whereas the three remaining isolates were genetically different. The one *Salmonella* Niakhar isolate that was multiresistant harboured a class I integron, *intI1* and two large plasmids.**

***Conclusions*: This study represents the first report of a ciprofloxacin-resistant *Salmonella* isolate from the animal arm of NARMS.**

Keywords: antimicrobial resistance, multiple drug resistance, integrons, PFGE, ribotyping

Introduction

Non-typhoid *Salmonella* is one of the leading causes of foodborne illness in the United States.[1,2] Gastroenteritis as a result of salmonellosis has been associated with the consumption of contaminated eggs, poultry, meat products, unpasteurized milk and juice, milk products, contaminated raw fruits and vegetables, direct contact with animals, including reptiles, exotic birds, rodents and insects, contaminated water and soil, and person-to-person contact.[1,3–5] Currently there are over 2500 different *Salmonella* serotypes.[6] Although salmonellosis is most often associated with a smaller number of serotypes,[7] all serotypes have the potential to cause disease.

Salmonella enterica serotype Niakhar belongs to serogroup V[6] and is not commonly isolated in the United States and abroad. *Salmonella* Niakhar was first isolated at Niakhar, Senegal, in 1970 from a febrile man with diarrhoea.[8] Since 1970, *Salmonella* Niakhar has been isolated exclusively from animals in the United States.[9–11]

Animal isolates in the United States are tested for susceptibility to antimicrobials as part of the animal arm of the National Antimicrobial Resistance Monitoring System—Enteric Bacteria (NARMS) located in Athens, GA, USA (http://www.ars.usda.gov/Main/docs.htm?docid=6750; 30 September 2005, date last accessed). Isolates originate from a wide variety of animal sources and are submitted to NARMS by veterinary diagnostic laboratories, on-farm studies, and raw product collected from federally inspected slaughter and processing plants.[12]

Until 2000, resistance was observed among all of the antimicrobials tested in NARMS with the exception of ciprofloxacin. Ciprofloxacin is primarily used to empirically treat gastroenteritis in human medicine, and similar antimicrobials (enrofloxacin and sarafloxacin) have been used in small animal medicine[13–15] as well as treatment for airsacculitis in poultry.[13,15] Ciprofloxacin belongs

*Corresponding author. Tel: +1-706-546-3685; Fax: +1-706-546-3066; E-mail: pcray@saa.ars.usda.gov

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to the fluoroquinolone class of antimicrobials and resistance occurs as a result of a two-step mutation in the *gyrA* gene.[16,17] Although multiple resistance in *Salmonella* appears to be increasing such as DT104 and Newport,[18,19] *Salmonella* isolates originating from animals in the United States have been susceptible to ciprofloxacin. Between 1997 and 2000, NARMS analysed a total of 22 383 *Salmonella* isolates from cattle, swine, cats, dogs, horses, turkey, exotics and chickens. Only five (0.02%) isolates were identified as *S. enterica* serotype Niakhar and originated from either on-farm studies or veterinary diagnostic laboratories. Until submission of the *Salmonella* Niakhar isolate in 2000, resistance to ciprofloxacin was not observed from any *Salmonella* serotype tested in the animal arm of NARMS. This study reports the phenotypic and genotypic characteristics of *Salmonella* Niakhar isolates from NARMS.

Materials and methods

Bacterial isolates

Five isolates of *S. enterica* serotype Niakhar were identified within the animal arm of NARMS from 1997 to 2000. The isolates originated from on-farm dairy cattle studies in 1997 (designated as isolates A and B), and from veterinary diagnostic laboratories in 2000 (designated as isolates C, D and E). All isolates were serotyped at the National Veterinary Services Laboratories (NVSL), located in Ames, IA, USA. Maintenance of NARMS isolates has been described.[12] Establishing and maintaining bacterial isolates for further study was accomplished by following the isolation procedures practised by NARMS.[12]

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using a custom-made panel of antimicrobials in the Sensititre™ semi-automated system (TREK Diagnostics, Inc., Westlake, OH, USA) as per the manufacturer's instructions. Antimicrobials or their classes were selected based on their use in human and veterinary medicine and, where possible, full range MICs were used (http://www.ars.usda.gov/Main/docs.htm?docid=6750; 30 September 2005, date last accessed). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* 29212 were used as quality control strains. Results were interpreted according to the NCCLS guidelines for broth microdilution methods[20,21] when available.[21]

Random amplification of polymorphic DNA analysis

Random amplification of polymorphic DNA analysis (RAPD) analysis was performed using PCR primers 1290 (5′-GTGGATGCGA-3′)[22] and 1254 (5′-CCGCAGCCAA-3′)[23] synthesized by OPERON (QIAGEN® Operon, Germantown, MD, USA). The whole cell DNA template for PCR was prepared according to the protocol of Hilton *et al*.[23] with the final 10 µL of reaction consisting of 2 mM $MgCl_2$ (Gene Mate Plastics, ISC BioExpress), 50 pmol of RAPD oligonucleotide primer (each primer contained forward and reverse oligonucleotides combined), 0.2 mM deoxynucleoside triphosphates (Boehringer Mannheim, Indianapolis, IN, USA) and 0.05 U of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA). The samples were prepared as described by the Idaho Technology Rapidcycler, Idaho Falls, ID, USA.[24] Program parameters for the Rapidcycler were (i) denaturation at 92°C for 30 s, (ii) annealing at 36°C for 7 s, and (iii) elongation at 72°C for 70 s, for a total of two cycles; immediately followed by (iv) denaturation at 92°C for 1 s, (v) annealing at 36°C for 7 s, and (vi) elongation at 72°C for 1 min, for a total of 38 cycles. DNA products for the RAPD–PCR were analysed by gel electrophoresis on a 1.5% agarose gel mixed with 1× Tris/acetic acid/EDTA (TAE) containing ethidium bromide (0.02 mg/L) at 100 V for 30 min. The 100 bp ladder (Roche Diagnostics) served as the molecular weight standard for determining the size of the PCR products.

