Evaluation of Various Methods for the Detection of Enteropathogenic *Escherichia coli* in Scouring Calves

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Neonatal diarrhea of calves (calf scours) is a complex disease which can be caused by a variety of infectious agents proliferating alone or in combination with other microorganisms, and these include viruses, bacteria and protozoa. *Escherichia coli* has long been included as a causative agent in this disease complex (1,2,3,4,5).

Only certain specific strains of *E. coli* are enteropathogenic or possess the ability to produce calf scours. Since *E. coli* is a normal inhabitant of the intestinal tract, it is difficult to differentiate enteropathogenic *E. coli* from nonpathogenic *E. coli* of the digestive tract using routine culture methods. culture methods.

Enteropathogenic *E. coli* (EEC) possess unique features, described as "virulence factors," enabling these bacteria to produce scours. One of these "virulence factors" is a surface structure termed a pilus or a "hair-like" protein projection which facilitates attachment and colonization of EEC on the surface of mucosal epithelial cells lining the lumen of the small intestine. This pilus in EEC of calves is termed K99 (6,7). Other "virulence factors" are toxins (enterotoxins) released by EEC colonized on mucosal epithelial cells. These enterotoxins include a heat-stable and a heat-labile toxin whose net effect to mucosal epithelium is a loss of water and electrolytes into the lumen of the small intestine resulting in diarrhea in the calf. (8,9).

These "virulence factors" (K99, heat-stable and heat-labile toxins) of EEC, along with antibiotic resistance and hemolytic properties, are determined by extrachromosomal genetic elements called plasmids, which can be transmitted from one *E. coli* to another by conjugation (10).

Enteropathogenic *E. coli* of calves have been suggested to belong to a relatively few serological groups which are identified as O groups as determined by somatic antigens or O antigens. This in contrast to other animal species, such as baby pigs, where a large number of O groups are associated with enteric disease (11,12) and in earlier studies in calves which implicated a wider spectrum of O groups (13).

Since specific necropsy or histologic lesions are not always readily apparent in calf scours caused by EEC and no conspicuous differences are observable between EEC and normal *E. coli* inhabitants of the intestine using standard microbiological techniques, a procedure which would readily identify EEC and implicate its involvement in calf scours would be a valuable diagnostic tool.

Materials and Methods

E. coli isolates were obtained from necropsy specimens submitted to the North Dakota State Veterinary Diagnostic Laboratory during the springs of 1979 and 1980. All isolates were identified as *E. coli* by their reactions on 5% sheep blood agar, MacConkey, SS and Tergitol-7 agar as well as reactions as required on Triple Sugar Iron, Urea, SIM and Simmons Citrate media (all Gibco Diagnostics, Madison, WI).

Antisera O and K were purchased from the Department of Veterinary Science, Pennsylvania State University, University Park, PA. The O antisera were used as pools designated D, E, F and G and monospecific sera. The O antisera contained in pools were: Pool D, 08, 09, 020ab, 0101 and 0157; Pool E, 0141, 0139 and 0149; Pool F, 015, 017, 075, 078, and 088; Pool G, 06 and 026. K antisera was used as monospecific antisera for K99 with K88 and normal rabbit serum as controls. Fluorescein labelled goat anti-rabbit IgG was purchased from Miles Biochemicals, Elkhart, IN.

Enterotoxin assays for heat-stable toxin (ST) were performed, using the infant mouse bioassay. Several passages of *E. coli* (various isolates per case) in Trypticase Soy Broth or Brain Heart Infusion Broth (both Gibco Diagnostics) were made prior to 24 hr growth in Casamino medium (with shaking). Culture supernates with Evans blue dye added were administered to five-day old mice by intragastric injections. Following an incubation period (1 to 4 hr at 25°C) the animals were sacrificed, small intestine examined for presence of dye, fluid distension, weight ratio of gut to body (greater than 0.085 considered positive reaction to ST) and compared to negative and positive ST *E. coli* cultures (14).

Determinations were made on several isolates per case for O antigens. A 24-hr growth in Trypticase Soy Broth was divided in two, with one portion steamed for 1 hr and the other autoclaved for 2 hr at 121°C. Slide agglutination tests were conducted on these samples, using O pools and specific O antigens. Slide agglutination studies for K99 were made on isolates following 24-hr growth in Trypticase Soy Broth and 24-hr growth in Minca-isovitolex broth (with shaking) (4,15).

Fluorescent antibody determination for K99 were done employing frozen sections of ileum (FTFA) as well as smears of ileum contents (DSFA). The indirect

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method was used and sections were stained with monospecific K99 antiserum and normal rabbit serum as a control followed by goat anti-rabbit IgG (16,17).

