Characterization of the relationship between rumen protozoa and enhanced *
Salmonella* virulence

**Principal Investigator:** Steve Carlson and Mark Rasmussen

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Background

Salmonella enterica serotype Typhimurium phagetype DT104 (DT104) is a multiple antibiotic-resistant pathogen that affects numerous hosts. In cattle, DT104 has been reported to be up to 13 times more deadly than antibiotic sensitive cohorts.

DT104 is resistant to five or more antibiotics. The standard biotype for DT104 is designated as AFSSuT indicating respective resistances to ampicillin (and other penicillin derivatives such as amoxicillin), florfenicol, streptomycin, sulfamethoxazole and tetracycline. The AFSSuT phenotype is directly related to the presence of an integron, a large DNA cassette that contains five antibiotic resistance genes. Additionally, some DT104 can acquire plasmids conferring resistance to ceftriaxone, gentamicin, kanamycin, apramycin or amikacin.

Our preliminary work revealed two interesting findings regarding DT104. First, the increase in DT104 lethality (virulence) appeared to be related to exposure to rumen protozoa (RPz) which are native microflora that ingest bacteria as a food source in the rumen. Second, DT104 can acquire antibiotic resistance plasmids while living inside RPz.

Our DT104-RPz model begins with DT104 being orally ingested thus entering the rumen. RPz then engulf the DT104 and provide a “safe haven” in which the pathogen can acquire new antibiotic resistance genes and can hyperactivate the expression of native virulence genes. The DT104-laden RPz then leave the rumen and enter the abomasum where they are in turn digested as part of the normal physiology of ruminants. RPz can also be lysed and digested in the rumen during rumen acidosis or “grain overload”. The DT104, now with new antibiotic resistance and virulence phenotypes, are then released in the abomasum. The pathogen is translocated to the intestines were it can invade the intestinal cells and cause enteritis with an accompanying systemic infection that is difficult to treat with most antibiotics.

Additional research was needed in that area to fully characterize the phenomenon. The complete characterization of the phenomenon will enable the initiation of studies examining preventative measures.

The stated objectives for this work were:

Characterize the relationship between rumen protozoa and enhanced virulence in multiple-antibiotic resistant Salmonella.

Methodology

(Step 1) Approximately 100 ml of post-prandial rumen fluid was removed from a 12 year-old non-lactating cow fed a standard hay and grain diet. Fluid was removed through a rumen fistula that was surgically introduced approximately 9 years previous. Rumen fluid was then filtered to remove large particulate matter and mixed with an equal volume of Coleman’s Buffer D. Protozoa were then allowed to settle for 2 hr under CO2. Settled protozoa were aspirated and washed twice with approximately 45 ml Coleman's Buffer D then centrifuged for 20 sec at 230 x g. Pelleted protozoa were resuspended in 30 ml Coleman's Buffer D under CO2. One ml was used for enumeration and 3 ml (approximately 105 RPz) were used in each assay.

The Salmonella/RPz mixture was then gently rolled for 16 hrs at 37oC in a sealed 5 ml glass tube. At the end of the 16 hour incubation period, extracellular Salmonella were killed using 300 µg/ml florfenicol. RPz were then centrifuged at 15,000 rpm for 2 min. then resuspended in 350 µl.
Lennox L broth. 25 µl was used for enumeration while 325 µl (approximately 4 x 108 CFU of *Salmonella*) was used for infection. The 325 µl was placed in a gelatin capsule and the capsule was orally introduced into 1-2 week-old calves (approximately 50-100 lbs. each) immediately followed by 500 ml of commercial milk replacer. Control calves were challenged with DT104 exposed to RPz buffer or DT104 present in non-RPz cells. Calves were monitored for changes in appetite, stool consistency and rectal temperature every 8-12 hours. At 36 hours post-infection, calves were euthanized using xylazine (1 mg/lb., intramuscular; Phoenix Laboratories) and pentobarbital (2.6 mg/lb., intravenous; Fort Dodge Laboratories) and tissues were aseptically removed. Tissues collected included the spleen and mesenteric lymphatic tissue (e.g., ileocecal lymph nodes, celiac lymph nodes and gut-associated lymphoid tissues).

Tissue samples (1.5-3 gm) taken from calves were homogenized with a rubber mallet and a stomacher. Homogenates were then subjected to the most-probable number enumeration procedure using a series of selective media preparations. The identity of *Salmonella* strains was confirmed using fluorescence, antisera/agglutination-based serogrouping, and PCR.

(Step 2) To create TnZeo, the zeocin resistance gene was PCR-amplified from pCRXL (Invitrogen), with overhanging SalI and BamHI sites engineered onto the forward and reverse primers. Amplicons were digested with SalI and BamHI restriction enzymes and cloned into the pMOD multiple-cloning site. The full-length zeocin resistance-bearing transposon (TnZeo) was obtained by PCR-amplification using primers that flank the 19-bp mosaic end sequences. Amplicons of TnZeo were then incubated with EZ::TM transposase, resulting in the EZ::TnZeo transposome.

(Step 3) Not necessary

(Step 4) Not necessary

(Step 5) The hilA promoter and floR were individually cloned from DT104 using PCR. Using recombinant PCR, the hilA promoter was fused to the 3’ end of floR and then cloned into pCR2.1. This plasmid was transformed into DT104, thus generating DT104/philAp-AsfloR, and florfenicol resistance was determined using serially dilutions of florfenicol. We found that the minimum inhibitory concentration of florfenicol was 8 µg/ml for DT104/philAp-AsfloR recovered from RPz.

