

## Characterization of *Escherichia coli* Obtained from Newborn Calves with Diarrhea<sup>1</sup>

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A total of 373 isolates of *Escherichia coli* were obtained from one or more calves with diarrhea in 155 herds during the 1974 calving season in Montana. Sixty-seven (18%) of the isolates representing 59 of the 155 herds were found to be enterotoxigenic as indicated by their ability to cause distention of the calf ligated intestinal segment. The 67 isolates of enterotoxigenic *E. coli* (ETEC) were placed in one of six different antigenic groups based upon agglutination of formalized whole cells in KO antiserum. Eighty-seven percent (58 of 67) of the ETEC had antigen 1, 2, or 3, whereas only 2.3% (7 of 310) of the non-ETEC (NETEC) had antigen 1, 2, or 3. This antigen numbering system was used for convenience and is not related to any established typing system. Antigens 1, 2, and 3 do not belong to any of the O groups 1 to 157 or K groups 1 to 93 of the International Schema. Colony color or morphology of ETEC and NETEC grown on Tergitol-7 agar with triphenyltetrazolium chloride added could not be used as an indicator of enterotoxigenicity, although there was a tendency for *E. coli* with the smooth and mucoid colony type to be enterotoxigenic whereas rough colonies were seldom enterotoxigenic. Among ETEC isolates with antigen 1, 2, or 3, there was good correlation between colony type and antigen number. All 14 ETEC isolates with antigen 1 had the smooth colony type, 17 of 19 ETEC isolates with antigen 2 had the smooth, mucoid colony type, and all 25 isolates of ETEC with antigen 3 had the intermediate colony type. Conversely, of the 310 isolates of NETEC, none had antigen 1 and the smooth colony type; none had antigen 2 and the smooth and mucoid colony type; and only one isolate of NETEC had antigen 3 and the intermediate colony type. Sixteen of the 67 isolates of ETEC (24%) were motile. Fifteen of the 16 motile isolates of ETEC had the intermediate colony type, and none of the ETEC with smooth or smooth and mucoid colonies were motile.

Enteropathogenic *Escherichia coli* apparently cause diarrhea (enteric colibacillosis) in many animal species including humans, calves, lambs, and pigs (6, 12, 13). Neonatal calf diarrhea experimentally induced by *E. coli* can be prevented by proper vaccination of the dam (9, 10). Establishment of the cause of field cases of diarrhea in young calves is of primary importance in attempts to effectively treat or prevent the disease. Although a number of different infectious agents have been incriminated in the etiology of the disease, there is a lack of definitive information concerning the cause of the majority of cases of diarrhea in newborn calves.

This study was conducted to establish the prevalence of enterotoxigenic *E. coli* (ETEC) in cases of calf diarrhea in Montana. *E. coli* isolated from calves with diarrhea were charac-

terized from the standpoint of ability to produce enterotoxin, colony appearance, motility, and antigen composition in an attempt to gain more information concerning the various strains of ETEC and to differentiate ETEC from non-ETEC (NETEC).

### MATERIALS AND METHODS

**Isolation of *E. coli*.** Isolates of *E. coli* were obtained from one or more calves (usually less than 2 months old) with diarrhea in 155 herds during the 1974 calving season in Montana. The majority of the *E. coli* isolates were obtained by direct culture of fecal specimens from the calves. *E. coli* isolates were initially streaked on Tergitol-7 (T-7) agar (Difco Laboratories, Detroit, Mich.) with 100 mg of triphenyltetrazolium chloride added per liter (11). After overnight incubation at 37 C, isolated colonies with distinct color or morphology were restreaked on T-7 plates and incubated overnight. Pure cultures were stored in the dark in screw-cap tubes at room temperature on tryptose agar slants. Typically, two to five

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isolates with distinct colony characteristics were obtained in pure culture from one or more calves in each herd. Standard biochemical tests were conducted to confirm that the isolates were *E. coli* (3). Enterotoxigenicity of *E. coli* isolates was determined using the calf ligated intestinal segment test (8).

