EVOLUTION OF MULTIPLE ANTIMICROBIAL DRUG RESISTANCE: CONSERVATION OF GENES ENCODING STREPTOMYCIN, SULFONAMIDE AND TETRACYCLINE RESISTANCE AMONG *ESCHERICHIA COLI* WITH INCREASING MULTI-DRUG RESISTANCE

By

RENU JOSEPH

A thesis submitted in partial fulfillment of
the requirement for the degree of

MASTER OF VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY

COLLEGE OF VETERINARY MEDICINE

DECEMBER 2007

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of RENU JOSEPH find it satisfactory and recommend that it be accepted.

____
Chair

____

____

ACKNOWLEDGEMENT

I would like to take this opportunity to convey my deepest appreciation to everyone who helped shape this amazing journey in my life. First and foremost, a very special thanks to my major advisor Dr. Thomas E Besser. His input as an excellent mentor and a good friend made my research and graduate school experience absolutely fascinating. I would also like to thank my committee, Dr. Douglas R. Call and Dr. Dale D Hancock. I sincerely appreciate all the time that you have invested in preparing me for one of the biggest steps in my life. Thank you for your patience and support. The ever-important support of fellow graduate students has been immeasurable and includes but is not limited to Joshua Daniels, Josh Whitworth, Sonja Lloyd and Bijay Adhikari. Thank you to Dr. Sri Srikumaran, Dr. Devendra Shah, Yubei Zhang and Russell McClanahan. Much appreciation to all my friends in Pullman, who have enriched my personal life in one way or another. My sincere gratitude to my best friends Dr. Drubo Jyothi Dey Laskar and Snigdha Laskar. Special thanks to the professors at the College of Veterinary Medicine, Mannuthy, India, who helped lay the foundation for my future.

EVOLUTION OF MULTIPLE ANTIMICROBIAL DRUG RESISTANCE: CONSERVATION OF GENES ENCODING STREPTOMYCIN, SULFONAMIDE AND TETRACYCLINE RESISTANCE AMONG *E. COLI* WITH INCREASING MULTI-DRUG RESISTANCE

Abstract

by Renu Joseph, MS
Washington State University
December 2007

Chair: Thomas E. Besser

Commensal *E. coli* may serve as a reservoir of resistance genes transferable to pathogens and may also at as foci for "assembly" of multi-drug resistance (MDR). MDR to streptomycin, sulfonamide and tetracycline (R-type SSuT) is particularly frequent among *E. coli* isolated from cattle, either alone (3-drug resistance) or in combination with additional resistance phenotypes (SSuT+). We hypothesized that SSuT+ strains are assembled from a backbone of conserved SSuT resistance determinants and therefore compared the locations (chromosomal vs. plasmid) of different resistance genes in *E. coli* isolated from clinically normal cattle. Streptomycin resistance conferring genes (s*trA, strB*) and a sulfonamide conferring gene (*sul2*) were largely conserved across the range of MDR commensal *E. coli.* Consistent with the hypothesis, an additional sulfonamide resistance gene, s*ul1,* was detected more frequently in isolates with higher level MDR but

the distribution of tetracycline resistance genes was inconsistent with the hypothesis as the frequency of *tet*(B) decreased and the frequency of *tet*(A) increased in R-type SSuT8+ isolates; relatively few high-level MDR isolates retained *tet*(B). The locations of the resistance genes were also unconserved, being universally non-plasmid encoded in the R-type SSuT but increasingly plasmid-encoded in isolates with increasing MDR. Together, these observations suggest that R-type SSuT+ isolates do not evolve directly from R-type SSuT isolates and that some other process must account for the conservation of the SSuT phenotype in SSuT+ isolates.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ………………………………………………...iii

ABSTRACT …………………………………………………….............. iv

LIST OF TABLES……………………………………………………... vii

LIST OF FIGURES……………………………………………………. viii

DEDICATION………………………………………………………..ix

CHAPTER

1. **INTRODUCTION .... ….1**
2. **MATERIALS AND METHODS...4**
3. **RESULTS 7**
4. **DISCUSSION………………………………………………………..9**
5. **REFERENCES ....13.**

.

LIST OF TABLES

1. PCR primers and expected amplicon sizes of PCR products………………….21

2. Genotypes of SSuT resistance determinants of *E. coli.* ………………………..22

3 Transformable resistance phenotypes of MDR E. coli isolated from cattle…....24

LIST OF FIGURES

1. Histogram showing percentages of *E. coli* R-Types containing *sul1* and *sul2*….. 18

2. Histogram showing percentages of *E. coli* R-Types containing *tet*(A) and *tet*(B)..19

3. Frequency of carriage of plasmid encoded resistance in *E. coli* R-Types……......20

DEDICATION

To my love, my husband Dr. Davis Maliakal. Thank You for your support.

To my parents T. P. Joseph and Dr. M. C. and my siblings, Dr. Remmy Joseph and Greeboy Joseph. Thank you for your unconditional love and steadfast belief in me.

INTRODUCTION

Transferable drug resistance poses a serious threat to the treatment of infectious diseases in both humans and animals (9, 18). Resistance to all currently known antimicrobial agents has been identified, including drugs such as expanded-spectrum cephalosporins and fluoroquinolones (3, 4), which are considered first-line drugs against important human diseases. The use of antimicrobial agents in either human or veterinary medicine imposes selective pressures favoring the evolution and dissemination of antibiotic resistant bacteria (13, 21, 37). Any antibiotic treatments, including those used in the food animals, should favor resistant bacteria by inhibiting or eradicating susceptible flora and thereby indirectly promoting expansion of resistant populations and potentially increase transmission of resistance elements..

Antimicrobial drug resistance can result from mutation of existing host genes, acquisition of foreign resistance genes, or a combination of these two mechanisms (29). Self-transmissible or mobilizable plasmids play very important roles in horizontal transmission of antimicrobial resistance genes and transfer of plasmids encoding multiple antibiotic resistance genes is considered the main mechanism for development of high level resistance in *E. coli* (8, 33). Due to accumulation of multiple antimicrobial resistance genes on plasmids and other mobile genetic elements, resistance to multiple antimicrobial drugs can be transferred to a susceptible recipient via single genetic events (16). One mechanism by which multiple drug resistance determinants may be accumulated are integrons, specialized genetic units that capture small mobile elements (cassettes) through site-specific recombination events catalyzed by an integron-encoded integrase gene *intI* (26, 30, 32). Sulfonamide resistance is also encoded by type I

integrons, as *sulI* is an integral part of 3' conserved segment of type I integron structure. However, most type I integrons carry only one or two additional genes, probably because of limited transcription efficiency from the 5' encoded promoter. Other than integrons, the mechanisms by which mobile elements encoding multiple drug resistance (MDR) are accumulated into chromosomal or mobile genetic elements are generally poorly characterized.