PFGE

A 24 h *Salmonella* PFGE procedure was performed as described by Pulse Net: The National Molecular Subtyping Network for Foodborne Disease Surveillance.[25] Bacterial genomic DNA plugs were digested using the restriction enzyme, *Xba*I (Promega, Madison, WI, USA). DNA standards were prepared from *S. enterica* serotype Newport AM01144. Digested DNA was separated using the CHEF-DRII PFGE system as per the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out for 19 h at 6 V, using 2.2 L of the buffer 0.5× Tris/borate/EDTA (TBE) at a temperature of 14°C, and an initial pulse time of 2.16 s followed by a final switch time of 63.8 s. BioNumerics software (Applied Maths Scientific Software Development, Belgium) was used to normalize the band patterns based on the molecular weight standards included on each gel. The Dice coefficient was used to statistically analyse the band pattern dissemination. A dendrogram was constructed to illustrate the genetic relatedness of the isolates.

Ribotyping

Ribotyping was performed using the automated RiboPrinter® microbial characterization system (DePont Qualicon™, Wilmington, DE, USA) as per the manufacturer's directions. In brief, colonies were picked from 24 h SBA plates, the cells were lysed and DNA was digested using the restriction enzyme, *Pvu*II. Samples were then loaded into the RiboPrinter®, and restriction fragments were separated by electrophoresis and simultaneously transferred onto a nylon membrane. The DNA probe for *Salmonella* species was then hybridized to the genomic DNA of each isolate on the membrane. Bound labelled antibodies were captured using a chemiluminescence detection system containing a charged-coupled device camera. Once analysed, the band patterns were manually assigned to ribogroups. Restriction patterns were combined by similarity to form an individualized ribogroup which could be used to establish genetic relatedness of the isolates.[26–29] Based on the results of normalized band patterns, Bionumerics software program version 3.5 constructed a dendrogram using Pearson's coefficient to determine genetic relatedness.

PCR amplification

DNA probes were generated using PCR specific primers for classes 1, 2, 3 and 4 integrons (*intI1*, *intI2*, *intI3* and *int4*, respectively) as described (Table 1). Oligonucleotides were synthesized by OPERON (QIAGEN® Operon). Template preparation from whole cell DNA was obtained as described previously.[23] Samples were prepared as described (Idaho Technology Rapidcycler). Rapidcycler program parameters for the integrons were (i) denaturation at 94°C for 15 s, (ii) annealing at 55°C for 15 s, and (iii) elongation at 72°C for 35 s, for a total of 30 cycles. The PCR products were purified using the CONCERT Rapid PCR Purification system (Gibco BRL, Life Technologies) and analysed by gel electrophoresis using 1.5% agarose mixed in 1× TAE plus ethidium bromide (0.02 mg/L) at 100 V for 30 min. The 100 bp ladder served as the molecular weight standard.

Southern transfer and hybridization

Following PFGE, the gel was placed in the CL-1000 Crosslinker (UVP Laboratory Products, Upland, CA, USA) where ultraviolet light at 60 mJ of energy was applied to separate DNA into single strands. The single-stranded DNA was transferred to a positively charged,

Table 1. PCR primers used to generate probes for detection of integrons and resistance genes

Gene	Template source	Oligonucleotide sequences[a]	Product size (bp)	Reference or source
intI1	*Salmonella* Typhimurium DT104	L: 5′-ACATGTGATGGCGACGCACGA-3′ R: 5′-ATTTCTGTCCTGGCTGGCGA-3′	568	Ploy *et al.*[60]
intI2	*E. coli* J53.3::Tn7	L: 5′-CACGGATATGCGACAAAAAGGT-3′ R: 5′-GTAGCAAACGAGTGACGAAATG-3′	788	Ploy *et al.*[60]
intI3	*E. coli* AK9873	L: 5′-GCCTCCGGCAGCGACTTTCAG-3′ R: 5′-ACGGATCTGCCAAACCTGACT-3′	979	Ploy *et al.*[60]
int4	*Vibrio cholerae*	C: 5′-TTCAACGCTCGCAACTAGAAC-3′ D: 5′-GTGTGGCAAGTCACGGTCTTT-3′	526	this study
intCS		F: 5′-GGCATCCAAGCAGCAAG-3′ R: 5′-AAGCAGACTTGACCTGA-3′	variable[c]	Levesque and Roy[59]
1290[b]		5′-GTGGATGCGA-3′	variable	Hopkins and Hilton[22]
1254[b]		5′-CCGCAGCCAA-3′	variable	Hilton *et al.*[23]

[a]L, C and F = forward primer; D and R = reverse primer.
[b]RAPD primer number.
[c]Range for band fragments to occur is variable.

nylon membrane (Roche Diagnostics) using the Model 785 Vacuum Blotter and Pump (Bio-Rad). The procedures for DNA–DNA hybridization and detection were carried out as described (Genius 3 kit, Boehringer Mannheim). Hybridization of DNA occurred at 65°C. Hybridizing DNA fragments were detected by using an anti-digoxigenin antibody–alkaline phosphatase conjugate mixed with a colour substrate solution of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Genius system, Boehringer Mannheim).

Plasmid analysis

For large plasmids, DNA was cut using the S1 nuclease enzyme.[30,31] Electrophoresis of the 1.2% agarose gel occurred in two blocks. Block 1 involved running the gel for 14 h at 6 V, including a pulse time of 45 s for both the initial and final time. In Block 2, the gel was run for 6 h at 6 V, having a pulse time of 25 s for both the initial and final time. For these two blocked times, 2.2 L of 0.5× TBE buffer was used at a temperature of 14°C. Following electrophoresis, Southern transfer and hybridization were carried out as described above. Small plasmid detection was conducted as described using the QIA prep spin miniprep kit (Qiagen, Inc., Valencia, CA, USA). Gel electrophoresis was done on a 0.8% agarose gel at 80 V for 45 min. Supercoil DNA (Invitrogen, Life Technologies) was used as the molecular weight standard. The gel was placed in ethidium bromide (0.01 mg/L) for 20 min to allow for staining, followed by 10 min in nanopure water to eliminate excess ethidium bromide.

