TRANSMISSION AND EPIDEMIOLOGY OF *SALMONELLA ENTERICA* IN COMMERCIAL DAIRY FARMS IN WASHINGTON STATE

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine

AUGUST 2008

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of BIJAY ADHIKARI find it satisfactory and recommend that it be accepted.

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Chair

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ACKNOWLEDGMENTS

The first person I would like to thank is my mentor Dr. Dale Hancock, who has been a principle-centred person in my study. His professional view on scientific research, enthusiasm, and mission for providing only high-quality guidance has made a deep impression on me. I owe him lots of gratitude for having helped me over the many hurdles of scientific research and as a guardian to my family.

I would like to thank my committee members Drs. Tom Besser, John Gay and Larry Fox, for their help, considerable time, efforts and guidance in this project, and for keeping a watchful eye on the progress of my work and for always being available when I needed their advice. Their role as committee member was remarkable and noteworthy. I would also like to thank my former advisor Dr. Rowland Cobbold who recruited me on this program and provided me with valuable comments and inputs. His efforts and keenness on my research work was significant.

I would like to thank Dr. Margaret Davis for her invaluable help in my research especially technical helps in genotyping of *Salmonella*. I would also like to thank Drs. William Sischo and Douglas Call for their suggestions and technical helps. The completion of this thesis is also due to the support provided by Russell McClanahan, Katherine Kaya Baker, Christina Sherman, Lisa Jones, Lindsey Tippett and other Field Disease Investigation staff. I thank you all for your help. Thanks are due to all the owners of 60 Washington dairy farms and two feed mills for their cooperation and agreed to allow us to conduct research in their property. Without their help this project may not have succeeded.

I would like to extend my thanks to friends and office-mate, Sonja Lloyd, Veerasak, Coocki, Letizia Tomassini, Solomon Ramabu and Dot Newkirk for their help in matters of academic complexities and technical discussion. I am thankful to Drs. Joshua Daniels, Cat Berge and Regina Finger for their help in data analysis, serotyping and thoughtful discussion.

I would like to extend my gratitude to the Washington State University for my admission, and to the Department of Veterinary Clinical Sciences accepting me as a graduate student, financial support and allowed to conduct necessary research work and to use departmental resources. This project was supported in part by National Institute of Health, Department of Health and Human Services under the contract number No1-AI-30055 and by the Agricultural Animal Health Program, College of Veterinary Medicine, Washington State University. I am thankful to these organizations.

I feel a deep sense of gratitude to my father and mother who formed part of my foresight, taught me the good things that really matters in life and always provide inspiration for my journey through life. I am grateful to my three brothers Jay Ram, Bhim and Achyut for rendering me the sense and the value of brotherhood, and always taught to be a nice person. I am glad to be their brother. I would like to give very special thanks to my wife Biju whose support and patient love greatly facilitated the completion of this work. With her love and support during the life mission and academic complexities made me who I am today. Thanks to our lovely sons Brishank and Barnan for his patience and for coping with me in every challenging situation during the study. My chain of gratitude would be incomplete if I were to forget to acknowledge the first cause of this chain, Jai guru.

TRANSMISSION AND EPIDEMIOLOGY OF *SALMONELLA ENTERICA* IN COMMERCIAL DAIRY FARMS IN WASHINGTON STATE

Abstract

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May 2008

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An estimated $1.3x10^6$ human salmonellosis cases and over 500 deaths occur annually in the United States, and livestock products are implicated. *Salmonella enterica* often exhibits emergence and clonal dissemination patterns indicating a major role for inter-herd transmission. This research highlights three features of *Salmonella enterica* in cattle. First, increased ceftazidime resistance was observed for a *Salmonella* Typhimurium clade designated WA-TYP035/187, in cattle and humans in the Pacific Northwest. bla_{CMY-2} plasmid diversity within minimum spanning tree branches of MLVA alleles suggested that resistance was acquired on multiple occasions followed by subclonal dissemination. Because this pattern of increasing resistance was very similar to that for the bovine-adapted serovar *S*. Dublin reported previously, we concluded that selection pressure in cattle due to ceftiofur use was the most likely explanation. Results presented in second chapter indicates that the rate of new MDR *Salmonella* strain introduction was 75.5 per 1000 herd-months (95% CI 50.8 – 119.4), which correlates to 0.91 new strains per farm per year. This indicates that Washington dairy farms acquisition of new MDR *Salmonella*

strains is a common event, though the acquisition rate varies greatly among farms. MDR *Salmonella* were rarely found in delivered feeds and then only at low concentrations. No transmission from feed was noted. Results presented in third chapter indicate that off-farm heifer raising with co-mingling with cattle from other sources was strongly associated with acquiring any new MDR *Salmonella* strains. (OR= 8.9, 95% CI: 2.4, 32.80 and the number of new strains acquired per farm OR= 2.3, 95% CI: 1.1, 4.7). Herd size, per 100 animals increment, was significantly associated with farm acquiring new strains (OR= 1.04, 95% CI, 1.01, 1.05 and with the number of new strains acquired (OR=1.02, 95% CI, 1.01, 1.03). Prior diagnosis of clinical salmonellosis was associated with the number of new strains introduced (OR=2.5, 95% CI, 1.3, 5.0). These studies indicate that MDR *Salmonella* transmission to dairy farms in Washington State is common, that selection pressure for ceftiofur resistance most likely occurs on farms, and that the most important management variable involved in transmission is off-farm heifer raising.

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DEDICATION

This dissertation/thesis is dedicated to parents, Chiranjibi Adhikari and Tulasa D. Adhikari, and to my wife Bijaya Aryal (Biju)

CHAPTER ONE

Diverse *bla*$_{\text{CMY-2}}$ encoding plasmids within a clonal lineage of *Salmonella enterica* serovar Typhimurium isolated from cattle and humans

CHAPTER 1

ABSTRACT:

Salmonella enterica serovar Typhimurium circulating in food animal populations and carrying resistance to antimicrobial agents represents a human health risk. Recently, a new clade of *S.* Typhimuriun, WA-TYP035/187, was reported in cattle and humans in the Pacific Northwest. The objective of this study was to describe possible mechanisms of acquisition of extended spectrum cephalosporin resistance in this clade. We characterized 129 (74 ceftazidime susceptible and 55 resistant) *S.* Typhimurium of this clone isolated from 1999 through 2006 from cattle and humans using multiple-locus variable number tandem repeat analysis (MLVA) and plasmid profiling. Ceftazidime resistance increased steadily in this WATYP035/187 from 0% (0/2) in 1999 to78% (18/23) in 2006 (χ^2 for linear trend, p-value < 0.001). The $bla_{\text{CMY-2}}$ plasmid diversity within minimum spanning tree branches of MLVA alleles suggests that resistance was acquired on multiple independent occasions by WA-TYP035/187. Given the lack of an obvious reservoir in species other than cattle and the parallel rise in ceftiofur resistance in the bovine-specific serovar *S.* Dublin reported in previous study (20), we conclude that the most likely source of increased ceftazidime resistance in this clade was selection pressure due to the use of ceftiofur in cattle.

INTRODUCTION

Salmonella enterica serovar Typhimurium is considered one of the leading causes of food-borne bacterial infections in the USA (15, 29). Development of resistance to broad-spectrum antimicrobials, particularly third-generation cephalosporins, limits the treatment options for human infections (3). Third-generation cephalosporin resistance in *Salmonella* is of particular concern because these drugs are commonly used in treatment of pediatric salmonellosis. Multidrug resistant (MDR) *Salmonella* limit the effectiveness of first-line antibiotics when treating pediatric infections and more likely to cause prolonged or severe illnesses than antimicrobial-susceptible strains (13).

The widespread use of antimicrobial agents in food animals has been implicated in the increasing antimicrobial resistance in food-borne pathogens isolated from humans (5). Cattle have been recognized as a reservoir for non-typhoid *Salmonella* and as a likely source of some human cases of MDR-salmonellosis (37, 38) . Tracking of *Salmonella* isolates from veterinary diagnostic laboratory submissions suggest that antimicrobial resistance is increasing in cattle isolates from the Northwestern USA (9, 10, 20).

Some bacterial plasmids carry genes encoding resistance to several antimicrobials, with the potential to complicate therapy of infections by pathogens. Frequent plasmid transfer between animal-associated *Salmonella* and *Escherichia coli*, and isolation of identical $bla_{\text{CMY-2}}$ plasmids in these organisms isolated from animals and humans have been reported (6, 45). Widespread use of ceftiofur, a veterinary extended-spectrum cephalosporin in cattle may select for and maintain ceftriaxone-resistant *Salmonella* in cattle (4, 11).

Recently, Davis et al. reported the emergence of a clade of *Salmonella enterica* serotype Typhimurium (19), identified by the highly similar *Xba* I PFGE patterns (WA-TYP035 and WA-

TYP187), To date, TYP035/187 isolates have largely been restricted to bovine and human hosts in the Pacific Northwest USA (Paula Fedorka-Cray, personal communication). This study investigates the increasing incidence of extended spectrum cephalosporin resistance in WA-TYP035/187, with the hypothesis that WA-TYP035/187 acquired extended spectrum cephalosporin resistance on multiple, independent occasions. Variable-number tandem repeat (VNTR) loci provide polymorphic markers that are the basis of a powerful molecular technique to discriminate isolates within clonal complexes (34, 42, 43). Theretherefore, we used MLVA method to describe genotype diversity within the WA- TYP035/187 clade.

MATERIALS AND METHODS

Isolates. *Salmonella enterica* serovar Typhimurium isolates were obtained from three sources: the Washington Animal Disease Diagnostic Laboratory (WADDL), the Zoonoses Research Unit (ZRU) and the Field Disease Investigation Unit (FDIU). WADDL receives animal disease diagnostic specimens primarily from the Pacific Northwest region of the United States. The FDIU has been receiving and banking isolates of *Salmonella* derived from field research projects conducted in cattle herds across the Pacific Northwest for over 20 years. Since 2004, the ZRU has obtained human clinical isolates of *Salmonella* from the Washington State Department of Public Health for comparison with strains circulating in the animal reservoirs. Analysis was limited to independent isolates from cattle herds, only the first in each serotype and resistance type within each herd and calendar year was included in the analysis.