The purpose of this study was to evaluate one possible mechanism for the accumulation of MDR determinants within *E. coli*. The distribution of specific antimicrobial resistance phenotype is non-random in that 1) the prevalence of resistance to older, more widely used antimicrobial drugs is consistently higher than resistance to more recently introduced or rarely used drugs, and 2) isolates carrying resistance to newer or less used drugs are consistently also resistant to many older drugs. For example, in a survey of over 5,000 *E. coli* isolates carried by bovine calves, Berge et al. (2005) demonstrated that a high prevalence of resistance to tetracycline, streptomycin and sulfonamides (>74% of isolates), intermediate prevalence of resistance to ampicillin, chloramphenicol and trimethoprim/sulfonamide (25% - 74%), and relatively low prevalence of resistance to amikacin, ceftiofur and amoxicillin/clavulanic acid (<25%). Importantly, essentially all isolates resistant to ampicillin, amoxicillin/clavulanic acid, chloramphenicol, trimethoprim/sulfonamide, amikacin or ceftiofur also retained SSuT resistance. Others have reported similar high prevalence of SSuT resistance (1, 19) and the retention of SSuT resistance in *E. coli* isolates with additional resistance phenotypes. Therefore, the genes encoding SSuT resistance represent an opportunity to test the hypothesis that highly MDR isolates evolved from R-type SSuT isolates by acquisition of

additional resistance determinants (in which case they would be expected to share the same SSuT resistance conferring genes and the chromosomal or plasmid location of these genes). If the MDR isolates evolved independently of SSuT isolates, the SSuT resistance determinants and the genetic location of these determinants would be expected to resemble a random distribution.

Analysis of the distribution of antimicrobial resistance genes at the population level would permit us to determine the relative importance of different mechanisms of dissemination and persistence of these traits. Most previous population-based studies of antimicrobial resistance have relied exclusively on phenotypic assays, and as a result these studies do not provide the ability to discriminate which resistance genes produce a given phenotype. In this study, we used resistance gene detection by PCR assays to identify conserved or unconserved streptomycin, sulfonamide and tetracycline resistance genotypes among *E. coli* isolates with SSuT resistance phenotypes accompanied by diverse degrees of multi-drug resistance.

MATERIALS AND METHODS

Selection of study isolates:

Study isolates were selected by random number table from 3,474 characterized *E. coli* isolates that were originally collected from 19 cattle farms in Oregon and Washington State between February 2002 and January 2004. The farms, which included two feedlots, two calf ranches, five beef cow-calf herds, and eleven dairy herds, were sampled on two occasions at six months or longer intervals and forty animals were sampled at each occasion. Individual fecal samples were diluted and spread-plated on MacConkey agar supplemented with 8 µg/ml tetracycline. Up to four red colonies were chosen from each animal, including diverse colony morphology isolates when present. Isolates were confirmed as *E. coli* by production of fluorescence after overnight incubation in EC-MUG broth medium. Each isolate was initially tested by agar dilution for resistance to breakpoint concentrations of the following antimicrobial drugs: ampicillin (≥32 µg/ml), gentamicin (≥16µg/ml), tetracycline (≥16 µg/ml), chloramphenicol (≥32 µg/ml), nalidixic acid (≥32 µg/ml), streptomycin (≥64 µg/ml), sulfonamide (≥512 µg/ml), amikacin (≥64 µg/ml), amoxicillin/clavulanic acid (≥32 and ≥16 µg/ml, respectively), ceftiofur (≥8 µg/ml), cephalothin (≥32 µg/ml), trimethoprim/sulfonamide (≥4 and ≥76 µg/ml, respectively). Initially, we selected 20 isolates each of resistance types (R-types) SSuT and MDR (8+) using a random number table method. R-type MDR (8+) included all isolates resistant to eight or more antimicrobial drugs including resistance to SSuT. Subsequently, we randomly selected an additional 10 isolates each of R-types T, ST-A,

SSuT-A, SSuT-C, SSuT-ASxt, SSuT-CSxt, SSuT-ACSxt, SSuT-ASxtG and SSuT-ACSxtG. The MDR (8+) group included the following R-types: SSuT-ACGSxtAmcCaz (n=2), SSuT-ACSxtAmcCaz (n=7), SSuT-AGSxtAmcCaz (n=1), SSuT-ACGSxtAmc (n=3), SSuT-ACSxtAmc (n=3), SSuT-ACGSxt (n=1), SSuT-ACAmc (n=2), SSuT-ACSxt (n=1).

Antimicrobial resistance profiles of *E. coli* isolates were confirmed by Kirby-Bauer agar diffusion method according to the Clinical and Laboratory Standards Institute guidelines (11) using the following 10 antimicrobial drug disks (potency): ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), streptomycin (10 µg), triple-sulfa (250 µg), trimethoprim-sulfamethoxazole (1.25 to 23.75 µg), tetracycline (30 µg), amoxicillin-clavulanic acid (20 to 10 µg), ceftazidime (30 µg) and nalidixic acid (30 µg). Mueller-Hinton agar was used as standard growth medium for Kirby-Bauer tests with inoculum density of 0.5 McFarland standards. Isolates whose phenotypes by agar diffusion did not agree with the breakpoint agar dilution test results were replaced by random selection from the isolate pool, and a total of 139 *E. coli* isolates were evaluated to obtain the study set of 130 isolates.

Plasmid profiles, isolation and transfer:

The presence of plasmid DNA was determined by plasmid profiling using Kado & Liu method (22). Plasmid electrophoresis conditions included 1.1% agarose gels run at 105 V, 3 h, in 0.5 % TAE buffer (40 mM Tris–acetate, 1 mM EDTA). Plasmid sizes were estimated relative to a BAC-Tracker Super coiled DNA ladder (EPICENTRE Biotechnologies, Madison, WI, USA).

