CHARACTERIZATION OF *SALMONELLA SPP.* ISOLATED FROM BEEF CATTLE FROM POST WEANING TO SLAUGHTER

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

ABSTRACT

Nesemeier, Brenton Fred, M.S., Department of Veterinary and Microbiological Sciences, College of Agriculture, Food Systems and Natural Resources, North Dakota State University, May 2010. Characterization of *Salmonella spp.* Isolated From Beef Cattle Post-Weaning To Slaughter. Major Professor: Dr. Margaret L. Khaitsa

The occurrence of *Salmonella* in cattle has been well documented, but little is known of tracking its prevalence and antimicrobial resistance (AMR) from post-weaning to slaughter. This study follows a longitudinal approach, allowing for the best analysis of *Salmonella* prevalence and AMR in cattle. It was carried out to monitor variation in *Salmonella* prevalence and AMR patterns in beef cattle from range (calves post weaning in North Dakota (ND)) and feedlot cattle up to slaughter (Nebraska). Two separate groups were analyzed, cattle which remained at the Dickinson Research Extension Center (DREC) throughout the course of the study and calves which initially were housed at the DREC, then transferred to a University of Nebraska Feedlot, where they remained until slaughter. Fecal samples were taken four times over a sampling period of eleven months, September 2008 – July 2009; a mid-line sponge sample was taken of the steers before slaughter. Laboratory culture of fecal and sponge samples for *Salmonella* followed a standard published procedure. National Antimicrobial Resistance Monitoring System (NARMS) panels were used for AMR testing of *Salmonella* isolates. Additionally, PCR was performed to determine the prevalence of the Integrase 1 gene in the

Salmonella isolates and presumptive integrase positive isolates were further analyzed for the presence of a conserved sequence. Overall, the prevalence of *Salmonella* ranged from 7.9% to 92.1% in adult cattle throughout the study. The prevalence of *Salmonella* in calves at post weaning ranged from 27.7% to 54.4%, with one month, December 2008, displaying 100% prevalence. At the final sampling of calves, which included a midline sponge sample along with a fecal grab, the prevalence of *Salmonella* was 45.8% and 46.8%, respectively. *Salmonella* isolates displayed the highest rate of resistance towards chloramphenicol (57.3%), streptomycin (54.7%) and tetracycline (54.7%) in both groups. Overall, the integrase 1 gene was isolated from 100 (50.0%) isolates, with 88 (44.0%) isolates harboring a conserved sequence. In conclusion, this study provided data on AMR patterns of *Salmonella* shed by beef cattle at the different stages of production. Also, an association between AMR towards the various antimicrobials tested and presence of integrase 1 on the *Salmonella* isolates recovered was investigated providing some information on the mechanisms of resistance to these antimicrobials. However, further research is necessary to quantify other resistance mechanisms that weren't explained by this study. Most importantly, this research contributes information to the scientific literature on *Salmonella* prevalence and AMR risk assessment in the beef cattle food chain that can allow for development of appropriate control measures.

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INTRODUCTION

Foodborne illnesses in the United States (US) are caused by a wide variety of microorganisms, and are estimated to cause 76 million illnesses, 325,000 hospitalizations and 5,000 deaths annually (37). Moreover, this number is thought to be an underestimation because of the relatively high number of infections that are not reported (37). Of all foodborne pathogens that affect humans, *Salmonella* is widely considered to be one of the most important. A FoodNet report estimated *Salmonella* related infections in the US to be at 1.4 million illnesses, 15,000 hospitalizations and 400 deaths annually (57).

One of the reasons *Salmonella* is widely associated with foodborne illnesses is its prevalence in a wide variety of environments including; vegetables, fruit, poultry, sheep, pigs, humans, and cattle (41, 54). Especially of concern is the presence of *Salmonella* on the hide/skin of cattle as cross contamination can occur between the hide and carcass and then to the various meat products during processing, ultimately attributing to illnesses and potentially death in humans. Moreover, the presence of *Salmonella* on cattle hide/skin has also been found to be correlated with the fecal shedding of *Salmonella* (36). Cattle infected with *Salmonella* may or may not show any classic symptoms of salmonellosis, which include bloody stools, rapid breathing, fever and/or dehydration, thereby making it difficult to treat and/or diagnose. The ability of *Salmonella* to remain undetected

in some cattle populations allows for the continued risk of fecal – oral transmission not only among individual cattle herds, but also between cattle and humans as well (52).

The ability of *Salmonella* to become resistant to antimicrobials has hampered efforts in treating illnesses caused by this pathogen and has made AMR tracking in production of food products, especially those from cattle, more important. Antibiotic resistance is the ability of microorganisms to evade the effects of an antibiotic, often times through the use of developed mechanisms, such as: efflux pumps, integrons and through the uptake of resistance genes (10). *Salmonella* has taken on this ability and is becoming more and more resistant to antibiotics at an alarming rate (11, 24). Data have indicated that the phenomenon of antimicrobial resistance can complicate and jeopardizes treatment of bacterial infections, including *Salmonella*, in both humans and animals (34). As previously stated, *Salmonella* accounts for approximately 1.4 million US cases annually of which roughly one fifth (272,000) are resistant to antibiotics (34). This in turn leads to longer hospital stays, multiple antibiotic treatment methods and a total cost in the US exceeding one billion dollars (48).

The ability of microorganisms to evade or to become resistant to antibiotics can be acquired by a number of different mechanisms. One of these mechanisms is through integrons, genes that consist of a central variable region that often

harbors antibiotic resistance gene cassettes (2). Gene cassettes conferring resistance have been identified for nearly every class of antibiotics and multidrug resistance is becoming an increasing threat (61). Additionally, integrons have been reported as common in many different types of bacteria including *Salmonella spp.* However, not many studies have quantified the relationship between AMR and the presence of integrons.

This research project set out to evaluate the prevalence of *Salmonella spp.* in beef cattle at different production stages from post-weaning to slaugher. Information obtained can be used to help to identify critical control points for reduction of fecal shedding of *Salmonella* in beef production that could be targeted for its control. Additionally AMR patterns and the presence of integron 1 in the *Salmonella* isolated will be evaluated and their association quantified. This data will provide insight in the AMR trends in *Salmonella* isolates from beef cattle during production, information that is vital in planning prudent antimicrobial use in both animal and public health.

HYPOTHESIS

1. The prevalence of fecal shedding of *Salmonella* in beef cattle remains constant from post weaning to slaughter.
2. Antimicrobial resistance patterns of *Salmonella* isolated from beef cattle at different stages of production are similar.
3. An association exists between presence of Integron 1 and AMR in the *Salmonella* isolated.

OBJECTIVES

1. To determine the prevalence of *Salmonella* in beef cattle from post weaning to slaughter.

2. To determine the rate of antimicrobial resistance and multidrug resistance in the *Salmonella* strains isolated from beef cattle at the different stages of production.

3. To determine the association between the presence of integron 1 and antimicrobial resistance in the isolated *Salmonella* strains.

LITERATURE REVIEW

Prevelance of *Salmonella* from Post-Weaning to Slaughter

The control of *Salmonella* at the pasture or farm level is important so that the determinants (stress, forage type, housing, seasonality etc.) that relate to increased shedding of *Salmonella* can be properly investigated and potentially reduced (30). According to a published study limited studies have been published corresponding to pasture based grazing systems and the prevelance of *Salmonella*. Yet, some studies (19, 20, 29) have indicated the presence of *Salmonella* in cattle at pasture and reported that approximately 80% of farms had at least one positive sample.

A longitutudal study evaluated the prevalence of *Salmonella* in feedlot cattle; they reported a rather low prevalence of *Salmonella* of 6.3% of the cattle sampled; they further stratified their data based on pen type and saw no statistical difference among the results (13). Another study which also evaluated the prevalence of *Salmonella* in the feces of feedlot cattle found contradictory results; in their study they tested 600 fecal samples from cattle ready to be sent to slaughter, and found that 192 (30.3%) of the samples tested were positive for *Salmonella* (27). The *Salmonella* prevalence reported in that same study was similar to that reported in a similarly published study who observed a prevalence of fecal shedding of *Salmonella* in feedlot cattle of 33.9% (16, 27). In addition to

the previously referenced studies, a feedlot study (62) assessed the levels of *Salmonella* in large US feedlots. In this study, fecal samples from the cattle pens were tested for *Salmonella* and approximately 22.3% of the pens had at least one positive sample, similar to other studies (16, 27, 50). A 2006 study evaluated the presence of *Salmonella* among feedlot cattle and the length of time on the feed lot and reported an increase of *Salmonella* from 0.7% (1/144) at the introduction of cattle to the feed lot in October 2003 to 62% (89 /144) at the final sampling in May 2004 (26).

Overall, studies that describe the *Salmonella* load on cattle hides are lacking, although this contamination is a major contributior to food borne pathogens being passed on to the consumer in the final product. A 2008 study examined the prevalence of *Salmonella* at harvest on cattle hides at four different abattoirs (27). Although they did not find a difference in prevalence of *Salmonella* among the four testing sites, they did find a high prevalence of *Salmonella* (69.6% , 752/1081) on the hides of the cattle. A similar study completed in 2005, also examined the prevalence of *Salmonella* in slaughter ready cattle; they found that prevalence of *Salmonella* in the fecal matter, regardless of sampling procedure, was as high as 40%. However, when the same cattle were sampled at slaughter they observed an increase of *Salmonella* prevalence from 37.3% to 84.2% (55). A prevalence study completed in 2008, looked at the prevalence of *Salmonella* on

the hides of cull cattle at slaughter; overall the researchers found a prevalence of 89.6% on cattle hides (8). They also found a prevelance of 50.2% and 0.8% on pre-evisveration and post-intervention carcasses, respectively. One possible source of the high prevelance of *Salmonella spp.* at slaughter is the lairage, an area in which cattle pass through before processing (3, 4). The same study reported that up to 30% of the increase in *Salmonella* prevalence at slaughter orginated from lairage (3, 4). The high levels of contamination on the hides at slaughter is important, because this is the most likely stage in the production process in which cross contamination between beef and ground beef and other beef products occurs (45).

Risk factors for *Salmonella* Shedding in Cattle

A 2001 study identified a positive association for *Salmonella* shedding in cattle herds with herd size, exposure to wild geese, exposure to wild rodents and the presence of poultry on neighboring farms (58). Although, their study comprised of a relatively small sample size and diversity among herds, they were able to determine that these associations or a combination of multiple associations were positively associated with *Salmonella* infection in cattle (58). Another study also evaluated *Salmonella* prevalence among feedlot cattle and reported a positive association between the length of time on feed at the feedlot and prevelance of *Salmonella* (26).

