ESCHERICHIA COLI IN BOVINE CALF SCOURS

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ABSTRACT

Scours is caused by inflammation of the intestinal tract of ruminants leading to significant mortality and morbidity rates. It is predominately found in neonatal ruminants where the disease can occur 36 hours after birth. One of the most common infectious agents linked to scours is pathogenic *Escherichia coli*. Therefore, it is important to understand the virulence factors, diagnostic assays, age of the animals infected, and the co-factors associated with an *E. coli* scours outbreak. These factors are important in both scours disease pathogenesis and potential food safety-related postharvest pathogens. Using the most frequently identified virulence factors, a new scours diagnostic assay could be created to detect and prevent disease in cattle. The present study determined that virulence factors *astA, fimC, fimH, int1, int2, irp2, papC* were identified over 15% percent of the time and could be implemented into a more specific multiplex PCR test for pathogenic *E. coli*.

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<u>INTRODUCTION</u>

Many deceased bovine calves presented at the NDSU Veterinary Diagnostic Laboratory (NDSU-VDL) with similar antemortem symptoms led the pathologist to request a scours panel to check for pathogens. Results of these tests indicate the fecal samples from some of the post-mortem specimens contained *Escherichia coli* (*E. coli*) resistant to many of the commonly used antibiotics such as ampicillin, ceftiofur, chlortetracycline, clindamycin, erythromycin, florfenicol, neomycin, oxytetracycline, penicillin, spectinomycin, sulphachlorpyridazine, sulphathiazole, tiamulin, tilmicosin, trimethoprim, and tylosin. These specimens were negative for *E. coli* virulence factors routinely tested for at the NDSU-VDL. Since the animal was sick it seemed inconsistent to have no pathogenic *E. coli* genes found. Our study investigated this inconsistency further.

In the present study, samples collected in 2004 were tested for 22 additional virulent *E. coli* strains to determine which of these were being detected in multi-drug resistant samples. These samples also showed no positive results for the six *E. coli* virulence factors tested for in the NDSU-VDL. A supposition was made that an *E. coli* virulence factor not included on the test was present in the calves and making them resistant to antibiotics and susceptible to disease. Testing for the 22 additional virulence factors would help define which other virulence genes were present in North Dakota and bordering Minnesota farms.

In addition to determining the specific gene present, additional genes could be added to the current NDSU-VDL test for *E. coli* virulence factors. The results of this study will not only help the NDSU-VDL provide more definitive results, but also help the producer and veterinarian narrow down a treatment and/or preventative option for the already ill or highly susceptible herds.

LITERATURE REVIEW

Infectious Disease Pathogenesis

All bacteria go through steps of survival inside a host which may lead to disease. For bacteria to survive inside a host, they must first find and recognize their specific host. If host attachment receptors are specific for a bacterium, the bacteria will attach and colonize the host and can lead to disease. If the host's immunity is not compromised or vaccines against a bacterium are given, the attachment and replication of the bacteria may be blocked. Thus, no disease will be caused. In the case of neonatal calf scours, many strains of *E. coli* cannot be considered harmless (1). If the host is immunosuppressed or they have not been treated with an effective vaccine, *E. coli* are able to attach, colonize and grow, thus leading to disease expression such as scours (11).

Once *E. coli* attach they need to obtain nutrients in order to survive and replicate. Their mechanisms for acquiring nutrients frequently cause disease in the host (7). *E. coli* is able to acquire specific nutrients such as amino acids, iron, trace elements, and a carbon source from their host. This is done by utilizing the host's nutrient supply. The primary nutrient is iron. If the iron is present, but not tightly bound, *E. coli* that produce iron acquisition genes that are more likely to access it and create an iron deficiency in the host (2). Bacteria growing in aerobic environments need iron to perform various tasks such as the oxygen decrease for synthesis of ATP, the decrease of the precursors of ribotides of DNA, and the creation of heme, to name a few. Because these are essential factors for the colonization of bacteria, the microorganism must obtain some form of iron for survival. This is done by requiring the microorganism to form molecules to compete with the hydroxyl ion of iron in its ferric state. If the iron is unavailable, siderophores will be dispersed and will carry the less tightly bound iron to the bacteria. The availability of the siderophores is governed by the gene *fur* (Ferric Uptake Regulation) and thus the two will compromise to bind the gene to the operon, usually Fe (II). Once binded, the siderophores are able to "carry" the iron back to the depleted bacteria (24).

As nutrients become available, *E. coli* will replicate and reproduce asexually. This is done by cell division, which form clones of themselves every time division occurs. A reoccurrence of all these steps happens with each clone, thus increasing the bacterial numbers to a capacity that eventually overwhelms the host, leading to a full blown scours disease (33). Following damage and death of the host's cells, the *E. coli* can be passed to a new host via infective feces, blood, respiratory secretions, feed and bedding (6).

<div align="center">Colibacillosis</div>

Colibacillosis is a diarrheal disease caused by the Gram-negative bacteria, *E. coli*. Colibacillosis has been linked to clinical symptoms, such as dehydration, diarrhea, fatigue, fever, malaise, and depression, in the calf between 3-5 days old. While *E. coli* is a common resident of the intestinal tract of calves, there are certain strains that are pathogenic. Pathogenic *E. coli* may cause diarrhea in ruminant neonates. If left untreated, the calf could develop colisepticemia, when the bacterial infection spreads into the bloodstream of the host (6). This could ultimately lead to death.

Garcia-Migura, et al. (11) performed research using a private herd in Toronto, Canada, and discovered the criteria for identifying a colibacillosis positive calf from those not infected. Their research identified a larger amount of *E. coli* in the small intestine along with lymph node involvement in calves displaying colibacillosis symptoms. Additionally, an absence of other commonly identified disease causing bacteria was also discovered in those calves. This study was used to determine a vaccine for the cows that would provide them with antibodies to combat the disease. The researchers found that cows who are vaccinated using specific *E. coli* K and O antigens have a lower mortality and morbidity rate in their offspring. This may indicate that the *E. coli* found in cases positive for colibacillosis in calves could possibly be of K or O serotype.

E. coli isolates are serologically separated according to their major surface antigens. The three major surface antigens are the O (somatic), H (flagellar), and K (capsulated) antigens. *Escherichia coli* can also be separated into categories based on properties specific to their characteristics (22). These include: virulence factors, specific clinical symptoms, and their distinct O:K:H serotypes (14). The H antigen is also commonly found in pathogenic *E. coli*, as well. This type

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uses its flagella to incorporate movement of the bacteria so they are able to gain as much nutrients as they need. The K serotype is a polysaccharide capsule that envelopes the bacteria whereas the O serotype is an actual part of the lipopolyssacharide molecule (12).