**Serologic study of *E. coli*.** KO antisera against 50 isolates of ETEC were prepared by repeated intravenous injections of rabbits with formalized and live whole cells according to standard procedures (4). Initially, the agglutination titer of the KO antiserum was determined by using doubling dilutions of each antiserum against the homologous formalized whole cell antigen in the microtube agglutination test. The end-point titer was usually 1:64 or 1:128. Since KO antisera prepared against six different isolates of ETEC agglutinated formalized cells of all 67 isolates of ETEC, these six antisera were used for screening 310 isolates of NETEC. For convenience, these six different antigenic groups are numbered 1 to 6. This numbering system is unrelated to any established typing system. Screening of *E. coli* isolates with the six different KO antisera was done by using the microagglutination test conducted in plastic microtiter plates with U-shaped bottoms. In this test, 0.05 ml of diluted antiserum (usually diluted 1:8) was mixed with 0.05 ml of formalized (0.5% formalin) cells. Procedures for the plate and the tube agglutination tests included incubation at 37 C for 2 h followed by overnight storage at 4 C and observation of agglutination with the aid of a light shining through the suspension from below. Whenever possible, to avoid H agglutination, antisera against nonmotile isolates of ETEC were used for screening of *E. coli* isolates.

Homologous O (prepared in rabbits by repeated intravenous injections with cells autoclaved 2.5 h) and KO antisera were used to determine the O inagglutinability and to establish the type of K antigen present for one isolate from each of the six different antigenic groups according to standard methods (4).

**Colony characteristics of *E. coli*.** Colony characteristics of *E. coli* isolates on T-7 agar plates were studied prior to initial storage on tryptose agar slants. Colonies were observed with the aid of a dissecting microscope and an intense light placed a few inches above the colonies. Colonies were examined primarily from the standpoint of color and morphologic characteristics to determine if any colony characteristics of ETEC are different from NETEC.

**Motility of ETEC.** Motility was determined according to a standard procedure (2) using semisolid motility medium (Difco Laboratories, Detroit, Mich.). The medium was stabbed with ETEC isolates previously stored on tryptose agar slants. Motility was determined for all ETEC isolates after incubation for 24 and 48 h at 25 and 37 C.

## RESULTS

Of the 373 *E. coli* isolates, 67 (18%) were found to be enterotoxigenic as indicated by their ability to cause distention of the ligated

intestinal segment of the young calf. These 67 isolates came from 59 of 155 different herds.

**Serologic study.** All 67 isolates of ETEC could be placed in one of the six different antigenic groups (Table 1), with 86% (58 of 67) of the isolates belonging to group 1, 2, or 3. Only 2.3% (7 of 310) of the NETEC isolates reacted strongly in groups 1, 2, or 3. There were too few isolates of ETEC in groups 4, 5, or 6 to clearly establish correlation with enterotoxigenicity, although it appears these groups are not closely correlated with enterotoxigenicity.

Fourteen of the 15 ETEC isolates obtained from calves with diarrheal disease during the 1972 calving season had antigen 1, 2, or 3. These 15 isolates were untypable with standard O groups 1 to 157 and K groups 1 to 93 antisera (serotyping results received from P. J. Glantz, Pennsylvania State Univ.). Four of the isolates gave a weak agglutination reaction with O9 or O101 antisera, indicating the O antigens are related to antigens O9 or O101. No attempt was made to obtain typing information for isolates with antigens 4, 5, or 6 because ETEC with these antigens were seldom found and because there appeared to be little relationship between presence of these antigens and enterotoxigenicity.

A number of isolates of NETEC reacted weakly in the agglutination test with more than one of the six KO antisera. These isolates had, in most cases, intermediate or rough colonies. ETEC isolates with antigen 1, 2, 3, or 4 did not react with any of the heterologous antisera 1

TABLE 1. *Antigen number of individual ETEC isolates obtained from calves with diarrheal disease*

Antigen no.	Isolate no. (MVRL) <sup>a</sup>
1	4, 46, 53, 80, 115, 116, 202, 203, 213, 215, 217, 224, 296, 332
2	77, 81, 86, 97, 101, 117, 122, 151, 154, 157, 209, 255, 280, 281, 284, 291, 336, 361, 364
3	18, 51, 55, 76, 82, 92, 99, 108, 109, 119, 120, 121, 127, 152, 235, 240, 246, 251, 295, 306, 316, 320, 322, 358, 360
4	74
5	90, 105, 134
6	110, 111, 128, 130, 204

<sup>a</sup> Montana Veterinary Research Laboratory number.

to 6. ETEC isolates with antigens 5 or 6 often reacted weakly with heterologous antisera 5 or 6 (indicating that antigens 5 and 6 are related) but did not react with antisera 1 to 4.