A 2005 study examined the presence of *Salmonella* in slaughter ready cattle. Overall, they found that prevalence of *Salmonella* in the fecal matter, regardless of sampling procedure, was as high as 40%. They reported that as the sample size (amount of fecal matter) being tested increased; the proportion of positive samples for *Salmonella* also increased (55). They concluded that increase in *Salmonella* as the fecal sample size increased is likely, due to the fact that *Salmonella* may not be present in the same ratio throughout the sample. The same study also examined the prevalence of *Salmonella* in slaughter ready cattle and they reported an increase of *Salmonella* prevalence from 37.3% at the feedlot to 84.2% at the slaughter facility (55). The authors attributed the increase in shedding to factors such as cross contamination during transportation or to stress factors such as overcrowding during transportation, which can led to increased levels of fecal shedding of *Salmonella* in cattle (55).

Several studies have also looked at the prevalence of *Salmonella* at the feedlot and compared it to the hide samples taken at slaughter, and in each of these studies the prevalence increased dramatically (3, 5, 16). The researchers attributed the rise in prevalence in part due to the close confined quarters of the cattle transportation method and also in part due to the stress involved with the transportation of cattle. Another study examined that the lairage enviroment as a possible source of contamination and spread of *Salmonella,* concluding that it

plays a major role in the spread of *Salmonella spp* (4). The same study further reported that bacteria persist in this enviroment, even after routine cleaning cycles have completed and also concluded that a significant portion of this contamination could be remedied through hide wash cabinets thereby reducing the risk of bacteria being passed onto the poroduct and eventually to the consumer.

Antimicrobial Resistance Among *Salmonella* Strains Isolated from Cattle

According to the World Health Organization (WHO), one potential reason why antimicrobial resistant strains of *Salmonella* emerge is in response to antimicrobial use in feed products (63). Also, another study outlined the reasons why antibiotic resistance occurs, and they included use of antibiotics for prophylactic and therapeutic reasons in animal and in some cases as growth enhancers; they further stated that this allows for reservoirs of antibiotic resistant bacteria to accumulate (1). It has also been observed that *Salmonella* strains are developing a means to keep the resistance even long after the antimicrobial is no longer being used (63). A 1994 study on *Salmonella* and antimicrobial resistance in cattle noted a overall 75% level of sensitivity to the antimicrobials tested, whereas in a more recent 2003 study, a 62.8% level of sensitivity was noted (13). The decrease in sensitivity of the *Salmonella spp.* is important, because the proportion of antimicrobial resistant isolates is increasing, making treatment more difficult.

In the US, the antimicrobials most often associated with antimicrobial resistant strains of *Salmonella* are tetracycline, streptomycin, sulfamethoxazole and ampicillin, with increasing resistance seen to ceftriaxone (11). Another study stated that *Salmonella* strains are increasingly resistant to sulfadiazine, spectinomycin and cholorophenicols (24). A study that evaluated the resistance levels of *Salmonella* to various antibiotics reported most resistance to tetracyline (13%), streptomycin (10.3%) and ampicillin (10.1%) (44). The researchers' findings agree with a previous study that examined resistance patterns of *Salmonella* (11). This study also looked at multidrug resistant (MDR) data and found that the presence of *Salmonella* resistant to 5 or more drugs was 25.3%, 13.3% and 4.2% among calves, sick cattle and healthy cattle, respectively.

Another study that examined the antimicrobial resistance patterns of 101 *Salmonella* isolates from cattle determined that 97% of the isolates were resistant to at least one antibiotic, whereas 20.5% were resistant to 2 or more of the antimicrobials (16). Results of this study concurred with previous studies where most resistance was reported in sulfamethoxazole (96.08%) and streptomycin (17.65%) (11, 24). A study of 92 *Salmonella* isolates from a cattle population indicated that 5.2% of the strains were resistant to two or more antibiotics (34). This study concurs with other previously mentioned studies in that it reported the highest rates of resistance to tetracycline (11, 44).

Integron 1 and its Association with *Salmonella*

There are five different integrons that have been reported; with class 1, 2 and 3 frequently associated with antimicrobial resistance (AMR) in the enviroment (22). The same authors further state that although class 2 and 3 are also associated with AMR in the enviroment, the most frequently associated and well studied integron is in fact their ancestor, the class 1 integron and more recently the class 2 integron (49). Class 1 integrons have been associated and are the most frequent carrier of AMR in a number of gram negative organisms, including the *Salmonella* species. Integrons are characterized by a 5' conserved segment, containing the integrase gene, a 3'conserved sequence and a central *attI* recombination site where the gene cassettes may be located (39). Integrons are important because of their ability to insert themselves into bacterial chromosones, often helping to confer resistance to different groups of antimicrobials such as; chloraphenicols, sulfonamides, streptomycins and tetracyclines. Also, integrons are believed to be able to cross the serotype and species barrier, often conferring resistance from multiple sources to *Salmonella* (18).

A study estimated the prevelance of class 1 integrons in *Salmonella* species from animals at 60% (93/151) of all isolates tested (23). The association of integron 1 and AMR in *Salmonella* species has been widely documented (60, 61); these authors stated that integrons are often associated with resistance and in many

instances resistance to one or more linked antimicrobial resistant genes, therefore, leading to MDR. The same study has indicated that 20% of MDR *Salmonella* strains posess integron 1 (61). A study of 59 *Salmonella* Dublin strains, isolated from various sources, found an association between AMR and integron 1 in cattle species. They found the prevalence of integron 1 was 20.3% (56). Furthermore, this study reported that all of the isolates that were resistant to ≤ 1 antimicrobials were *int*-negative, whereas those that were resistant ≥ two antimicrobials were all *int*-positive (56). These data suggest an association between integron 1 presence and AMR in *Salmonella spp.* However, not many studies have quantified this relationship

MATERIALS & METHODS

Study Design

This was a longitudinal study involving 48 cattle and 48 spring born calves originating from two regions of North Dakota. The study was conducted over a 9 month sampling period (September 2008 – June 2009). The 48 adult cattle were kept at North Dakota State University Research Extension Center (DREC) at Dickinson, North Dakota throughout the study period. The cattle were initially fed on pasture and then they were transferred to a winter dry lot for the winter month and later returned to pasture after the May 2009 sampling. The 48 calves were originally kept at DREC on pasture post weaning for up to 4 months (September 2008 to October/December 2008). In both October and December 2008, 24 calves, on two occasions, were transferred to a feed lot, a finishing stage in which the animals are held until slaughter, at Nebraska Panhandle, Scottsdale, NE where the 48 calves were kept for 4-6 months before being sent to slaughter. The 48 cattle were sampled four times on the following dates: September 22, 2008; November 03, 2008; February 05, 2009 (38); and May 05 (8) & May 06 (30), 2008 (Figure 1). The calves were sampled five times on the following dates: September 22, 2008 (48); October 15, 2008 (24); November 03, 2008 (24); December 16, 2008 (24), February 19, 2009 (47) and June/July (48) (Figure 1). Two sampling times (September 22, 2008 and November 03, 2008) were synchronized for both the

calves and cattle while the other sampling times were different due to difficulty in sampling logistics in the field. The June/July 2009 sampling for the calves occurred when each calf was at proper weight for slaughter. As this did not occur at the same time for all calves, the final sampling period occurred over a two month period and involved a total of three separate samplings.

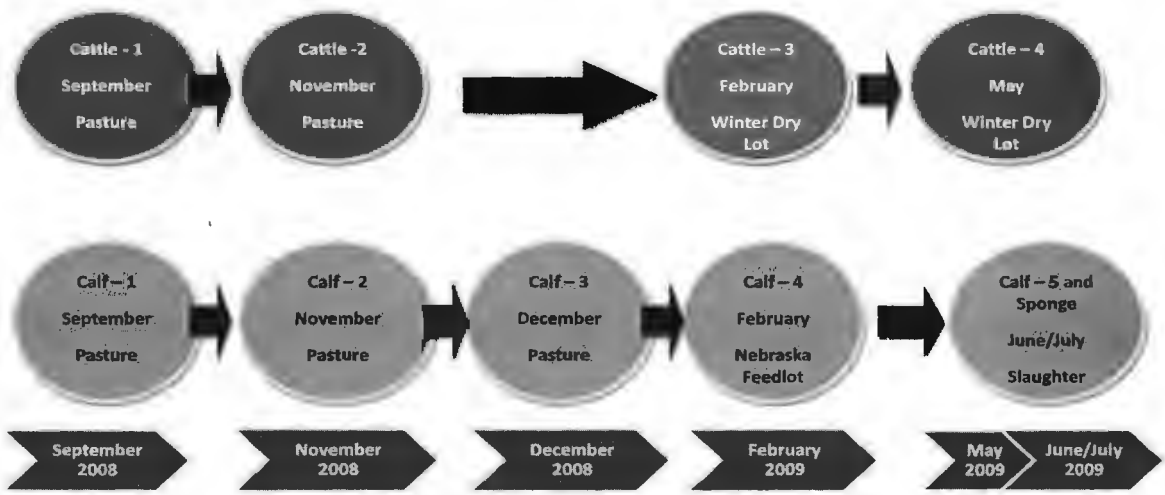

Figure 1: Representation of sampling procedure for cattle and calves in the study.

Housing and Feeding of Cattle

Forty eight cattle and 48 calves were held at the DREC at the onset of this study. The cattle grazed native pasture from the beginning of the study (September 2008) until November 3, 2008. The cattle were fed an alfalfa grass mixed diet from November 3, 2008 through April 29 and 30th 2009 while they were housed in a winter dry lot, an alternative feeding style in which the cows were fed similarly to that of a feed lot until pastures were ready. An analysis of the dry matter forage was taken and it consisted of moisture 9.1%, crude protein 13.3%, total digestible nutrients (TDN) 51.6%, neutral detergent fiber 58.5% acid detergent fiber 39.7%, calcium 0.95% and phosphorus 0.28%. All 48 calves were worked either April 29th or 30th, where they were branded and received a booster vaccination for Clostridia organisms and an initial multi-viral vaccine (5 Way (Modified live) Bovi Shield Gold 5: IBR, BVD Types 1 & 2, PI3 and bovine respiratory syncitial virus). They were also worked again two weeks prior to their weaning date. The calves were broken up into two groups, early weaned and conventionally weaned. The early weaned calves were weaned on August 13, 2008 and transferred to a feedlot pen for 14 days until they were transferred to replicated unharvested fields where they grazed from August 27, 2008 until October 14, 2008 when they were shipped to the UNL Panhandle RE Center Feedlot, Scottsbluff NE for finishing and final harvest. The conventionally weaned

calves were handled identically and were shipped to the UNL feedlot on November 3, 2008 where they were housed in typical feed lot pens consisting of concrete bunks, continuous water flow and steel or cable fences; no bedding was provided . While at the UNL feed lot the calves were given progressive diets that consisted of alfalfa hay, corn (cracked), corn (silage) and supplements. They were housed here until they reached final weight at which point they were then slaughtered.