Plasmid DNA was isolated from donor cells using a Plasmid Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Electroporation of plasmid DNA into competent (*E. coli* DH10B, Gene Hog, Invitrogen, Carlsbad, CA) cells was performed in 1 mm cuvettes using a GenePulser (BioRad, Hercules, CA). Transformants were immediately placed into SOC medium (1 ml) and incubated for 1 h at 37°C with agitation (300 rpm). Transformants were then plated onto selective media containing tetracycline (16 μg/ml prepared from stock solution of 16 mg/ml), ampicillin (8 μg/ml prepared from stock solution of 8 mg/ml) and chloramphenicol (12.5 μg/ml prepared from stock solution of 16 mg/ml) and incubated at 37°C for overnight. Plasmid profiles of resultant transformants were performed as described above.

<u>PCR for streptomycin, sulfonamide, and tetracycline determinants:</u>

Total DNA was extracted from the bacteria using a commercial kit (DNeasy Tissue Kit, Qiagen) according to the manufacturer's directions. The PCR mixture consisted of 5 μl of 10× buffer, 1.5 mM MgCl (*tet*(A)), 2 mM $MgCl_2$ (*sul1, sul2, strA, strB* and *tet*(B)), 2 U Platinum Taq polymerase (Invitrogen), 0.2 mM of each of the deoxyribonucleoside triphosphates (dNTPs), 0.5 μM primer pairs (Table 1), 0.1 μg of template DNA, and double-deionized water for 50 μl total volume. The mixture was subjected to 35 PCR cycles in an ICycler thermocycler (BioRad). The parameters for the amplification cycles were as follows: Initial denaturation for 3 min at 95°C, denaturation for 45 s at 95°C, annealing of primers for 30 s, and primer extension for 1 min at 72°C. Annealing temperatures for *sul1, sul2* and *tet*(B) was 60°C, and for *strA, strB* and *tet*(A) was 58°C, 57°C and 59°C respectively. There was a final extension step of 10 min at 72°C. Electrophoresis of amplification products was performed on 1% agarose gels for 1

h at 100 V. Gels were stained with ethidium bromide and photographed over UV transillumination (17).

RESULTS:

We examined the distribution of genes encoding streptomycin, sulfonamide and tetracycline resistance in *E. coli* isolated from cattle and expressing diverse multi-drug resistance phenotypes (R-types). First, we compared the distribution of streptomycin, sulfonamide and tetracycline resistance determinants in isolates resistant to only those three antimicrobial drugs (R-type SSuT) with those of isolates resistant to five or more antimicrobial drugs in addition to SSuT (R-type SSuT8+, Table 2). If SSuT8+ isolates evolved from SSuT isolates, we expected to see conservation of the SSuT resistance determinants between these groups. Consistent with this hypothesis, one or both determinants associated with streptomycin resistance (*strA* and *strB*) were present in all SSuT and SSuT8+ isolates. Similarly, *strA* and/or *strB* were uniformly present in other streptomycin resistant isolates with diverse intermediate levels of resistance (Table 2). Lastly, *sul2* was uniformly detected in all sulfonamide resistant R-types tested, with the exception of a single SSuT8+ isolate.

The sulfonamide determinant *sul1*, frequently associated with the presence of class I integrons, was detected in addition to *sul2* in all MDR8+ isolates, but was never detected in R-type SSuT isolates. As expected, *sul1* was not detected in R-type T or AST isolates. The presence of *sul1* was found to be statistically associated with an increasing degree of multi-drug resistance (Fig. 1, r = 0.81, $P = 0.025$).

In contrast to nearly universal detection of the streptomycin resistance genes *strA* and *strB* among streptomycin resistant isolates and to the frequent co-existence of *sul1*

with *sul2* among sulfonamide resistant isolates with increased multi-drug resistance, tetracycline resistance determinants were differentially present among tetracycline resistant isolates (Fig. 2). Isolates having an SSuT-only phenotype exclusively harbored the *tet*(B) gene, whereas SSuT8+ isolates were more likely to encode *tet*(A) compared with *tet*(B) (55% vs. 30%, respectfully; Table 2. Tetracycline resistance gene *tet*(A) in isolates with increasing degrees of MDR (Table2, slope for trend= 1.83, *t* for trend= 2.73, *P*= 0.0232). Detection of both *tet*(A) and *tet*(B) within single isolates was only encountered twice out of 130 tetracycline resistant isolates tested. In five cases neither *tet*(A) nor *tet*(B) was detected, including CSSuTSxt (2 of 10 isolates) and SSuT8+ (3 of 20 isolates).

Major differences were also observed between isolates of different R-types in detection of transformable (plasmid-encoded) resistance (Table 3): Less than half of SSuT-only isolates harbored detectable plasmids and DNA preparations from these isolates were uniformly unable to transform recipients with selectable resistance to sulfonamide or tetracycline, consistent with a chromosomal location of the SSuT resistance determinants. In contrast, SSuT8+ isolates invariably harbored one or more large plasmids and DNA preparations from SSuT8+ isolates were uniformly able to transform recipients with selectable resistance that generally included at least R-type SSuT and most often (15 of 20) were resistant to five or more antimicrobial drugs. Isolates with intermediate degrees of multi-drug resistance had intermediate frequency of plasmid carriage and presence of detectable plasmid transferable antimicrobial drug resistance (slope = 2.847, *t* for trend= 4.64, *p*= 0.0012) (Table 3, Fig. 3). Plasmid profiles of transformants and parent strains revealed single plasmids in all transformants that

matched one of the parent strain plasmids. Most R-plasmids conferred S, Su, and T resistance along with resistance to additional antimicrobial drugs (Table 3).

DISCUSSION:

We used PCR-based genotyping assays to investigate the evolutionary and epidemiologic relatedness between SSuT and multidrug resistant non- pathogenic *E. coli*. We employed genotypic molecular methods rather than phenotyping because they offered the ability to discriminate similarly MDR isolates by the distribution of specific SSuT resistant determinants. Most previous studies on multidrug resistance evolution have been based on phenotyping and therefore were not generally able to distinguish identical phenotypes encoded by different genetic determinants (15).