Results and Discussion

Initial efforts in evaluating techniques for diagnosing EEC in scouring calves included heat-stable (ST) toxin demonstration, frozen tissue fluorescent antibody examination (FTFA) of sections of ileum for K99 antigen, O antigen serotyping and agglutination testing for K99 antigen on isolated *E. coli*. These examinations were applied to a total of 251 cases received during the spring calving of 1979 and included either whole calves or tissues (128), with the balance as fecal samples from scouring calves (Figure 1).
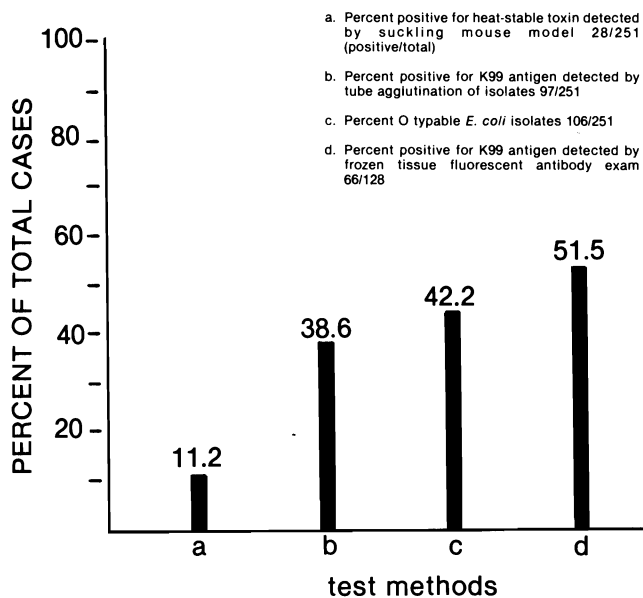

a. Percent positive for heat-stable toxin detected by suckling mouse model 28/251 (positive/total)

b. Percent positive for K99 antigen detected by tube agglutination of isolates 97/251

c. Percent O typable *E. coli* isolates 106/251

d. Percent positive for K99 antigen detected by frozen tissue fluorescent antibody exam 66/128

Figure 1. Evaluation of methods of demonstrating *E. coli* potential enteropathogenicity.

Isolates of *E. coli* which were determined to be capable of elaborating ST by the suckling mouse model were recovered from only 11.2% of the 1979 cases. This figure coincides with ST demonstration in another study (18), but represents only a small proportion of diagnostic cases suspected to have been caused by an EEC. The significance of demonstrating an *E. coli* with the capability to produce a toxin involved in the mechanisms of producing scours is good evidence of enteropathogenicity. However, the percentage of *E. coli* recovered which elaborated ST suggests other models of demonstrating this toxin may be more sensitive, as is apparently the case in other species (19), and these methods, which include isolated loops of intestines, are expensive and unwieldy for routine diagnostic work. In addition, only 25% of the *E. coli* isolates determined to be ST positive were discovered to be typable by O antigen. These belonged to group 017, 0101, 08 and 09 which are suggested as common O types expected in calf scours by recent reports (12). Also, of the ST positive *E. coli* isolates, 14 were positive for K99 by agglutination

studies and 14 were negative. Because of the limited recovery of ST and poor correlation with other tests, this technique was not repeated in the 1980 studies.

The recovery of an O typable *E. coli* isolate occurred in 42.2% of cases (Figure 1). An interesting spectrum of O types was recorded (Table 1) implying a wider spectrum of *E. coli* O types in calf scours than recently suggested (11,12,18,19). In addition, a number of these were K99 antigen positive by agglutination studies (Table 1). Although the limitations of this test are apparent, i.e., use of additional pools and specifics would increase the numbers of O typable *E. coli* and a percentage of *E. coli* involved in calf scours may not as yet be serologically identified, the data recovered are useful epidemiological information on *E. coli* involved in calf scours in North Dakota. These studies will be enhanced with the completion of spring 1980 O antigen results.

The K99 antigen determinations from the calf scour studies of spring 1979 samples were the most promising techniques (Figure 1). The indirect fluorescent antibody examination of frozen sections of ileum allowed the observation of *E. coli* attached to epithelial cells of the villi of the ileum, which presented good evidence of the involvement of this bacteria as a causative agent of scours. Additionally, the test is simple, inexpensive and can be completed within hours of receiving the sample.

Table 1. *E. coli* O and K99 antigen agglutination studies

O Pool	O Specific	Isolates Recovered	Isolates K99 Antigen Positive
D	8	15	7
	9	16	8
	101	5	2
	157	1	0
	20ab	1	0
E	139	7	0
	141	5	2
	149	3	2
F	15	1	1
	17	34	20
	75	9	2
	78	1	0
	88	3	0
G	6	2	1
	26	3	0

Good correlation was determined on K99 positive samples between FTFA and K99 agglutination of isolates of *E. coli* recovered from these tissues. However, a percentage was negative on frozen tissue, but positive on agglutination. An explanation for a portion of these was the damage to the mucosa of intestinal sections due to post mortem changes and freezing and thawing artifacts. In these cases, with the mucosa sluff-

ing into the lumen of the intestine, K99 positive *E. coli* were lost from the sections. To circumvent this problem, the continuation of this study employed paired smears of ileum contents and a frozen section of ileum for K99 fluorescent antibody examination followed by K99 agglutination of isolates.