Sampling Procedure

Sampling of cattle was conducted in accordance with the guidelines established by the Institute of Animal Care and Use Committee (IACUC) as outlined in a previous study (25). The cattle were briefly restrained in a chute and approximately 25 g of feces were removed from the rectum using a clean set of polythene gloves and placed in a sterile cup which was placed in a cooler containing ice for transport; this procedure was repeated for each animal. This process was completed for each subsequent sampling of both groups of cattle. Mid-line sponge samples were obtained from the hides of live cattle before they were sent to slaughter using a sterile sponge moistened with 20 ml of sterile water and completed in a process previously described by another study (28). Only the calves which were sent to the Nebraska feedlot had mid-line sponge samples taken and not the cattle that remained at DREC.

Fecal Samples

The fecal samples were transported back to the laboratory at North Dakota State University, Fargo, ND within 24 hours of sampling. Once the samples, approximately 10 g, reached the lab they were transferred, using a sterile swab, to Buffered Peptone Water (BPW), (EMB Chemicals Inc., Gibbstown, N.J.) for 18-24 hours for enrichment. Positive and negative controls were obtained from the NDSU Veterinary Diagnostic Laboratory (VDL) and used for the duration of the experiment. Immunomagnetic beads were used to retrieve the *Salmonella* from the enrichment phase. This procedure involved transferring 1 ml of enrichment broth into 1.5 ml microcentrifuge tubes labeled individually for each animal. Each tube contained 20 µl of each of the respective anti-*Salmonella* immunomagnetic beads, which were processed following the manufacturer's instructions. The tubes were rotated for ten minutes on an electric rotator machine. The beads were washed 3 times with phosphate buffered saline and Tween20 (PBST) using a magnetic separator rack to capture the beads, reconstituted in 100 µl of PBST, and vortexed. After vortexing, 100 µl of aliquot (immunomagnetic beads and captured bacterial cells) were transferred to Rappaport Vasiliadis R10 broth (Becton, Dickinson and Company, Sparks, M.D.) and incubated over night at 42°C. Samples were streaked onto modified brilliant green (Becton Dickinson, Sparks, Md) and mannitol lysine crystal violet brilliant green agar (MLCB) (Oxoid LTD., Basingstoke,

UK) agars and incubated overnight at 37°C. A single presumptive positive colony was streaked out on tryptic soy agar (TSA) (Becton Dickinson). A single colony was then stabbed on Triple Iron Sugar (Becton Dickinson) slants. Colonies displaying hydrogen sulfide production were identified using API20E strips (Biomerieux, France) according to the manufacturer's instructions.

Sponge Samples

The sponge samples were placed in a sterile bag and transported back to the laboratory at NDSU in a cooler with ice packs within 24 hours. Once the sponge samples reached the laboratory they were transferred to sterile stomacher bag containing 100 ml of Buffered Peptone water and the sample was homogenized in a stomacher for 90s. Following homogenization, 10 ml of homogenate was added to an equal volume of 2X Buffered Peptone water and incubated overnight at 37°C. Following incubation, the previously described procedure of isolation, selection and verification was completed.

Antimicrobial Resistance

Antimicrobial resistance (AMR) of isolated *Salmonella* strains was performed using National Antimicrobial Resistance Monitoring System (NARMS) CMV1AGNF panel according to the manufacturers' instructions (Sensititre, Trek Diagnostics, Westlake, Ohio, USA). Each isolate was screened for resistance using full range minimum inhibitory concentrations (MICs). The 15 different

antimicrobials that were used with their corresponding MICs include amikacin (0.5–64 μg=mL), amoxicillin/clavulanic acid (1=0.5–32=16 μg=mL), ampicillin (2–32 μg=mL), cefoxitin (0.5–32 μg=mL), ceftiofur (0.12–8 μg=mL), ceftriaxone (0.25–64 μg=mL), chloramphenicol (2–32 μg=mL), ciprofloxacin (0.015–4 μg=mL), gentamicin (0.25–16 μg=mL), kanamycin (6–64μg=mL), nalidixic acid (0.5–32 μg=mL), streptomycin (32–64 μg=mL), sulfizoxazole (16–512 μg=mL), tetracycline (4-32 μg=mL) and trimethoprim-sulfamethoxazole (0.12=2.4–4=76 μg=mL). The breakpoints of the antimicrobials as determined by the United States Department of Agriculture assisted in the determination of whether or not resistance was observed. The breakpoints used in this study are as follows in μg/ml: amikacin (≥ 64), amoxicillin/clavulanic acid (Amox_cla) (≥ 32/16), ampicillin (≥ 32), cefoxitin (≥ 32), ceftiofur (≥ 8), ceftriaxone (≥ 64), chloramphenicol (≥ 32), ciprofloxacin (≥ 4), gentamicin (≥ 16), kanamycin (≥ 64), nalidixic acid (≥ 32), streptomycin (≥ 64), sulfizoxazole (≥ 512), tetracycline (≥ 16) and trimethoprim-sulfamethoxazole (≥4/76) (53).

DNA Extraction

The bacterial DNA used in polymerase chain reaction (PCR) were prepared from the *Salmonella* isolates using the single cell lysing buffer (SCLB) protocol (35). A single isolated colony was suspended in 40 μl of SCLB buffer in a 0.2 microcentrifuge tube. The SCLB master mix consisted of 10 μl of 20 mg/ml

proteinase K (Amresco) and 990 µl of 10 mM Tris HCL and 1mM EDTA (TE) buffer solution (Amresco). The lysis procedure consisted of lysing the cells at 80°C for 10 minutes and then cooling the cells to 55°C for 10 minutes in a thermocycler (Eppendorf, Westbury, NY). Following this, 80 µl of sterile double distilled water was added to the suspension and then centrifuged for 30 seconds at 4500 x g. The samples were stored at -20°C until further analysis could be completed.

Integrase 1 Detection

In order to determine whether or not Integron 1 was present in the *Salmonella* isolates, polymerase chain reaction (PCR) was performed. Forward and reverse primers were used encoding for the Integrase gene. The primer sequences comprised of *Int*1 - Forward: 5′-TCT CGG GTA ACA TCA AGG-3′ and *Int*1-Reverse: 5′-AGG AGA TCC GAA GAC CTC-3 (Sigma-Aldrich, Texas USA). The PCR mastermix contained the following concentration; - 5X PCR buffer, 10 pmol/L each primer, 0.2 mM dNTPs and 2.5 U Taq Polymerase (Promega); 23 µl of the mastermix was placed in a sterile PCR tube and 2 µl template DNA was added. The following PCR parameters were used: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 30 s at 72°C. Twenty – five µl of PCR product was mixed with 5 µl of Envision loading dye (Amresco, Solon, OH) and subjected to horizontal electrophoresis at 75 volts in a 1.5% agarose (Amresco) gel in 1X Tris-Borate-EDTA (TBE) Buffer. All PCR reactions included both positive (V1) (23) and negative

controls (sterile water). The expected base size of Integron 1 positive isolates is approximately 280 base pairs; the *int* positive isolates were subjected to further analysis as described in the following procedure.

Conserved Sequence Detection and Sequencing

The *Salmonella* isolates determined to contain the Integrase 1 gene were tested for the presence of the conserved sequence of Integron 1. Specific primers: *Int1* CS: 5' GGCATCCAAGCAGCAAG and 3' AAGCAGACTTGACCTGA for the conserved sequence within the integrons were used (6). The same PCR protocol as described above was used in the conserved sequence analysis. The PCR amplicons were visualized using horizontal gel electrophoresis at 50 volts in a 1.5% agarose gel with 1X Tris-acetate-EDTA (TAE) buffer (Amresco). The PCR reaction and electrophoresis contained both positive (V1) (23) and negative controls (sterile water). Following electrophoresis, the amplicons were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The DNA band from the gel was excised and placed in a 1.5 ml microcentrifuge tube. To every 10 mg of gel slice, 10 µl of membrane binding solution was added; the solution was vortexed and held at 55-60°C until the gel was completely dissolved. A SV minicolumn was inserted into collection tube, in which the dissolved gel mixture was added and incubated at room temperature for 1 minute. It was centrifuged at 16,000 x g for 1 minute, the flowthrough was discarded and the minicolumn was

reinserted into collection tube. A washing step then followed in which 700 µl membrane wash solution was added and centrifuged at 16,000 x g for one minute, the flowthrough was again discarded and the minicolumn was reinserted into the collection tube. This process was completed a second time with 500 µl of membrane wash solution and centrifuged for 5 minutes. The collection tube was then opened and the column assembly was recentrifuged for 1 minute with the microcentrifuge tube open to allow for evaporation of the ethanol. The minicolumn was carefully transferred to a sterile 1.5 microcentrifuge tube and 50 µl of Nuclease –Free water was added. The solution was incubated for 1 minute at room temperature and then centrifuged at 16,000 x g for 1 minute; the minicolumn was discarded and the DNA was stored at -20°C until the purified product was sent for sequencing at Macrogen.

Statistical Analysis

The generated data sets were entered into a Microsoft Excel spreadsheet for further statistical and graphical representation. In order to accurately assess the patterns, distribution and trends in the data, a comparison of the *Salmonella* prevalence observed at different sampling times throughout the study was conducted. In order to accurately assess the significance of Integron 1 and its association with AMR, various statistical tests were performed including Fishers' exact test and a Chi square test. These tests were completed with the aid of EpiInfo

3.5.1. The information generated from Microsoft Excel 2007 and EpiInfo 3.5.1 determined if a difference exists in the prevalence of fecal shedding of *Salmonella* in beef cattle at different stages of production. It also deteremined whether the AMR patterns of *Salmonella* isolated from beef cattle at different stages of production differed and if an association existed between the presence of Integron 1 and AMR in the *Salmonella* isolated.

RESULTS

Salmonella Prevalence in the Cattle and Calves

A total of 410 fecal samples (Table 1) were processed over the period of study, (238 (58.1%) from calves and 172 (42.0%) from cattle). Of the 238 calf fecal samples, 117 (49.2%) tested positive for *Salmonella*. Of 172 fecal samples collected from cows, 83 (48.3%) tested positive for *Salmonella* (Table 1). Overall prevalence between the two groups of cattle was 48.8% (200/410) with 49.2% of total calf samples and 48.3% of total cattle samples testing positive for *Salmonella* (Table 1). Biochemical tests, including API20E (Biomerieux) confirmed the presence of *Salmonella* in the isolates tested. All 200 susceptive *Salmonella* positive isolates were subjected to API20E with 197 (98.5%) isolates displaying excellent *Salmonella* identification (Table 2) and 3 (1.5%) isolates displaying great *Salmonella* identification as according to information provided with the API20E strips.