Over representation of the SSuT resistance phenotypes were observed among multiply drug resistant *E. coli* strains from cattle from which our study set was selected (data not shown).SSuT resistance is of increasing interest because of reported associations between these resistances with other fitness traits. For example, a fitness advantage was recently reported for a plasmid isolated from human clinical *E. coli* carrying the *sul2* and *strA/strB* genes. Plasmid p9123 in *E. coli* which did not encode resistance to any commonly used antibacterial agents was maintained in host bacteria because of the improvement of the inherent fitness of the host strain (14). A similar fitness advantage was reported in a study in SSuT strains in E. coli from calves by Khachatryan et al (2004) (23).

We performed a population based study to investigate the evolution of multi-drug resistance from SSuT by random selection of 130 study isolates, stratified into 11 different R-types ranging from singly resistant isolates to isolates resistant to ten or more

antimicrobial drugs, including nine R-types that included SSuT resistance, and analyzed the SSuT resistance determinants and the frequency of plasmid carriage and plasmid encoded resistance. While the hypothesis that highly multi-drug resistant isolates evolved from R-type SSuT isolates was supported by the conservation of the streptomycin resistance determinants *strA* and *strB* as well as the sulfonamide resistance determinant *sul2* across the range of R-types studied, the differing distributions of *tet*(A) and *tet*(B) as well as the difference in the genetic location of SSuT resistance determinants between SSuT and MDR8+ R-Types strongly suggests this hypothesis is incorrect and that a more complex mechanism is required. One possible alternative hypothesis is that MDR evolve by acquisition of additional resistance genes, but acquisition of some combinations of resistance genes may result in adverse fitness effects that trigger strong selection for the subsequent loss of the unfavorable combination. For example, it is possible that simultaneous expression of both *tet*(A) and *tet*(B) efflux pumps is not compatible and thus when genes are linked to these tetracycline genes they cannot re-assort in a random fashion.

The evidence presented here does not support an adverse fitness effect of carriage of multiple *sul* alleles but rather suggests that acquisition of *sul1* is routinely found with increasing MDR. In contrast, the distribution of *tet* determinants suggest strong pressures exist against carriage of multiple efflux pumps, since detection of more than one *tet* gene was rare (2 of 130 study isolates). While the presence of both *tet*(A) and *tet*(B) in single isolate has been reported previously (35), a general negative association between *tet*(A) and *tet*(B) has been reported (27) and suggested to be due to plasmid incompatibilities (27).

As in our study isolates, *tet*(B) has been reported by others to be the most widespread of the tetracycline resistance determinants (31) and to be frequently chromosomally encoded (34). The plasmid incompatibility argument against simultaneous *tet*(A) and *tet*(B) is not supported by our data because the chromosomally encoded *tet*(B) allele, even if due to an integrated plasmid, should not be displaced by a plasmid encoded *tet*(A) allele as was detected in the majority (55%) of MDR (8+) isolates. R-type AGSSuTSxt was the exception to this tendency, as all ten isolates carried only *tet*(B), suggesting that the process which resulted in the evolution of this R-type differed from that of most others.

The carriage of linked resistance determinants has been suggested to represent the logical outcome of evolutionary pressures (5), and numerous examples of linked resistance determinants, often carried by mobile genetic elements, has been previously described including for the SSuT resistance determinants. The linkage of *strA-strB* genes is particularly strong, probably because the combination results in a higher degree of resistance than either determinant alone (10).S*trA-strB* genes have been reported within integrons, transposons (e.g., Tn*5393*) and on broad-host-range plasmids RSF1010, pBP1, pSTR1 (9, 35, 36). *Sul2* is often associated with *strA-strB* in small multicopy or large transmissible multi-resistance plasmids as well as in chromosome (14). *Sul1* is an integral component of Class 1 integrons, which are frequently associated with multiple drug resistance in pathogens and in commensal *E. coli* (7, 24). Class 1 integrons are commonly associated with various transposons including Tn*21*, Tn*1696*, and Tn*1412* of the Tn*3* family. Similarly, tetracycline genes have been associated with classic transposons and integrons, located on a diverse group of plasmids as well as in the chromosome (20, 28).

For example, *tet*(A) is encoded in transposon Tn1721 and *tet*(B) in Tn10 (2, 12). In this study, we did not attempt to delineate the presence of possible mobile elements associated with the SSuT resistance determinants, but recognize that identifying these determinants in the context of molecular markers of transposons or other mobile elements is a logical extension that may clarify some of the questions raised by our findings.

We used plasmid purification, electroporation, and antimicrobial drug selection to determine the presence of R-plasmids in our study isolates. In general, we observed a tendency for increased likelihood of R-plasmid detection in isolates with higher numbers of antimicrobial resistance phenotypes. The frequent association of MDR resistance determinants and R-plasmids, compared to the chromosomal location of resistance genes on SSuT isolates, tends to support the concept that strongly favorable determinants will eventually become chromosomally encoded (5). In fact, we cannot exclude the possibility that the MDR isolates in our study carried chromosomally encoded SSuT resistance determinants in addition to plasmid encoded multi-drug resistance.

In conclusion, different resistance determinants either were (streptomycin and sulfonamide resistance due to *strA, strB, sul2)* or were not (tetracycline resistance due to *tet*(A) and *tet*(B)) conserved among isolates with diverse multi-drug resistant phenotypes. Also, the chromosomal or plasmid locations of the resistance genes were not conserved among isolates with diverse drug resistance phenotypes. Based on our findings, if SSuT+ resistance evolved from SSuT resistance, factors in addition to simple acquisition of additional resistance determinants must be involved to account for transition of tetracycline resistance determinants and for movement of SSuT resistance determinants onto R-plasmids.