Phenotypic characterization. All confirmed *S*. Typhimurium isolates were serotyped at the USDA National Veterinary Services Laboratory, Ames, Iowa. The Kirby-Bauer agar diffusion method (7) was used according to Clinical and Laboratory Standards Institute (CLSI) guidelines (30, 31) to test antimicrobial susceptibility phenotypes of isolates using the

following antimicrobial disks: ampicillin (A, 10 μg), ceftazidime (Caz, 30 μg), chloramphenicol (C, 30 μg), gentamicin (G, 10 μg), amoxicillin-clavulanic acid (Amc, 20/10 μg), kanamycin (K, 30 μg), streptomycin (S, 10 μg), tetracycline (T, 30 μg), triple-sulfa (a combination of sulfadiazine, sulfamethazine, and sulfamerazine) (Su, 250 μg), and trimethoprim-sulfamethoxazole (Sxt, 1.25/23.75 μg) (BD Diagnostics, Sparks, MD, USA). *Salmonella* isolates were considered antimicrobial resistant based on the following resistance-susceptible zone size of inhibition cut-point (mm): A <13, C < 12, G <12, K <13, S <11, Su < 10, T <14, Sxt <10, Amc <13, Sul <12 and Caz < 12. Isolates resistance to two or more antimicrobials were considered MDR.

Pulsed-Field Gel Electrophoresis. All isolates of *S.* Typhimurium were subjected to pulsed-field gel electrophoresis (PFGE) to examine genetic diversity. Isolates were grown overnight on blood agar plates, and PFGE was performed following *Xba*I restriction using the PulseNet protocol for *Salmonella* and using *S.* Braenderup, H9812, as a standard (37).The gels were analyzed using BioNumerics 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium).

Plasmid profiles. Plasmids were detected using plasmid profiling method described previously (24). Briefly, bacterial cells were grown overnight in blood agar media at 37 °C, harvested by centrifugation, then suspended in 60 μL of lysis buffer (40 mM Tris acetate, 2 mM EDTA [pH 12.6]), incubated for 30 min at 55 °C, and containing plasmids extracted from 100 μL phenol-chloroform (1:1 [vol/vol]) supernatant. After centrifugation, the supernatant was subjected to agarose gel electrophoresis for plasmid isoaltion. Plasmid electrophoresis was performed in 1% agarose gels run at 0.6 V cm^{-2}, 4 h, in TAE buffer (40 mM Tris–acetate, 1 mM EDTA). Plasmid sizes were estimated relative to a BAC-Tracker Supercoiled DNA ladder

(EPICENTRE Biotechnologies, Madison, WI, USA) using Bionumerics (Applied Maths) software.

Multilocus Variable Number of Tandem Repeats Typing. Four VNTR loci (STTR9, STTR6, STTR5, STTR10pl) used in MLVA typing as described previously (27). Briefly, isolates were grown overnight on blood agar plates and one colony was selected to prepare a PCR template using the boiled cell lysate method. Multiplex PCR was performed with a PCR kit (Qiagen Hilden, Germany) in a total of 25 μL and 2.50 pmol each of primers STTR9-F, STTR9-R, STTR6-F, and STTR6-R and 1.25 pmol each of primers STTR5-F, STTR5-R, STTR10pl-F, and STTR10pl-R. These forward and reverse primers were used to amplify repeat sequence of four VNTR loci, STTR9, STTR6, STTR5, STTR10pl, respectively. Amplification was performed with an iCycler thermocycler (Bio-Rad, Hercules, CA) as follows: one cycle of 15 min at 94 °C, followed by 25 cycles of 30 sec at 94 °C, 1 min at 60 °C, and 1.5 min at 72 °C and finally an extension step of 10 min at 72 °C. Then, 19.357 μL of Hi-Di formamide (Applied Biosystems, Foster, CA), 0.125 μL Liz600 size standard (Applied Biosystems), and 0.5 μL DNA template were mixed for capillary electrophoresis. The final PCR products were separated in capillary electrophoresis using an ABI-3730 DNA analyzer (Applied Biosystems) at the Laboratory for Biotechnology and Bioanalysis at Washington State University. Data were preprocessed with GeneMarker (SoftGenetics, State College, PA, USA) software.

Plasmid transformation and PCR: Plasmids were isolated by electroporation (GenePulser, Biorad, Hercules, CA) into *E. coli* competent cells, DH10B, using Plasmid Mini Kit (Qiagen, Valencia, CA) and previously described methods (22). Electroporants were immediately placed into 200 μL super optimal broth and incubated shaken at 300 rpm for 1 h at 37 °C. Transformants were then plated onto selective media containing 8μg/mL ceftazidime.

Antimicrobial susceptibility and plasmid profiles of transformants were performed as described earlier, except that electrophoresis conditions included 0.95% (w/v) agarose gels run at 100 V for 3 hr in 1% TAE buffer (40 mM Tris–acetate, 1 mM EDTA). The presence of bla_{CMY-2} gene was confirmed by polymerase chain reaction (46).

Data Analysis. Allele sizes from MLVA were entered into BioNumerics as character values and a dendrogram was generated using the categorical coefficient and the Ward algorithm. Following the fragment analysis, each locus was assigned a variant score based on the fragment size. Isolates were assigned MLVA types based on compilation of the variant score of the four loci. In the dendrogram, *S.* Typhimurium PFGE type WA-TYP035/187 isolates were split into 39 types. Population modeling of the isolates was performed in a minimum spanning tree (MST) method using MLVA profiles, using the categorical coefficient to calculate the distance matrix based on following priority rules as described previously (27): the highest number of single-locus variants (SLVs; when two types have an equal distance to a linkage position in the tree, the type that has the highest number of SLVs is linked first), the highest number of SLVs and double-locus variants (DLVs), the highest number of entries is linked first), and the most frequent state (the types that have the highest rank are linked first). Plasmid diversity within MST clusters were calculated using the following formula to generate the Shannon-Wiener diversity index (H), $= -\sum P_i \ln P_i$, where P_i is the frequency of the i^{th} type divided by the number of strains with this particular type (28).

RESULTS

One hundred-twenty-nine isolates of *Salmonella* Typhimurium PFGE type WA-TYP035/187 were obtained from bovine and human sources, of which 40 of 100 (40%) bovine

and 15 of 29 (52%) human isolates were resistant to ceftazidime. The earliest two isolates of WA-TYP035/187 were isolated in 1999 from cattle and were ceftazidime sensitive. Ceftazidime resistance in WA-TYP035 and WA-TYP187 isolates was first observed in 2000 and 2004 respectively. Isolates resistance to ceftazidime increased from 0% in 1999 (0/2) to 78% (18/23) in 2006 (Table 1). This increment was significant (Chi square test for linear trend p-value < 0.001). Ninety percent (9/10), 8% (1/13) and 83% (5/6) of human clinical isolates of *Salmonella* Typhumurium WA-TYP035 obtained from the Washington Department of Public Health in 2004, 2005 and 2006 were resistant to ceftazidime, respectively.

MDR TYP035/187 exhibited diverse patterns of antimicrobial resistance. Ceftazidime resistant isolates from both bovine and human sources exhibited resistance to from three to nine of the antimicrobials tested. The most frequently occurring pattern of resistance, to ampicillin, kamamycin, streptomycin, tetracycline, amoxicillin-clavulanic acid, triple-sulfa and ceftazidime (AKSTAmcSuCaz) comprised 30% and 27% of bovine and human isolates, respectively (Table 2).

PFGE generated with the XbaI restriction enzyme showed that ceftazidime resistant and sensitive isolates of WA-TYP035 and WA-TYP187 clones were indistinguishable (data not shown). Multilocus variable number tandem repeat analysis (MLVA) was used to further dissect the genetic diversity within the WA-TYP035/187 clade. Initially five different MLVA loci were selected as described (27). However, in a preliminary study of VNTR locus stability, the VNTR locus STTR3 demonstrated a lack of stability during a 30-day passage study (Call *et al.* unpub. data), which led us to abandon that locus because of its potential to generate error. VNTR locus STTR9 had two different alleles (160 and 169 bp). Locus STTR6 had nine different alleles (303, 309, 314, 320, 326, 332, 338, 344 and 362 bp), locus STTR10p had eight different alleles (323,

341, 366, 372, 379, 385, 391 and 397 bp) and locus STTR5 had six different alleles (262, 268, 274, 280, 286 and 298 bp).

The MLVA differentiated WA-TYP035/187 into 39 different types. A minimum spanning tree (MST) was constructed using MLVA data to visualize relationships between the various genotypes of the clade. Isolates were grouped into seven branches (Branch-1 to Branch-7) in the MST dendrogram (Fig. 1). The MST branch 1 contained primarily WA-TYP187 isolates and branch-2 to branch-7 contained primarily WA-TYP035 isolates. Ceftazidime-resistant isolates were detected in all but one of seven MST branches radiating from the center node. Ceftazidime resistant isolates from bovine and human sources were grouped together in three branches (Branch-1, Branch-3 and Branch-6).

All 55 ceftazidime-resistant isolates of the WA-TYP035/187 clade were subjected to plasmid profiling and results are given in Table 3. Three isolates (5.5%) each harbored three plasmids ranging from 88 kb to 161 kb in size, 12 isolates (22%) harbored two plasmids ranging from 97 kb to 153 kb in size and 40 isolates (72.5%) harbored a single plasmid ranging 84 kb and 195 kb in size. $bla_{\text{CMY-2}}$ plasmids were isolated after transformations into *E. coli* competent cells. PCR amplification of plasmid DNA using $bla_{\text{CMY-2}}$ –specific primers revealed that the gene was present in all isolates and was carried on plasmids ranging between 84 kb and 195 kb size (Table 3).

The Shannon-Wiener diversity index for plasmids within MST branch-1 was 0.47, whereas plasmids in MST branch-6 had a considerably higher diversity index (1.73) (Table 3).