The prevalence of *Salmonella* in calves remained constant throughout the first two sampling periods at 47.9% and 54.2% (September 2008 and November 2008) of the study (Figure 2). The December 2008 sampling of the 24 remaining calves at the DREC, Dickinson ND, saw the prevalence of *Salmonella* rise to 100% before they were sent to the Nebraskan feedlot (Figure 2). After that, the prevalence of *Salmonella* in calves returned to levels similar to what was observed

at the beginning of the study. The prevalence of *Salmonella* in cattle initially had a downward trend bottoming at 7.9% at sampling time 4 (February 2009). The highest *Salmonella* prevalence was 92.1% during the May 2009 sampling before cattle were moved out to pasture from the winter feedlot (Figure 2).

Table 1: Prevalence of *Salmonella* throughout study in cattle and calves

Calves	Sample Size	# Positives	Percentage Positive	Sample Month
	48	23	47.9%	September
	24	13	54.2%	November
	24	24	100.0%	December
	47	13	27.7%	February
	47	22	46.8%	July
	48	22	45.8%	July - Sponge
	238	117	49.2%	Total
Cattle	Sample Size	# Positives	Percentage Positive	Sample Month
	48	27	56.30%	September
	48	18	37.50%	November
	0	0	0%	December
	38	3	7.90%	February
	38	35	92.10%	May
	172	83	48.3%	Total
Total	410	200	48.8%	

Table 2: API 20E results

ONPG	ADC	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL
-	+	+	+	+	+	-	-	-	-	-
GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	
+	+	+	+	+	-	+	-	+	-	

+ = positive

- = negative

See Table 3 for explanation of other abbreviations

Table 3: Explanation of API 20E results

TESTS	SUBSTRATE	REACTION TESTED
ONPG	ONPG	beta-galactosidase
ADH	arginine	arginine dihydrolase
LDC	lysine	lysine decarboxylase
ODC	ornithine	ornithine decarboxylase
CIT	citrate	citrate utilization
H2S	Na thiosulfate	H2S production
URE	urea	urea hydrolysis
TDA	tryptophan	deaminase
IND	tryptophan	indole production
VP	Na pyruvate	acetoin production
GEL	charcoal gelatin	gelatinase
GLU	glucose	fermentation/oxidation
MAN	mannitol	fermentation/oxidation
INO	inositol	fermentation/oxidation
SOR	sorbitol	fermentation/oxidation
RHA	rhamnose	fermentation/oxidation
SAC	sucrose	fermentation/oxidation
MEL	melibiose	fermentation/oxidation
AMY	amygdalin	fermentation/oxidation
ARA	arabinose	fermentation/oxidation
OX	oxidase	oxidase

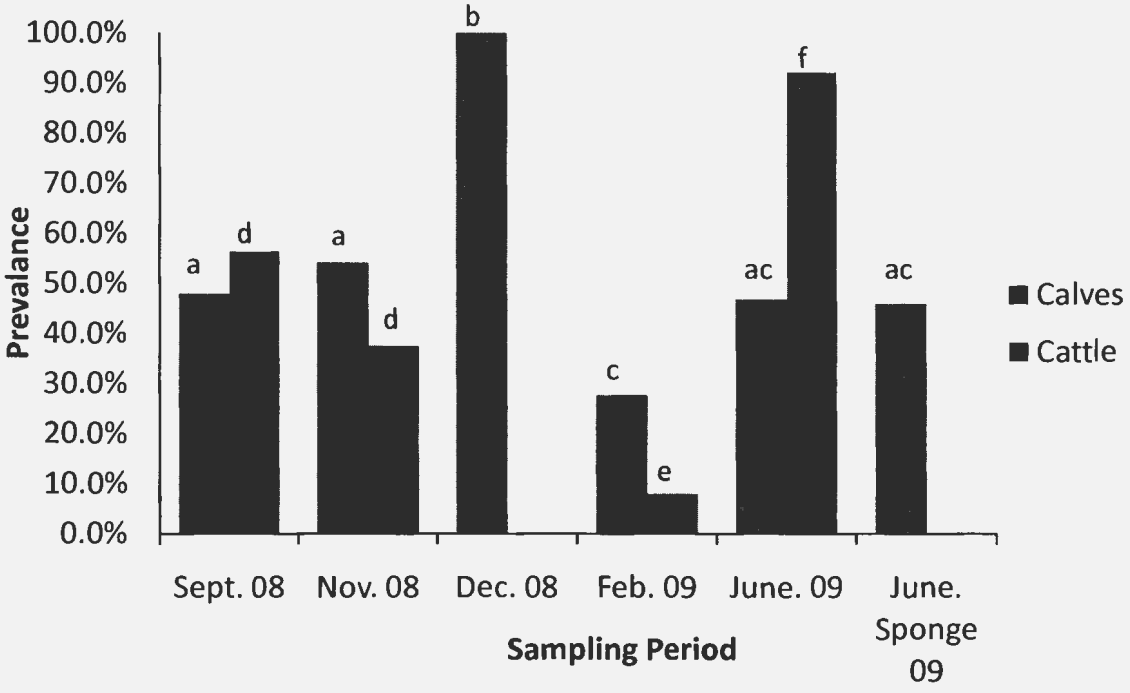

Figure 2: *Salmonella* prevalence in cattle and calves. This shows the samplings throughout the 5 sampling periods and the sponge sampling. Different letters a, b and c refer to statistically different prevalences ($p>0.05$) of *Salmonella* in calves whereas letters d, e and f refer to the same in the cattle. (Sept = September, Nov = November, Dec = December, Feb = February)

Antimicrobial Resistance Patterns in the *Salmonella* Isolates

All 200 *Salmonella* isolates were examined for antimicrobial susceptibility. A total of 116 (58.0%) of all isolates displayed resistance to at least one antimicrobial being tested. A total of 69/117 (59.0%) of the *Salmonella* isolated from calves displayed resistance to at least one antimicrobial throughout the duration of the study (Figure 3). The highest rate of resistance was seen to tetracycline (54.7%), sulfizoxazole (54.7%), streptomycin (54.7%), chloramphenicol (57.3%), ampicillin (54.7%) and Amox_Cla (47.0%), (Figure 3). The December 2009 sampling of the calves saw a slight increase in the rate of chloramphenicol resistance (12.5%), where 4 additional isolates displayed resistance to chloramphenicol as compared to the other antimicrobials being tested. A single isolate from the February 2009 displayed resistance to cefoxitin. Figure 3 shows the resistance patterns from the onset of the study, September 2008, to the completion of the study, June/July 2009. The highest rates of resistance was seen in the June/July 2009 samplings before the calves were slaughtered, whereas the lowest rates of resistance occurred in the November 2008 when 24 calves were sampled at the DREC.

A total of 47/83 (56.6%) of the *Salmonella* isolated from cattle displayed resistance to at least one antimicrobials during the duration of the study. The highest rates of resistance were seen towards tetracycline (56.6%), sulfizoxazole (56.6%), streptomycin (56.6%), chloramphenicol (56.6%), ampicillin (55.4%), and

Amox_Cla 45.8%), (Figure 4). The highest rate of resistance was seen during the first sampling (September 2008), when the cattle were at pasture and the lowest rate of resistance was seen in the November 2008 sampling taken immediately before cattle were moved to a winter dry lot.

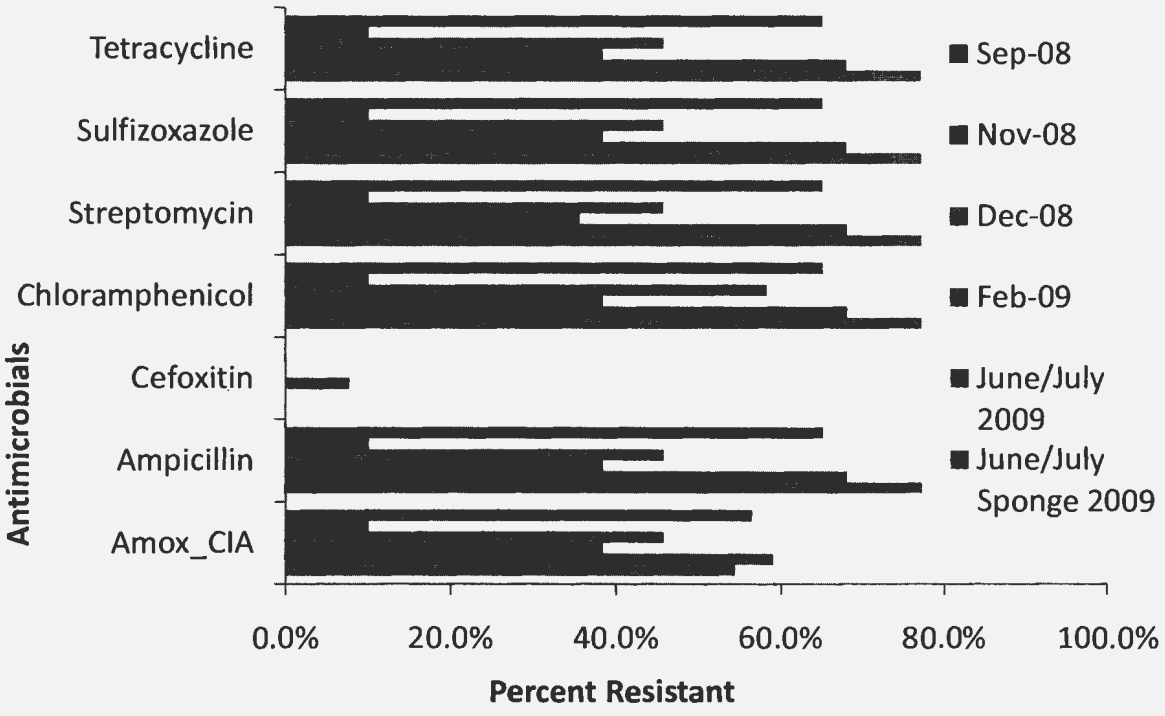

Figure 3: Comparison of the resistance patterns displayed in calves throughout the study. A total of 15 antimicrobials were tested with the 8 antimicrobials displaying no resistance omitted. (Sep = September, Nov = November, Dec = December, Feb = February)