REFERENCES

1. **Aalbaek, B., J. Rasmussen, B. Nielsen, and J. E. Olsen.** 1991. Prevalence of antibiotic-resistant *Escherichia coli* in Danish pigs and cattle. APMIS **99:**1103-1110.
2. **Allmeier, H., B. Cresnar, M. Greck, and R. Schmitt.** 1992. Complete nucleotide sequence of Tn1721—gene organization and a novel gene-product with features of a chemotaxis protein. Gene **111:**11-20.
3. **Anderson, A. D., J. M. Nelson, S. Rossiter, and F. J. Angulo.** 2003. Public health consequences of use of antimicrobial agents in food animals in the United States. Microb. Drug Resist. **9:**373-379.
4. **Bradford, P. A., P. J. Petersen, I. M. Fingerman, and D. G. White.** 1999. Characterization of expanded-spectrum cephalosporin resistance in *E. coli* isolates associated with bovine calf diarrhoeal disease. J. Antimicrob. Chemother. **44:**607-610.
5. **Bergstrom, C. T., M. Lipsitch and B. R. Levin**, 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics **155**: 1505–1519.
6. **Call, D. R., M. K. Bakko, M. J. Krug, and M. C. Roberts.** 2003. Identifying antimicrobial resistance genes with DNA microarrays. Antimicrob. Agents Chemother. **47:**3290–3295.
7. **Carattoli, A.** 2001. Importance of integrons in the diffusion of resistance. Vet. Res. **32:**243–259.

8. **Carattoli, A., L. Villa, C. Pezzella, E. Bordi, and P. Visca.** 2003. Expanding drug resistance through integron acquisition by IncFI plasmids of *Salmonella enterica* Typhimurium. Emerg. Infect. Dis. **7**:444-447.

9. **Catry, B., H. Laevens, L. A. Devriese, G. Opsomer, and A. DeKruif.** 2003. Antimicrobial resistance in livestock. J. Vet. Pharmacol. Therap. **26:**81-93.

10. **Chiou, C. S., and A. L. Jones.** 1995. Expresssion and identification of the *strA-strB* gene pair from streptomycin-resistant *Erwinia amylovora*. Gene **152:**47-51.

11. **Clinical and Laboratory Standards Institute.** 2005. Performance standards for antimicrobial susceptibility testing, 15th informational supplement, vol. 25, no. 1. M100-S15. Clinical and Laboratory Standards Institute, Wayne, Pa.

12. **Coleman, D. C., I. Chopra, S. W. Shales, T. G. Howe, and T. J. Foster.** 1983. Analysis of tetracycline resistance encoded by transposon Tn10: deletion mapping of tetracycline-sensitive point mutations and identification of two structural genes. J. Bacteriol. **153:**921-929.

13. **DeFrancesco, K. A., R. N. Cobbold, D. H. Rice, T. E. Besser, and D. D. Hancock.** 2004. Antimicrobial resistance of commensal *Escherichia coli* from dairy cattle associated with recent multi-resistant salmonellosis outbreaks. Vet. Microbiol. **98:**55–61.

14. **Enne, V. I., P. M. Bennett, D. M. Livermore, and L. M. C. Hall.** 2004. Enhancement of host fitness by the *sul2*-coding plasmid p9123 in the absence of selective pressure. J. Antimicrob. Chemother. **53:**958-963.

15. **Foley, S. L., D. G. White, P. F. McDermott, R. D. Walker, B. Rhodes, P. J. Fedorka-Cray, S. Simjee, and S. Zhao.** 2006. Comparison of subtyping

methods for differentiating *Salmonella enterica* serovar Typhimurium isolates obtained from food animal sources. J. Clin. Microbiol. **44:**3569-3577.

16. **Harbottle, H**., S. **Thakur**, S. **Zhao, and** D.G. **White.** 2006. Genetics of antimicrobial resistance. Animal Biotechnol. **17**:111-124.
17. **Harris, S., and D. B. Jones.** 1997. Optimisation of PCR. J. Biomed. Sci. **54:**166-173.
18. **Hayes, J.D.** 1999. Costs of eliminating sub-therapeutic use of antibiotics. In: Committee on Drug Use in Food Animals, Panel on Animal Health, Food Safety, and Public Health, National Research Council, pp. 179-187.
19. **Hoyle, D. V., C. M. Yates, M. E. Chase-Topping, E. J. Turner, S. E. Davies, J. C. Low, G. J. Gunn, M. E. Woolhouse, and S. G. Amyes.** 2005. Molecular epidemiology of antimicrobial-resistant commensal *Escherichia coli* strains in a cohort of newborn calves. Appl. Environ Microbiol. **71:**6680–6688.
20. **Jones, C. S., D. J. Osborne, and J. Stanley.** 1992. Enterobacterial tetracycline resistance in relation to plasmid incompatibility. Mol. Cell. Probes **6:**313-317.
21. **Jorgensen, S. E., and B. Halling-Sorensen.** 2000. Drugs in the environment. Chemosphere **40:**691-699.
22. **Kado, C. I., and S. T. Liu. 1981.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
23. **Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call.** 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. **70:**752-757**.**

24. **Leverstein-van Hall, M. A., M. B. HE, T. D. AR, A. Paauw, A. C. Fluit, and J. Verhoef.** 2003. Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. J. Infect. Dis. **187:**251–259**.**

25. **Livermore, D. M., M. Warner, L. M. C. Hall, V. I. Enne, S. J. Projan, P. M. Dunman, S. L. Wooster, and G. Harrison.** 2001. Antibiotic resistance in bacteria from magpies (*Pica pica*) and rabbits (*Oryctolagus cuniculus*) from west Wales. Environ. Microbiol. **3:**658–661.

26. **Martinez-Freijo, P., A. C. Fluit, F.-J. Schmitz, J. Verhoef, and M. E. Jones.** 1999. Many class I integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. Antimicrob. Agents Chemother. **43:**686–689.

27. **Maynard, C., J. M. Fairbrother, S. Bekal, F. Sanschagrin, R. C. Levesque, R. Brousseau, L. Masson, and J. Harel.** 2003. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. Antimicrob. Agents Chemother. **47:**3214-3221.

28. **Mendez, B., C. Tachibana, and S. B. Levy.** 1980. Heterogeneity of tetracycline resistance determinants. Plasmid **3:**99-108.

29. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.

30. **Recchia, G. D., and R. M. Hall.** 1995. Gene cassettes: a new class of mobile element. Microbiol. **141:**3015–3027.

31. **Roberts, M. C.** 1996. Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. **19:**1–24.

32. **Rodríguez, I., M. C. Martínez, M. C. Mendoza, and M. R. Rodicio.** 2000. Class 1 and class 2 integrons in non-prevalent serovars of *Salmonella enterica*: structure and association with transposons and plasmids: J. Antimicrob. Chemother. **58:**1124-1132.