DISCUSSION

We used the molecular genotyping methods including PFGE, PCR MLVA and plasmid profiling to describe the distribution of extended spectrum cephalosporin resistance in a newly described clade of *S*. Typhimurium. Phenotypic characterization based on antimicrobial susceptibility testing provided resistance profiles of this clade resistant to ceftazidime as a marker for resistance or decreased susceptibility to ceftiofur and ceftriaxone. Ceftiofur is a broad-spectrum third generation cephalosporin antibiotic, which is primarily used for veterinary use (32) and is closely related to ceftriaxone, which is commonly used for treatment of human pedriatic salmonellosis (16).

We found that ceftazidime resistance increased steadily in the *S.* Typhimurium WA-TYP035/187 clade isolated from bovine source from 1999 to 2006. About half of the human clinical isolates of this clade were also resistant to ceftazidime. Although ceftazidime resistance was frequent in human isolates collected after 2004, the ceftazidime resistance WA-TYP035/187 clade appeared to become prevalent earlier among bovine isolates (19). A trend of increasing ceftazidime resistance was also reported in cattle-adapted *Salmonella* serovar Dublin isolates in the same region and during the same time period (20). The parallel increases in ceftazidime resistance in *S.* Typhimurium WA-TYP035/187 clade and the cattle host adapted *S.* Dublin strongly support the hypothesis that the increasing resistance was driven by ceftiofur use in cattle. In support of this view, an association was found between ceftiofur use in herds and isolation of *E. coli* with reduced susceptibility to ceftriaxone, a broad-spectrum cephalosporin (41).

Increase in ceftiofur resistance has been reported for several serotypes of *Salmonella* isolates from both cattle and poultry. In a recent study, Frye and Fedorka-Cray (21) reported an

increase from 4.0% in 1999 to 9% in 2003 in ceftiofur resistance in *Salmonella enterica* isolated from farm animals, suggesting that the increased prevalence in resistance to ceftiofur was mainly due to the acquisition and the spread of $bla_{\text{CMY-2}}$ plasmids among *Salmonella* animal isolates (21).

MLVA differentiated the WA-TYP035/187 clade isolates into 39 different MLVA types indicating that the MLVA technique provides further discrimination within this clonal group. This result is consistent with observations by other investigators that the MLVA method is more discriminatory than PFGE (12) and the method has been proposed as an alternative to PFGE for genotyping clonal groups of bacteria (17, 25, 26, 27, 35).

$Bla_{\text{CMY-2}}$ plasmids diversity was observed within the MST branches of the WA-TYP035/187 clade. Branch-6 isolates contained a highly diverse size of $bla_{\text{CMY-2}}$ plasmids, suggesting that ceftazidime-resistant *Salmonella* WA-TYP035/187 experienced multiple independent acquisitions of plasmids encoding an identical $bla_{\text{CMY-2}}$ gene. On the other hand, the MST branch-1, where 82% (18/22) of the isolates of WA-TYP187 had identical $bla_{\text{CMY-2}}$ plasmids indicates a clonal dissemination in this PFGE type as this cluster predominantly contains isolates of WA-TYP187. Thus, our observations within this clonal complex are consistent with previous findings among more diverse *Salmonella* that ceftiofur resistance likely evolved by multiple independent acquisitions of $bla_{\text{CMY-2}}$ encoding plasmids followed by clonal spread of the resistant subtypes (2, 14, 44).

Stability in MLVA profiles during isolation and passage is essential for reliable genotyping and subsequent data interpretation. Relatively little is known about the stability of VNTR loci. Vogler et al. found a good correlation between VNTR diversity, repeat copy number, and mutation rate in pathogenic bacteria (42). Other studies have described MLVA

profiles to be stable over time, for example in *Coxiella burnetii* (43) and *Salmonella enterica* (13). One study found that MLVA is comparable to MLST for detecting genetically-related epidemic *Enterococcus* isolates (40). Frequent mutations can produce some gain or loss in plasmid DNA. In contrast Daniels *et al.* reported that 26 of 35 *S.* Newport isolates from different Northwestern USA herds in over two years all had the same plasmid pRFLP type, suggesting that the bla_{CMY2} plasmids in this serovar were relatively stable (18). The diverse size of bla_{CMY2} plasmids ranging from 84kb to 190kb found in the present study suggests that plasmids diversified but it is not definitely known whether this process occurred within the WA-TYP035/187 clade or whether the clade acquired diverse plasmids on multiple occasions.

Ceftiofur is an extended-spectrum cephalosporin that has been approved for therapeutic use in veterinary medicine in the United States (23). Although data is lacking on the true magnitude of ceftiofur use in the US dairy farms, several studies have reported that ceftiofur was frequently used in dairy herds (39, 47). A recent questionnaire survey of 381 commercial Washington State dairies showed that ceftiofur was one of the most commonly used drugs in these farms (36). Antimicrobial use provides a strong selective pressure favoring the fitness of resistant bacteria, resulting in the subsequent spread of these drug-resistant pathogens (1, 8, 33). Widespread use of ceftiofur in cattle has been shown to select for and maintain ceftriaxone-resistant *Salmonella* in cattle (4, 11).

In conclusion, plasmid diversity in the MST branches detected in the present study suggests that either resistance was acquired on multiple independent times in WA-TYP035/187 evolution or that the resistance plasmids acquired rapidly diversify in their size presumably due to insertions or deletions. With the lack of an obvious reservoir in species other than cattle, we conclude that ceftazidime resistance was acquired on multiple independent occasions and that the

most likely cause of the increased prevalence of third-generation cephalosporin resistance in this clone was selection pressure from the use of ceftiofur in cattle. To understand the epidemic nature of this clone, its occurrence and spread should be investigated further in coordination with public health laboratories.

ACKNOWLEDGEMENTS

This work supported in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. N01-AI-30055.

The skilful technical assistance of Katherine Kaya Baker is gratefully acknowledged. We thank Lindsay Tippett, Lisa Jones and staff of the FDIU, WSU and Kaye Eckmann, Kathryn MacDonald, and David Boyle of the Washington State Department of Public Health, and Derek Pouchnik of School Molecular Bioscience, WSU for their technical assistance.

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Table 1. Ceftazidime resistance in bovine and human isolates of *Salmonella* Typhimurium PFGE type WA-TYP035/187

Year	Bovine isolates (%)		Human isolates (%)	
	WA-TYP035/187	Ceftazidime resistant	WA-TYP035/187	Ceftazidime resistant*
1999	2	0 (0)	NA	NA
2000	16	2 (12)	NA	NA
2001	19	1 (5)	NA	NA
2002	10	1 (10)	NA	NA
2003	5	3 (60)	NA	NA
2004	13	7 (54)	10	9 (90)
2005	12	8 (67)	13	1 (8)
2006	23	18 78)	6	5 (83)
Total	100	40(40)	29	15(29)

*NA - Not applicable

Table 2. Antimicrobial resistance patterns of *Salmonella* Typhimurium PFGE subtypes WA-TYP035/187 isolates from cattle and humans during 2000 through 2006, resistant to ceftazidime

Resistance type*	BOVINE							Total	HUMAN			Total
	2000	2001	2002	2003	2004	2005	2006		2004	2005	2006	
AAmcCaz							1	1				
ACSTSuCaz					1			1				
AKSTSuCaz					3		1	4	3			3
ACKSxtSTSu		1						1				
ACKSTSuCaz				2				2	3			3
AKTAmcSuCaz						2		2				
ACKSxtSTSuCaz	2		1	1	3	1		8	2			2
ACSTAmcSuCaz							1	1				
AKSTAmcSuCaz							12	12			4	4
ACKSTAmcSuCaz						1	1	2				
AKSTAmcSuCaz						2	1	3				
ACKSTAmcSuCaz						1		1		1		1
ACKSxtSTAmcSuCaz						1		1	1		1	2
AGKSxtSTAmcSuCaz							1	1				
Total	2	1	1	3	7	8	18	40	9	1	5	15

*A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, sulfonamide; T, tetracycline; Sxt, trimethoprim-sulfadimethoxazole; Caz, ceftazidime; Amc, amoxycilin-clavulanic acid

Table 3. Plasmid profiles of *Salmonella* Typhimurium PFGE subtype WA-TYP035/187 isolates resistant to ceftazidime in different minimum spanning tree clusters.

Source	bla_{CMY-2} plasmid size (kb)	Number of isolates in each MST branch*						Total
		Branch 1	Branch 3	Branch 4	Branch 5	Branch 6	Branch 7	
Bovine	84	4	1					5
	101					3	4	7
	110				1			1
	125					2		2
	150					3		3
	169					2		2
	175	18				3		21
	180			1				1
	195					1		1
	Shannon-Wiener diversity index (H)	0.47	0	0	0	1.73	0	1.61
Human	90					3		3
	101					1		1
	125					2		2
	175	4						4
	195		1			1		2
	Shannon-Wiener diversity index (H)	0	0			1.28		1.52