Bovine Calf Scours

Calf scours is a multifactorial disease that involves the dam, the calf, the calving facility, and the overall management of the herd. It is vital for a pregnant cow to receive optimum nutrition in order to ensure a healthy calf at birth. Their diet should include ample amounts of proteins and calories in order to guarantee a sufficient amount of brown fat in the offspring to supply energy that will increase calf vigor upon birth. Higher vigor, along with a healthy calf, will provide the energy needed for the calf to want to stand and nurse. Once the calf has received colostrum, the calf will obtain the acquired immunity that will assist in combating many pathogenic bacteria (3). It can affect up to 70 percent of a herd's offspring, with up to a 50 percent mortality rate. Because scours predominately affects calves, it is the cow-calf operations that are impacted the hardest. A scours outbreak can lead to a financial disaster for ranchers and may even lead to their closure (15).

Escherichia coli

Infectious agents can be bacteria, viruses, protozoans, parasites, or fungi. In scours, the most common infectious agent is pathogenic *E. coli*. A ruminant can become exposed to pathogenic *E. coli* as early as passage through the birth canal. Lactobacilli and enterococci are considered to be good bacteria and found in the first 30-40 feet of the small intestine and abomasum of a calf. If an abnormal presentation occurs along with trauma, the producer needs to supplement the calf with the dam's colostrum in order to safeguard its health so the acquired immunity enters the calf as soon as possible. The calf may not want to nurse due to the stress it endured therefore esophageal tubing should be performed (35). During the first few hours of a calf's life, it is crucial for them to receive adequate amounts of colostrum (37). Once the calf starts breathing, the small intestine starts to mature, beginning intestinal cell turnover, abosomasal acidity, and development of intestinal secretions and intra-epithelial vacuoles used in digestion. The intestine only absorbs macromolecules for 24 hours before the closure begins. This initial process is known as passive immunity. This is why

it is so important for the calf to receive immunoglobulins that are found in colostrum as soon as possible (31).

Colostrum is filled with immunoglobulins which play a role in limiting the growth and the colonization rates of *E. coli* as well as other infectious causes such as *Salmonella*, Coronavirus, Rotavirus, and *Cryptosporidium* (37). If the animal does not have maternal antibodies and has been exposed to pathogenic *E. coli* or other infectious agents the small intestine will quickly become colonized with the pathogen. This is also known as failure of passive transfer (31). The overwhelming pathogenic bacterial load results in increased financial losses for the producer. This is due to reduced weight gain, escalation of medical costs, or death of the infected calves (34).

Diarrheagenic *E. coli* cause diarrhea as well as extra-intestinal infections in both humans and animals of almost all species of warm-blooded vertebraes. All these groups shed *E. coli* into the feces of the infected host which aids in spreading to other hosts. There are six categories of *E. coli* that are known to cause disease. These six categories are: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC) and uropathogenic *E. coli* (UPEC) (6,22).

Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) are able to create lesions in intestinal epithelial cells in warm blooded mammals. EPEC uses humans as its primary host and is the number one killer in children under five years of age in developing countries. This is due to its ability to cause severe life-threatening diarrhea in the third world. Bacteria in this category do not produce shiga toxins and are not associated with hemolytic uremic syndrome (26). Despite humans being the primary host, this particular *E. coli* are a cause of disease and death in calves, piglets, rabbits, and both wild and domestic birds (26).

Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) are the main causative agent of traveler's diarrhea. The bacteria in this subcategory are known to affect the small intestine, and produce a heat-stable or heat-labile enterotoxin to create buildup of fluid and loose fecal matter (21). This particular type of

E. coli causes watery diarrhea in its host. It secretes toxins into the intestines. The most common genes produced are the heat-stable and heat-labile toxins (ST and LT)(19).

Enteroinvasive (EIEC) and Enterohemorrhagic (EHEC) *E. coli*

Enteroinvasive *E. coli* (EIEC) are associated with *Shigella* spp. Like *Shigella* spp., the EIEC are associated with the possession of a large plasmid, which encrypts the outer membrane proteins involved in the process. Bacteria categorized as EIEC are known to invade the colon and disrupt and grow inside epithelial cells which ultimately lead to cell death (21).

Enterohemorrhagic *E. coli* (EHEC) causes bloody intestine in its host. These bacterial strains secrete verotoxins which are lethal to African green kidney (vero) cells. They are also known as shigatoxins, due to the activity of the *Stx* (shiga toxin) gene in the *Shigella* bacteria. Thus, the EHEC producing strains can also be referred to as *Stx*-producing *E. coli*. The multiple strains placed into this subcategory are linked to infections that can be quite severe or even fatal. There are over 200 serotypes belonging to this EHEC subgroup (21).

Uropathogenic *Escherichia coli* (UPEC)

Uropathogenic *E. coli* (UPEC) are known to cause Urinary Tract Infections. They do this by infecting the kidney of the host and utilizing their expressed fimbriae to attach and colonize. The main fimbriae of concern is that of the P-fimbriae, which is encrypted by the *pap* gene, and is known to be an adhesion resistant to mannose. This fimbriae uses its adhesion unit to attach to the uroepithelial cells. Once adhered, it can successfully cause renal failure leading to a urinary tract infection. Along with the P-fimbriae, there are other virulence strains expressed. These include the fimbrial adhesion I, hemolysin, cytotoxic necrotonizing factor and the S fimbriae (18).

Virulence Gene Classification

Virulence genes are known to facilitate a microorganisms ability to establish itself on or within a host; in this instance, a calf. This will allow the pathogen to express toxins, adhesins, and enzymes enhancing the ability of the bacterium to cause disease. In this study, each virulence gene was categorized into one of these four categories: 1) Adhesion, 2) Toxin, 3) Iron Regulating, and 4) Antibiotic Resistance. The listed categories are explained in more depth below.

Adhesion Genes

Bacterial adhesion genes are bacteria that express adhesion proteins that reside inside a biofilm (26). This allows the bacterial population to adhere to each other and to surfaces. The specific bacteria that form biofilms must have flagella or another form of motility. They utilize their forms of motility to move along the surface of the intestine to find a nutrient rich area. Type 1 pili, required for the initial surface attachment, is encoded by the gene *fim* and has specific phenotypes that aid in its flagellar production. The *fim* gene clusters that are located on the operon, *fimH,* have lesions that affect the growth of the tip of the Type 1 pili. This will cause this particular gene to function as an adhesin specific to mannose and therefore cooperate with residues of mannose on the cells to enable cystitis (28). Other genes tested that produce adhesion operons are that of *papC, ibeA, sfa, fimC, afa, tsh, est1*, and *neuC.*

Toxin Genes

Toxin genes are known to cause bacterial death and significant defects in bacterial growth leading up to a decreased gene expression in each cell (28). This affects normal growth of the *E. coli* which can, in turn, cause a new, highly resistant strain of *E. coli* to form in the intestine. Toxin activity can be caused by a cytolethal distending toxin (CDT) due to its deadly effect on cells. This particular protein is said to distend the cells prior to killing them off. However, this protein is heat sensitive and causes differing distentions based on whether the toxins are heat-labile, heat-stable, or shiga-like (30).