In these 1980 calf scour cases a smaller number of samples were processed, reflecting the milder spring, more favorable calving and calf rearing conditions. From a total of 92 cases processed, 46% (42/92) were determined to be K99 positive by agglutination (Table 2) with 44% (39/88) K99 positive by fluorescent antibody examination on direct smear (DSFA). From the latter group, 25 were also examined by FTFA for K99 and 72% (18/25) were positive (Table 2).

Table 2. K99 demonstration by agglutination or fluorescent antibody examination

TEST METHOD	TEST RESULT		
	Positive	Negative	Total
K99 antigen by agglutination	42	50	92
Frozen tissue fluorescent antibody examination	18	7	25
Direct smear fluorescent antibody examination	39	49	88

Excellent correlation between these three tests was observed (Table 3). From the 42 cases positive for K99 by agglutination, only one case was negative by either fluorescent antibody method. In the samples positive for K99 by DSFA, all samples determined K99 positive by the other two methods correlated. The FTFA K99 cases were positive in two cases when DSFA studies were negative and in two other samples when K99 agglutination was negative.

In the instance of the negative K99 fluorescent antibody examination with positive results on agglutina-

tion, a possible explanation would be that the *E. coli* isolate had the genetic potential to produce K99. Although it was not expressing K99 antigen in the animal, (EEC was not involved in the enteric disease in this animal) it was induced to do so on media (see materials and methods). The several cases that were positive for K99 by FTFA studies and negative by agglutination for K99 could possibly be the exact opposite of the above, but K99 antigen expression could not be induced on media. Finally, the samples which were positive by FTFA studies for K99, but negative by DSFA, indicate the importance of using both techniques to thoroughly examine scouring calf intestines to implicate the involvement of enteropathogenic *E. coli*.

SUMMARY

1. Examination of *E. coli* isolates from intestinal contents of scouring calves has implicated a broad range of O serotypes determined to be potentially enteropathogenic by the presence of K99 antigen.

2. A protocol for determining the involvement of enteropathogenic *E. coli* has been evolved utilizing fluorescent antibody examination for K99 antigens (by frozen section and direct smear) and by K99 agglutination on *E. coli* isolates.

3. The results from this two-year study have enhanced the veterinary diagnostic laboratories accuracy and expediency in diagnosing enteropathogenic *E. coli* calf scours.

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Table 3. Comparison of samples positive for K99 by agglutination and fluorescent antibody examination of frozen sections or direct smears

	Negative Frozen Tissues Fluorescent Antibody	Negative Direct Smear Fluorescent Antibody	Negative Agglutination
Positive agglutination (42)	1/18	1/39	———
Positive direct smear fluorescent antibody exam (39)	0/18	———	0/39
Positive frozen tissue fluorescent antibody exam (18)	———	2/18	2/18

other hand, confining cows and calves to confined lots exposes newborn calves to every organism present in the cow herd.

In the soil samples which were collected from the various cooperators, tremendous differences exist in the number and type of "bugs" found in the area which cows normally frequented to areas which rarely, if ever, had been used by the cow herd. This points to a need for early cleaning of calving lots to give the summer sun a chance to kill as many of the organisms as possible. Consideration should also be given to disinfecting the soil in calving lots with some product such as lime.

Several questions loom large in my mind after the first year of the calf scours study.

(1) Many producers have gone to earlier or later calving to help prevent calf scours and avoid the wet conditions generally found in late February and March. Does this mean that there is less stress associated with cold windy weather (early February) or the hot, dry and dusty weather that we experienced in April this year? Isn't stress just as devastating no matter what form it comes in? OR—Is there some particular aspect of the management system and/or the cows nutritional requirements that change during this period?

(2) The calving period is proceeding smoothly, the weather changes and there is a scours outbreak. What caused the outbreak? Stress! Why didn't all calves get it? Weren't they all exposed to the same amount of stress? Haven't all cows been exposed to the same disease prevention program? Why didn't the neighbor five miles away with the same kind of cows and same type calving pasture have any scours?

If these questions seem unanswerable, it is because with our present knowledge and understanding of calf scours they are unanswerable. I believe that it is only through the continuation of ongoing research projects and the cooperation and input of cooperators such as those we worked with, that we can someday hope to comprehend and then control calf scours.

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