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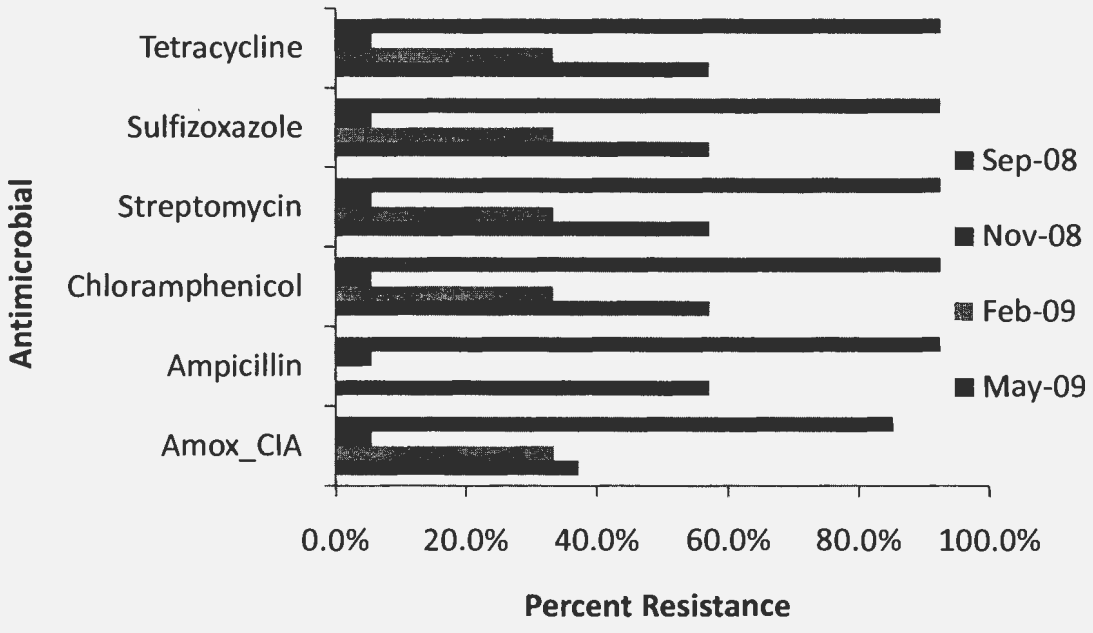

Figure 4: Comparison of the resistance patterns displayed in cattle throughout the study. A total of 15 antimicrobials were tested with the 9 antimicrobials displaying no resistance omitted. (Sep = September, Nov = November, Feb = February)

Multiple drug resistance patterns, resistance to >3 antimicrobials was also analyzed in this study. A total of 111/200 (55.5%) of all *Salmonella* isolated displayed some incidence of multiple drug resistance, whereas the remaining 4/200 (2.00%) isolates displayed resistance to only one antimicrobial, chloramphenicol or cefoxitin. The antimicrobial pattern displaying the highest prevalence of multiple drug resistance in cattle included tetracycline, sulfizoxazole, streptomycin and chloramphenicol and ampicillin (55.4%) and 2nd highest pattern also included amox_cla (45.8%) with the previously listed antimicrobials. In calves the highest prevalence of MDR was seen to tetracycline, sulfizoxazole, streptomycin, chloramphenicol and ampicillin (54.7%) and the 2nd highest pattern included amox_cla (47.0%) along with the previously listed antimicrobials (Figure 5). The majority (54.7% in calves, 56.6% cattle) of *Salmonella* isolates were predominately resistant to 5 to 6 antimicrobials or to 0 antimicrobials (41.8% in calves, 44.4% in cattle), with 5 isolates (from calves) displaying resistance to only one antimicrobial (Figure 5).

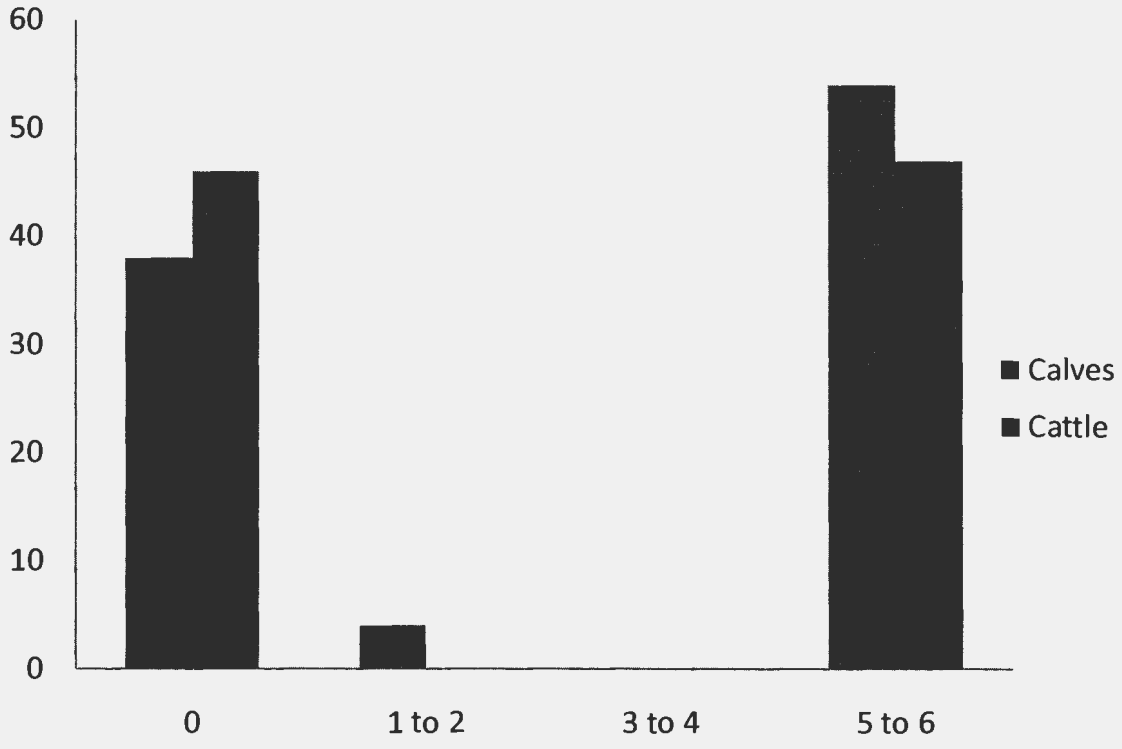

Figure 5: Multiple drug resistant (MDR) data from the study. The Y axis corresponds to the number of *Salmonella* isolates, whereas the X axis refers to the number of antimicrobials the isolate was resistant to. The predominant patterns indicate that the *Salmonella* isolates were predominately resistant pansusceptible (no antimicrobials) or resistant to 5 to 6 antimicrobials.

Integrase 1 Analysis

A total of 200 *Salmonella* isolates were used in the analysis for the presence of the Integrase 1 gene. The isolates were analyzed for the presence of the class 1 integrons by polyermase chain reaction (PCR). Of the isolates analyzed a total of 98 (49.0%) contained *Int*1. Figure 6 shows an image of the gel that was used in the analysis for presence of *Int*1.

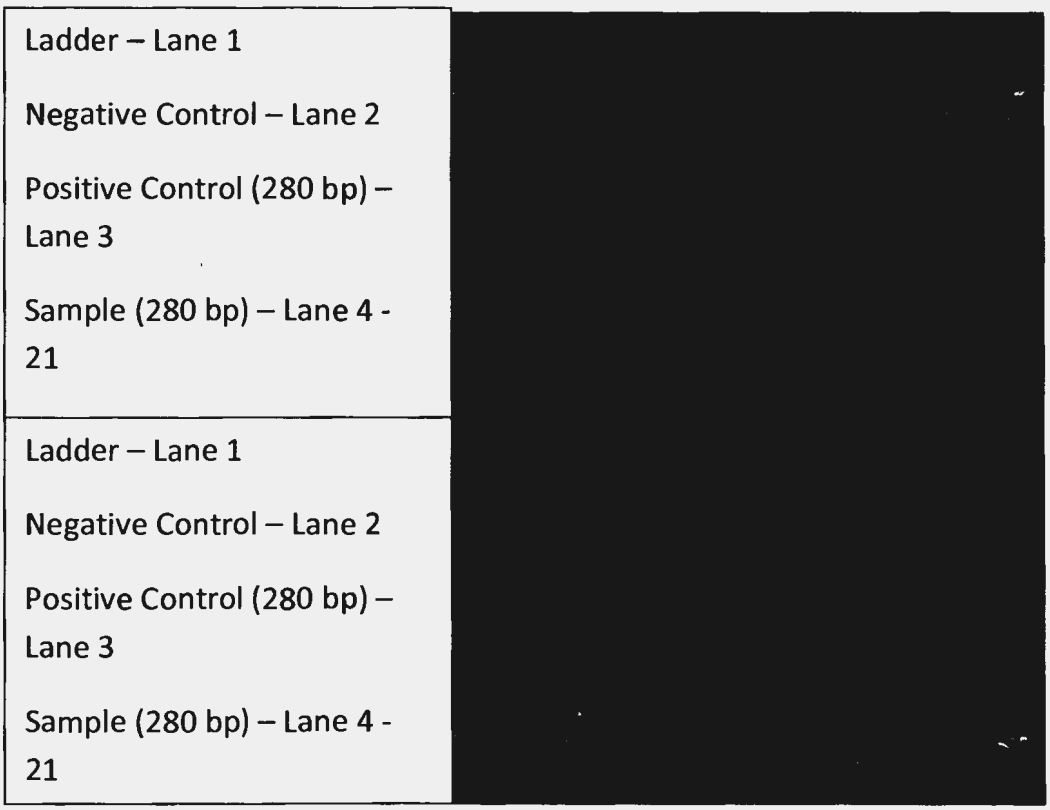

Figure 6: Gel image of the Integrase 1 analysis. Lane one on both the top and bottom contained the ladder, lanes 2 on both contain the negative control and lanes 3 contain the positive control. The remaining lanes contain the *Salmonella* isolates with the *Intl*1 primer amplication.

Analysis of the data indicated that a significant association between the presence of Integrase 1 gene and resistance to the tested antimicrobials existed. Tables 4 and 5 summarize statistical information generated with its respective chi square, odds ratio (95% CI), P value, relative risk and attributable fraction. In table 4, ampicillin is one of the antimicrobials highlighted. Approximately, 84.9% of integrase positive isolates displayed resistance to ampicillin. The statistical data generated from this information is a X^2 of independence of 41.7, an odds ratio of 16.9 with its 95% CI (6.6-43.2), a significant p value of <0.0001, relative risk of 5.16 and an attitutable fraction of 80.6%. This information leads to the conclusion that the odds of *Salmonella* isolates being resistant to ampicillin were 16.9 times higher if they possessed the integrase 1 gene as compared to the absence of the integrase 1 gene. A relative risk of 5.16 implies that among *Salmonella* isolates possessing the integrase 1 gene, the risk of showing resistance to ampicillin was 5.16 times greater than that of the isolates without the integrase 1 gene. An attributable fraction of 80.6% implies that amoung *Salmonella* isolates resistant to ampicillin, 80.6% of the resistance to ampicillin was associated with the presence of the integrase 1 gene. Also, table 4 shows an interesting relationship between cefoxitin and presence of integrase 1. A negative association is show between the presence of integrase 1 and cefoxitin. Table 5 shows similar informationto that in table 4 but only for cattle instead of calves.