E. coli capable of producing CDT will attach and efface the microvilli (AE) causing lesions via establishment of microcolonies, destruction of microvilli, and have massive amounts of actin microfilaments at their attachment site. Because of the large quantities of actin, a fluorescent stain can be used to determine the attachment and effacement activity. Performing this type of procedure has identified the presence of the *eae* toxin (25).

Shiga-like toxin producing *E. coli* (STEC) are known to cause food poisoning as well as severe/possibly fatal illness. Shiga-like toxin producing strains known to be pathogenic contain the phage-encoded cytotoxins called shiga-toxins (*stx1* and *stx2*) also known as verotoxins, and a protein

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called intimin. Intimin is responsible for the AE in the intestinal mucosa. The genes important to test were the shiga-toxins (*stx1* and *stx2*) as well as *eae* (Intimin)(27). These were tested along with other toxin-producing genes, *astA, vat, cnf,* and *elt1*.

Iron-Regulating Genes

Iron is an essential mineral that is utilized in a calf's metabolism and plays an important role in many of their systems. Nitrogen fixation and oxygen transport are just a few examples of what this mineral contributes to. The iron-dependent repression is facilitated by the Ferric Uptake Regulation (FUR), which uses genes involved in the previously mentioned systems. The bacterium creates a transport system through the use of siderophores (22). According to K.D Krewulak's research, siderophores solubilize the iron prior to transport then use a combination of receptors and transporters to create iron metabolism for the bacteria. This creates an advantage to the bacteria because it can utilize iron that was produced at another organism's expense or utilize other iron-acquisition systems that could otherwise steal iron in an inappropriate form. *E. coli* contains. The other way microorganisms will deal with iron deficiency is to obtain the iron extracellularly from lactoferrin, transferrin, and precipitated ferric hydroxides or by from hemoglobin intracellularly. The iron uptake process is established using the permeable outer membrane of Gram-negative bacteria. The trimeric β-barrel proteins housed in the outer membrane allow for solutes less than 600 Da. Because the lactoferrin, and transferrin are more than the 600 Da, they must find a way to execute the iron externally. The external binding of these molecules is done utilizing the proteins, TbpB/TbpA and LbpB/LbpA, by an N-terminal lipid anchor. The protein TbpB will perform as the first site for binding of the transferrin and make the additional binding to the TbpA go much easier. The TbpA and TbpB have large loops at the surface that separate the region surrounding the binding sites of the transferrin and lactoferrin to separate and release any present iron into the periplasm. Once in the periplasm, the iron is bound to the FbpA protein and transported to the cytoplasmic membrane and then into the cytoplasm using the cytoplasmic membrane transporters, FbpB and FbpC. Once out, the iron can be utilized by the bacteria for survival procedures. The iron regulating genes tested for in this study include: *irp1, irp2, iucD,* and Hly.

<center>Antibiotic Resistance Genes</center>

Antibiotics were founded by Alexander Fleming in 1928. Further research has found that the bacteria have been developing resistance to antibiotics. The bacteria can develop the resistance buy mutating and/or using horizontal gene transfer to swap DNA from other bacteria. Normally, the antibiotic will secure itself to a protein making the protein unable to function correctly. However, if the bacteria have some sort of mutation in their DNA that code for that protein, the particular antibiotic will be unable to fasten itself to the abnormal mutated protein and thus the mutated bacteria will live and keep reproducing more of the mutant bacteria. Because these mutants can live in the environment where the antibiotic is present, the bacteria will thrive and rapidly reproduce when the antibiotic is present and the illness will persevere (29).

Many physicians, veterinarians, and producers utilize antibiotic therapy in order to treat clinically sick neonates, which can in turn lead to an increased chance of antibiotic resistant bacteria. It has been noted that bacterial plasmids keep virulence factor encoding genes. These genes are used to encrypt virulence factors for scours. These genes can also be used in bacterial conjugation as well as antibiotic resistance. Presently, a number of the scours-related *E. coli* strains have developed resistance to numerous antibiotics, including but not limited to streptomycin, sulfisoxazole, ceftiofur, and tetracycline. Those particular antibiotics are screened using a sensitivity test in the NDSU-VDL Microbiology laboratory. Antibiotic resistant strains have the ability to inhabit the intestinal tract of antibiotic treated animals. Some bacteria live in biofilms, increasing the prevalence of antibiotic resistance (26).

Environmental changes to the surface of the intestinal cell are accepted by the biofilm to activate changes to permit the bacteria to undertake interactions to create antibiotic resistance. Newer commercially used antibiotics, such as aminoglycosides, fluoroquinolones, and expanded-spectrum cephalosporins, have been found to be quite effective against *E. coli* that cause scours in ill calves. However, because none of these antibiotics carry a label claim for use to treat enteric disease in calves, they can only be given as Extra Label Care Use in the United States under the Animal Medicinal Drug Use Act (36). Ceftiofur is another antimicrobial drug approved to treat respiratory

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disease in bovine and swine but does not have the label claim to treat scours. However, it is still often used to treat enteric diseases caused by multiresistant *E. coli* strains (36).

<center>**Preventative Measures**</center>

Two cattle producers in north central North Dakota found combating colibacillosis to be a difficult task (20, 34). Local veterinarians were able to help with preventative vaccination including ScourGuard 4KC (Zoetis, Kalamazoo, MI). This was administered to the cattle and heifers two to three weeks prior to their calving date. ScourGuard 4KC contains killed viruses of the serotypes G1 and G10 bovine rotavirus, coronavirus, enterotoxigenic strains of *E. coli* particularly those with K99 pili adhesion factors, and *Clostridium perfringens* type C. This vaccine is given to cows prior to calving ensuring the colostrum has these specific antibodies to help the nursing offspring build immunity to the previous mentioned toxins (Dr. Richard Lagasse, Rugby Veterinary Service, Rugby, ND, Personal Communication).