33. **Salyers, A. A., and C. F. Amabile-Cuevas.** 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents Chemother. **41:**2321–2325.

34. **Sawant, A. A**., N. V. **Hegde**, B. A. **Straley**, S. C. **Donaldson**, B. C. **Love**, S. J. **Knabel**, B. M. **Jayarao.** 2007. Antimicrobial-resistant enteric bacteria from dairy cattle. Appl. Environ. Microbiol. **73:**156-63.

35. **Sunde, M**., and M. **Norström.** 2006. The prevalence of, associations between and conjugal transfer of antibiotic resistance genes in *Escherichia coli* isolated from Norwegian meat and meat products. J. Antimicrob. Chemother. **58:**741-742.

36. **Sundin, G. W., and C. L. Bender.** 1996. Dissemination of the *strA-strB* streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. Mol Ecol **5:**133–143.

37. **Winckler, C., and A. Grafe.** 2001. Use of veterinary drugs in intensive animal production: evidence for persistence of tetracycline in pig slurry. J. Soils Sedim. **1:**66-70.

FIG. 1.

Histogram showing percentages of *E. coli* R-Types obtained from cattle containing sulfonamide resistance genes as determined by PCR using primers for *sul1* and *sul2*. Open bars = *sul1*; Filled bars = sul2.

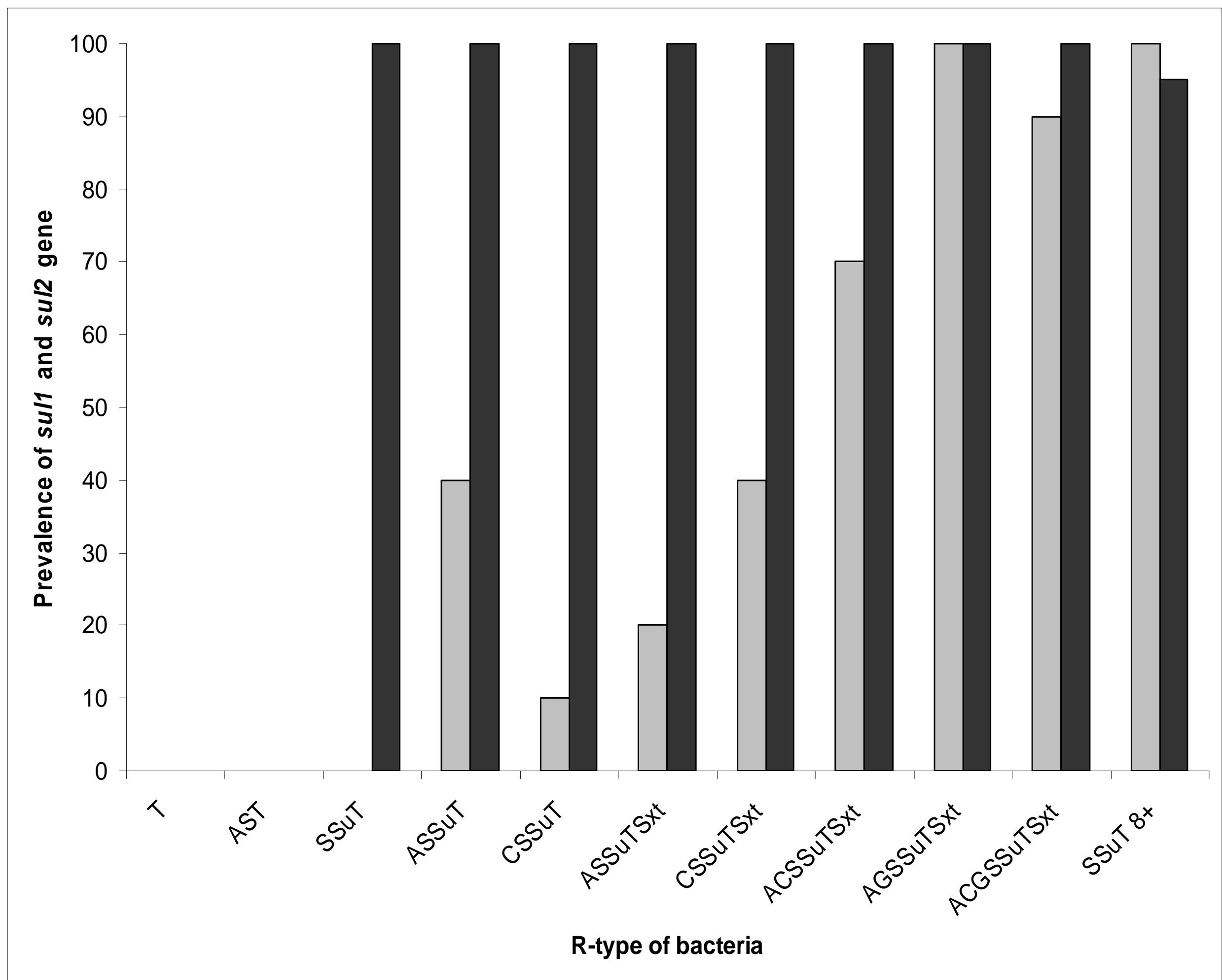

FIG. 2.

Histogram showing percentages of *E. coli* R-Types obtained from cattle containing Tetracycline resistance genes as determined by PCR using primers for *tet*(A) and *tet*(B). Open bars = *tet*(A); Filled bars = *tet*(B).

