Feedlot, Cow-Calf & Dairy Combined Session

Dr. Otto Radostits and

Dr. Terry Church, *Presiding*

Escherichia Coli Enterotoxin in Calf Diarrhea

C. L. Gyles, *D.V.M.*
Department of Veterinary Microbiology & Immunology
Ontario Veterinary College, University of Guelph
Guelph, Ontario N1G 2W1

It is a pleasure for me to participate in this presentation and to continue this session by a discussion of the role of *Escherichia coli* enterotoxin in the production of diarrhea in calves. At the outset I would emphasize that *E. coli* is only one of several infectious agents implicated in calf scours, and that in addition, there are non-infectious causes of diarrhea in young calves. One of the major difficulties, at a practical and theoretical level, in implicating *E. coli* in diarrhea in calves is that this bacterium is a part of the normal flora and, when fecal samples from normal calves or diarrheic calves are cultured routinely, large numbers of *E. coli* are recovered since this organism is the dominant aerobic organism in the feces. Culture by itself, then, tells us very little about the involvement of *E. coli* in the disease process. Many of the very early studies attempting to implicate *E. coli* in calf diarrhea failed because the *E. coli* that were recovered from the scouring calves and used in the experiments were not capable of causing diarrhea. There were several other reasons for some of these failures which will become evident as we continue with our discussion.

In a culture of feces from a calf with *E. coli* diarrhea we note a profuse or heavy growth of *E. coli* of a single morphological type, and this occurs because the enteropathogenic *E. coli* tends to dominate the *E. coli* flora of the feces when it is the cause of disease. Typically, these *E. coli* are very mucoid—they produce an abundant amount of capsular material and this mucoid material tends to cause the colonies close to each other to merge. This characteristic appearance is helpful, but is certainly not definitive. It is possible to get a similar appearance when in fact *E. coli* is not causing diarrhea and sometimes *E. coli* causes diarrhea without assuming this marked dominance I have depicted. People have turned to serological typing of *E. coli* in an attempt to distinguish between the enteropathogenic ones capable of causing disease and the non-enteropathogenic ones which are unable to cause disease.

There is a limited number of 0 groups among enteropathogenic *E. coli:* 08, 09, 020 and 0101 are the types which are commonly implicated. There is also a fairly limited number of K antigens as well, and we find K99 antigen as a common feature of serological identification of these bacteria. Although the antigen identified as K99 has a K designation which suggests capsular antigen, it is in fact a fimbrial antigen.

After years of study, we now have a reasonable understanding of the process of development of diarrhea by enteropathogenic *E. coli* in a calf. There are still many gaps in our knowledge, but we have a good overview of the processes involved, and it appears that there are two major aspects to the disease problem. One involves colonization of the intestine and the other production of enterotoxins. One really cannot discuss one without the other. Let us start off by talking about the role of colonization in the production of diarrhea.

Many factors are involved in colonization. By colonization I mean the process by which enteropathogenic *E. coli* are able to attain very large numbers in the small intestine, an area in which normally *E. coli* are found in very small numbers. The inoculum size is important; the numbers of bacteria ingested by the calf, certainly under experimental conditions, need to be large in order to produce disease. In the field there is some evidence that for some strains of *E. coli* the numbers ingested need not be very large.

The age of the calf is a critical factor. This is attested to by the age range of calves in which we see *E. coli* diarrhea in the field - very young calves, most within the first few days of life. Similarly, it is not difficult to produce the experimental disease in calves less than one day of age, but after one to two days it becomes very difficult to produce disease despite introducing large numbers of organisms into the intestine of the calves. There is good evidence that not all strains of enteropathogenic *E. coli* are alike in their ability to produce disease. Some are very virulent, others are less virulent. Some of the virulence factors are known; others are still poorly understood. Gastric pH appears to be a very important factor in colonization and hence in disease. In the very young calf, gastric acid secretion is not well developed and gastric pH tends to be high. As the calf gets older, gastric pH becomes lower and the ability of gastric secretion to destroy ingested bacteria becomes more effective as the calf

gets older. We know, too, that gastric pH varies markedly with the time of feeding and with the quantity of feed. Normally in a young calf the gastric pH just before feeding is very low, of the order of 2-3. Immediately after feeding the pH rises to approximately 6, and slowly falls back to the pre-feeding levels of 2-3. Thus, much of the bactericidal effect of low gastric pH is lost on ingestion of large quantities of fluid.