Table 4: Association between AMR and presence of integrase 1 gene in *Salmonella* isolated from calves

	Integrase 1 + % Resistant	X^2	OR (95% CI)	P_V	RR	AF
Amox_CLA	79.0%	38.2	13.5 (5.8-32.7)	<0.0001	3.46	71.0%
Chloramphenicol	86.0%	40.1	16.7 (6.4-43.9)	<0.0001	5.63	82.1%
Cefoxitin	1.6%	0.9	0 (Undefined)	0.5202	0.98	-2.0%
Ampicillin	84.9%	41.7	16.9 (6.6-43.2)	<0.0001	5.16	80.6%
Streptomycin	84.9%	41.7	16.9 (6.6-43.2)	<0.0001	5.16	80.6%
Sulfizoxazole	84.9%	41.7	16.9 (6.6-43.2)	<0.0001	5.16	80.6%
Tetracycline	84.9%	41.7	16.9 (6.6-43.2)	<0.0001	5.16	80.6%

+ = Positive
X^2 = chisquare value
OR = Odds Ratio
CI = Confidence Interval
Pv = p-value
RR = Relative Risk
AF = Attributable Fraction

Table 5: Association between AMR and presence of integrase 1 gene in *Salmonella* isolated from cattle

	Integrase 1 + % Resistant	X^2	OR (95% CI)	P_V	RR	AF
Amox_CLA	77.8%	31.7	18.7 (6.1-57.2)	<0.0001	3. 6	72.2%
Chloramphenicol	91.7%	45.4	53.6 (15.2-218.7)	<0.0001	11.3	91.2%
Cefoxitin	0.0%	0	0 (Undefined)	NA	0	0
Ampicillin	91.7%	45.4	53.6 (15.2-218.7)	<0.0001	11.3	91.2%
Streptomycin	91.7%	45.4	53.6 (15.2-218.7)	<0.0001	11.3	91.2%
Sulfizoxazole	91.7%	45.4	53.6 (15.2-218.7)	<0.0001	11.3	91.2%
Tetracycline	91.7%	45.4	53.6 (15.2-218.7)	<0.0001	11.3	91.2%

+ = Positive
X^2 = chisquare value
OR = Odds ratio
Pv = p-value
RR= Relative Risk
AF = Attributable Fraction
NA = Not Available

Conserved Sequence Anaylsis

All Integron 1 positive isolates were subjected to conserved sequence analysis. One hundred total isolates were tested in duplicates and 88 (88% of integrase postive isolates, comprising of 44% of total isolates) possessed the conserved sequence. All 88 isolates had product size of 1000 bp. Figure 7 shows the gel image of this analysis. A representative sample of 8 PCR products were cleaned and sent to Macrogen, USA (Maryland, US) for sequencing. Upon receiving results a BLAST search was completed. Results indicated the presence of the *aadA* resistance gene, which is associated with streptomycin/spectinomycin resistance, in all 8 representative samples.

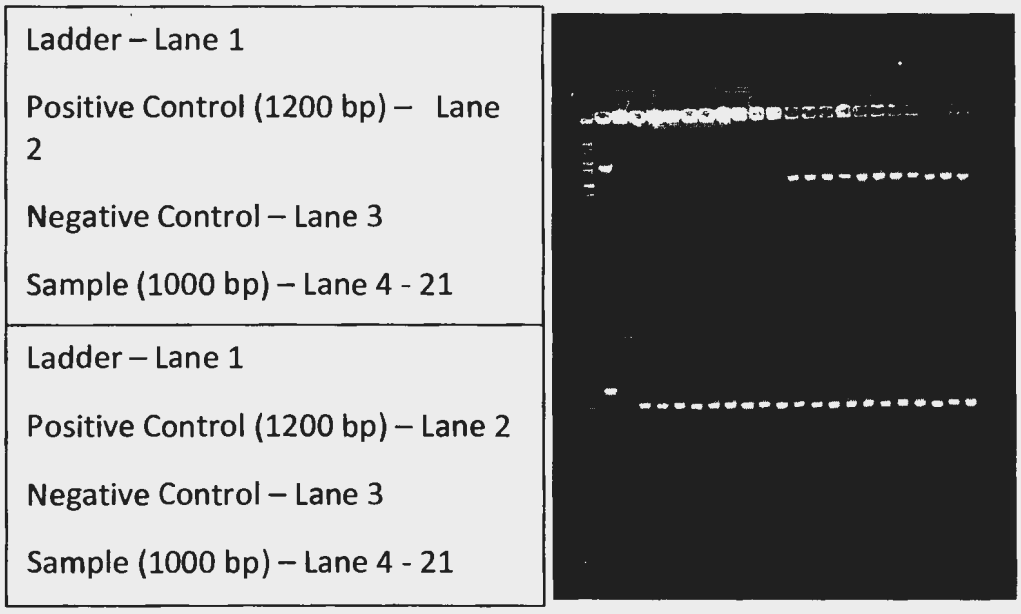

Figure 7: Gel image of conserved sequence of integron 1. Lane one on both the top and bottom contain the ladder, lanes 2 on both contain the postive control and lanes 3 contain the positive control. The remaining lanes contain the *Salmonella* isolates with the CS primers for amplication.

DISCUSSION

In this study, we initially observed *Salmonella* prevalence to differ in cattle to that of calves in the first two sampling periods (September 2008 (56.3% and 47.9%) and November 2008 (37.5% and 54.2%)), respectively, when they were being kept at pasture. This was surprising as *Salmonella* shedding in both cattle and calves housed at pasture have been reported to be low ranging from about 2% to 3% (29, 30). However, it has been reported in the literature that it is difficult to measure the prevalence of *Salmonella* at pasture, because cattle tend to shed *Salmonella* sporadically (29, 30). A downward spike was seen in the February 2009 sampling period in which the cattle were kept on a winter dry lot. The final sampling taken at the dry lot, immediately before the cattle were put out to pasture saw a large spike in the prevalence of *Salmonella*, indicating an apparent large increase in the fecal shedding of *Salmonella spp.* The increase in *Salmonella* prevalence observed in cattle at the dry lot could have been attributed to the concentration of cattle in the dry lot. Studies have indicated that *Salmonella* fecal shedding increases in cattle when they are moved from pasture to a housed setting (26). Also, other factors could be attributed to the spike in *Salmonella* prevalence observed in cattle; including seasonal variation, stress or recent transportation of the cattle to and from pasture (30). Some studies have indicated the presence of

Salmonella in cattle at pasture and reported that approximately 80% of farms had at least one positive sample (19, 29).

A study of dairy cattle indicated that *Salmonella spp.* was most likely to be isolated in the summer, fall or spring rather than during the winter months with the summer period having the greatest odds of *Salmonella* isolation (20). Another study of dairy cattle analyzed the seasonal variation on fecal shedding of *Salmonella* in cows and reported similar results, in which, fecal shedding was more common in the May-July time frame as compared to the February – April time frame (59). Although it has been reported that the fecal shedding of dairy cattle is higher than that of beef cattle, the similar seasonal variations are seen in our study (30, 59). An increase in the prevalence of *Salmonella* was observed in the May 2009 sampling, which is the start of the summer season, as well as a decrease in presence of *Salmonella* in the winter season (February 2009) sampling. The drop in the prevalence of *Salmonella* in February also concurs with another study from an Irish abattoir, which saw no *Salmonella* during the February sampling; however, like our study their results indicated that the prevalence of *Salmonella* was highest during the August-October period (36). Overall, our data indicate that fecal shedding of *Salmonella spp.* is lowest in the winter season, increasing in the fall, spring and summer seasons.

In calves, we observed an increase in *Salmonella* prevalence in the December 2008 sampling, before they were shipped to the feedlot and also before the calves were sent to slaughter. Studies have indicated that transport stress prior to slaughter affects the proportion of animals that are contaminated with pathogens, such as *Salmonella spp.* (12, 21, 42). The increase of fecal shedding of *Salmonella* by steers before slaughter is a significant food safety concern because of the risk for humans acquiring infection through the consumption of contaminated beef. Studies have reported a correlation between fecal shedding of *Salmonella* by cattle and contamination of carcasses at slaughter (3, 4, 8, 55).

While at the feedlot (February 2009 and June/July 2009 sampling) the prevalence of *Salmonella* fecal shedding in the calves increased from 27.7% to 46.8%. This result concurs with a longitudinal study of *Salmonella* fecal shedding in feedlot cattle which reported an increase in *Salmonella* prevalence from 0.7% on arrival at the feedlot to 62% at slaughter (26). Some studies have suggested that an increase in the prevalence of *Salmonella spp.* could be attributed to many factors including; environmental factors, stress, other wildlife and/or feed type (13, 16, 27, 58). One or any combination of these various factors could have attributed to the erratic shedding of *Salmonella spp.* in this study.

The second portion of this experiment dealt with analyzing the antimicrobial resistance patterns (AMR) found in the *Salmonella* isolates. Overall, AMR patterns

were similar in both the cattle and calves with highest rates of resistance observed against tetracycline, streptomycin, chloramphenicol, ampicillin, sulfizoxazole and amoxicillin-clavulanic acid (amox_cla). These results were similar to reports from a recent study of antimicrobial resistance in US beef cattle at the feed lot or stocker phase of production; in that study, similar AMR profiles was seen against ampicillin, chloramphenicol, sulfamethoxazole, tetracycline and streptomycin (46). Multiple studies have looked at antimicrobial resistance in *Salmonella spp.* isolated from cattle; they report that the greatest resistance is seen to tetracycline, streptomycin, ampicillin, sulfamethoxazole and increasing resistance to other antibiotics; ceftriaxone, spectinomycin and chloramphenicols (11, 24, 44). The similarity between antimicrobial resistant strains of *Salmonella* in cattle humans suggests that some of the resistance could be transferred horizontally among similar species.

One of the more interesting findings in this study was the high rate of resistance seen towards chloramphenicol in both the cattle and the calves, as this antimicrobial is not approved for use in US cattle operations. According to an online study, a similar drug florfenicol, a chloramphenicol analog that shares its resistance loci, is currently licensed as a therapeutic drug for cattle in the US. This study suggests that the close relationship between florfenicol and chloramphenicol may play a role in the increased rates of resistance to chloramphenicol (14).

Another study that examined the relationship between the two antimicrobials (chloramphenicol and florfenicol) indicated that *flo_{st}*, a gene which confers resistance to chloramphenicol and florfenicol, has become a common genotype of *Salmonella* and confers resistance to both antimicrobials in the DT104 (14). Furthermore, the study reports that use of florfenicol may further compromise the use of chloramphenicol as a possible treatment for salmonellosis in cattle (7, 40).

Overall, the AMR patterns appeared to have a similar distribution in both groups. The resistance patterns indicated that the final sampling, June/July 2009 samplings, in calves and the first sampling, September 2008, in cattle as having the highest proportion of AMR isolates. The lowest AMR prevalence was observed in both groups in the November 2008 sampling. The similar prevalence of resistance could be attributed to similarity in the fecal shedding of *Salmonella* and the proportion of resistant isolates being shed.