Conjugation

Salmonella Niakhar isolate D possessing resistance to nine antimicrobials served as the donor strain. One isolate of *Salmonella* Heidelberg and one isolate of *Salmonella* Cerro retrieved from the 2001 *Salmonella* NARMS isolates were used as recipient strains. Both of these strains were resistant to apramycin, an antimicrobial to which isolate D developed susceptibility. Nine antimicrobials (ampicillin, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole) served as the selective markers based on the antibiotic resistance phenotype of isolate D. Conjugation was achieved by mating a fresh culture of the donor strain (*Salmonella* Niakhar) and recipient strains (*Salmonella* Heidelberg and *Salmonella* Cerro separately) in tubes containing 5 mL of TSB for 18–24 h in a 37°C aeration incubator. The transconjugants were evaluated by the results obtained from three donor/recipient dilutions: 1 : 1, 1 : 2 and 1 : 5. The serotype combinations were centrifuged for 2 min at 4950 rpm so that the serotypes would have close contact with one another during the incubation period. After the incubation period of the mating mixture, 100 μL of the donor/recipients at 1 : 1, 1 : 2 and 1 : 5 dilutions were spread equally using a sterilized bacterial cell spreader (VWR Scientific, West Chester, PA, USA) on the appropriately labelled TSA plates containing combinations of the selective antibiotics [32 μg of apramycin and 32 μg of ampicillin; 32 μg of apramycin and 32 μg of chloramphenicol; 32 μg of apramycin and 64 μg of kanamycin; 32 μg of apramycin and 32 μg of nalidixic acid; 32 μg of apramycin and 64 μg of streptomycin; 32 μg of apramycin and 512 μg of sulfamethoxazole; 32 μg of apramycin and 16 μg of tetracycline; 32 μg of apramycin, 4 μg of trimethoprim and 76 μg of sulfamethoxazole; 32 μg of apramycin (served as control); and the appropriate amount of all previously mentioned antimicrobials with the exception of apramycin in a TSB plate (served as other controls)]. Thirty-six hours later, transconjugants were examined for antimicrobial susceptibility profiles. PFGE analyses, as described earlier, were performed and evaluated on *Salmonella* Niakhar, *Salmonella* Heidelberg, *Salmonella* Cerro and transconjugants.

Sequencing

A probe was generated from PCR primers of the 5′ (*intCSF*) and 3′ (*intCSR*) conserved segment of *intI1* as described (Table 1). The PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) was used to amplify the conserved segment with the following program parameters: (i) 94°C for 5 min, (ii) denaturation at 94°C for 30 s, (iii) annealing at 55°C for 30 s, and (iv) elongation at 72°C for 2.5 min, continuous for 30 cycles. After 30 cycles, elongation occurred again at 72°C for 5 min. Using a gel containing 1.5% agarose, electrophoresis was conducted for 30 min at 100 V. DNA was purified using the Concert Rapid PCR Purification System protocol. Primers and templates were sent to the Molecular Genetics Instrumentation Facility (University of GA, Athens, GA, USA) to evaluate the DNA sequence of the conserved region of *intI1*. Assembly of sequence contigs for the 5′–3′ region of the integron was performed using GENE

RUNNER software (Hastings Software, Inc.). Analyses of the sequence in order to determine the antibiotic resistance genes located within the gene cassette of the integron were accomplished using BLAST software (National Center for Biotechnology Information, Bethesda, MD, USA).

Results

Antimicrobial resistance

Demographics, source and antimicrobial resistance patterns for each isolate are shown in Table 2. Isolates A, B and E were susceptible to all antimicrobials evaluated, whereas isolate C was only resistant to ampicillin. However, isolate D exhibited multiple resistance (defined as resistance to two or more antimicrobials) to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim/sulfamethoxazole.

RAPD analyses

RAPD–PCR results are shown in Figure 1. RAPD–PCR primers 1290 (lanes 2–8) and 1254 (lanes 10–16) were used. Using RAPD primer 1290, isolates A (lane 3) and B (lane 4) were genetically similar, whereas isolates C, D and E (lanes 5, 7 and 6, respectively) were genetically unrelated to all isolates. Using RAPD primer 1254, isolates A (lane 11) and B (lane 12) were genetically similar, whereas isolates C, E and D (lanes 13, 14 and 15, respectively) were genetically different. *E. coli* (lanes 2 and 10) and *Salmonella* Typhimurium DT104 (lanes 8 and 16) isolates were used to represent a distant and similar banding pattern, respectively.

PFGE

E. coli (lane 2) and *Salmonella* Typhimurium DT104 (lane 8) isolates served as controls as described for Figure 1. Using visual and dendrogram analyses (Figure 2a and b), isolates A and B were genetically identical to one another. Isolates C, D and E (Figure 2a) were genetically unrelated. A greater than seven band pattern difference between isolates was used to classify isolates as dissimilar.[32] Dendrogram results (Figure 2b) indicated that isolates C and D exhibited 77% similarity, whereas isolate E and *E. coli* exhibited 79% similarity, and *Salmonella* Typhimurium DT104 exhibited 68% similarity to all isolates including *E. coli*.

Ribotyping analysis

Ribotype patterns were compared using Pearson's coefficient and a dendrogram was constructed (Figure 3). Banding patterns showed a high degree of relatedness (95%) between isolates A and B.

Table 2. Demographics, source and antimicrobial resistance patterns for each *S. enterica* serotype Niakhar isolate

Isolate	Year	Source	Location	Region[a]	Antimicrobial resistance pattern[b]
A	1997	on-farm—dairy cattle	Southeastern United States	2	none
B	1997	on-farm—dairy cattle	Western United States	5	none
C	2000	veterinary diagnostic laboratory—cattle	Northeastern United States	1	AMP
D	2000	veterinary diagnostic laboratory—cattle	North Central Midwestern United States	3	AMP, CHL, CIP, KAN, NAL, STR, SUL, TET, SXT
E	2000	veterinary diagnostic laboratory—dog	South Central United States	4	none

[a]Identification of state by region: region 1 = ME, VT, NH, NY, MA, CT, RI, PA, MD, DE, NJ, OH, IN, MI; region 2 = VA, KY, TN, NC, SC, GA, AL, WV, FL, PR; region 3 = ND, SD, NE, KS, MN, IA, MO, WI, IL; region 4 = OK, AR, LA, TX, MS; region 5 = WA, MT, OR, ID, WY, CO, UT, NM, AZ, NV, CA.
[b]AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole.