Cathy Balsiger, the lead herdsman/calf specialist at a local dairy barn in west-central Minnesota, has administered the vaccine Guardian (Merck, Summit, NJ), into her herd to decrease the chance of scours in the calves. She gives this vaccine, composed of a cell-free extract of K99 pilus of *E. coli,* two inactivated coronaviruses, two G serotypes of rotaviruses, and a bacterin-toxoid of *Clostridium perfringens* Types C and D to her 1350 dairy cows subcutaneously at dry off time, which is 50-60 days prior to calving, to create a stronger immunity to the above toxins in the offspring. This vaccine has reduced the prevalence of scours she had in the past, to less than 1 percent death loss (Cathy Balsiger, BGR Dairy, Lake Park, MN, Personal Communication). Dr. Richard Lagasse recommends Scour Bos 4 (Novartis Animal Health, Larchwood, IA) and Scour Bos 9 (Novartis) vaccinations to his clientele. Scour Bos 9 is given intramuscularly (IM) in the neck 8-16 weeks prior to calving. A revaccination with Scour Bos 4 should be given IM in the neck one month prior to calving to prevent infection. These vaccinations contain killed viruses of rotavirus, coronavirus, and bacterial species *Clostridium perfringens* Type C, and K99 pili *E. coli* (Dr. Richard Lagasse, Rugby Veterinary Service, Rugby, ND, Personal Communication) (Table 1). There are also vaccinations and oral medications to administer to newborns and include: Calf Guard (Zoetis),

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Bovine Ecolizer + C20 (Novartis Animal Health, Larchwood, IA), Bovine Ecolizer (Novartis), and Bar-Guard 99 (Boehringer-Ingelheim, St. Joseph, MO). There are other medications that aid in the prevention of scours, as well. The vaccinations and medications listed below were recommended by the attending veterinarian to prevent infection (Dr. Richard Lagasse, Rugby Veterinary Service, Rugby, ND, Personal Communication) (Table 1).

Table 1. Vaccine Recommendations for Bred Cows and Calves.

Vaccinations given to Bred Cows	Vaccinations Given to Calves
Scour Guard 4KC	Calf Guard
Guardian	Bovine Ecolizer +C20
Scour Bos	Bovine Ecolizer
	Bar Guard 99

Calf Guard (Zoetis) can be given to both cows and calves to protect against rotavirus and coronavirus. Cows receive two doses 3-6 weeks apart in late gestation. Calves are to receive an oral dose within 4 hours of birth. Bovine Ecolizer + C20 (Novartis) is another oral medicine given to calves immediately after birth, no later than four hours and protects against *Clostridium perfringens* Type C as well as K99 *E. coli*. Bovine Ecolizer (Novartis), an oral medication that contains K99 *E. coli* antibodies, is given to calves within 12 hours of birth to prevent scours. Bar-Guard 99 (Boehringer Ingelheim) is an oral medicine that contains whole culture K99 pili hyperimmunized blood from horses and should be given within 12 hours of birth for optimal results (Dr. Richard Lagasse, Rugby Veterinary Service, Rugby, ND, Personal Communication)(Table 1).

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Food Safety Distresses

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If a calf raised for veal becomes infected and is given antibiotics, a potential food safety threat is created since antibiotic resistance remains a large problem. If the calf displays severe infection after the administration of antibiotics, the producer may choose to slaughter early. After slaughter, leftover antibiotic residue may be present in the meat, posing a direct threat to the

consumer. This is against FDA regulations. Therefore, the producer, who assumes the risk, could receive a fine if proven guilty (13).

Conjugative plasmids are said to not carry genes fundamental to host cell growth in conditions that are not under stress. However, Wang, et al (39) found that almost all plasmids bestow particular phenotypes including but not limited to antibiotic resistance genes. Plasmids are very adaptable to DNA and will transfer the genetic information found in DNA. He noted in his discovery that prior to his research, *E. coli* isolates that had the extended-spectrum ß-lactamase-encoding genes mapped on plasmids had been found in food-producing animals and humans that were in good health. His research supported this finding (39). This indicates that plasmids can pose a problematic source for individuals who are found to be healthy implying that there is a risk in normal flora.

Escherichia coli contamination from the herd of cattle is a food safety concern as well. To prevent the shedding of *E. coli,* some producers add chlorine to animals' drinking water to help reduce any spread of the pathogen to other animals. The FSIS has no law prohibiting this procedure, since it is not harmful to the meat supply (9). Due to recent drought in some areas of North Dakota and Minnesota, additional drugs have been used to help livestock gain weight faster. Problems can arise since safety experts may not test for all of the drugs when the animal is slaughtered leading to meat that contains drug residue potentially causing harm to the consumer.

Overall, food safety plays an integral role in the livestock industry, since it remains an important part of the economy. An important aspect of food safety involves keeping the cattle healthy and disease-free in order to prevent costly outbreaks to humans. The latest outbreak known by the Center of Disease Control was in June of 2014 where people were sickened in four different states. Of these 12, 58 percent were hospitalized. Once the undercooked ground beef from various restaurants from the Wolverine Packing Company was found to be the source, 1.8 million pounds of it was indicated to be contaminated and a recall was released. Recalls such as this can be quite costly but precautions as simple as cooking ground beef to 160 degrees Fahrenheit should always be taken to decrease the chance of becoming ill. By reducing the amount of illness in the herd and cooking raw

meat to appropriate temperatures, one can safeguard a safe, enjoyable, consumable product at the

table.

Seventy-five highly resistant isolates obtained from clinical cases submitted to the North

Dakota State University-Veterinary Diagnostic laboratory (NDSU-VDL) from the year 2004 were

chosen for study. The age of the calf, location of farm or ranch (county and state), month in which

infected, final diagnosis, and any other statistics were noted. This data was compiled and analyzed.