Adherence of the bacteria to the intestinal wall is a critical part of the colonization process. Normally, bacteria which cannot adhere are washed through the intestinal lumen and do not have an opportunity to establish themselves. However, those bacteria which can adhere can attain very large numbers if they can multiply in the intestine. Thus the ability to adhere and multiply rapidly are important characteristics of bovine enteropathogenic *E. coli.*

In sections taken from the middle small intestine of young calves and viewed by scanning electron microscopy, the villi are long, the epithelial cells are intact, and we cannot see any evidence of bacteria, attesting to the low numbers of bacteria normally present in the small intestine. In the lower small intestine, the villi tend to be a little shorter, a little flatter and more tongue-shaped, and few or no bacteria are observed. The villi in the middle small intestine of calves infected with enteropathogenic *E. coli* have their surfaces completely covered by *E. coli.* These bacteria are firmly adherent to the villus epithelial surface: most of them adhere in a side-on manner, but some appear end-on as well.

There have been several studies which clearly demonstrate that the K99 antigen plays a dominant role in colonization of the calf intestine by enteropathogenic *E. coli* and for this reason the K99 antigen has been studied very extensively. We know that the K99 antigen exists as fimbriae which are protein structures which extend from the surface of the bacteria. Several aspects of the expression of K99 *in vitro* are interesting. For example, if bacteria which have the ability to produce K99 antigen are cultured *in vitro* at temperatures significantly below body temperature, 30° and below, the K99 antigen is not expressed. Similarly, several components of laboratory media will repress the K99 antigen; these include glucose and the amino acid alinine. This kind of information may be useful in helping to protect against calf scours due to *E. coli.* The production of K99 antigen is determined by a plasmid, which is simply an extrachromosomal piece of DNA which can be readily transferred from one bacterium to another. This means there is a potential that this factor which involves virulence can be transferred to new types of *E. coli* to convert non-pathogenic *E. coli* to pathogenicity provided they have the other attributes of virulence required for full pathogenicity. The K99 antigen is produced *in vivo* and in strains in which there is difficulty in getting the K99 to be expressed *in vitro,* one can readily get this K99 to be expressed *in vivo.*

We have reason to believe that not only is the K99 antigen important in colonization but the polysaccharide capsule produced by these bacteria also plays a significant role in colonization. The bovine enteropathogenic *E. coli* are organisms which produce mucoid colonies because of an abundant amount of capsular material. If one examines the role of the polysaccharide capsule in pathogenicity, it is quite clear it is very important. We do know that the polysaccharide is produced in abundant quantities in the intestine. When we conducted fluorescent antibody studies involving the capsular polysaccharide, we demonstrate very bright fluorescence because of the very large amounts of capsular polysaccharide produced. Furthermore, by transmission electron microscopy and studies involving direct staining of the polysaccharide capsule, we can show that these are very closely involved in the attachment of the organisms to the intestinal villi. There is some evidence, in fact, that perhaps both the polysaccharide K antigen and the fimbrial K99 combine to mediate adherence. These studies involve looking at polysaccharide K production *in vivo* by use of ruthenium red staining, a method which specifically stains capsular polysaccharide, and by use of specific ferritin-labelled antibody which indentifies the capsular polysaccharide. But perhaps the most telling experiments were those in which mutants which lacked one or the other of the K antigens were investigated for their pathogenicity. If we studied wild type *E. coli* which are quite capable of producing diarrhea and then used mutants which lacked either the K99 or the capsular polysaccharide antigen, we found that no disease was produced as a result of infecting calves with strains which lacked either the K99 or the polysaccharide K antigen. Further, we could demonstrate that this failure to produce disease was associated with a failure to colonize the small intestine. So much for the colonization aspect of development of diarrhea.

The second area of considerable importance is the production of enterotoxin, and initially, I want to make quite clear the distinction between enterotoxins and endotoxins. The term endotoxin has been around for a very long time and has so often been associated with production of disease in gram-negative bacteria that some confusion exists. Endotoxin is a large molecular weight lipopolysaccharide structure which is a part of the cell wall of gram negative bacteria. This substance is present in the cell wall of both pathogenic and non-pathogenic *E. coli* and although there was some belief some time ago that it was important in diarrheal disease, we now know that this plays no recognized role in production of diarrhea. It is important in septicemic disease because it can cause a number of very important effects such as fever, intravascular coagulation and shock when it is found in the bloodstream and in the organs.