*All MST branch-2 isolate were sensitive to ceftazidime

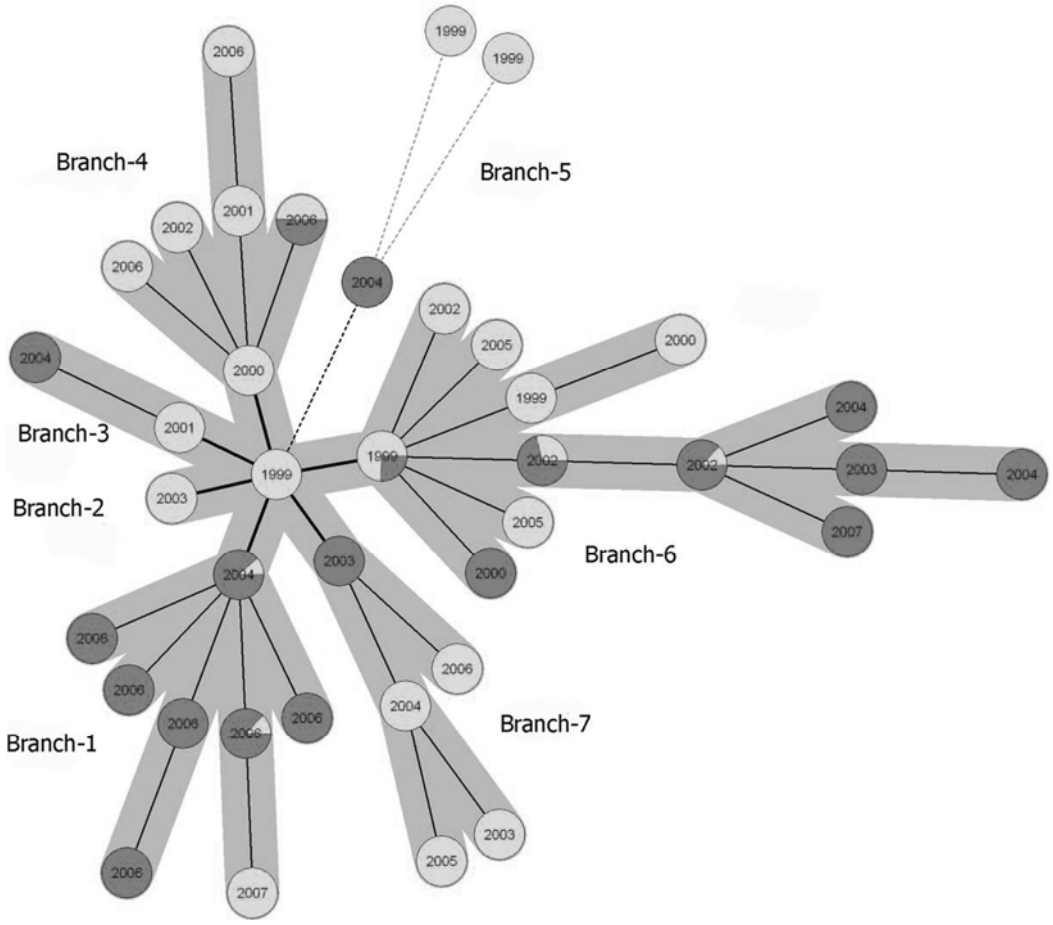

Fig 1. Minimum spanning tree of MLVA data from the *Salmonella* Typhimurium WA-TYP035/187 clade. Each circle represents a unique MLVA type and the year each type was first detected in the same lineages is printed within circle. Heavy short lines connect MLVA types that differ by a single locus, thin longer lines connect two-locus variants, and dotted lines indicate the most likely connection between types that differ by more than two loci. MST branch 1 includes all but one WA-TYP187 as well as a few WA-TYP035 isolates. MST branches branch 2-7 include primarily WA-TYP035 isolates plus one WA-TYP187 isolate. Dark grey: ceftazidime resistant, Light grey: ceftazidime susceptible; the relative sizes of the wedges is proportional to the frequency of ceftazidime resistance.

CHAPTER 2

Title: Introduction of new multidrug resistant *Salmonella enterica* strains into commercial dairies

CHAPTER 2

ABSTRACT

To estimate the rate of introduction of new multidrug resistant (MDR) *Salmonella enterica* strains into commercial dairy farms, we conducted a longitudinal observational study of 59 dairies in Washington State. Samples were collected on these farms over seven visits at approximately four month intervals over a period of 15 to 21 months, resulting in an average study period of 20 months. Samples were cultured for *Salmonella* spp. and serogroup, serotype and antimicrobial susceptibility patterns were identified for each isolate. Pulsed-field gel electrophoresis (PFGE) was used to genotype all MDR *Salmonella* isolates from feces to determine whether a particular strain was new to a farm. The rate of new MDR *Salmonella* strain introduction was 75.5 per 1000 herd-months (95% CI 50.8 – 119.4). The most commonly introduced MDR *Salmonella* serovars were Typhimurium (30/1000 herd months, 36% of total introductions), Newport (14/1000 herd months, 21% of total introductions), Dublin (11/1000 herd months, 15% of total introductions) and Infantis (xx/1000 herd months, 8% of total introductions) were calculated. Thirty-three (56%) of 59 farms had at least one new MDR *Salmonella* introduction during the study period. The number of new MDR *Salmonella* strains acquired by farms ranged from zero to eight. These data indicate that acquisition of new MDR *Salmonella* strains by dairy farm was a common event, through the rate of acquisition varies greatly among farms.

Key words: MDR *Salmonella*, dairy farm, incidence rate, introduction

INTRODUCTION

Salmonella enterica is a common zoonotic pathogen that is one of the leading causes of food-borne bacterial infections in the USA (CDC 2005; Mead et al., 1999) and other countries (Helms et al., 2005). An estimated 1.3 million human clinical salmonellosis cases and over 500 human deaths occur annually in the United States (ERS/USDA 2001; Mead et al., 1999). Domestic livestock are considered the main reservoir for foodborne salmonellosis , including strains that are multidrug resistance (**MDR**) (CDC 1996; Sanchez et al., 2002).

Pathogenic, zoonotic bacteria demonstrating antimicrobial resistance are an emerging problem worldwide (White et al., 2002). Transmission of zoonotic MDR *Salmonella* is of particular concern because of limited effectiveness of first-line antibiotics when treating cases, particularly pediatric infections, and drug-resistant strains are more likely to cause prolonged or severe illnesses than drug-susceptible strains (Butaye et al., 2006). Reports of *Salmonella* transmission from animals to humans involving either meat or milk from cattle (Spika et al., 1987), meat from poultry (McPherson et al., 2006) or infection via contact with infected cattle (Bezanson et al., 1983; Fey et al., 2000) have been documented. MDR is a characteristic of many newly emerging strains that spread by clonal expansion (Davis et al., 2007a; Davis et al., 2002; Hancock et al., 2000; Velge et al., 2005).

The epidemiology of *Salmonella* in dairy cattle has been studied previously (Edrington et al., 2008; Wells et al., 2001). Dairy farms are susceptible to acquire one or more infectious diseases over time. The transport of infected animals and movements of animal dealers and contractors have been described as the primary route of *Salmonella* transmission into farms (Evans 1996; Wray et al., 1990; Zansky et al., 2002). Other studies have shown that *Salmonella* transmission can occur into farms by people, equipment, physical sources and environment

contamination (Langvad et al., 2006; Nielsen et al., 2007a). Direct animal contacts with cattle from other farms can result in the introduction of *Salmonella* spp. into dairy farms (van Schaik et al., 2002). The number of cattle purchased from test-positive herds shown to be associated with recipient herds changing from *Salmonella* test negative status to positive (Nielsen et al., 2007b). Other source of *Salmonella* transmission include livestock feed (Anderson et al., 2001; Davis et al., 2003).

Relatively few studies have been performed to examine the rate at which *Salmonella* spp are introduced into commercial dairy farms. Recently, incidence rate of *Salmonella* spp was measured in dairy farms in the UK. The authors reported an incidence of 0.43 cases of *salmonellosis* per farm-year at risk for any serovar of *Salmonella* (Davison et al., 2006).

Infection of animals with zoonotic pathogen results in a risk of pathogen transmission to humans (FAO/WHO 2007). For interventions to minimize pathogen burden, it is important to better understand the magnitude at which transmission of *Salmonella* occurs between farms. No data is currently available on the incidences of MDR *Salmonella* strain introduction into dairy farms in North America. The purpose of this study was to estimate the rate at which multidrug resistant *Salmonella* strain being introduced in commercial dairy farms in the Northwestern USA.

MATERIALS AND METHODS

Study farms

A total of 59 commercial dairy farms in the Northwestern USA were selected based on diagnostic samples submission for *Salmonella* diagnosis with the Washington Animal Disease Diagnostic Laboratory (WADDL). Herds were enrolled via herd veterinarian and willingness to

participate in the study. Study farms were located in western, south-central and central Washington and contained 173,253 dairy cattle of all ages.

Sampling plan

Each farm was visited and sampled seven times at two to four-month intervals over a period of 15 to 21 months. All sampling occurred between August 2005 and December 2007. Pooled fecal samples were collected from cattle on all study farms. Each fecal pool consisted of 10 individual fresh fecal pat samples of approximately 50g contained in individually labeled sterile Whirlpak bags using sterile tongue depressors. An average of 16 fecal pools were collected per farm at each visit from different groups of animals (lactating cow (5-9), dry cow (2-3), close-up (2-3), heifer (1-2), maternity/hospital (1-2) and calves (1)). A higher proportion of samples was collected from certain target populations (lactating cows, calves with diarrhea and recent history of antimicrobial treatment, common hospital-maternity pens) to increase sensitivity of detection of new MDR-*Salmonella* strains (Hancock 1996; Warnick et al., 2003). This sampling method was estimated to provide at least 95% positive herd detection sensitivity with expected herd prevalence of 8% (Jordan, 2005). Samples of all available feed types were collected from respective farms and from two feed mills that supplied feeds to most farms in the study. Pooled feed samples comprised of five samples of 10g each collected from different areas of each feed storage area, using sterile techniques. Feed samples were taken with sterile gloves after removing 1-2 inches of the top surface layer of feed. A total of 3941 feeds were sampled from the study farms and feed mills. At each dairy farm visit, one slurry sample (50 mL) was collected from lagoons or other manure storage areas, and one discarded milk filter was collected; these samples were comprised to the pooled samples that represent potential diversity of *Salmonella* isolates on the farm. A total of 410 slurry samples and 341 milk filters were

collected. Samples were transported to the laboratory at 4°C and within 24 hours of collection for microbiological processing.