(Step 6) The EZ::TnZeo transposome was electrottransformed into DT104/philAp-AsfloR using approximately 1010 bacteria and 0.1µg of transposome in 0.2 cm cuvettes, 2.5 kV and 25°C in the BioRad GENE PULSER II. Transformants pools were incubated with RPz then recovered and grown on plates containing 32 µg/ml florfenicol. One florfenicol-resistant colony was isolated and expanded for genomic DNA isolation (G NOME™ DNA Kit, BIO 101). DNA was digested with EcoRV then self-ligated with T4 DNA ligase. Inverse PCR was performed on the self-ligated fragments. Subsequently, the amplicons were gel-purified, cloned into a vector for amplification purposes. Purified DNA was then prepared and submitted for DNA sequencing.

(Step 7) For in vivo studies with DT104 lacking SO13, calves were infected as described in Step 1. Additionally, however, *Salmonella* virulence was assessed in 6-8 week-old calves that were defaunated using 0.7 mg/kg of the surfactant dioctyl sodium sulphosuccinate via oral drench.

(Step 8) Antibiotic-resistant *Salmonella* and ceftriaxone-resistant *Klebsiella* were co-incubated in the presence of RPz in vitro, using RPz derived from a fistulated cow, or in vivo using 6-8 week old calves. Following 24-36 hours of co-incubation, ceftriaxone-resistant *Salmonella* were sought and enumerated using *Salmonella*-selective media. Putative ceftriaxone-resistant *Salmonella* were confirmed as such using PCR.

Besides the transfer of an antibiotic resistance plasmid bearing a ceftriaxone resistance gene, we also assessed the *Klebsiella* to *Salmonella* transfer of a colicin-encoding plasmid in vivo. Co-
incubations were as described above. Additional experiments included assessing the Klebsiella to Salmonella transfer events in defaunated calves.

Findings

(A) In vivo characterization of RPz-mediated hypervirulence for DT104

Our bovine infectivity experiments showed conclusively that DT104 is more virulent after exposure to RPz. Specifically, calves infected with RPz-exposed DT104 had 40-80 times greater pathogen load when compared to calves infected with DT104 not exposed to RPz. Clinical signs, e.g. diarrhea, dehydration and pyrexia, were more robust and had an earlier onset in calves infected with DT104 exposed to RPz.

(B) Identification of a DT104 gene involved in the RPz-mediated hypervirulence

Using the novel hilA-floR reporter system and the novel zeocin transposon, we were able to identify a DT104 that upregulates invasion of DT104 resulting from exposure to RPz. This gene is designated as SO13 and it is present in the DT104 integron designated as SGI1. Besides containing five antibiotic resistance genes, SGI1 contains 20 other genes some of which do not yet have an ascribed function. It appears that SO13, one of the SGI1 genes without a known function, expression is activated while DT104 is inside RPz and that the SO13 protein can enhance the expression of hilA. Since hilA is the key regulator of Salmonella invasion, i.e. the process whereby Salmonella physically penetrates intestinal cells in order to gain access to the systemic circulation, the enhancement of hilA expression leads to and enhancement of invasion with a resulting augmentation of virulence. Studies addressing the molecular basis for the SO13-mediated upregulation of hilA expression are currently underway.

(C) Salmonella acquires antibiotic resistance genes within RPz

Using in vitro assays with RPz and in vivo assays with calves, we have found that Salmonella can procure antibiotic resistance genes while inside RPz. Specifically, Salmonella can acquire ceftriaxone resistance plasmids by conjugating with another bacterium that does not survive within RPz. Since Salmonella can survive within RPz and since we estimate that there are approximately 1015 RPz present in the rumens of cattle in the U.S., RPz can unfortunately serve as an origin for multi-resistant Salmonella. This specific transfer event is especially troubling since: ceftriaxone is the “last line of defense” for treating multi-resistant Salmonella in children; ceftriaxone resistance can lead to cross-resistance to ceftiofur, an important antibiotic used to treat respiratory infections in cattle. Fortunately, this problem may be preventable as discussed in the next subsection.

(D) Rumen defaunation prevents RPz-mediated hypervirulence and gene acquisition for DT104

Using in vivo assays with calves, we found that defaunation of the bovine rumen would prevent DT104 hypervirulence and Salmonella gene exchange. The defaunation agent used was dioctyl sodium sulphosuccinate which, unfortunately, has some short-term toxicity issues related to its surfactant properties. However, we are in the process of identifying a plant essential oil that can defaunate the bovine rumen without perturbing rumination and without causing toxicosis. Most essential oils are non-toxic and have anti-RPz activities while some oils do not have an impact upon rumination. It would also be beneficial to identify an essential oil with anti-Salmonella and anti-E. coli O157:H7 properties. We believe that cilantro oil may be a good candidate since cilantro leaves possess potent anti-Salmonella compounds. For the next NCBA grant cycle, we plan on submitting a proposal in which cilantro oil and other oils are examined for their effects upon RPz, rumination, and Salmonella and E. coli O157:H7.
Implications
The results of this study show that hypervirulence, i.e. the ability to cause a more profound disease, in DT104 is related to exposure to protozoa normally present in the first stomach (rumen) of cattle. These rumen protozoa hyperactivate disease-causing processes in DT104 and a single gene was found to underlie this phenomenon. Additionally, this research shows that rumen protozoa also promote antibiotic resistance gene exchange whereby Salmonella can acquire new antibiotic resistances. Fortunately, eliminating the rumen protozoa prevented both of these phenomena thus this elimination step may soon become a way to curb problems associated with Salmonella in cattle.

For more information contact:
National Cattlemen's Beef Association
A Contractor to the Beef Checkoff
9110 East Nichols Avenue
Centennial, Colorado 80112-3450
(303) 694-0305