The following figures show the geographical location where the isolates were located (Figure 1).
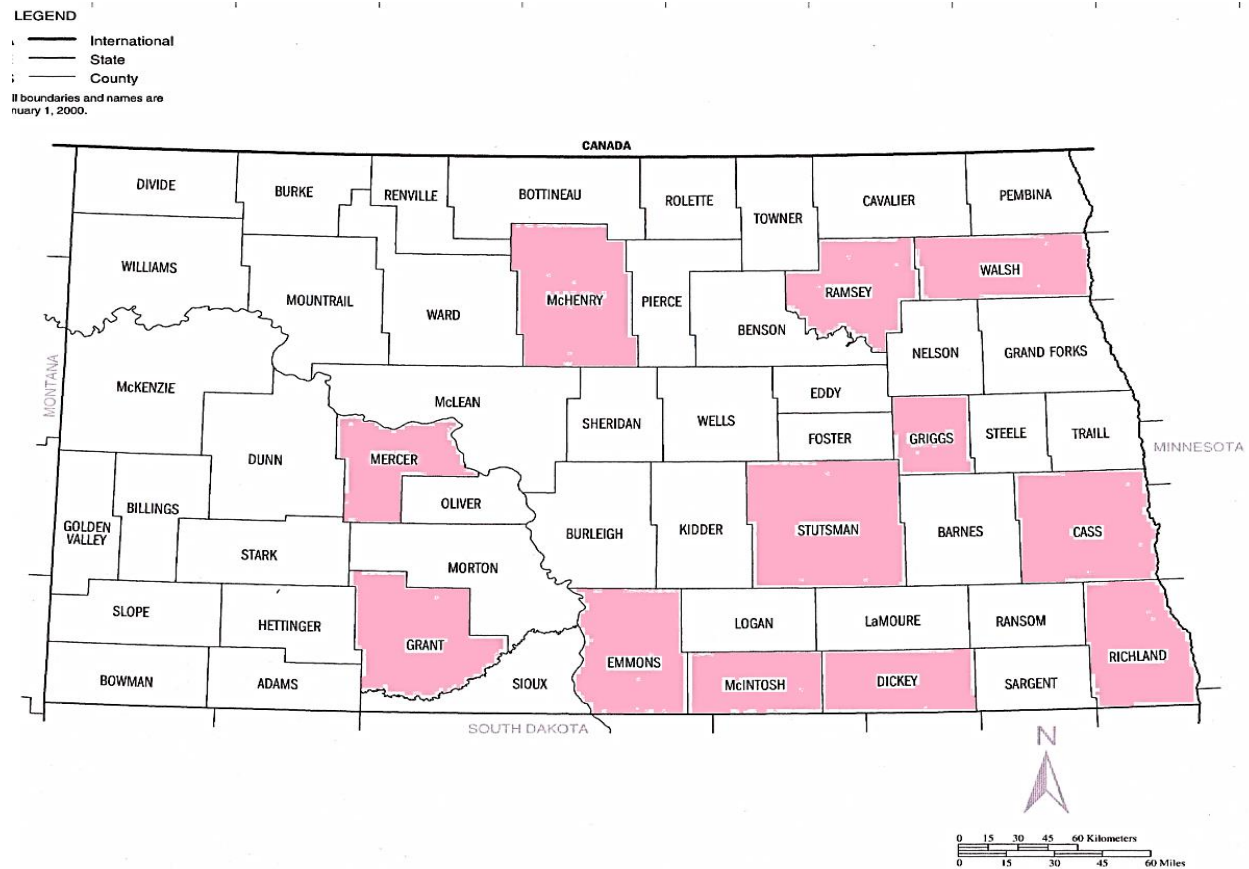

Figure 1. Map of North Dakota with Positive Scours Cases Highlighted.

Further analysis indicated a high percentage of calves being affected in March which is

consistent with the spring calving season that is found in North Dakota (Figure 2). Due to the

extreme low temperatures, many cows and older calves are housed in the same building/area as cows

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and newborn calves. The housing unit can become dirty very quickly and the producer must clean
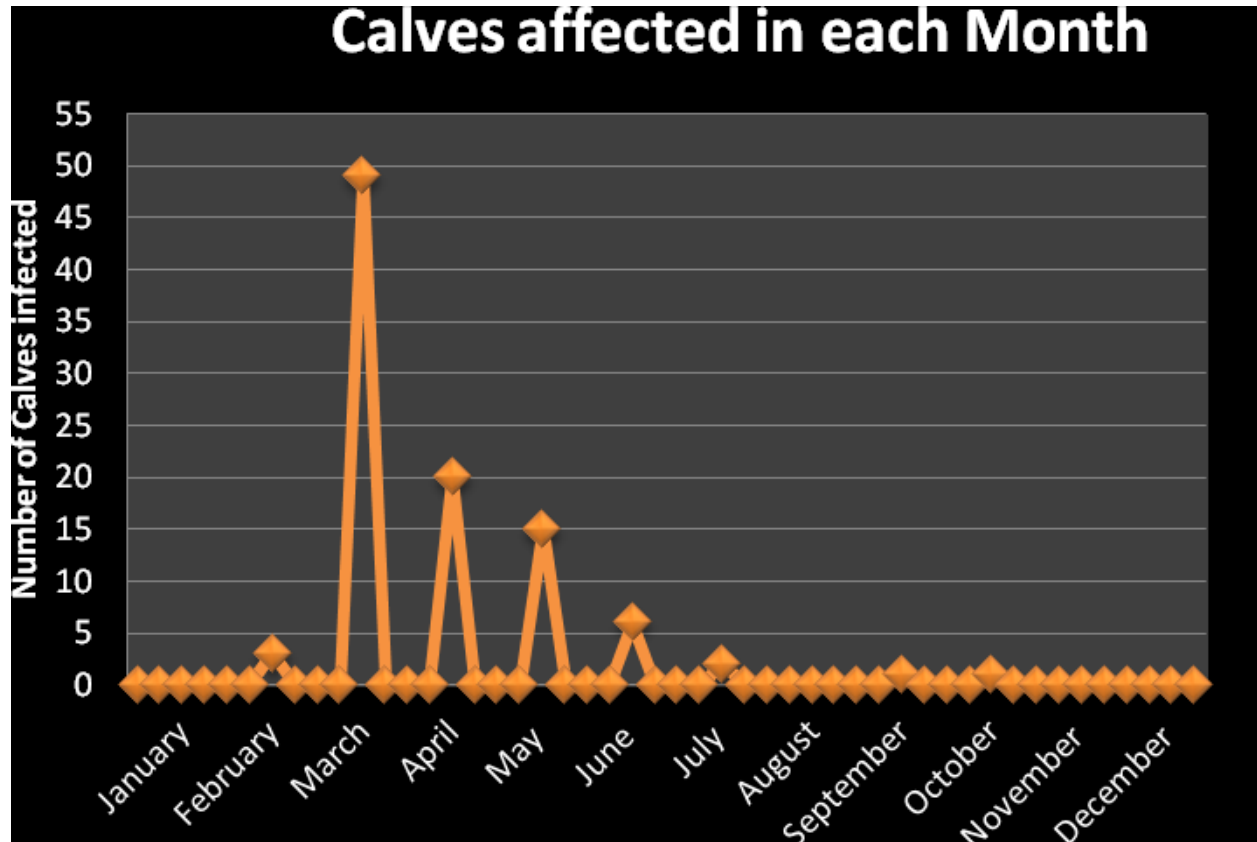
the area often (38).

Figure 2. Infection of Calves Per Month Breakdown.

The NDSU-VDL currently performs the polymerase chain reaction (PCR) test for only six

virulence factors, including: *K99*, *F41*, *Sta*, Intimin, *stx-1,* and *stx-2* (8). Many of these virulence

factors were not amplified during PCR despite *E. coli* being isolated from the sample. This may be

due to the overwhelming amount of other pathogenic virulence factors that may be present in the

animal. If other factors are shown to be useful in the typing of *E. coli*, a new diagnostic test utilizing

these factors could be developed.