Culture and identification

Fecal samples and milk filters were enriched in Tetrathionate broth (**TB**; Hardy Diagnostics, Santa Maria, CA) and Rappaport-Vassilidas broth (R10; Hardy Diagnostics, Santa Maria, CA) before plating onto XLT-4 agar plates (Hardy Diagnostics, Santa Maria, CA) and MacConkey agar supplemented with ampicillin (256 µg/mL), chloramphenicol (8 µg/mL) and streptomycin (32 µg/mL) (Hardy Diagnostics, Santa Maria, CA and antibiotics from Sigma-Aldrich, Dallas, TX) and incubated for 24 hrs at 37°C. Pooled feed and slurry sub-samples (25g) were pre-enriched in 225 buffered peptone water (**BPW**; Hardy Diagnostics, Santa Maria, CA) at 37°C before performing parallel enrichments in TB and R10 broth at 42°C. Selective enrichment was performed using a mixture of 1 mL enrich sample and 9 mL TET broth, and a mixture of 100 µL enrich sample and 10 mL R10 broth. Three suspect *Salmonella* colonies from XLT-4 and MACacs were inoculated onto lysine iron agar (**LIA**) and incubated for 24 hrs at 37°C. Transfer positive suspects on triple sugar iron (**TSI**) to urea by streaking across the agar slant without stabbing into the agar. The lid of Urea tube was loosened to allow gas exchange and incubated both suspect TSI and urea tubes 18-24 hours at 37°C. Urea negative reaction is a presumptive identification of *Salmonella* spp. One isolate from XLT-4 media that was confirmed by biochemical and serogroup tests by use of a commercial polyvalent A-I and Vi antisera as well as individual serogroups B, C1, C2, C3, D1 and E (Difco Laboratory, Detroit, MI). Isolates were then banked in BHI broth containing 25–30% buffered glycerol at 80°C. Isolates were serotyped at the National Veterinary Service Laboratory, Ames, Iowa. Isolates were serotyped at the Clinical Laboratory Standard Institute (**CLSI**), Ames, Iowa. A 3-tube most probable number

(**MPN**) method was used for determining the level of *Salmonella* contamination of feed (Blodgett 2006).

Antimicrobial susceptibility testing

Susceptibility testing was done by a disk diffusion method (Bauer, 1966) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (NCCLS 2003a; NCCLS 2003b) to test antimicrobial susceptibility of isolates to the following antimicrobial drugs: ampicillin (A, 10 μg), ceftazidime (Caz, 30 μg), chloramphenicol (C, 30 μg), gentamicin (G, 10 μg), amoxicillin-clavulanic acid (Amc, 20/10 μg), kanamycin (K, 30 μg), streptomycin (S, 10 μg), tetracycline (T, 30 μg), triple-sulfa (a combination of sulfadiazine, sulfamethazine, and sulfamerazine) (Su, 250 μg), and trimethoprim-sulfamethoxazole (Sxt, 1.25/23.75 μg) (BD Diagnostics, Sparks, MD, USA). These antimicrobials were chosen to reflect the most relevant to both human and veterinary medicine Isolates resistant to two or more antimicrobials were considered MDR.

Pulsed-Field Gel Electrophoresis

All MDR *Salmonella* isolates from fecal samples were analyzed using PFGE following *Xba*I restriction digestion based on the PulseNet protocol for *Salmonella* and using *S.* Braenderup, H9812 as a standard (Ribot et al., 2002).Gel images were analyzed using BioNumerics v. 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium). Independent strains were defined by PFGE profiles that differed by at least two bands.

Data analysis

Descriptive statistics for the number and incidence rate of MDR *Salmonella* were performed in Microsoft Excel (Microsoft, Redmond, WA) and SAS v.9.1 software (SAS Inc., Cary, NC, USA). The incidence rate of new introduction of MDR *Salmonella* strains was calculated based on number of new strains introduced and the number of herd-months at risk. For

the purpose of this paper, a strain found in a farm was considered a newly introduced strain if the strain was isolated at the second or later sampling visit and had not been previously observed on that farm in previous sampling visits to the farm or in diagnostic case reports from clinical cases for at least three years before the commencement of the study. In addition, if a strain was isolated in slurry or milk filter samples prior to its isolation in feces, it was not considered a new introduction.

RESULTS

Description of new strain introduction

The current study collected a range of fecal and environmental samples from 60 Washington commercial dairy farms containing 173,253 cattle. During the study period one of the farms went out of business and was excluded from the analysis. Characteristics of the study farms are presented in Table 1. The median total herd size and lactating cows populations were 1800 (range 231 – 17775) and 750 (range 100 – 7150) respectively. Fifty-three percent of the farms introduced new animals during the study period.

A total of 70 new introductions of MDR *Salmonella* strains were observed in the 59 dairy farms from January 2006 through December 2007 (Table 2). Of the new strains, 14, 10, 8, 3 and 2 were belonged to serovar Typhimurium, Newport, Dublin, Montevideo and Uganda respectively. Serovars Ohio, Orianenburg, Hadar, Infantis, Meleagridis and others were detected as new introductions in a single herd each. New strains T5, D1, I1 and N1 were acquired by 13 (22%), 3 (5%), 3 (5%) and 2 (3%) farms. Fifty-eight (83%) strains were resistant to five or more antimicrobials tested including ceftazidime, a third generation cephalosporin.

On 26 (44%) of farms, no newly introduced strains were observed while 33 (56%) farms acquired at least one new introduction of MDR *Salmonella* during the study (Fig. 1). The number of new introductions of MDR *Salmonella* on these farms varied from 0 to 8. Eighteen farms acquired one new MDR *Salmonella* strains, twelve farms acquired two or three new MDR *Salmonella* strains, and six and eight new introductions were observed in one and two farms respectively. The incidence rate of introduction of MDR *Salmonella* strains in Northwestern commercial dairy farms was 75.5 per 1000 herd-month (95% CI 50.8 – 119.4). The rates of new strain introduction for MDR *Salmonella* serovar Typhimurium, Newport and Dublin were 30, 14 and 11 per 1000 herd-month, respectively.

Table 3 summarizes the temporal and geographical distribution of newly introduced MDR *Salmonella* serotypes isolated from commercial dairies between January 2006 and December 2007. Of the 59 farms, new strains of serovar Typhimurium were acquired by 27 (39%) farms followed by Newport (17%), Dublin (16%), Infantis, Montevideo and Uganda (4%). Newly introduced strains of serovar I 4,12: nonmotile, Infantis and Newport were isolated from farms in four counties of the Washington State. Strains of serovar Typhimurium and Oranienburg isolated from farm in three and two counties.

MDR *Salmonella* contamination in feed

A total of 4582 pooled feed samples from commercial dairy farms and 665 feed samples from two feed mills were collected during the study period. Of those, 3.5% (158) of feeds were positive for any *Salmonella*. Of those positive, 83%, 8% and 9% were pan susceptible, one antimicrobial resistant and MDR *Salmonella* strains, respectively. Overall, the prevalence of pan susceptible and MDR *Salmonella* in feeds were 2.5% and 0.5% respectively. Feeds from 12 (19%) dairy farms yielded 12 MDR *Salmonella* serovars collected on various dates (Table 4). Of

665 feeds from two feed mills, only one sample was positive for MDR *Salmonella*. The positive feed mill sample was a total mixed grain feed that contained MDR *Salmonella* serovar Bardo. MDR *Salmonella* Typhimurium were isolated from several types of feeds on farms, including straw, corn silage, calf grains and haylage. MDR *S.* Dublin and *S.* Newport were isolated from calf grain, bakery waste, and cannery waste at these farms respectively. *Salmonella* Saint-Paul was the only serovar isolated from cotton seed at a single farm. MDR *Salmonella* feed isolates were resistant to two to seven antimicrobials.

Quantitative analysis of feeds positive for MDR *Salmonella* showed that none of the samples were positive for *Salmonella* in triplicate dilution series using inoculum quantities of 0.1, 0.01 and 0.001 g after 48h enrichment in TB. Thus, the concentration of MDR *Salmonella* for all types of feeds was less than 3 MPN/g. These feeds were positive only by the selective enrichment of 25 g feed.

DISCUSSION

Dairy cattle are an important part of human food chain. Of the several challenges at the dairy industries, *Salmonella* transmission between farms and to human food remains a potential risk (Langvad et al., 2006; Van Kessel et al., 2004). Little data currently exists describing the rate of any *Salmonella* introductions into dairy farms and data is lacking on the rate of introduction of new MDR *Salmonella* into commercial dairies.

We found that the rate of new MDR *Salmonella* strain introduction was 75.5 per 1000 herd-month at risk, which can be translated to 0.91 new strains per dairy farm per year at risk. This is higher than 0.43 cases per farm-year at risk reported in England and Wales (Davison et al., 2005). This is likely due to differences in herd size, feeding and other variables between British dairies and Western USA dairies. The most common *Salmonella* serovars found in the

present study were Typhimurium, Newport and Dublin, whereas Davison and others reported Dublin, Agama and Typhimurium were the most common serovars in their study (Davison et al., 2005).

Our finding of the most common *Salmonella* serovars in the order of Typhimurium, Newport and Dublin may be consistent with the emergence, reemergence and increasing isolation of these serovars in previous studies in the Northwestern commercial dairies (Berge et al., 2004; Davis et al., 2007a; Davis et al., 2007b; Velge et al., 2005). The MDR *Salmonella* strain designated T5 was acquired by 13 farms. This strain belonged to *Salmonella* Typhimurium PFGE subtype TYP035/187, which recently emerged and disseminated in dairy farms in the Pacific Northwest USA and which has been increasingly seen in human clinical cases (Davis et al., 2007a).

Although some studies have reported evidence of *Salmonella* introduction in cattle via feeds (Davis et al., 2003; Jones et al., 1982; Lindqvist et al., 1999), several other studies have failed to show the direct evidence indicating feed as a potential source of *Salmonella* infection in cattle (Richardson 1975; Williams 1975; Wray and Sojka 1977). In the current study, large majorities (88%) of the feed Salmonella isolates were pan susceptible and very small proportion showed MDR characteristics. A low prevalence of MDR *Salmonella* was detected in feeds collected at various dairy farms and no feed was positive for new MDR *Salmonella* strain introduced on the same farm. The infectious dose for *Salmonella* organisms in healthy adult cattle has been estimated to be 10^9 to 10^{11} cells (Smith et al., 1979; Spier et al., 1991). However, reduction in dry matter intake can influence the survival and multiplication of *Salmonella* in the gut environment (Chambers and Lysons 1979; Mattila et al., 1988). In the current study, the level of feed contamination with MDR *Salmonella* as measured by the 3-tube MPN method was less

than 3 MPN/g for all feeds in which MDR strain detected using each 25g feed. This indicates that low level of contamination of MDR *Salmonella* in feed is below the estimated infectious dose threshold. This does not mean, however, that higher contamination levels do not occur, as has been documented in some studies (Anderson et al., 1997). Dairy farm operators should recognize the importance of biosecurity of dairy farm feedstuffs and prevent feed contamination by *Salmonella*.