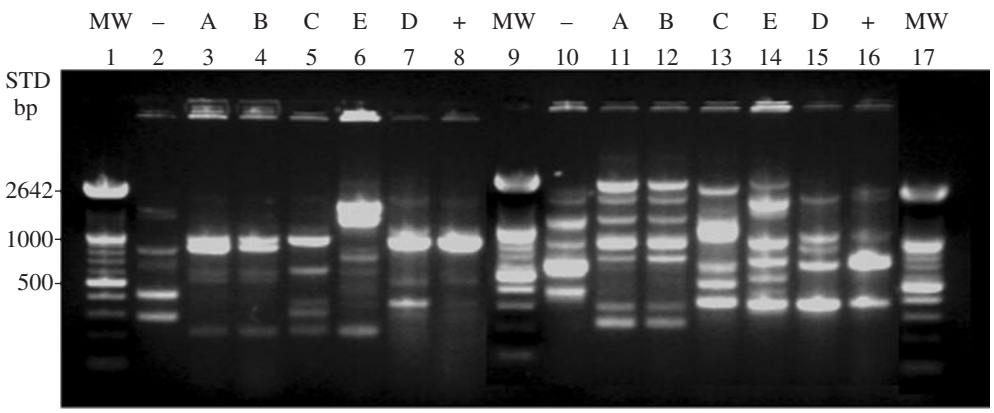

Figure 1. Comparison of genetic relatedness of the five Niakhar isolates using RAPD-PCR analysis. RAPD–PCR primers were 1290 (lanes 2–8) and 1254 (lanes 10–16). Standard sizes in base pairs are located to the left of the figure. Lanes 1, 9 and 17, 100 bp ladder; lanes 2 and 10, *E. coli* (negative control); lanes 3 and 11, isolate A; lanes 4 and 12, isolate B; lanes 5 and 13, isolate C; lanes 6 and 14, isolate E; lanes 7 and 15, isolate D; and lanes 8 and 16, *Salmonella* Typhimurium DT104 (positive control).

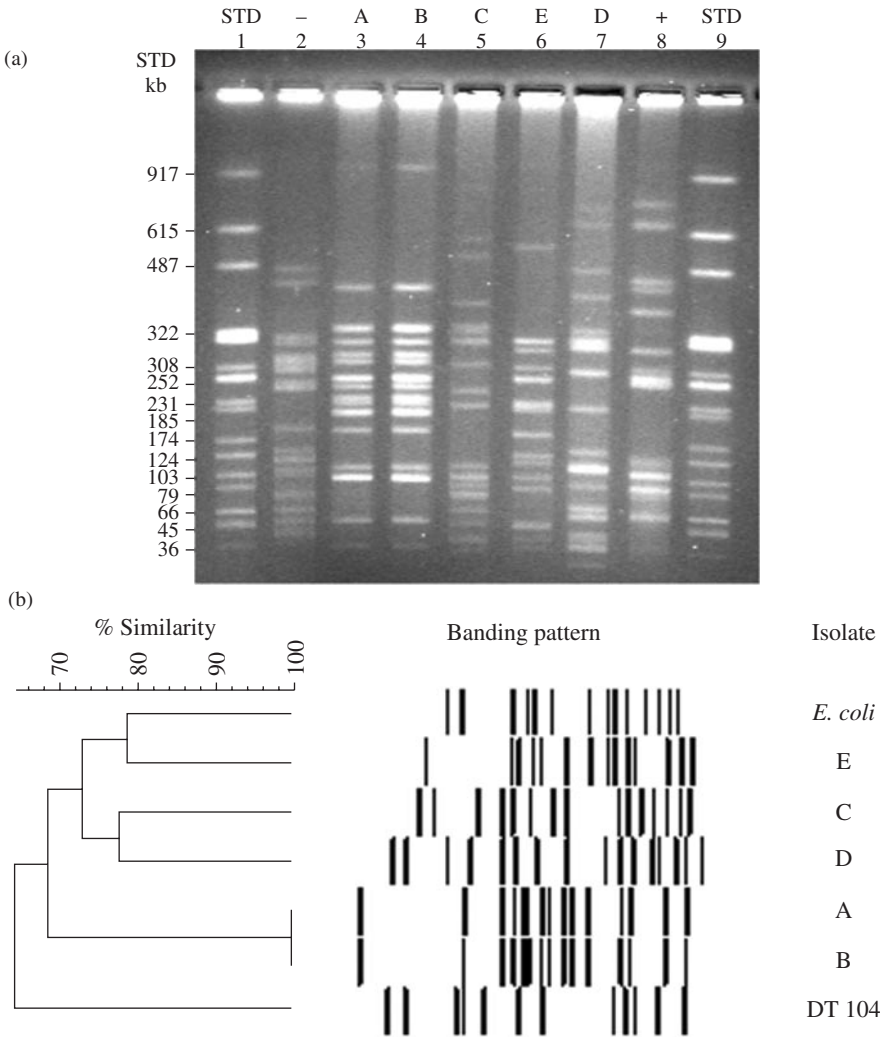

Figure 2. Comparison of genetic relatedness of five Niakhar isolates using PFGE. DNA was digested using *Xba*I. (a) Standard sizes in kilobases are indicated to the left of the figure. Lanes 1 and 9, *S. enterica* serotype Newport standard strain AM01144; lane 2, *E. coli* (negative control); lane 3, isolate A; lane 4, isolate B; lane 5, isolate C; lane 6, isolate E; lane 7, isolate D; and lane 8, *Salmonella* Typhimurium DT104 (positive control). (b) A dendrogram was constructed from the PFGE band patterns using BioNumerics software.