There are essentially two types of enterotoxins produced by *E. coli* responsible for diarrheal disease in man and animals. One is referred to as LT because it is a heat-labile type of toxin readily inactivated by mild heat treatment. The other is referred to as ST for stable toxin because it resists a considerable amount of heating. LT is a large molecular weight protein which is highly antigenic and readily neutralized by specific antibody. In contrast, ST is a low

usable milk 24 hrs. sooner!*

when you treat Udder Edema with

Lasix®
(furosemide)

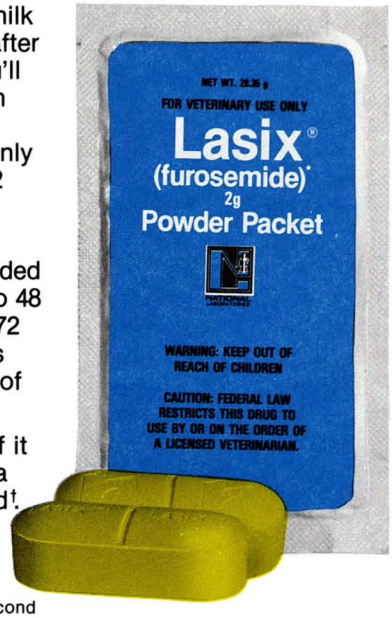
Powder Packet or Bol-O-Tabs® (2 g)

* It's the Lasix® advantage. Since milk must be withheld only 48 hours after the last treatment with Lasix, you'll have 2 extra usable milkings from each cow treated. Compare this with Naquasone, another commonly used remedy for udder edema: 72 hours (6 milkings) is the required withdrawal period.

And consider this: the recommended treatment period for Lasix is 24 to 48 hours. For Naquasone, it's 48 to 72 hours. In many edema cases, this could mean an additional saving of *2 TO 4 MORE MILKINGS!*

ANOTHER IMPORTANT POINT: if it is necessary to treat udder edema before calving, Lasix may be used†. *Naquasone may not be used during pregnancy because it contains the corticosteroid Dexamethasone.*

†Lasix is contraindicated only during the second trimester of pregnancy.



Safe- No risk of abortion.

Effective- Two-day therapy rapidly relieves edema, thereby lessening the risk of permanent udder damage.

Convenient- Empty contents of one packet per cow daily for two days as a top dressing on grain mixture.

Palatable- Readily accepted by cows.

Economical- No stress and associated milk loss with a feed top dressing. Milk production maintained following "milk-out" period.

AVAILABLE ONLY FROM LICENSED VETERINARIANS

BRIEF SUMMARY
(For full prescribing information, see package insert.)

Lasix® Powder Packet (2gm) Bol-O-Tabs®

CAUTION: Federal law restricts this drug to use by or on the order of a licensed veterinarian.

INDICATIONS
Cattle:
Lasix® (furosemide) is indicated for the treatment of physiologic parturient edema of the mammary gland and associated structures.

CONTRAINDICATIONS - PRECAUTIONS
Lasix® (furosemide) is a highly effective diuretic which if given in excessive amounts, may result in dehydration and electrolyte imbalance. Therefore, the dosage and schedule may have to be adjusted to the patient's needs. The animal should be observed for early signs of electrolyte imbalance, and corrective measures administered. Early signs of electrolyte imbalance are: increased thirst, lethargy, drowsiness or restlessness, fatigue, oliguria, gastrointestinal disturbances and tachycardia. Special attention should be given to potassium levels. Lasix® (furosemide) may lower serum calcium levels and cause tetany in rare cases of animals having an existing hypocalcemic tendency.

Although diabetes mellitus is a rarely reported disease in animals, active or latent diabetes mellitus may on rare occasions be exacerbated by Lasix® (furosemide).

Electrolyte balance should be monitored prior to surgery in patients receiving Lasix® (furosemide). Imbalances must be corrected by administration of suitable fluid therapy.