In conclusion, results suggest that new MDR *Salmonella* strains are frequently introduced in commercial dairies, WA and that feed probably plays lesser role in *Salmonella* transmission to dairies than other modes of introduction such as cattle introduction. Further studies are needed to investigate specific risk factors for the new MDR *Salmonella* introduction onto commercial dairies.

ACKNOWLEDGEMENTS

This project was supported in part by National Institute of Health, Department of Health and Human Services under the contract number NO1-AI-30055 and by the Agricultural Animal Health Program, College of Veterinary Medicine, Washington State University. The skillful technical assistance of Dr. Regina Finger and Katherine Kaya Baker is gratefully acknowledged. We also thank Lindsay Tippett, Lisa Jones and other staff of the FDIU, WSU for their technical help.

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Table 1. Attributes of Northwestern commercial dairy farms enrolled in the study

Items	Study farms (n=59)	Range
Median herd size	1800	231-17775
Median number of lactating cows	750	100 - 7150
Median dry cows	130	0 - 1409
Median calves <3 months on-farm	80	0 - 3000
Median calves <3 months off-farm	0	0 - 685
Median heifers from 3-20 months on-farm	230	0 - 3500
Median heifers from 3-20 months off-farm	40	0 - 5600
Median heifers from 20 months to calving off-farm	100	0 - 700
% of farms introduced new animals during study	53	-
% of farms had off-farm raised cattle	47	-

Table 2. Characteristics of newly introduced MDR *Salmonella* strains isolated from fecal samples from 59 commercial dairies from August 2005 through December 2007

Arbitrary strain type designation	Serotype	PFGE type**	Resistant type*	No. of farms
A	I 4,12:Nonmotile	wTYP014	AKTSu	1
B	I Rough: 6,7:-[†]	wNEW012	AAmc	1
C	I Rough:6,8:- [†]	wNEW035	AAmc	1
CC	I 9,12:Nonmotile	w9_12_N003	ACGKSTAmcSuCaz	1
E	I Rough: 9,12:- [†]	wD1_001	ACSxtSTAmcSul	1
F	I Rough:- [†]	wINF001	ACKSTAmcSulCaz	1
D1	Dublin	w9,12:N002	ACSTAmcSulCaz	1
		wDUB002	ACSTSu	1
		wDUB002	AKAmcSuCaz	1
D2	Dublin	wDUB003	ACGKTAmcSuCaz	1
D3	Dublin	wDUB004	ACGKTAmcSuCaz	2
D4	Dublin	wDUB007	ACGKSTAmcSuCaz	1
D5	Dublin	wDUB008	AAmcSuCaz	1
D6	Dublin	wDUB009	ACSTSu	1
D7	Dublin	wDUB012	ACKTAmcSuCaz	1
D8	Dublin	wDUB016	ACGKSTAmcSuCaz	1
G1	Meleagridis	wMEL001	AAmcCaz	1
H1	Hadar	wHAD001	ST	1
H2	Hadar	wHAD004	ST	1
I1	Infantis	wINF001	ACSTAmcSuCaz	2
			ACSTAmcSulCaz	1
N1	Newport	NEW005	ACSTAmcSu	1
			ACSTAmcSuCaz	1
N10	Newport	wNEW036	ACSTAmcSuCaz	1
N2	Newport	NEW046	ASTAmcSuCaz	2
N3	Newport	NEW076	ASTAmcSuCaz	1
N4	Newport	wNEW005	ACSTAmcSuCaz	1
N5	Newport	wNEW010	ACSTAmcSuCaz	1
N6	Newport	wNEW013	ACSTAmcSuCaz	1
N7	Newport	wNEW015	ACSTAmcSuCaz	1
N8	Newport	wNEW016	ACSTAmcSuCaz	1
N9	Newport	wNEW018	ACSTAmcSuCaz	1
O	Ohio	wOHI001	TSu	1
R	Oranienburg	wORA001	AAmcCaz	1

*A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, sulfonamide; T, tetracycline; Caz, ceftazidime; Amc, moxicillin-clavulanic acid; Sul, sulfisoxazole; Sxt, trimethoprim-sulfadimethoxazole; **PFGE type as designated my WA Dept. of Public Health except types starting with letter w indicates new PFGE type designated at WSU; [†] Popoff, 2001

Table 2 continued

Strain designation	Serotype	PFGE type	Resistant type*	No. of farms
T1	Typhimurium	TYP004	ACSTSu	1
T2	Typhimurium	TYP012	ASSu	1
T3	Typhimurium	TYP016	AKSTSul	1
T4	Typhimurium	TYP139	AKSTSu	1
T5	Typhimurium	TYP035/187	AKSTAmcSuCaz	7
	Typhimurium	TYP035/187	AKSTAmcSulCaz	1
	Typhimurium	TYP035/187	AKSTSu	3
	Typhimurium	TYP035/187	AKSTSuCaz	1
	Typhimurium	TYP035/187	AKSxtSTAmcSulCaz	1
T6	Typhimurium	TYP139	AKSTAmcSulCaz	1
T7	Typhimurium	wTYP002	ACSTSul	1
T8	Typhimurium	wTYP013	AKT	2
T9	Typhimurium	wTYP013a	KSTSu	1
T10	Typhimurium	wTYP062	AKSTSul	1
T11	Typhimurium	wTYP093	AKSTAmcSulCaz	1
T12	Typhimurium	wTYP108	ACKSTAmcSulCaz	1
T13	Typhimurium	wTYP113	AKSTAmcSulCaz	1
T14	Typhimurium	wTYP114	AKSTAmcSulCaz	1
U1	Uganda	wUGA001	CKSxtSTSu	2
U2	Uganda	wUGA010	CKSxtSTNalSul	1
V1	Montevideo	wMON006	ACSTAmcSulCaz	1
V2	Montevideo	wMON007	ACSxtSTAmcSu	1
V3	Montevideo	wMON022	ACGKSTAmcSuCaz	1

Table 3. Temporal and geographical distribution of newly introduced MDR *Salmonella* serotypes of a strain isolated from the study farms in Washington State

Serotype	Jan-Apr 2006	May-Aug 2006	Sep-Dec 2006	Jan-Apr 2007	May-Aug 2007	Sep-Dec 2007	No. of counties	No. of farms
Typhimurium	1	2	7	6	4	7	3	27 (38.6)
Newport	7	2		1	2		4	12 (17.1)
Dublin	2			8		1	1	11 (15.7)
Infantis				2	1		4	3 (4.3)
Montevideo			1	1	1		1	3 (4.3)
Uganda			2		1		1	3 (4.3)
Hadar			1		1		1	2 (2.9)
9,12:Nonmotile				1			1	1 (1.4)
I 4,12:Nonmotile				1			4	1 (1.4)
I 6,7:Rough					1		1	1 (1.4)
I 6,8:Rough						1	1	1 (1.4)
I 9,12:Rough						1	1	1 (1.4)
Meleagridis					1		1	1 (1.4)
Ohio		1					1	1 (1.4)
Oranienburg			1				2	1 (1.4)
Rough						1	1	1 (1.4)

Table 4. Attributes of multidrug resistant *Salmonella* serotypes isolated from feeds

Isolate ID	Farm	Serotype	Source	Date collected	Resistance type*
10646	A	Dublin	Calf Grain	8/24/2005	ACSTAmcSu
14583	AAA	Typhimurium	Straw	7/9/2007	AKSTSul
10828	F	Tennessee	Cracked Corn	9/19/2005	SSu
14709	GG	Infantis	Soybean meal	8/13/2007	ACSTAmcSul
14711	GG	Infantis	TMG	8/13/2007	ACSTAmcSulCaz
13591	HH**	Bardo	TMG	12/5/2006	ASTAmcSuCaz
14627	III	Newport	Cannery waste	7/16/2007	ACSTAmcSul
12712	LL	Saint-Paul	Cotton seed	7/17/2006	ACSTAmcSuCaz
14015	P	Dublin	Bakery waste	8/24/2005	ACSTAmcSu
13348	PP	Oranienburg	Mineral	10/17/2006	AAmcCaz
14160	SS	Typhimurium	Straw	4/9/2007	AKSTAmcSuCaz
14754	TT	Typhimurium	Corn silagea	8/27/2007	AKSTAmcSulCaz
13384	VV	Typhimurium	Calf Grain	10/23/2006	ACSTSu
13390	YY	Typhimurium	Haylage	10/25/2006	ACSTSu