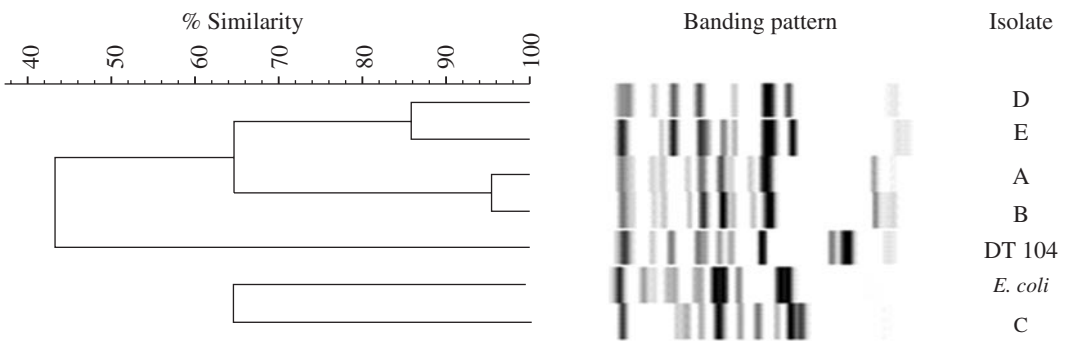

Figure 3. Comparison of genetic relatedness among five Niakhar isolates using automated ribotyping (Qualicon). Ribotype profiles were obtained after digestion with *Pvu*II enzyme. From the band patterns, a dendrogram was constructed to illustrate the genetic relatedness of the isolates.

Isolates D and E were also related although to a lesser degree (86%). Isolate C showed only a 37% similarity to the four other *Salmonella* Niakhar isolates. Collectively, isolates A and B and isolates D and E were even less related (65% similarity). *Salmonella* Typhimurium DT104 was 43% similar to isolates A, B, D and E, whereas *E. coli* was only 37% genetically similar. However, isolate C was more closely related to *E. coli* than the four other isolates (65% similarity). These results are very similar

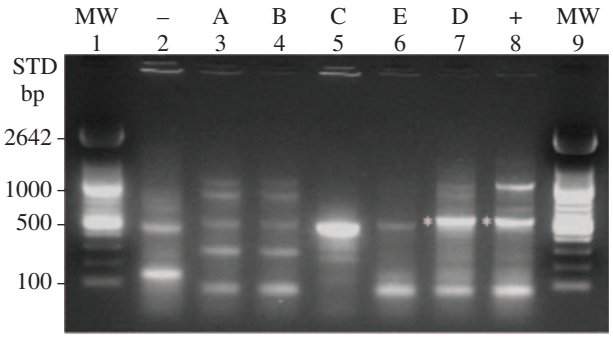

Figure 4. Detection of the *intI1* gene in the chromosomal DNA of the Niakhar isolates using PCR. Standard sizes in base pairs are located to the left of the figure. Lanes 1 and 9, Roche Diagnostics 100 bp ladder standard; lane 2, *E. coli* (negative control); lane 3, isolate A; lane 4, isolate B; lane 5, isolate C; lane 6, isolate E; lane 7, isolate D; and lane 8, *Salmonella* Typhimurium DT104 (positive control). Asterisk = identification of the *intI1* gene in the multiresistant *Salmonella* Niakhar isolate, isolate D.

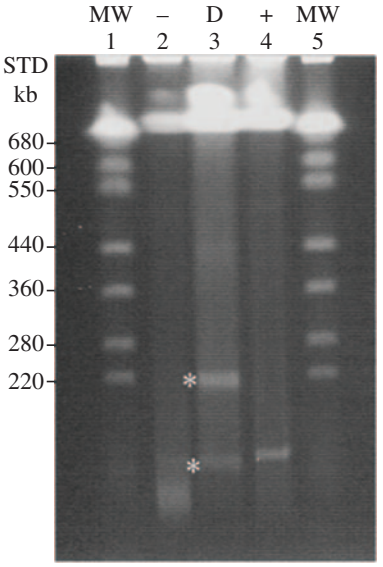

Figure 5. Detection of large plasmids in a Niakhar isolate. S1 enzyme was used to cut circular DNA plasmids. Standard sizes in kilobases are indicated to the left of the figure. Lanes 1 and 5, *Saccharomyces cerevisiae* chromosomal DNA standard marker; lane 2, *E. coli* (negative control); lane 3, isolate D; and lane 4, *Salmonella* Typhimurium DT104 (positive control). Asterisks indicate the identification of large plasmids in the multiresistant *Salmonella* Niakhar isolate, isolate D.

to those (isolates A and B) obtained by PFGE dendrogram, which indicated a high degree of relatedness between isolates A and B.

Location of antimicrobial resistance genes

The integrons (*intI1*, *intI2*, *intI3* and *int4*) and their expected base pair size are shown in Table 1. After probing, only isolate D possessed an integron, *intI1*. Figure 4 shows the presence of *intI1* amplifying at the 568 bp PCR fragment for isolate D and *Salmonella* Typhimurium DT104 (lanes 7 and 8). The integron from isolate D was localized to an ~12 kb *Xba*I fragment using PFGE. Use of Southern blotting showed that *intI1* was located on the chromosome of isolate D (data not shown; J. D. Tankson, P. Fedorka Cray and C. Jackson). Using PCR primers to the 5′ (*intCSF*) and 3′ (*intCSR*) conserved region of *intI1*, a 1.2 kb fragment was amplified from isolate D. Sequencing of the 5′ and 3′ conserved region revealed that it contained *aadA-1*, a spectinomycin resistance gene, conferring resistance to the aminoglycoside antibiotic class. Therefore, the spectinomycin resistance gene was the only resistance gene located within the 5′ and 3′ conserved region of the integron.

Only isolate D contained two large plasmids which localized to ~220 and 60 kb fragments (Figure 5). There were no small plasmids isolated in any of the Niakhar isolates. Further work is warranted in order to determine the location of the other eight resistance genes. Work is in progress for determining the resistance genes located within the two plasmids.

Discussion

S. enterica serotype Niakhar is infrequently isolated in both the United States and elsewhere. In this study, the significance of the serotype lies not with its infrequent isolation, but rather with its acquisition of resistance to ciprofloxacin. Interestingly, prior to 2000, the animal arm of NARMS did not report resistance to ciprofloxacin among *Salmonella* serotypes. Conversely, the human arm of NARMS reported a 0.1–0.4% resistance to ciprofloxacin from 1988 to 2000 (www.cdc.gov/narms/reports.htm; 30 September 2005, date last accessed). However, none of the ciprofloxacin-resistant human isolates was identified as *Salmonella* Niakhar (www.cdc.gov/narms/reports.htm; 30 September 2005, date last accessed). Therefore, this is a first observation of ciprofloxacin resistance among the NARMS animal collection of isolates.