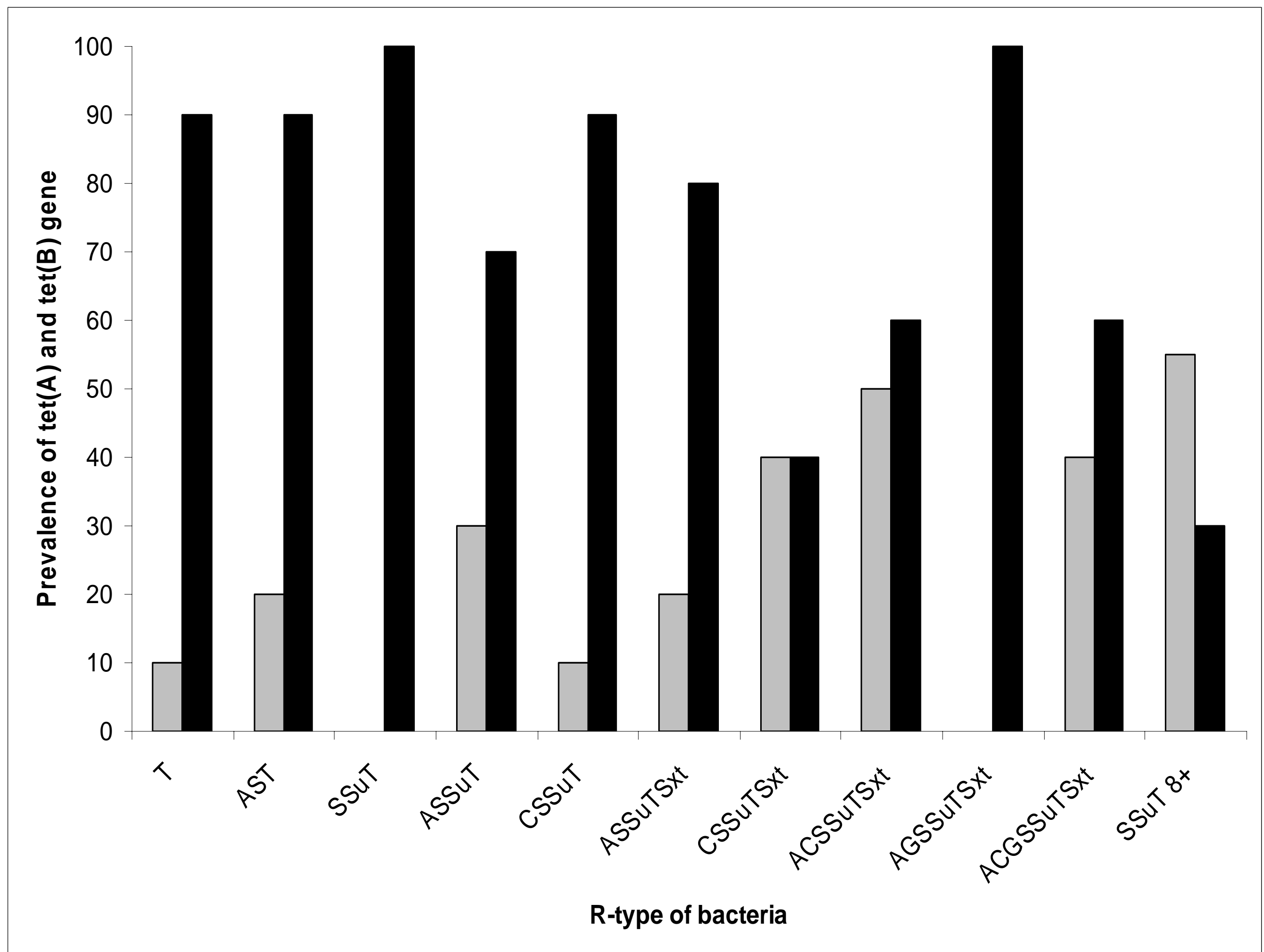

FIG.3.

Frequency of carriage of plasmid encoded resistance in *E. coli* R-Types under study as determined by electroporation experiments and selective plating on tetracycline, chloramphenicol and ampicillin plates.