The 2004 NDSU-VDL isolates were screened for the following virulence genes: *astA, cnf, hly*

EHEC*, hlyA, papC, eaeA, vat, irp1, int1, ibeA, fimH, stx1, stx2, sfa, fimC, afa, tsh, est1, elt1, iucD*,

and *neuC (*8). These virulence factors have been shown to have significance in the bovine and avian

intestine, as well as a source for foodborne illness in consumption of raw meat. Screening provides evidence as to which of the virulence is potentially pathogenic. Phylogenetic grouping was completed to determine the group each isolate falls into. Shiga toxins (*stx-1* and *stx-2*) have been shown to be harmful to public health.

The above virulence factors were screened using PCR. Each isolate was performed in duplicate in order to decrease the potential for human error. Positive strains, negative strains, and water were used as controls in each case. In order to determine the sizes of the amplified bands a 100 base pair ladder was used. Each isolate ran on a thermal cycler with parameters for the selected virulence factor.

Polymerase Chain Reaction Analysis Testing

The isolates were plated onto MacConkley agar and incubated at 37°C for 24 hours. Lennox broth (900 µL) was placed into 1.5 mL tubes and a separate colony of *E. coli* was added to each tube and incubated for 24 hours at 37°C with shaking. After 24 hours, 600 µL of glycerol was added to each tube, vortexed, and frozen in an -80°C freezer until needed for the addition of the individual primer master mix for the thermal cycler.

The PCR master mix consisted of 5 µL of 5x Green GoTaq Reaction Buffer (Promega, Madison, Wisconsin), 0.2 µL dNTP mix (Promega), 0.25 µL Forward Primer (Sigma Aldrich, St. Louis, Missouri), 0.25 µL Reverse Primer (Sigma Aldrich), 5.0 µL 5x GoTaq DNA Polymerase (Promega), and 17.175 sterile water. A master mix with the corresponding primers was made for all 22 virulence factors.

Table 2. List of Primers and Their Gene Products Used in This Study.

Primer	Direction (5'-> 3')	Gene	Bp size	Reference
Chua.1	Forward	*chuA*	279	3
Chua.2	Reverse			
yjaa.1	Forward	*yjaA*	211	3
TspE4C2.1	Forward	*TspE4C2*	152	3
TspE4C2.2	Reverse			
Int1-F	Forward	*Int1*	280	10
Int1-R	Reverse			
Slt-F	Forward	*Stx1*	614	17
Slt-R	Reverse			
SltII-F	Forward	*Stx2*	779	17
SltII-R	Reverse			
Sfa/foc-F	Forward	*Sfa/foc*	410	41
Sfa/foc-R	Reverse			
Afa-F	Forward	*afa*	750	41
Afa-R	Reverse			
IbeA-F	Forward	*ibeA*	171	41
IbeA-R	Reverse			
Irp1.A	Forward	*Irp1*	1691	2
Irp1.B	Reverse			
St-1B	Forward	*Est-1 a/b*	123	33
St-1C	Reverse			
LT-I/1	Forward	*Elt-1 a/b*	365	33
LT-I/2	Reverse			
EAST-1F	Forward	*astA*	110	33
EAST-1R	Reverse			
EHLY1	Forward	*hlyEHEC*	889	33
EHLY2	Reverse			
EHLYA-F	Forward	*hlyA*	534	41
EHLYA-R	Reverse			
AERA-F	Forward	*iucD*	692	33
AERA-R	Reverse			
TSH-F	Forward	*tsh*	804	33
TSH-R	Reverse			
PAPC-F	Forward	*papC*	482	33
PAPC-R	Reverse			
FIMC-F	Forward	*fimC*	476	33
FIMC-R	Reverse			
EAEA-F	Forward	*eaeA*	384	41
EAEA-R	Reverse			
VAT-F	Forward	*vat*	981	17
VAT-R	Reverse			
fimH-F	Forward	*fimH*	508	41
fimH-R	Reverse			
neuC-F	Forward	*neuC*	675	41
neuC-R	Reverse			
CNF-F	Forward	*cnf*	693	43
CNF-R	Reverse			

Corresponding to each program run on the thermal cycler, a series of temperature and time adjustments were completed (Table 2). All of these programs have steps that denature the template, anneal the primer, and extend the primer. The first step in all the programs begins at a temperature of 94°C or higher for 15 seconds to 2 minutes to denature the template. This step separates the DNA strands from one another, which then gives one the single-stranded DNA to replicate using the thermostable DNA polymerase. The next step drops the temperature to 40-60°C to allow for oligonucleotide primers to form associations with the denatured target DNA. These function as primers for the DNA polymerase. This step takes approximately 15-60 seconds. Lastly, the synthesis of new DNA starts as the temperatures is raised to the optimum for the DNA polymerase. This varies between programs but generally lasts about 1-2 minutes. The program will return to 94°C until stopped and samples are removed.

Gel Amplification

Following the thermocycler reaction, 10 microliters of sample from each reaction tube was placed in a 2% agarose gel. The gel was placed in a gel-casting tray in 5X TBE (trio/borate/EDTA) electrophoresis apparatus. The electrical field was adjusted to 150 volts and ran for 30 to 60 minutes depending on the size of the gel. The presence of bands and their sizes, which corresponded to the amp icon size expected using the specific primers was observed. The presence of virulence factors and the percentage of positive isolates were calculated. Those virulence factors found in many samples were deemed reasonable for use on a diagnostic test. Each step of the cycle was optimized for each template and primer pair combination. If the temperature during the annealing and extension processes was similar, the two steps can be combined and both the annealing and extending will takes place at the same time. After 20-40 cycles, the amplified band may be run on a gel to find particular band patterns. These band patterns signify whether the sample was positive or negative for that particular factor.
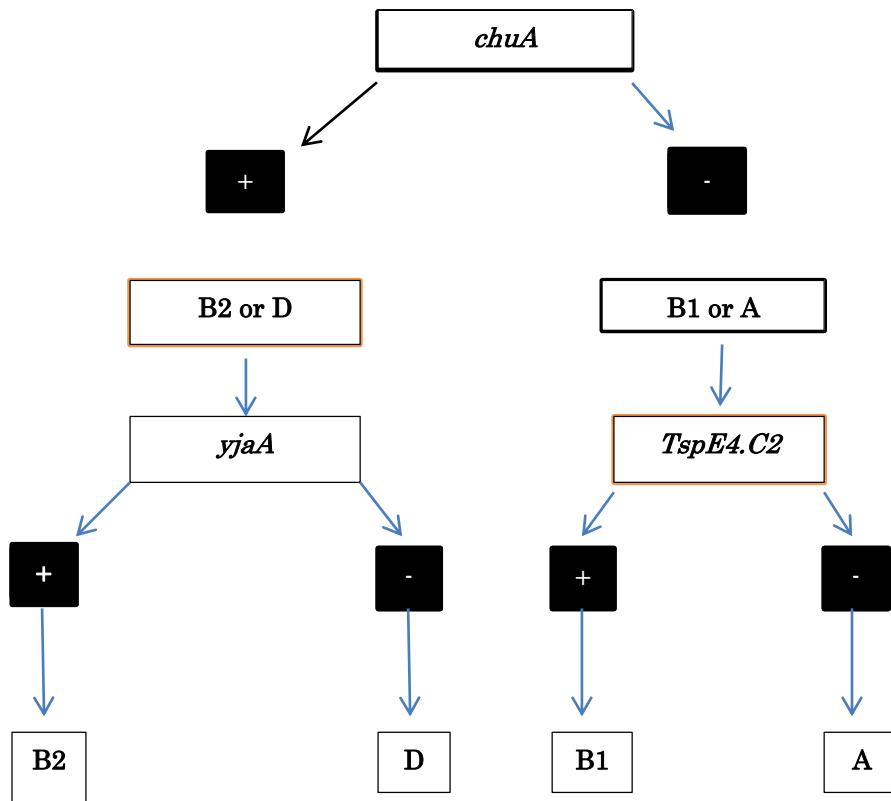
Figure 3. Phylogenetic Typing Diagram Clarification. (5)