In our study, we observed 55.5% of total *Salmonella spp.* isolates showing multiple drug resistance, resistance to 2 or more antimicrobials. Multiple drug resistance (MDR) has been on the rise with the continued use of antimicrobials in feed products. A 2002 study reported that MDR increased by as much as 35% in isolated *Salmonella spp.* from 1999 to 2000 (51). The 55.5% of total *Salmonella spp* with MDR reported in this study represents a continued increase in MDR from that

reported in the year 2002 underscoring the need for continued surveillance of AMR in *Salmonella* globally including factors responsible for the observed increase.

The final portion of the study analyzed the relationship between the presence of Integron 1 and antimicrobial resistance of the *Salmonella* isolated. Although not all resistance profiles could be attributed to Integrons in our study, a high percentage of resistance (71.1% to 91.1%) was attributed to the presence of integrons as measured by the attributable fraction. Overall, 100 (50%) of the *Salmonella* isolates tested contained the Integrase 1 gene, 88 (88%) of which were determined to have Integron 1 gene sequences. This is important, because these isolates in the future would have the ability to harbor resistance gene cassettes. Studies have indicated that the lack of integrons may be explained by the lack of gene cassettes or by a defective integrase (17).

The percentage (50%) of integrase 1 gene presence reported in this study was higher than the 20% to 30.8%, of *Salmonella* isolates, containing the integron 1 gene sequence reported by other studies (15, 24), and lower than that of another study that found that integron 1 was present in 60% (93/151) of isolated *Salmonella* (23). The discrepancy in integron mediated resistance may be attributed to various factors including; different levels of fecal shedding and/or due to the horizontal transfer of integron positive isolates to other species.

From the conserved sequence analysis, it was determined that the gene cassette carried by the integron gene in this study was the *aadA2* gene, which is approximately 500 bp in length and encodes resistance to streptomycin-spectinomycin. An earlier study (24) of resistance profiles of various *Salmonella* isolates reported that this gene is usually found in 1000 base pair amplicons which concurs with our study. Additionally, a 2003 also reported *aadA2* gene sequence in integron 1 positive, *Salmonella* isolates (15). Studies have reported that the *aadA* gene is a common gene cassette in integrons (38, 64).

One of the most common antimicrobials to which resistance is reported is tetracycline. Although our study showed a relationship between tetracycline resistance and the presence of integrase 1 gene, further analysis for presence of the integron gene was negative. According to a recent study, the most common mechanism in which tetracycline resistance is conferred is through the *tetA* gene, with some resistance being conferred by the *tetB* or *tetG* genes (24). This study also reports that tetracycline resistance is usually acquired by horizontal gene transfer; given how often resistance to tetracycline is seen (24).

Resistance to ampicillin was determined in our study to not be carried by class one integrons although other studies have indicated that ampicillin resistance is mediated though the *pse-1* and *oxa-1* genes, which are known to be integron mediated and to produce an amplicons of about 1200 bps (47). Another study (15)

that examined how resistance to ampicillin is mediated, reported that ampicillin resistance was often acquired by TEM Beta-lactamases, genes that are not often associated with integrons. Although some integron mediated resistance to ampicillin was observed in that study, it was not the sole mechanism in which resistance was conferred (15). Although ampicillin resistance in our study was not found to be carried by integrons, future research is warranted in order to determine the mechanism for ampicillin resistance. Additionally, a statistical association between chloramphenicol resistance and the presence of integron 1 was reported in this study. However, the previously described gene $flo_{st,}$ known to carry resistance for chloramphenicol is generally not carried by Integrons (14). Further research is therefore needed in order to determine the mechanism for chloramphenicol resistance in our *Salmonella* isolates as the sequence analysis did not suggest presence of the gene flo_{st} on the integron.

CONCLUSION

In conclusion, this study provided important information on variation in the prevalence of *Salmonella* in beef cattle from pasture (calves and cattle) to the feedlot and at slaughter (calves). Additionally, the study provided data on AMR patterns of *Salmonella* shed by beef cattle at the different stages of production with the highest rates of resistance reported towards tetracycline, sulfizoxazole, streptomycin, chloramphenicol, ampicillin, and amox_cla. Also, an association between AMR towards the various antimicrobials tested and presence of integrase 1, on the *Salmonella* isolates recovered was investigated. A relationship between the presence of integron 1 and resistance was only established for streptomycin. Further research would be necessary in order to determine specific mechanisms in which the other resistance was conferred and also where the integron was integrated into the bacterial DNA. This study was able to contribute significant data towards risk assessment of *Salmonella* shedding and AMR trends in beef production, information that will allow for development of appropriate control measures for this important foodborne pathogen in the beef industry.

REFERENCES

1. Ahmed, A.M., Ishida, Y. and Shimamota T. (2009). Molecular characterization of antimicrobial resistance in *Salmonella* isolated from animals in Japan. *Journal of Applied Microbiology*. 106: 402-409.
2. Amita, C., Chowdhury, S.R., Thunapathra, M., Ramamurthy, T., Balakrish Nair, G. and Ghosh, A. (2004). Class I integrons and SXT elements in El Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. Available from: URL: http://www.cdc.gov/ncidod/EID/vol9no4/02-0317.htm
3. Arthur, T.M., Bosilevac, J.M., Brichta-Harhay, D. M., Kalchayanand, N., King, D.A., Shackelford, S. D., Wheeler, T.L. and Koohmaraie, M. (2007). Effects of a minimal hide wash cabinet on the levels and prevalence of *Escherichia coli* O157:H7 and *Salmonella* on the hides of beef cattle at slaughter. *Journal of Food Protection.* 70:1076-1079
4. Arthur, T.M. Bosilevac, J.M., Brichta-Harhay, D. M., Kalchayanand, N., King, D.A., Shackelford, S. D., Wheeler, T.L. and Koohmaraie, M. (2008). Source tracking of *Escherichia coli* 0157:H7 and *Salmonella* -contamination in the lairage environment at commercial U.S. beef processing plants and identification of an effective intervention. *Journal of Food Protection.* 71:1752-1760.

5. Barham, A. R. Barham, B.L., Johnson, A.K., Allen, D.M., Blanton Jr., J.R. and Miller, M.F. (2002). Effects of the transportation of beef cattle from the feedyard to the packing plant on the prevalence levels of *Escherichia coli* 0157 and *Salmonella* spp. *Journal of Food Protection.* 65:280-283
6. Bass, L., C.A. Liebert, M.D. Lee, A.O. Summers, D.G. White, S.G. Thayer, and J.J. Maurer (1999). Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli. Antimicrobial Agents and Chemotherapy* 43:2925-2929
7. Bolton, L.F., Kelley, L.C., Lee, M.D., Fedorka-Cray, P.J., Maurer, J.J. (1999). Detection of multidrug-resistant *Salmonella enterica* serotype typhimurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. *J. Clin. Microbiol.* 1999 37: 1348-1351
8. Brichta-Harhay, D.M. Guerini, M.N., Arthur, T.M., Bosilevac, J.M., Kalchayanand, N, Shackelford, S.D., Wheeler, T.L. and Koohmaraie, M. (2008). *Salmonella* and *E. coli* 0157:H7 contamination on hides and carcasses of cull cattle presented for slaughter in the United States – An evaluation of prevalence and load using immunomagnetic separation and direct plating methods. *Appl. Environ. Micobiol.* 74(20):6289-6297.

9. Cabrera, R., Ruiz, J., Marco, F., Oliveira, I., Arroyo, M., Aladuena, A., Usera, M.A., Jimenez D., M. Teresa, Gascon, J. and Vila, J. (2004). Mechanism of resistance to several antimicrobial agents in *Salmonella* clinical isolates causing traveler's diarrhea. *Antimicrob. Agents Chemother.* 48: 3934-3939
10. Centers for Disease Control and Prevention. (2008). About antibiotic resistance. Available at URL: http://www.cdc.gov/drugresistance/community/anitbiotic-resistance-faqs.htm
11. Chen, S., Zhao, S., White, D.G., Schroeder, C.M., Lu, R., Yang, H., McDermott, P.F., Ayers, S. and Meng, J. (2004). Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. *Appl. Environ. Microbiol.* 70: 1-7
12. Corrier, D. E, C. W. Purdy, and J. R. DeLoach. (1990). Effects of marketing stress on fecal excretion of *Salmonella* spp. in feeder calves. *Am. J. Vet. Res.* 51:866–869.
13. Dagataz, D.A., Fedorka-Cray, P.J., Ladely, S.R., Kopral, C.A., Ferris, K.E., and Headrick, M.L. (2003). Prevalence and antimicrobial susceptibility of *Salmonella* spp. Isolates from US cattle in feedlots in 1999 and 2000. *Journal of Applied Microbiology*. 95:753-761

14. Davis, M.A., Hancock, D.D., Bessner, T.E., Rice, D. H., Gay, J. M., Gay, C., Gearhart, L. and DiGiacomo, R. (1999). Changes in antimicrobial resistance among *Salmonella enterica* Serovar Typhimurium isolates from humans and cattle in the northwestern United States, 1982-1997. *Emerging Infectious Diseases. 5:6: 802-806*

15. Ebner, P., Garner, K. and Mathew, A. (2004). Class 1 integrons in various *Salmonella enterica* servovars isolated from animals and identification of genomic island SGI1 in *Salmonella enterica* var. *Meleagridis*. *J. Antimicrob. Chemother.* 53: 1004-1009

16. Fluckey, W.M, Longerhan, G.H., Warner, R. and Brashears M.M. (2007). Antimicrobial drug resistance of *Salmonella* and *Escherichia coli* isolates from cattle feces, hides and carcasses. *Journal of Food Protection*. 70:551-556

17. Fluit, A. C., Schmitz, F.J. (1999). Class 1 integrons, gene cassettes, mobility and epidemiology. *European Journal of Clinical Microbiology and Infectious Diseases.* 18 (11): 1435-1473

18. Foley, S. L., Lynne, A. M. (2008). Food animal-associated Salmonella challenges: pathogenicity and antimicrobial resistance. *J. Anim Sci.* 86: E173-187

19. Fossler, C.P., Wells, S.J., Kaneene, J.B., Ruegg, P.L., Warnick, L.D., Bender, J.B., Eberly, L.E., Godden, S.M. and Halbert L.W. (2005). Herd-level factors associated with isolation of *Salmonella* in a multi-state study of conventional and organic dairy farms I. *Salmonella* shedding in cows. *Prev Vet Med* 70, 257–277.

20. Fossler, C.P., Wells, S.J., Kaneene, J.B., Ruegg, P.L., Warnick, L.D., Eberly, L.E., Godden, S.M.., Halbert, L.W., Campbell, A.M., Bolin, C.A. and Geiger Zwald, A.M. (2005). Cattle and environmental sample-level factors associated with the presence of *Salmonella* in a multi-state study of conventional and organic dairy farms. *Preventative Veterinary Medicine.* 67: 39-53

21. Frost, A. J., D. O'Boyle, and J. L. Samuel. (1988). The isolation of *Salmonella* spp. from feed lot cattle managed under different conditions before slaughter. *Aust. Vet. J.* 65:224–225.