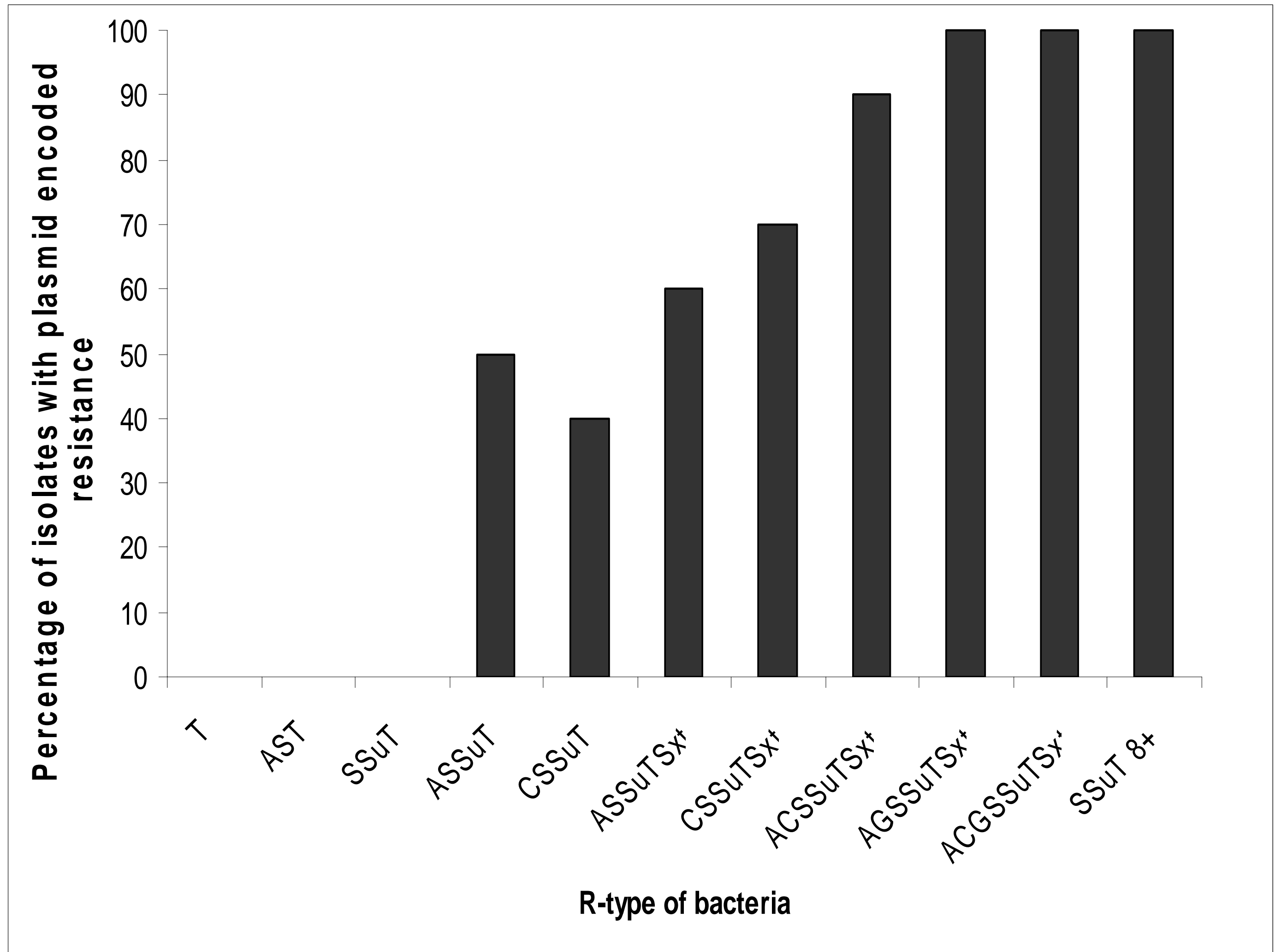

TABLE1: PCR primers used in this study and expected amplicon sizes of PCR products.

Primer	Sequence	Amplicon Size	Ref
sul1-F	CCGATATTGCTGAGGCGGACT	250	25
sul1-R	CCAACGCCGACTTCAGCTT		
sul2-F	TCAAGGCAGATGGCATTCC	400	25
sul2-R	ATCGAAGCGCAGCCGCAAT		
tet(A)-F	TTGGCATTCTGCATTCACTC	500	6
tet(A)-R	GTATAGCTTGCCGGAAGTCG		
tet(B)-F	CAGTGCTGTTGT TGTCATTAA	600	6
tet(B)-R	GCTTGGAATACTGAGTGTAA		
strA-F	CAACTGGCAGGAGGAACA	600	25
strA-R	CGCAGATAGAAGGCAAGG		
strB-F	TTCTCATTGCGGACACCT	450	25
strB-R	GGCATTGCTCATCATTTG		

TABLE 2. Antibiotic resistance genotypes for commensal *E. coli* collected from cattle in Oregon and Washington State.

Phenotype	N	Resistance determinant					
		sulfonamide		streptomycin		tetracycline	
		sul1	*sul2*	*strA*	*strB*	*tet*(A)	*tet*(B)
T	10	0	0	0	0	0	9
		0	0	0	0	1	0
AST	10	0	0	8	8	0	8
		0	0	1	1	1	0
		0	0	1	1	1	1
SSuT	20	0	17	17	17	0	17
		0	3	0	3	0	3
ASSuT	10	0	5	5	5	0	5
		2	2	2	2	0	2
		1	1	1	0	1	0
		1	1	1	1	1	0
		0	1	1	1	1	0
CSSuT	10	0	9	9	9	0	9
		1	1	1	0	1	0
ASSuTSxt	10	0	7	7	7	0	7
		0	1	1	1	1	0
		1	1	1	1	1	0
		1	1	1	1	0	1
CSSuTSxt	10	0	4	4	4	0	4
		3	3	3	3	3	0
		1	1	1	1	0	0
		0	1	1	1	1	0
		0	1	1	1	0	0
ACSSuTSxt	10	5	5	5	5	0	5
		1	1	1	1	1	1
		1	1	1	1	1	0
		0	3	3	3	3	0
AGSSuTSxt	10	10	10	10	10	0	10
ACGSSuTSxt	10	6	6	6	6	0	6
		3	3	3	3	3	0
		0	1	1	1	1	0
MDR (8+)	20	10	10	10	10	10	0
		6	6	6	6	0	6
		3	3	3	3	0	0
		1	0	1	1	1	0

0 = absence, 1 = presence of PCR product for the designated gene. Resistance phenotypes are indicated by concatenation of drug resistances: T = tetracycline, S = streptomycin, Su = sulfonamide, A = ampicillin, Sxt = sulfa / trimethoprim, C = chloramphenicol, G = gentamicin, Amc = amoxicillin/clavulanic acid, Caz = ceftazidime.

TABLE 3: Transformable resistance phenotypes of MDR E. coli isolated from cattle.

Resistance phenotype[1]		
Parent strains	Transformants	N
T	Not detected	0 / 10
AST	Not detected	0 / 10
SSuT	Not detected	0 / 20
ASSuT	A(S)SuT[2]	5 / 10[3]
CSSuT	C(S)SuT	4 / 10
ASSuTSxt	A(S)SuTSxt	1 / 10
	ASSuT	5 / 10
CSSuTSxt	C(S)SuTSxt	4 / 10
	(S)SuT	2 / 10
	(S)T	1 / 10
ACSSuTSxt	AC(S)SuTSxt	2 / 10
	AC(S)Su	3 / 10
	A(S)	4 / 10
AGSSuTSxt	A(S)SuT	7 / 10
	(S)SuT	1 / 10
	A(S)Su	1 / 10
	A(S)	1 / 10
ACGSSuTSxt	AG(S)SuTSxt	5 / 10
	A(S)SuTSxt	2 / 10
	A(S)SuT	1 / 10
	AC(S)Su	1 / 10
	A(S)	1 (10)
MDR8+	AC(S)SuTSxtAmcCaz	7 / 20
	AC(S)SuTAmcCaz	1 / 20
	AC(S)SuTSxtAmc	1 / 20
	ACG(S)SuTCaz	1 / 20
	A(S)SuTSxtAmc	1 / 20
	AC(S)SuTAmc	1 / 20
	AG(S)SuTSxt	1 / 20
	A(S)SuTSxt	2 / 20
	A(S)SuT	1 / 20
	AC(S)Su	1 / 20
	(S)SuT	1 / 20
	(S)T	2 / 20

[1] Resistance phenotypes are indicated by concatenation of drug resistances: T = tetracycline, S = streptomycin, Su = sulfonamide, A = ampicillin, Sxt = sulfa /

trimethoprim, C = chloramphenicol, G = gentamicin, Amc = amoxicillin/clavulanic acid, Caz = ceftazidime.

[1] The DH10b recipient strain is streptomycin resistant, so transfer of the streptomycin resistance phenotype is not confirmed as indicated by the brackets around ‘S’.

[2] N of transformant R-type is lesser than N of Parent R-type as Plasmid borne resistance determinants were not detected in some of the isolates.