*see Table 2 for abbreviations;
**sampled at feed mill prior to shipment to farm

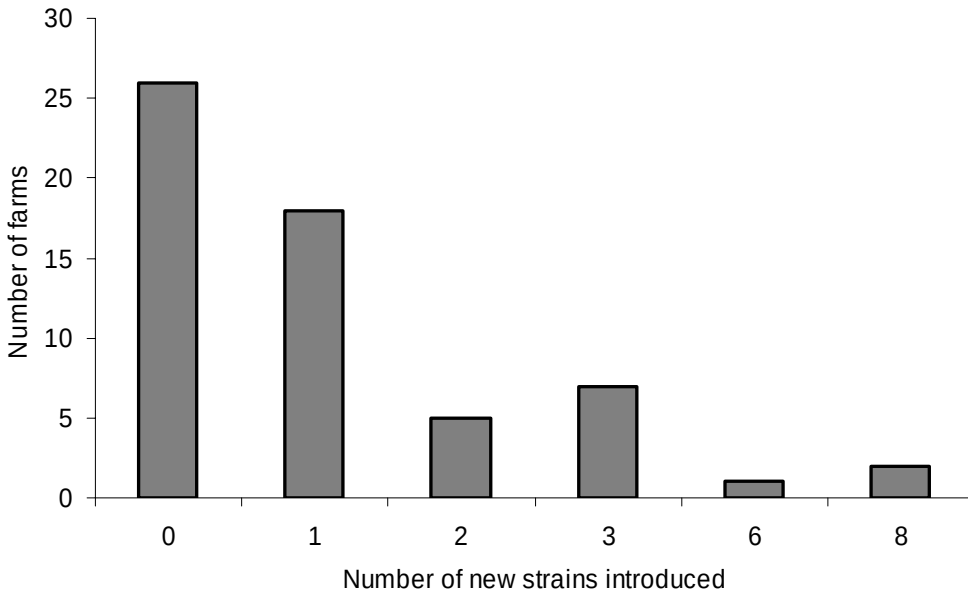

Fig 1. Distribution of 59 dairy farms based on the number of new MDR *Salmonella* strains acquired over 15 to 21 months follow-up period

CHAPTER THREE

Title: Herd-level factors associated with new multidrug resistant *Salmonella* strains introduction into commercial dairies

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ABSTRACT

Samples were collected from 59 commercial dairy farms for 15 to 21 months to determine the rate of introduction of new multidrug resistant (MDR) *Salmonella* strains. Farm management data were collected by on-site inspections and questionnaires on herd management practices before and after the study. A logistic regression analysis was used to evaluate the associations between potential herd-level factors and whether farm had new MDR strains introduced during the study period. Negative binomial regression was used to test association between potential herd-level factors and the number of new MDR *Salmonella* strains introduced. The logistic regression revealed that off-farm contract heifer raising where they co-mingled with cattle from other sources (OR= 8.9, 95% CI: 2.4, 32.80) and herd size, per 100 animal increment (OR= 1.04, 95% CI, 1.01, 1.05) were significantly associated with new MDR *Salmonella* introduction. The negative binomial regression similarly revealed that off-farm contract heifer raising where they co-mingled with cattle from other sources (RR= 2.3, 95% CI: 1.1, 4.7), herd size, per 100 animals (RR=1.02, 95% CI, 1.01, 1.03) and clinical salmonellosis diagnosed before start of the study (RR=2.5, 95% CI, 1.3, 5.0) were significantly associated with the number of new MDR *Salmonella* strains introduced.

Key words: *Salmonella*, dairy farms, herd, Herd-level, risk factors, MDR, introduction

INTRODUCTION

An estimated 1.3 million human clinical salmonellosis cases and over 500 associated human deaths occurs annually in the United States (ERS/USDA 2001; Mead et al., 1999). *Salmonella enterica* subsp. *enterica* are often found in livestock and poultry, including dairy cattle. Consumption of meat and milk produced from dairy farms have resulted in human salmonellosis cases (CDC 2006; Mazurek et al., 2004; Van Kessel et al., 2007), as has direct contact with infected dairy cattle (Besser et al., 1997; Bezanson et al., 1983; Calvert et al., 1998; Wall et al., 1994). Multidrug resistant (MDR) *Salmonella* strains are of particular concern because they can complicate treatment of human salmonellosis cases (Butaye et al., 2006).

Several emerging MDR *Salmonella* strains have been spread globally via clonal expansion (Davis et al., 2007; Davis et al., 2002; Hancock et al., 2000; Velge et al., 2005), which suggests that inter-farm transmission may be key element in the epidemiology of MDR *Salmonella*. The spread of emerging MDR *Salmonella* between regions may be due to international travel as suggested by Davis and colleagues (Davis et al., 1999) or may have been facilitated by the use of antimicrobials, international and national trade of infected animals is thought to play a major role in global spread. Several risk factors have been implicated in MDR *Salmonella* transmission between farms. The transport of *Salmonella* infected animals into farms has been recognized as a primary route of *Salmonella* introduction into farms (Evans 1996; Vanselow et al., 2007; Wray et al., 1990; Zansky et al., 2002). Some evidence suggests that this includes off farm raising of heifers (Hegde et al., 2005). Approximately 12.5% of U. S. dairy farms send their heifer calves off-farm to be reared at dedicated contracted calf ranches where

they are commingled with multiple rearing facilities (USDA 2007; Wolf 2003) and in some areas this practice may be more common.

The purpose of this study was to determine the role of animal movement, including off-farm rearing of heifers, in the inter-herd transmission of MDR *Salmonella*.

MATERIALS AND METHODS

Study farms

Sixty commercial dairy farms in the Northwestern USA were selected via their herd veterinarians who regularly submitted samples to Washington Disease Diagnostic Laboratory for suspected salmonellosis. Study farms were located in western, south-central and central Washington and contained 173,253 dairy cattle of all ages. Management data on cattle movement into farms, along with selected potential confounders were collected via a standardized questionnaire and by on-site inspection (Table 1).

Sampling plan

Each farm was visited and sampled seven times at two to four-month intervals over a period ranging from 15 to 21 months for different farms. All sampling occurred between August 2005 and December 2007. Pooled fecal samples consisting of approximately 50g of 10 individual fresh fecal pats were collected in sterile plastic bags using sterile tongue depressors. An average of 16 fecal pools were collected per farm at each visit from different pens housing different groups of animals (lactating cow (5-9), dry cow (2-3), close-up (2-3), heifer (1-2), maternity/hospital (1-2) and calves (1)). A higher proportion of samples were collected from certain herd-sub populations (calves, hospital pens, cattle with diarrhea or cattle with a recent history of antimicrobial treatment and common hospital-maternity pens) to increase sensitivity of detection of new MDR-*Salmonella* strains, as reported in previous studies (Hancock 1996;

Warnick et al., 2003a). This sampling method was estimated to provide at least 95% herd sensitivity of detecting MDR *Salmonella* shedding at a prevalence of 5% (Jordan 2005).

Culture and identification

Samples were transported in coolers with enough ice or ice packs to maintain temperature of 4^{o}C and brought into the laboratory within 24h of collection for microbiological processing. Fecal samples were enriched in Tetrathionate broth (TB; Hardy Diagnostics, Santa Maria, CA) and Rappaport-Vassilidas broth (R10; Hardy Diagnostics, Santa Maria, CA) before plating onto XLT-4 agar plates (Xylose Lactose Tergitol™ 4; Hardy Diagnostics, Santa Maria, CA) and incubated 24h at 37^{o}C. Selective enrichment was performed using mixture of 1 mL of BPW-enriched sample and 9 mL TB broth and a mixture of 100 µL and 10 mL in R10 broth. Suspect *Salmonella* based on colony morphology (black or red colonies with a black centre) on XLT-4 media were then inoculated onto lysine iron agar (LIA) and incubated 24h at 37^{o}C. *Salmonella* suspect growth from LIA was transferred to triple sugar iron (TSI) agar and urea agar tube. Urea negative reaction is a presumptive identification of *Salmonella* spp. One biochemically confirmed isolate was serogrouped using polyvalent A-I, Vi, B, C1, C2, C3, D1 and E (Difco Laboratory, Detroit, MI). Isolates were banked in BHI broth containing 25–30% buffered glycerol at -80^{o}C. All isolates newly introduced into the farms were serotyped at the National Veterinary Service Laboratory, Ames, Iowa.

Antimicrobial susceptibility testing

The Kirby-Bauer agar diffusion technique was used (NCCLS 2003a; NCCLS 2003b) to test antimicrobial susceptibility of isolates to the following antimicrobial drugs: ampicillin (A, 10 µg), ceftazidime (Caz, 30 µg), chloramphenicol (C, 30 µg), gentamicin (G, 10 µg), amoxicillin-clavulanic acid (Amc, 20/10 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), tetracycline (T,

30 µg), sulfisoxazole (Su, 250 µg), andsulfamethoxazole/trimethoprim- (Sxt, 1.25/23.75 µg) (BD Diagnostics, Sparks, MD, USA). *Salmonella* isolates were considered antimicrobial resistant based on the following resistance-susceptible zone size of inhibition cut-point (mm): A <14, C < 13, G <13, K <14, S <12, Su < 11, T <15, Sxt <11, Amc <14, Sul <13 and Caz < 15. Isolates resistant to two or more antimicrobials were classified as MDR.

Pulsed-Field Gel Electrophoresis

All MDR *Salmonella* isolates from fecal samples were analyzed using PFGE following *Xba*I restriction digestion based on the PulseNet protocol for *Salmonella* and using *S.* Braenderup, H9812 as a standard (Hunter et al., 2005). Electrophoresis was carried out on a Rio-Rad CHEF mapper XA system (Bio-Rad, Hercules, CA) using the following parameters: 6 v/cm; 120^{o} angle, initial switch time 2.2 seconds, final switch 63.8 seconds, run time 28 hours; 14^{o}C. Gel images were analyzed using BioNumerics v. 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium).

New MDR Salmonella strain introduction

For the purpose of this paper, independent strains were defined by PFGE profiles that differed by at least two bands. First farm visit and previous diagnostic samples were considered baseline data. Thus, a MDR *Salmonella* strain found in a farm was considered a new introduction if the strain was isolated between second and seventh sampling visit and had not been previously observed on that farm in previous visits to the farm or in diagnostic case reports from clinical cases.

Data analysis

A herd was defined as positive for a new MDR *Salmonella* strain if at least one sample had positive result of fecal bacteriologic culture of a MDR strain that had not been observed with

previous sampling periods or in previous diagnostic laboratory samples. Univariate analysis was performed to evaluate unadjusted association between individual variable and new MDR introduction and variables with p-value <0.25 were entered into multivariable models in a stepwise manner. Logistic regression analyses was used to evaluate the associations between potential herd-level factors and probability of whether farm had any new MDR strain introduced during study period controlling sample size effect. Negative binomial regression was used to test the same factors and the number of new MDR *Salmonella* strain introduced during study period. The effects; off-farm heifer raising, prior salmonellosis diagnosed and herd size and interaction terms were maintained in the final multivariate model if they were significant with a p-value of <0.05. All analyses were performed in SAS v.9.1 software (SAS Inc., Cary, NC, USA) and Microsoft Excel (Microsoft, Redmond, WA).