Dairy cattle were implicated as the source for isolates A and B. Although both isolates originated from on-farm collections (http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/index.htm; 30 September 2005, date last accessed), they were in disparate geographic locations (Southeastern and Western United States). It is interesting to note that they were pan-susceptible even though cattle are long-lived and likely to have seen at least some type of antimicrobial in feed for either prophylaxis or therapeutic means. Further support can also be inferred from isolates C and E. Although these isolates were also from disparate regions of the United States (Northeastern and South Central Midwest), they originated from clinical submissions, one from cattle (isolate C) and one from a dog (isolate E). Isolate C was only resistant to ampicillin whereas isolate E was pan-susceptible. Since diagnostic submissions are most probably a result of overt clinical illness (morbidity and/or mortality), it is probable that both animals were previously treated with some class of antimicrobial. Even though the status of the cow meat was unknown, seriously ill cattle are not sold as food for human consumption. Therefore, the probability of this submission making it through the preparation process to be sold for human consumption is relatively low. However, a dog is usually considered a companion for man and is likely to have been treated when illness occurred. Additionally, household pets (dogs, cats, etc.) are common on the farm and freely move around animal production environments. Therefore, they may also serve as vectors to those who come into contact with them, including both animals and humans. Although it is difficult to estimate antimicrobial exposure, the lack of resistance (especially clinical submissions) suggests that the development of resistance within a serotype may be dependent upon factors other than

antimicrobial use or exposure. These may include acquisition of transmissible elements[33,34] or a newly introduced clone.

However, it is interesting to note that isolate D originated in cattle and it is multiresistant to antimicrobials implicated in both the multiresistant *Salmonella* Typhimurium DT104 and *Salmonella* Newport serotypes.[19,35–39] Submissions from the diagnostic laboratories, as observed for isolates C, D and E, typically include major species identification (cattle, swine, dog, chicken, turkey, etc.) but rarely include differentiation within species such as dairy and beef cattle, or chick, broiler or broiler breeder for chicken isolates. The major reservoir for *Salmonella* Typhimurium DT104 and *Salmonella* Newport is cattle, particularly dairy cattle.[19,37,40–42] Because cattle are less confined during production than other food animals, it is probable that isolates A, B, C and D were introduced into the herd by a vector such as a bird, fly, rodent or other wildlife or through movement of cattle during transport. Birds, flies, rodents and wildlife are known vectors of *Salmonella*[1,43,44] and the conditions associated with cattle production increase the likelihood of contact between cattle and a vector(s). It is less likely that direct use of antimicrobials affected the multiresistance observed for this *Salmonella* Niakhar isolate. Evidence to support this includes the rarity of the serotype, the rarity of resistance within the serotype, and the observation that only one isolate was obtained by source, region and year. This further suggests that unlike *Salmonella* Typhimurium DT104 and *Salmonella* Newport[35,35,41,45–47], *Salmonella* Niakhar is not a virulent serotype and would be less likely to expand within a population.

Annual data compiled by NVSL also confirmed Niakhar is an uncommon serotype. NVSL reported Niakhar was isolated in 1993, 1997 and 2000 (one isolate, two isolates and two isolates, respectively).[9] Even though the NVSL report of Niakhar isolation did not coincide with NARMS,[10,11] it is important to recognize that the NARMS programme does not test every animal isolate submitted to NVSL for antimicrobial resistance. Therefore, while this observation of ciprofloxacin resistance is unique, it is only unique among the isolates tested. Although there were no reports of *Salmonella* Niakhar in 2001 or 2002, further monitoring in both animal and human populations is warranted as this may be an emerging and clinically significant serotype.

PFGE analysis suggests that Niakhar isolates from regions 5 and 2 (isolates A and B, respectively) are identical or genetically similar. Niakhar isolates from regions 1, 3 and 4 (isolates C, D and E, respectively) are genetically different from one another. Therefore, even though these isolates are from different geographic regions, the serotype is not clonal. Literature has emphasized that when comparing different fingerprinting methods, PFGE has a higher discriminatory power.[48–50] It has also been shown that ribotyping with *Pvu*II has less discriminatory power than PFGE.[51,52] According to these three DNA-based typing methods, the qualifier for differentiation is isolates A and B. Isolates A and B, both originating from on-farm dairy cattle, were genetically similar yet were recovered from different regions of the United States (regions 5 and 2, respectively) suggesting that either movement within the dairy population at-large may be occurring or dissemination may be owing to a vector(s). The three other isolates, all diagnostic submissions, were also from different geographic regions (regions 1, 3 and 4), and they were not genetically related. Genetic diversity between different sources (cattle versus dog) would not be unexpected. Genetic diversity among similar sources suggests that regional influences may affect resistance development. Regardless, these data support the observation that multiple clones of *Salmonella* Niakhar can be isolated throughout the United States.

Antimicrobial resistance patterns (Table 2) indicated that three isolates (isolates A, B and E) were pan-susceptible, one isolate (isolate C) was resistant to ampicillin and one isolate (isolate D) was resistant to nine antimicrobials. Multiple resistance determinants are most often associated with the presence of plasmids and integrons. Plasmids and integrons are defined as mobile, extrachromosomal pieces of DNA that have the ability to horizontally disseminate antimicrobial resistance genes (and multiresistance genes) rapidly and efficiently between serotypes, bacterial species or bacterial genera.[53–55] Integrons contain gene cassettes that harbour the antimicrobial resistance genes.[55–60] Enterobacteriaceae have been reported to carry integrons, especially class I (*intI1*) integrons.[61,62] Isolate D, which was multiresistant, harboured *intI1* and two large plasmids. This is similar to reports for *Salmonella* Typhimurium DT104 which also harbours plasmids and integrons.[63–65] However, unlike DT104, most of the *Salmonella* Niakhar resistance genes are not chromosomally integrated, but located on plasmids. However, further characterization of these genes is warranted.