Phylogenetic Typing Method

Determination of which gene goes where is done by a triplex PCR technique using genes, *chuA* and *yjaA*, along with an unknown DNA fragment. The technique for this test was done just like the others, using *chuA* as the initial determination gene. Those that produced a positive result were rerun with the same technique using primers for the gene, *yjaA*. A positive result in this step determined a final identification of group B2, whereas a negative result was determined as group D. A negative *chuA* gene result was rerun with primers detecting a region of DNA denoted and a new reaction was rerun using TspE4.C2(5). If the TspE4.C2 reaction was positive the resulting group was B1; if the isolate was negative for TspE4.C2 the resulting isolated was placed in group A (Figure 3).

<u>RESULTS AND DISCUSSION</u>

The results of the PCR testing are given in Tables 3-7. Field isolates of *E. coli* for five categories of potential virulence factors with each possessing different distinguishing features. For the purpose of this study, the virulence factors were classified based on specific characteristics for the particular gene. These include: adhesion, toxin production, iron regulation, presence of integrons/antibiotic resistance.

Table 3. Phylogenetic Typing Results Identified.

Phylogenetic Groups	Percent Positive
Group A	75
Group D	17
Group B1	3

Previously, the Phylogenetic Grouping (ECOR) was used to categorize various phylogenies of *E. coli* isolates. At that time, data indicated that more pathogenetic isolates fell into ECOR groups D and B2. However, additional data over the last decade has determined that phylogenetic grouping is less accurate in terms of normal flora vs. pathogenetic isolates. Our data supports the latter (Table 3). Although the majority of the tested isolates fell into ECOR groups A and B1, the normal flora is generally nonpathogenic. However, in the 2004 diagnostic lab cases, these calves were very ill with 100% mortality. This analysis led to looking more closely into what virulence factors were carried in the feces sample from the deceased bovine. The virulence factors picked for this study were chosen based on their virulence and antibiotic resistance profiles.

Table 4. Adhesion Gene Percent Positive Results.

Adhesion Genes	Percent Positive
hly EHEC	2.67
hlyA	5.3
est1	10.67
papC	36
int1	66.67
int2	41.3

The adhesion virulence factors genes tested were *neuC,,tsh, sfa, ibeA, hlyEHEC, hlyA, est1, papC, fimC*, and *fimH*. The first four genes (*neuC, tsh, sfa,* and *ibeA*) were negative in the samples tested and not put into the above table. The other genes had between 2.67% positive to 92% positive. The lowest percent positive was found in the gene *hly EHEC,* whereas the highest percent positive was *fimH*. The others had the following percent positives: *hlyA* with 5.3%, *est1* with 10.67%, *papC* with 36%, and *fimC* with 85.3% (Table 4).

These results indicate that *fimH* was the most prevalent factor found. This is in large part due to the genes ability to utilize its type 1 fimbriae to facilitate mannose binding to target host cells. It is most commonly found in urinary *E. coli* where it adheres to a host cell and gains replacements of amino acids which in turn increase the tropism for the uroepithelium and other components of the lower membrane. These replacement acid chains are monomannose and therefore have a mutation that helps the bacteria to colonize in the urinary tract and extraintestinal virulence of *E. coli*. This process helps the gene to become highly pathogenic and resistant to many antibiotics (35). Showing a second high of percent positives was that of *fimC*. This gene encodes for fimbriae that allow for attachment, however they do not utilize mannose. They create tight attachments to the host receptors and cause disease. Thirdly, the percent positives of papC were noted to be high in virulent

isolates. This virulence factor is known for its P pilus (pyelonephritis-associated pilus) and utilizes this pili to adhere to kidney tissues and allow for gram-negative bacteria to set up house-keeping to create mutations that allow for colonization (Table 4)(38).

Table 5. Iron Regulating Gene Percent Positive Results.

Iron Regulating Genes	Percent Positive
iucD	8
irp1	44
irp2	42.67

The Iron Regulating genes that were tested were *iucD, irp1,* and *irp2*. Of these three genes, *irp1* had the highest amount of positives found (44%) closely followed by *irp2* with 42.67%, and coming up the lowest was iucD with 8% positives (Table 5). Iron is the bacterial growth-limiting factor in all bacteria. The results of the iron regulating genes indicated many calves had these bacteria present. Iron that is not tightly bound allows the bacteria to multiply significantly causing a bacterial overload. The bacteria will then multiply and colonize. In this study, *irp1* was found to be the most significant iron acquisition gene.

During depletion or low iron, iron acquisition genes will bind to mRNA to reduce the translation of iron metabolism genes. The *irp1* will bind to an iron-sulfur cluster to conserve the energy production whereas the *irp2* gene will be corrupted. The other iron-regulating gene found to carry a significant amount of positives was that of the gene, *irp2*. This gene is less likely to be found in great abundance in cells due to its ability to be degraded. It functions the same as *irp1* but does not bind to an iron sulfuric cluster to regulate energy (16). Lastly, the iron-regulating gene found was *iucD*. This particular gene is known to be categorized as aerobactin that is formed when iron is being depleted in the tissues and fluids of the host. This gene is normally found on plasmids and is known for its product's ability to sift ferric acid from transferrin. The enzyme, aerobactin, is used to catalyze the initial step of the iron pathway (16). These genes all deplete the host of iron and energy, leaving the host drained and more susceptible to infection (Table 5).