22. Gillings, M, Boucher, Y., Labbate, M., Holmes, A., Krishnan, S. Holley, M. and Stokes H.W. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *J. Bacteriol.* 190: 5095-5100

23. Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Philips, B., Register, B., Grady, M., Liebert, C., Summers, A.O, White, D.G. and Maurer, J.J. (2001). Incidence of class 1 and 2 integrases in clinical and commensal bacteria from

livestock, companion animals and exotics. *Antimicrob. Agents Chemother.* 45: 723-726

24. Guerra, Beatriz, Soto, Sara, Cal, Santiago, Mendoza, M. Carmen. (2000). Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. *Antimicrob. Agents Chemother.* 44: 2166-2169

25. Khaitsa, M.L., Doetkott, D.K., Kegobe, R.B., Bauer, M.L., Gibbs, P.S. and Lardy., G.P. (2005). Comparison of two sampling methods for *Escherichia coli* 0157:H7 detection in feedlot cattle. *Journal of Food Protection.* 68:1724-1728

26. Khaitsa, M.L., Kegobe, R.B., Bauer, M.L., Gibbs, P.S., Lardy, G.P. and Doetkott, D.K. (2007). A longitudinal study of *Salmonella* shedding and antimicrobial resistance patterns in North Dakota feedlot cattle. *Journal of Food Protection.* 70(2):476-481

27. Kunze, D., Loneragan, G.H., Platt, T.M., Miller, M.F., Besser, T.E., Koohmaraie, M., Stephens, T. and Brashears, M.M. (2008). *Salmonella enterica* burden in harvest ready cattle populations from the southern high plains of the United States. *Appl. Environ. Microbiol.* 74: 345-351

28. Li, Q., Sherwood, J. S. and Logue, C. M. (2004). The prevalence of *Listeria, Salmonella, Escherichia coli* and *E. coli* 0157:H7 on bison carcasses during processing. *Journal of Food Microbiology.* 21: 791-799

29. Looper, M.L., Rosenkrans Jr, C., Aiken, G. And Edrington, T. (2003). *Escherichia coli* and *Salmonella* in beef cattle grazing tall fescue. *Ark Agric Exp Stat Rep.* 509, 58–60.

30. Looper, M.L., Edrington, T. S., Flores, R., Rosencrans Jr, C.F., Nihsen, M.E., Aiken, G.E. and USDA, ARS. (2006) Prevalence of *Escherichia coli* 0157:H7 and *Salmonella* in beef steers consuming different forage diets. *Letters in Applied Microbiology*. 42: 583-588

31. Looper, M.L., Edrington, T.S. and Rosencrans Jr., C.F. (2009). Influence of body condition and forage type on prevalence of *Escherichia coli* and *Salmonella* in grazing beef cows. *Letters in Applied Microbiology*. 49: 361-365

32. Lopes, V, Wedel, S.D, Bender, J.B., Smith, K.E., Leano, F.T., Boxrud, D.J., Lauer, D.C., Velayudhan, B.T. and Nagaraja, K.V. (2006). Emergence of multidrug-resistant *Salmonella* enteric serotype newport in Minnesota. *Clinical Infectious Diseases.* 2006; 43:210-213.

33. Losinger W.C, Garber L.P, Smith M.A, Hurd, H.S., Biehl, L.G., Fedorka-Cray, P.J., Thomas, L.A. and Ferris, K. (1997) Management and nutritional factors associated with the detection of *Salmonella sp.* from cattle fecal specimens from feedlot operations in the United States. *Prev Vet Med.* 31: 231-244.

34. Lundin, J.L., Dargatz, D.A., Wagner, B. A., Lombard, J.E., Hill., A. E., Ladely, S.R. and Fedorka-Cray, P. J. (2008). Antimicrobial drug resistance of fecal *Escherichia coli* and *Salmonella* spp. isolates from United States dairy cows. *Foodborne Pathogens and Disease*. 5:1:7-19

35. Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology.* 3: 208-218

36. McEvoy, J.M., Doherty, A.M., Sheridan, J.J., Blair, I.S. and McDowell, D.A. (2003). The prevalence of *Salmonella spp.* in bovine faecal, rumen and carcass samples at a commercial abattoir. *Journal of Applied Microbiology.* 94: 693-700.

37. Mead, P. S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607-625

38. Meyer, E., Lunke, C., Kist M., Schwab, F. and Frank, U. (2007). Antimicrobial resistance in *Escherichia coli* strains isolated from food, animals and humans in Germany. *Infection* 36: 59-61

39. Mulvey M.R., Boyd, D.A., Olson, A.B., Doublet, B. and Cloeckaert A. (2006). The genetics of *Salmonella* genomic island 1. *Microbes and Infections,* 8(7):1915-1922.

40. Nogrady, N., Gado, I., Zsolt-Fekete, P. and Paszti, J. (2005). Chloramphenicol resistance genes in *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolated from human and animal sources in Hungary. *Vet. Med.* 50 (4): 164-170

41. Oloya, J., Theis, M., Doetkott, D., Dyer, N., Gibbs, P. and Khaitsa, M. (2007). Evaluation of *Salmonella* occurrence in domestic animals and humans in North Dakota (2000-2005). *Foodborne Pathogens and Disease.* 4:4: 551-563

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

43. Raked, N., Gassama, A., Denis, F., and Ploy, M.C. (2003). Characterization of a novel complex class 1 Integron in *Salmonella keurmassar.* Abstr Intersci Conf Antimicrob Agents Chemother Intersci Conf *Antimicrob Agents Chemother.* Sept. 14-17. Abstract: C1-1180

44. Ray, K.A., Warnick, L.D., Mitchell, R.M., Kaneene, J.B., Ruegg, P.L., Wells, S.J., Fossler, C.P., Halbert, L.W., May, K. (2007). Presence of antimicrobial resistance among *Salmonella* on midwest and northeast USA dairy farms. *Preventative Veterinary Medicine*. 79:204-223

45. Reid, C.A., Small, A., Avery, S.M. and Bunic, S. (2001). Presence of foodborne pathogens on cattle hides. *Food Control.* 13: 411-415

46. Riley, D.G., Loneragan, G. H., Phillips, W.A., Gray, J.T. and Fedorka-Cray, P.J. (2007). Fecal Shedding of foodborne pathogens by Florida-born heifers and steers in U.S. beef production segments. *Journal of Food Production. 71 (4): 807-810*

47. Rodriguez, I., Rodicio, M.R., Herrera-Leon, S., Echeita, A. and Mendoza M.C. (2008). Class 1 integrons in multidrug-resistant non-typhodial *Salmonella enterica* isolated in Spain between 2002 and 2004. *Journal of Antimicrobial Agents.* 32: 158-164.

48. Rogers, L. (2009). Food safety and the antibiotic resistance crisis. The Pew Charitable Trusts. Available at URL: www.saveantibiotics.org. Accessed on May 18, 2009.

49. Sandvang, D., Aarestrup, F.M. and Jensen, L.B. (1998). Characterization of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiology Letters.* 160: 37-41

50. Tabe, E., Oloya, J., Doetkott, D.K., Bauer, M.L, Gibbs, P.S. and Khaitsa, M.L. (2008). Comparative effect of direct-fed microbials on fecal shedding of *Escherichia coli* 0157:H7 and *Salmonella* in naturally infected feedlot cattle. *Journal of Food Protection. 71:3:539-544*

51. Threlfall, E.J. (2002). Antimicrobial drug resistance in *Salmonella:* problems and perspectives in food- and water-borne infections. *FEMS Microbiology Reviews.* 26: 141-148

52. University of Michigan (2007). Cattle Health: What is *Salmonella*? Retrieved at URL: http://www.cattlenetwork.com/content.asp?contentid=99407. Accessed on May 18, 2009.

53. United States Department of Agriculture, (2009). National antimicrobial resistance monitoring system animal isolates. Available at URL: http://www.ars.usda.gov/Main/docs.htm?docid=6750&page=3

54. Uyttendaele, D.T., P. and Debevere J. (1999). Incidence of *Salmonella, Campylobacter jejuni, Campylobacter coli* and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. *Journal of Food Protection. 62(7):735-740.*

55. Vimont, AJ, Kiermeier, A, Padula, DJ, Holds, GL, Pointon, AM (2005). The adequacy of sample type/weight and incubation period on detection of *Salmonella spp.* in slaughter cattle. *J Vet Diagn Invest* 17: 430-435

56. Vo. A. T., Duijkeren, E.V., Fluitt, A.D., Heck, A., Verbruggen, A., Zwaluw, K. and Gaastra W. (2006). Class 1 integrons in Dutch *Salmonella enterica* serovar Dublin isolates from clinical cases of bovine salmonellosis. *Veterinary Microbiology. 117 (2-4)*: 192-200

57. Voetsch, A. C. Van Glider, T.J., Angulo F.J., Marley, M.M., Shallow, S., Marcus, R., Cieslak, P.R., Deneen, V.C and Tauxe, R.V. (2004). FoodNet estimate of the burden of illness caused by nontyphodial *Salmonella* infections in the United States. *Clinical Infectious Diseases.* 2004; 38(Suppl 3):S127-34

58. Warnick, L.D., Crofton, L.M., Pelzer, K.D. and Hawkins, M.J. (2001) Risk factors for clinical salmonellosis in Virginia, USA cattle herds. *Preventative Veterinary Medicine.* 49: 259-275

59. Wells, S.J. Fedorka-Cray, P.J., Dargatz, D.A., Ferris, K. and Green, A. (2001). Fecal shedding of *Salmonella* by dairy cows on farm and at cull farm markets. *Journal of Food Protection.* 64 (1): 3-11.

60. White, D., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D. and Meng, J. (2001). **T**he Isolation of antibiotic-resistant *Salmonella* from retail ground meats. *N Engl J Med.* 345: 1147-1154

61. White, P. A., McIver, C.J. and Rawlinson W. D. (2001). Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* **45:**2658-2661. [PubMed].

62. Whittier, W.D. (2002) *Salmonella* in Cattle. Virginia State University. Available from Virginia Cooperative Education URL: http://www.ext.vt.edu/news/periodicals/livestock/aps-02_05/aps-093.html

63. World Health Organization (2005). Drug resistant *Salmonella* fact sheet. Available at URL: http://www.who.int/mediacentre/factsheets/fs139/en/

64. Zhao S., White, D.G., MacDermott, P.E., Friedman, S., English, L., Ayers, S., Meng, J., Maurer, J.J., Holland, R. and Walker R.D. (2001). Identification and expression of cephamycinase *bla* (CMY) genes in *E. coli* and *Salmonella* isolates from food animals and ground meat. *Antimicrobial Agents and Chemotherapy* . 45:3647-3650