RESULTS

Of the 60 dairy farms, one farm went out of business and was excluded from the analysis. The rate of introduction of new MDR *Salmonella* strains introduction into dairy farms was described in our previous study (Adhikari et al. manuscript in preparation). Briefly, 71 new MDR *Salmonella* strains were introduced at a rate of 75.5 per 1000 herd-months (95% CI 50.8 – 119.4) (Table 2). In 26 farms no new MDR strains were introduced and in most of the remainder only a single MDR strain was introduced. Two farms had eight new MDR strains introduced.

Results of univariate analyses are shown in Table 3. Three variables were significant ($p<0.05$) in univariate analyses for factors associated with any new MDR strain introduction; namely, contract heifer raising off-farm where co-mingled with cattle from other sources (unadjusted odds ratio (OR) =6.6), salmonellosis diagnosed in the herd in the last three years

(unadjusted OR= 5.4) and herd size in 100 animal increments (OR=1.00015) were significantly associated with any new MDR *Salmonella* introduction into commercial dairies. These same variables were also the only ones significantly associated with the number of new MDR strains introduced (unadjusted OR= 2.0, 3.3 and 1.02 respectively). Additional variables, namely any cattle purchased and log of number of cattle purchased were also tested in univariate analyses. However, these variables were not significant at p-value 0.05.

Multivariable logistic regression showed that off-farm raised heifers co-mingled with cattle from other sources (adjusted OR=8.9, 95% CI, 2.4 – 32.8) and herd size in 100 animal increment (adjusted OR= 1.04, 95% CI, 1.01 – 1.05) were significantly associated with farm status on MDR *Salmonella* strain introduction into dairies (Table 4). Multivariable negative binomial regression revealed that factors significantly associated with the number of new MDR *Salmonella* strain introductions were off-farm raised heifers co-mingled with cattle from other sources (adjusted OR=2.3, 95% CI, 1.1 – 4.7), herd size in 100 animal increment (adjusted OR= 1.02, 95% CI, 1.01 – 1.03) and clinical salmonellosis diagnosed in the herd in the last three years before the start of the study (adjusted OR= 2.5, 95% CI, 1.3 – 5.0). No interaction terms were found to be significant.

DISCUSSION

In the present study, off-farm raised heifers co-mingled with cattle from other sources was found to be a significant risk factor for new MDR *Salmonella* strain introduction into commercial dairy farms. This corroborates results reported by Hegde et al. (Hegde et al., 2005), but is in conflict with another study (Edrington et al., 2008). Resolving this issue is important in

that 49% of dairy farms raised their heifers on contract calf ranches and it is a common practice elsewhere in the United States (Fulwider et al., 2008).

The present study failed to find a significant effect of cattle purchase (cow, bulls, calf and heifer) on new MDR introduction into commercial dairy farms. This is in conflict with several previous studies (Evans and Davies 1996; Wray et al., 1990; Zansky et al., 2002). It is possible that the high rate of heifers raising off-farm obviated effects of cattle purchases, since it is likely that those that contract raise their heifers are more likely to purchase animals, than those farms that are concerned by biosecurity involved in cattle introductions.

The present study found that prior diagnosis of clinical salmonellosis was significantly associated risk factor for the number of new MDR *Salmonella* strains introduced. That is, farms with a history of clinical salmonellosis were at increased risk of acquiring new strains. This is likely a matter of reverse causality: the greater the number of strains introduced, the more likely it is that one or more of them will result in clinical salmonellosis. Given that the association was still significant after adjustment for other variables, this suggests the existence of important risk factors for introduction of new strains that were now found in the present study. Studies suggest that recent antimicrobial treatment can increase the probability of isolating *Salmonella* in calves, heifers and cows (Berge et al. 2005, Warnick et al., 2003b). It is possible that antimicrobial use at the farm level increases the susceptibility of dairy herds to acquire new strains.

Our study indicated that herd size was significantly associated with herd status of new MDR *Salmonella* strain introduction and the number of new MDR introductions, which suggest that larger dairies are more likely to acquire new MDR introduction. This finding corroborates another study which found that the number of cattle introduction into dairy farms was a risk factor for introduction of *Salmonella* (Nielsen et al., 2007).Several previous studies also found an

association between herd size and *Salmonella* prevalence (Huston et al., 2002; Kabagambe et al., 2000; Vaessen et al., 1998; Warnick et al., 2001; Warnick et al., 2003b).

Purchased feed has also been implicated in the transmission of *Salmonella* into dairy farms (Davis et al., 2003; Jones et al., 1982; Lindqvist et al., 1999). However, as previously reported MDR *Salmonella* were only very rarely found in feeds in the farms used in the present study (Dr. Dale Hancock, per. Cumm.). Furthermore, even when present the concentration of *Salmonella* in feed was very low and not associated with *Salmonella* introduction.

In conclusion, the findings of the present study strongly implicate off-farm rearing of cattle in transmission of MDR *Salmonella* strains between farms, but also suggest that other important risk factors remain undiscovered. Further research is needed to clarify whether and how off-farm rearing of heifers can be done without imposing risk of *Salmonella* introduction.

ACKNOWLEDGEMENTS

This project was supported in part by National Institute of Health, Department of Health and Human Services under the contract number No1-AI-30055 and by the Agricultural Animal Health Program, College of Veterinary Medicine, Washington State University. The skilful technical assistance of Katherine Kaya Baker is gratefully acknowledged. We also thank Lindsay Tippett, Lisa Jones and other staff of the FDIU, WSU for their technical help.

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Table 1. Management practices included in the analysis on the status of MDR strain introduction on Washington state dairy farms

SN	Farm management related questions
1	Numbers of cattle in the following categories: Lactating cows Dry cows Calves < 3 months of age raised on premises Calves < 3 months of age raised off premises Heifers 3-20 months raised on premises Heifers 3-20 months raised off premises Heifers 20 months to calving raised on premises
2	How many cattle of following types have been purchased during the study period? Number of cows (lactating and dry) Number of Bulls Number of Calves Number of Heifers
3	Did any off-farm raised calves and heifers co-mingle with cattle from other sources?
4	Have any salmonellosis diagnosed during the past 3 years before start of the study?
5	Are heifers and cows in the same close-up pen?
6	Where do cows calve?
7	Are any other cows in contact with maternity cows in this location (e.g., sick cows)?
8	Where are cows moved after calving?
9	Are any other cows in contact with post-partum cows at this location (e.g., sick cows)?

Table 2. MDR *Salmonella* new strain introduction into 59 commercial dairies in the Northwestern USA

Number of new MDR *Salmonella* strains introduced	Number of farms (%)
0	26 (44)
1	18 (31)
2	5 (8)
3	7 (12)
6	1 (2)
8	2 (3)

Table 3. Univariate analysis of herd-level factors associated with whether any new MDR or number new MDR *Salmonella* strain introduction into dairy farms

Variables	Coding	Univariate association with			
		Any new MDR introduction		No. new MDR introduction	
		p-value	OR	p-value	RR
Any cows purchased					
	Yes	0.86	0.88	0.65	1.30
	No	*	1.00	*	1.00
Any bulls purchased					
	Yes	0.66	1.28	0.45	0.70
	No	*	1.00	*	1.00
Any calves purchased					
	Yes	0.81	0.71	0.83	0.80
	No	*	1.00	*	1.00
Any heifers purchased					
	Yes	0.10	0.36	0.99	1.00
	No	*	1.00	*	1.00
Any cattle purchased					
	Yes	0.38	0.63	0.31	0.69
	No	*	1.00	*	1.00
Herd size	per 100 animals	0.24	1.00	0.02	1.00
Number Cows purchased[a]					
	No.	0.54	0.92	0.52	1.00
Number Bulls purchased[a]					
	No.	0.07	0.88	0.46	0.99
Number Calves purchased[a]					
	No.	0.75	1.07	0.42	0.98
Number Heifers purchased[a]					
	No.	0.92	0.99	0.94	1.00
Total cattle purchased					
	No.	0.51	0.99	0.84	1.00
Common hospital-maternity pen					
	Yes	0.77	0.84	0.97	1.00
	No	*	1.00	*	1.00
Clinical salmonellosis in the last 3 years before start of the study					
	Yes	0.01	5.40	<0.01	3.30
	No	*	1.00	*	1.00
Off-farm raised heifers co-mingled with cattle from other sources					

	Yes	<0.01	6.60	0.05	2.00
	No	*	1.00	*	1.00
Heifers and cows in the same close-up pen					
	Yes	0.54	0.70	0.57	1.20
	No	*	1.00	*	1.00

CI = confidence interval, OR = odds ratio, RR =risk ration, - = not applicable, a = number in log10 scale

Table 4. Logistic regression (LR) and negative binomial (NB) models of herd-level factors associated with introduction of MDR *Salmonella* into dairy farms

Model	Variables and coding	OR	$\chi 2$	95% CI for OR	p-value
Any new MDR *Salmonella* introduction (LR)					
Off-farm co-mingled	Yes	8.9	10.7	2.4 - 32.8	0.001
	No	1	-	Reference	-
Herd size	Per 100 increase	1.04	4.7	1.01 - 1.05	0.031
No. of new MDR *Salmonella* introduction (NB)	Variables and coding	RR	$\chi 2$	95% CI for OR	p-value
Off-farm co-mingled	Yes	2.3	5.5	1.1 - 4.7	0.019
	No	1	-	Reference	-
*Sal in last 3yr	Yes	2.5	6.8	1.3 - 5.0	0.009
	No	1	-	Reference	-
Herd size	Herd size	1.02	9.3	1.01 - 1.03	0.002

CI = confidence interval, OR = odds ratio, RR=risk ratio, - = not applicable, * clinical salmonellosis in the last three years before the start of the study