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Transparency declarations

None to declare.

References

1. Koplan JP, Hughes JM, Cohen ML *et al.* Diagnosis and management of foodborne illnesses. *Morb Mortal Wkly Rep* 2001; **50**: 1–27.

2. Shallow S, Samuel M, McNees A *et al.* Preliminary FoodNet data on the incidence of foodborne illnesses—selected sites, United States, 2000. *Morb Mortal Wkly Rep* 2001; **50**: 241–3.

3. Enriquez C, Nwachuku N, Gerba CP. Direct exposure to animal enteric pathogens. *Rev Environ Health* 2001; **16**: 117–31.

4. Oosterom J. Epidemiological studies and proposed preventive measures in the fight against human salmonellosis. *Int J Food Microbiol* 1991; **12**: 41–51.

5. Warwick C, Lambiris AJ, Westwood D *et al.* Reptile-related Salmonellosis. *J R Soc Med* 2001; **94**: 124–6.

6. Holt JG, Krieg NR, Sneath PHA *et al.* Facultatively anaerobic Gram-negative rods. In: *Bergey's Manual of Determinative Bacteriology*. Baltimore: Lippincott Williams & Wilkins, 1994; 175–89.

7. Mead PS, Slutsker L, Dietz V *et al.* Food-related illness and death in the United States. *Emerg Infect Dis* 1999; **5**: 607–25.

8. Kelterborn F. *Catalogue of Salmonella first isolations 1965–1984*. Dorbrecht/Bosten/Lancaster: Martinus Nijhoff Publishers, 1987.

9. United States Animal Health Association. In: *Proceedings of the Ninety-seventh annual meeting of the United States Animal Health Association, 1993*. Sahara Hotel, Las Vegas, NV, USA.

10. United States Animal Health Association. In: *Proceedings One Hundred and First Annual Meeting of the United States Animal Health Association*, 1997. Galt House Hotel, Louisville, KY, USA.

11. United States Animal Health Association. In: *Proceedings One hundredth fourth Annual Meeting of the United States Animal Health Association, 2000*. Sheraton Civic Center Hotel, Birmingham, AL, USA.

12. Fedorka-Cray P, Englen MD, Gray JT *et al*. Programs for monitoring antimicrobial resistance. *Anim Biotechnol* 2002; **13**: 43–55.

13. Barnes HJ, Gross WB. Colibacillosis. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM, eds. *Diseases of Poultry*. Ames, Iowa: Iowa State University Press, 1997; 131–41.

14. Plumb DC. *Veterinary Drug Handbook, Pocket edn*. White Bear Lake, MN: Iowa State Press, 2002.

15. World Health Organization. *Use of Quinolones in Food Animals and Potential Impact on Human Health. WHO/EMC/ZDI/98.10. 1-18*. Geneva, Switzerland, 1998.

16. Griggs DJ, Gensberg K, Piddock LJV. Mutations in gyrA gene of quinolone-resistant *Salmonella* serotypes isolated from humans and animals. *Antimicrob Agents Chemother* 1996; **40**: 1009–13.

17. Heisig P, Kratz E, Halle E *et al*. Identification of DNA gyrase A mutations in ciprofloxacin-resistant isolates of *Salmonella typhimurium* from men and cattle in Germany. *Microb Drug Resist* 1995; **1**: 211–8.

18. Hosek G, Leschinsky DD, Irons S *et al*. Multidrug-resistant Salmoella serotype Typhimurium—United States, 1996. *Morb Mortal Wkly Rep* 1997; **46**: 308–10.

19. Zansky S, Wallace B, Schoonmaker-Bopp D *et al*. Outbreak of multi-drug resistant *Salmonella* Newport—United States, January–April 2002. *J Am Med Assoc* 2002; **288**: 951–3.

20. National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial disk tests. Approved standard M2-A7*. NCCLS, Wayne, PA, 1997.

21. National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial disk tests. Approved standard M2-A7*. NCCLS, Wayne, PA, 2000.

22. Hopkins KL, Hilton AC. Restriction endonuclease analysis of RAPD-PCR anplicons derived from Shiga-like toxin-producing *Escherichia coli* O157 isolates. *J Med Microbiol* 2001; **50**: 90–5.

23. Hilton AC, Banks JG, Penn CW. Optimization of RAPD for fingerprinting *Salmonella*. *Lett Appl Microbiol* 1997; **24**: 243–8.

24. Wittwer CT, Fillmore GC, Hillyard DR. Automated polymerase chain reaction capillary tubes with hot air. *Nucleic Acids Res* 1989; **17**: 4353–7.

25. Swaminathan B, Barrett TJ, Hunter SB *et al*. PulseNet: The molecular subtyping network for foodborne disease surveillance, United States. *Emerg Infect Dis* 2001; **7**: 382–9.

26. Bruce JL. Automated system rapidly identifies and characterizes microrganisms in food. *Food Technol* 1996; **50**: 77–81.

27. Clermont O, Cordevant C, Bonacorsi S *et al*. Automated ribotyping provides rapid phylogenetic subgroup affiliation of clinical extraintestinal pathogenic *Escherichia coli* strains. *J Clin Microbiol* 2001; **39**: 4549–53.

28. De Cesare A, Bruce JL, Dambaugh TR *et al*. Automated ribotyping using different enzymes to improve discrimination of Listeria monocytogenes isolates, with a particular focus on serotype 4b strains. *J Clin Microbiol* 2001; **39**: 3002–5.

29. Sethi MR. Fully automated microbial characterization and identification for industrial microbiologists. *Am Lab* 1997; **5**: 31–35.

30. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. *Anal Biochem* 1995; **226**: 235–40.

31. Mitsuda T, Muto T, Yamada M *et al*. Epidemiological study of a food-borne outbreak of enterotoxigenic *Escherichia coli* 025:NM by pulsed-field gel electrophoresis and randomly amplified polymorphic DNA analysis. *J Clin Microbiol* 1998; **36**: 652–6.

32. 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**: 2233–9.