Lasix® (furosemide) is contraindicated in anuria. Therapy should be discontinued in cases of progressive renal disease if increasing azotemia and oliguria occur during the treatment. Sudden alterations of fluid and electrolyte imbalance in an animal with cirrhosis may precipitate hepatic coma; therefore, observation during period of therapy is necessary. In hepatic coma and in states of electrolyte depletion, therapy should not be instituted until the basic condition is improved or corrected. Potassium supplementation may be necessary in cases routinely treated with potassium-depleting steroids

WARNINGS
Lasix® (furosemide) is a highly effective diuretic and, as with any diuretic, if given in excessive amounts may lead to excessive diuresis that could result in electrolyte imbalance, dehydration and reduction of plasma volume, enhancing the risk of circulatory collapse, thrombosis and embolism. Therefore, the animal should be observed for early signs of fluid depletion with electrolyte imbalance, and corrective measures administered. Excessive loss of potassium in patients receiving digitalis or its glycosides may precipitate digitalis toxicity. Caution should be exercised in animals administered potassium-depleting steroids.

Sulfonamide diuretics have been reported to decrease arterial responsiveness to pressor amines and to enhance the effect of tubocurarine. Caution should be exercised in administering curare or its derivatives to patients undergoing therapy with Lasix® (furosemide) and it is advisable to discontinue Lasix® (furosemide) for one day prior to any elective surgery.

CATTLE: Milk taken from animals during treatment and for 48 hours (four milkings) after the last treatment must not be used for food. Cattle must not be slaughtered for food within 48 hours following last treatment.

Lasix® (furosemide) is not indicated during the second trimester of pregnancy.

DOSAGE AND ADMINISTRATION
The usual dose of Lasix® (furosemide) is 1 to 2 mg/lb body weight (approximately 2.5 to 5 mg/kg). A prompt diuresis usually ensues from the initial treatment. Diuresis may be initiated with Lasix® (furosemide) Injection 5% and maintained by oral treatment following a 12-hour interval.

DOSAGE:
Oral: CATTLE
The contents of 1 packet (2g) per cow daily to be administered with the animal's individual concentrate ration. **Treatment not to exceed 48 hours postparturition.**

Parenteral: CATTLE
The individual dose administered intramuscularly or intravenously is 500 mg (10 ml) once daily or 250 mg (5 ml) twice daily at 12-hour intervals. **Treatment not to exceed 48 hours postparturition.**

HOW SUPPLIED
Parenteral:
Lasix® (furosemide) Injection 5% (50 mg/ml)
Each ml contains: 50 mg furosemide as a diethanol-amine salt preserved and stabilized with myristyl-gamma-picolinium chloride 0.02%, EDTA sodium 0.1%, sodium sulfite 0.1% with sodium chloride 0.2% in distilled water, pH adjusted with sodium hydroxide.
Available in 50 ml multidose vials.

Oral:
Lasix® (furosemide) 2g Powder Packet
Each packet contains 2g of furosemide: 4-chloro-N-furfuryl-5-sulfamoylanthranilic acid plus inert ingredients.
Available in boxes of 12 packets each.

Lasix® (furosemide) 2 g Bol-O-Tabs®
Each bolus contains 2 g of furosemide: 4-chloro-N-furfuryl-5 sulfamoylanthranilic acid.

Available in boxes with 12 Bol-O-Tabs® each.
Store at controlled room temperature (59°-86°F).
Avoid exposure to light.
Tablets with 50 mg or 12.5 mg each are available for use in small animals.

Lasix® (furosemide) 2g Powder Packet
Manufactured By:
Hoechst-Roussel Pharmaceuticals Inc.
Somerville, New Jersey 08876

Lasix® (furosemide) Bol-O-Tabs®
Manufactured By:
Hoechst-Roussel Pharmaceuticals Inc.
Somerville, New Jersey 08876

Lasix® (furosemide) Injection 5%
Manufactured By:
Taylor Pharmacal Co.
Decatur, Illinois 62525

Distributed by

AMERICAN HOECHST CORPORATION
ANIMAL HEALTH DIVISION
Somerville, New Jersey 08876

N226-181
Printed in U.S.A.

molecular weight toxin which is very poorly antigenic. Typically, in bovine enteropathogenic *E. coli* ST, but not LT, is produced. The production of both types of toxin is governed by transmissible plasmids in much the same way as the K99 fimbriae are coded for by plasmids. So again there is potential for plasmids coding for enterotoxins to be transferred from pathogenic to nonpathogenic types of *E. coli,* but very few new serotypes of bovine enteropathogenic organisms have developed. In recent years a number of workers have shown that these plasmids may sometimes code for drug resistance which implies that use of these drugs may select for production of enterotoxin as well.