Table 6. Toxin Gene Percent Positive Results.

Toxin Genes	Percent Positive
Stx1	4
Stx2	1.3
eaeA	5.3
Cnf	6.67
astA	22.67

The toxin genes analyzed were *vat, stx1, stx2, eaeA, cnf,* and *astA*. The gene with the most percent positives at 22.67% was *astA.* Following with 7% positive was *cnf* and lastly at 5.5% was *eaeA.* Toxin genes are known to cause host cell damage and/or death. Of the toxin genes tested, the gene *astA* showed the highest positive samples (Figure 4). According to Jacek Osek, the protein astA is found to have the presence of the colonization of factor F4, also known as the porcine fimbrial factor. This is known as the adhesion in ETEC to hold the most significance in pigs, but can also be found in other species, such as humans and cattle. The second high toxin gene found was the cytotoxic necrotizing factor (*cnf*). This gene's product is known to cause acute infections in the bladder and kidneys. It also triggers Rho GTPases which create mutations that increase the capacity of the particular *E. coli* strain. It will kill the neutrophils in the kidneys leaving the site open for toxic infection (31). The third most prevalent toxin gene was that of *eaeA*. This gene is called the attachment and effacement protein. Along with attaching to the intestine, it's product creates a mutation in the target gene that will kill off the microvilli. This causes a lack of absorption in the intestine leading to diarrhea (Table 6)(41).

Table 7. Presence of Integrons Percent Positive Results.

Integrons	Percent Positive
Int1	66.67
Int2	41.3

Two genes (*int1* and *int2*) were tested that are categorized this way. Of these two genes, *int1* had 66.67% positive while *int2* had 41.3% positive (Table 7). Integrons can be found on transposons and plasmids. Conjugation can also allow for antibiotic resistance. This is done by undergoing a mating process with another bacterium or by receiving resistant characteristics by the head of a virus (13). Two DNA components (*int1* and *int2*) were tested that are categorized this way. These two carry multidrug resistant *E. coli* strains. Knowing how notorious these specific DNA elements are for providing antibiotic resistance mechanism to bacteria and explains why they are both fairly high for percent positives (Table 7).

NDSU-VDL currently cultures the fecal matter of presented specimens to determine if *E. coli* is present. If the sample is hemolytic, it will go to the PCR lab to type the specific *E. coli* present. The *E.coli* test looks for only six virulence factors including *K99, F41, Sta, Intimin, Stx-1, and Stx-2.* Because assays for these factors often yielded negative results, it seemed pertinent to look into what factors other labs across the Midwest are using.

University of Minnesota Veterinary Diagnostic Lab performs aerobic culture to detect *E. coli*. If sample is hemolytic, the PCR lab is contacted and the sample is then sent through a PCR typing screen testing for the following virulence factors: *eae* (Intimin), F18 (cDNA encoding equine), K88 (marker for ETEC strains), F41 (adhesion), K99 (ETEC fimbriae), LT (Heat-Labile Toxin), STa (Heat-labile enterotoxin), STb(Heat-stabile enterotoxin), Stx2e (Shiga toxin 2e). The *eae* gene is the most common gene detected in the specimens that are tested. Another test this particular diagnostic lab performs is a full fecal work-up to rule out group A and B rotaviruses (using PCR), bovine coronavirus (using PCR) and *Salmonella* (using enrichment cultures) (Dr. Jeremy Schefers, University of Minnesota Veterinary Diagnostic Lab, St. Paul, MN, Personal Communication).

Iowa State University Veterinary Diagnostic lab has a calf diarrhea panel that tests for K99 *E. coli, Salmonella,* Coronavirus, Rotavirus *and Cryptospiridia*. This is done using a fecal sample from the sick animal. If addition testing is requested by the pathologist, a culture may be performed. For *E. coli*, they do not look into other virulence factors other than K99 (Dr. Timothy Frana, Iowa State University Veterinary Diagnostic Lab, Ames, IA, Personal Communication).

The present study could be used to identify virulent strains of *E. coli* that could potentially be included in a vaccine to be given to a pregnant cow to prevent scours. If the correct virulence factors are placed into an injectable or oral form of vaccine, producers would be able to administer this to their pregnant herd as a preventative measure to protect the fetus against some of the types of *E. coli* that can cause scours.

The present study utilized samples received from North Dakota and Northwestern Minnesota that had been sent in to the NDSU-VDL to define a cause of death in scouring calves. Many farms had more than one calf lost indicating a possible outbreak. This testing was performed to determine what other *E. coli* virulence factors could be in regional isolates that would enhance the detection capabilities of an *E. coli* multiplex PCR.

Iowa State University and University of Minnesota-Veterinary Diagnostic laboratories typed their *E. coli* samples using *eaeA, F18, F41, K99, LT, Sta, Stb, and Stx2*. NDSU tests for *K99, F41, Sta, Stx1, and Stx2*. Upon analyzing all the factors that are being tested in the three labs, one virulence factor was common. This factor was *K99*. North Dakota and Minnesota both test for *eaeA, Sta, Stx2, and F41.* This indicates that these genes are the most prevalent in the Midwest.

Any virulence factors noted in this study showing more than 15 percent positives could be added to a more specific diagnostic test. The reason for including those isolates positive per 15% of the time was due to the fact that a multiple PCR is only able to test a limited number of genes. The factors found to be more than 15 percent positives are: *astA, fimC, fimH, int1, irp1, irp2*, and *papC*. These factors were noted in only North Dakota and Northwestern Minnesota isolates; other geographical areas may have different virulence factors as other areas have miscellaneous bacterial growth. An example of this would be that of weather adaptations. In North Dakota, we have winter, therefore pathogens can freeze and become fewer and/or become more resistant to cold. In Texas, it is warmer and does not get as cold, therefore, those bacteria are more accustomed to warm temperatures.

Based on this study, it would be pertinent to add *fimC* and *fimH* to the current diagnostic test used by the NDSU Veterinary Diagnostic Lab as these two showed the highest percent positives indicating a high percent are multi-drug resistant and pathogenic. Possible future studies could implement more virulence factors into preventative medicines to help lower fatalities in North Dakota and northwestern Minnesota cattle herds. This would help to lessen incidence of scours by

preventing the problem from occurring and would also reduce the resistance of antibiotics since the calves would be protected from infections.

In addition to the previously mentioned future studies, another implementation could be taking the virulence factors mentioned above that were identified 15% percent of the time, culture them, and place the isolates directly into the calf's milk. This would be best done with bottle calves as it would be easier to administer orally. If the calf became ill a fecal sample should be taken to analyze for the presence of pathogens.

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