33. Datta N. Bacterial resistance to antibiotics. *Ciba Found Symp* 1984; **102**: 204–18.

34. Davies JE. Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found Symp* 1997; **207**: 15–27.

35. Cloeckaert A, Schwarz S. Molecular characterization, spread and evolution of multidrug resistance in *Salmonella enterica* Typhimurium DT104. *Vet Res* 2001; **32**: 301–10.

36. Molbak K, Baggesen DL, Aaerestrup FM *et al*. An outbreak of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104. *N Engl J Med* 1999; **341**: 1420–5.

37. Threlfall EJ, Frost JA, Ward LR *et al*. Epidemic in cattle and humans of *Salmonella typhimurium* DT 104 with chromosomally integrated multiple drug resistance. *Vet Rec* 1994; **134**: 577.

38. Threlfall EJ, Ward LR, Rowe B. Increasing incidence of resistance to trimethoprim and ciprofloxacin in epidemic *Salmonella typhimurium* DT104 in England and Wales. *Euro Surveill* 1997; **2**: 81–4.

39. van Duijkeren E, Wannet WJ, Heck ME *et al*. Sero types, phage types and antibiotic susceptibilities of *Salmonella* strains isolated from horses in The Netherlands from 1993 to 2000. *Vet Microbiol* 2002; **86**: 203–12.

40. Clegg FG, Chiejina SN, Duncan AL *et al*. Outbreaks of *Salmonella* Newport infection in dairy herds and their relationship to management and contamination of the environment. *Vet Rec* 1983; **112**: 580–4.

41. Poppe C, Smart N, Khakhria R *et al*. *Salmonella typhimurium* DT104: a virulent and drug-resistant pathogen. *Can Vet J* 1998; **39**: 559–65.

42. Ribot EM, Wierzba RK, Angulo FJ *et al*. *Salmonella enterica* serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. *Emerg Infect Dis* 2002; **8**: 387–91.

43. Bailey JS, Stern NJ, Fedorda-Cray P *et al*. Sources and movement of *Salmonella* through integrated poultry operations: A multistate epidemiological investigation. *J Food Prot* 2001; **64**: 1690–7.

44. Baumler AJ, Hargis BM, Tsolis RM. Tracing the origins of *Salmonella* outbreaks. *Science* 2000; **287**: 50–2.

45. Beltran P, Musser JM, Helmuth R *et al*. Toward a population genetic analysis of *Salmonella*: genetic diversity and relationships among strains of serotypes *S. choleraesuis*, *S. derby*, *S. dublin*, *S. enteritidis*, *S. heidelberg*, *S. infantis*, *S. newport*, and *S. typhimurium*. *Proc Natl Acad Sci USA* 1988; **85**: 7753–7.

46. Malik P, Sharma VD, Chandra R. Cytotoxigenicity in *Salmonella* serovars. *Indian J Exp Biol* 1995; **33**: 177–81.

47. Popoff MY, Miras I, Coynault C *et al*. Molecular relationships between virulence plasmids of *Salmonella* serotypes Typhimurium and Dublin and large plasmids of other *Salmonella* serotypes. *Ann Microbiol* 1984; **135A**: 389–98.

48. Allison LJ, Carter PE, Thomson-Carter FM. Characterization of a recurrent clonal type of *Escherichia coli* O157:H7 causing a major outbreaks of infection in Scotland. *J Clin Microbiol* 2000; **38**: 1632–5.

49. Kumao T, Ba-Thein W, Hayashi H. Molecular subtyping methods for detection of *Salmonella enterica* serovar Oranienburg outbreaks. *J Clin Microbiol* 2002; **40**: 2057–61.

50. Louie M, Read S, Louie L *et al*. Molecular typing methods to investigate transmission of Esherichia coli O157:H7 from cattle to humans. *Epidemiol Infect* 1999; **123**: 17–24.

51. Hermans PWW, Sluijter M, Hoogenboezem T *et al*. Comparative study of five different DNA fingerprint techniques for molecular typing of Streptococcus pneumoniae strains. *J Clin Microbiol* 1995; **33**: 1606–12.

52. Quale JM, Landman D, Flores C *et al*. Comparison of automated ribotyping to pulsed-field gel electrophoresis for genetic fingerprinting of Streptococcus pneumoniae. *J Clin Microbiol* 2001; **39**: 4175–7.

53. Carattoli A. Importance of integrons in the diffusion of resistance. *Vet Res* 2001; **32**: 243–59.

54. Davies J. Inactivation of antibiotics and the dissemination of resistance genes. *Science* 1994; **264**: 375–82.

55. Recchia GD, Hall RM. Gene cassettes: a new class of mobile element. *Microbiology* 1995; **141**: 3015–27.

56. Fluit A, Schmitz FJ. Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur J Clin Microbiol Infect Dis* 1999; **18**: 761–70.

57. Hall RM, Collis CM. Mobile gene cssettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol* 1995; **15**: 593–600.

58. Hall RM, Collis CM. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resist Updat* 1998; **1**: 109–19.

59. Levesque C, Roy PH. PCR analysis of integrons. In: Persing DH, Smith TF, Tenover FC, White TJ, eds. *Diagnostic Molecular Microbiology: Principles and Applications*. Washington, DC: ASM Press, 1993: 590–4.

60. Ploy MC, Denis F, Courvalin P *et al*. Molecular characterization of integrons I Acinetobacter baumannii: description of a hybrid class 2 integron. *Antimicrob Agents Chemother* 2000; **44**: 2684–8.

61. Jones ME, Peters E, Weersink A *et al*. Wide-spread occurrence of integrons causing multiple resistance in bacteria. *Lancet* 1997; **349**: 1742–3.

62. Sallen B, Rajoharison A, Desvarenne S *et al*. Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of Enterobacteriaceae. *Microb Drug Resist* 1995; **1**: 195–202.

63. Ng LK, Mulvey MR, Martin I *et al*. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. *Antimicrob Agents Chemother* 1999; **43**: 3018–21.

64. Poppe C, Ziebell K, Martin L *et al*. Diversity in antimicrobial resistance and other characteristics among *Salmonella Typhimurium* DT104 isolates. *Microb Drug Resist* 2002; **8**: 107–22.

65. Ridley A, Threlfall EJ. Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella typhimurium* DT104. *Microb Drug Resist* 1998; **4**: 113–8.