A number of assays are available for detection and quantitation of heat-stable enterotoxin. The first type of assay that was developed was the gut loop assay in which you put either the *E. coli* itself or ST preparations from the *E. coli* inside ligated segments of small intestine - and you look for the effect of the enterotoxin, manifested as an outpouring of fluid into the small intestine. The toxin has the effect of causing fluid accumulation and distension of the segment of the intestine. Strains that cannot cause diarrhea will cause no fluid accumulation at all. Another assay is the infant mouse assay which is more convenient and involves giving the toxin preparation to 4-day-old suckling mice by mouth or into the stomach. The mice are kept at room temperature for about 3-4 hours, killed and observed. There is tremendous distension of the intestine with fluid in response to the toxin. A number of workers, including our colleagues in Saskatoon, have purified this toxin and have shown that it is very active in nanogram quantities. One can quantitate the effect of the toxin by calculating the ratio of weight of intestine to weight of the remaining carcass of the mouse.

The end result of the two processes, that is colonization of the intestine and production of enterotoxin by the *E. coli* in close association with the intestinal epithelial cell, is that there is an outpouring of fluid into the intestine which we see as diarrhea. The fluid is essentially normal intestinal contents - we see some cells and simply a tremendous amount of fluid in infected animals. Sometimes we do note that blood tends to appear in the fluid and we do observe some mild histological change in some infected calves.

Very little is known about the mechanism of action of heat-stable toxin, although much is known about the mechanism of action of heat-labile toxin. What is known is that the activity of the heat-stable toxin is associated with stimulation of the enzyme guanyl cyclase and a consequent accumulation and increased levels of cyclic guanyl monophosphate in the intestinal epithelial cells. By unknown means these effect changes in the intestinal ion transport which we see as hypersecretion and excessive passage of ions and water to the intestinal lumen.

Having said what little we know about enterotoxins, the question arises - what are the potential uses to be put to our knowledge about *E. coli* enterotoxin in calf diarrhea? There are a number which spring to mind. First, as we understand more and more about the mechanism of action of the enterotoxin, our chances of developing drugs that would prevent or reverse the reaction will improve. There are a number of drugs, such as chlorpromazine and nicotinic acid, which have been shown to prevent or to reverse the outpouring of fluid caused by *E. coli* heat-stable enterotoxin. There is also the possibility that we might use this information to aid us in making more precise and more rapid diagnoses of *E. coli* diarrhea in calves. We know, for example, that it is possible to have a calf which is excreting enteropathogenic *E. coli* in the feces despite the fact that the enteropathogenic *E. coli* may not be causing diarrhea in the calf. These findings are very obvious in a recent study we did in collaboration with Dr. Butler, in which calves were infected with an enteropathogenic *E. coli* and were given colostrum containing antibodies against that type of *E. coli.* The calves did not develop diarrhea but excreted enteropathogenic *E. coli* in large numbers for a long time. The question arises - what if such a calf becomes infected with, say, a rotavirus? One would recover rotavirus plus an enteropathogenic *E. coli,* but the enteropathogenic *E. coli* may not be contributing to the diarrhea in these cases.

For this and other reasons, then, we decided to look at the possibility that detection of *E. coli* enterotoxin in the feces might be useful as an indicator of *E. coli* diarrhea. These studies are in progress. They indicate that we can sometimes detect *E. coli* enterotoxin in the feces of calves with *E. coli* diarrhea, but that we cannot do this consistently. We believe that the reason for the inconsistency may be that the levels of *E. coli* enterotoxin are very small and that we are very much at the margin of detection. Hence we are experimenting now with alternative methods for detection. We have tried the use of an enzyme-linked immunoabsorbant assay developed by workers in Belgium but the results proved to be no more sensitive than the infant mouse assay. We have turned to developing methods which will extract and concentrate enterotoxin very simply so that we can proceed to use this as an aid to a diagnosis of *E. coli* diarrhea.