Unheated antigens of six isolates of ETEC (one isolate from each of the six antigenic groups) were O inagglutinable with homologous O antisera, indicating the presence of K antigens. Heating the live cells at 100 C for 1 h did not remove the O inagglutinability for isolates in groups 1, 2, 5, or 6, indicating the K antigens were the A type. Absorption experiments indicated that isolates in groups 3 and 4 have K antigen of the L type.

**Colony characteristics.** The colony morphology of *E. coli* isolates was observed to be smooth, smooth and mucoid, intermediate, or rough. Smooth and mucoid colonies were differentiated from smooth colonies by the presence of capsular material on the surface of mucoid colonies after overnight growth on T-7 agar that formed a thread or strand of mucus-like material when a wire loop was withdrawn from the colony. The intermediate colony type varied from colonies having slightly irregular margins to colonies obviously rough over the entire surface. Smooth, smooth and mucoid, and intermediate colonies were about the same size and appeared markedly convex. Rough colonies were larger in diameter than the other colony types and appeared nearly flat with irregular margins. Color of colonies varied between isolates and included various shades of pink, red, yellow, and brown centers with clear to translucent edges. We did not observe any color or morphologic characteristics that would allow accurate differentiation of ETEC from NETEC. There was a tendency for smooth and mucoid colonies to be enterotoxigenic and rough colonies were usually not enterotoxigenic (Table 2). Among ETEC isolates with antigens 1, 2, or 3, there was good correlation between colony type and antigen number (Table 3).

There was a tendency for one or more colony types different from the original isolate (usually rougher) to appear on T-7 agar after storage of

TABLE 3. Number of isolates of ETEC and NETEC with each colony type and antigen number<sup>a</sup>

Colony type	Antigen no.					
	1	2	3	4	5	6
Smooth and mucoid . . . . .	0,0	17,0	0,0	0,0	0,0	0,0
Smooth . . . . .	14,0	0,4	0,1	0,0	0,5	3,8
Intermediate . . . . .	0,0	1,1	25,1	1,0	2,2	2,3
Rough . . . . .	0,0	1,0	0,0	0,0	1,0	0,0

<sup>a</sup>The first number in each column represents the number of isolates of ETEC and the second number represents NETEC isolates.

*E. coli* on tryptose agar. These mutant colonies from ETEC usually retained the enterotoxigenicity of the original isolate. All ETEC were routinely stored in sterile defibrinated bovine blood at -20 C (as well as on tryptose agar slants) to retard the development of rough mutants and to retain viability of ETEC for a number of years. There may be less tendency for large numbers of rough mutants to develop in the calf as we seldom found rough isolates from field specimens to be enterotoxigenic.

**Motility of ETEC.** Sixteen of the 67 isolates of ETEC (24%) were motile. The number of motile isolates of ETEC in groups 1 through 6 were 0, 1, 9, 1, 3, and 2, respectively. Fifteen of the motile isolates had the intermediate colony type and one isolate had the rough colony type. There were no motile isolates of ETEC with the smooth or smooth, mucoid colony type. Motility of isolates of NETEC was not determined.

## DISCUSSION

Apparently there is little information concerning the antigenic composition of bovine strains of ETEC. As mentioned by Glantz (5), K antigens of *E. coli* isolates with standard O antigens are often untypable. It is of interest that the five bovine ETEC isolates that we obtained from outside Montana belong to antigenic groups 1 or 2, indicating that ETEC in calves throughout the world may be similar in this respect. Following is a list of the five isolates, their origin and their antigen no: B44, England, 2; 60/1577, Canada, 1; 3267-2, California, 1; 4042, Canada, 2; 466A, California, 2. The porcine and human isolates of ETEC that we studied (8) did not have these six different antigens.

The six ETEC isolates studied all demonstrated O inagglutinability with unheated antigens indicating the presence of K antigen.

TABLE 2. Colony type of isolates of ETEC and NETEC

Isolates	Smooth and mucoid		Smooth		Intermediate		Rough	
	No.	%	No.	%	No.	%	No.	%
ETEC (67)	17	25	17	25	31	46	2	3
NETEC (310)	12	4	91	30	163	53	40	13

Smooth or smooth, mucoid colonies (isolates in groups 1 and 2) would be expected to have K antigen. We have not, however, established the presence of K antigen for all of the ETEC isolates.

Often, attempts to correlate enteropathogenicity of *E. coli* isolates with antigenic composition have failed (1, 7). This may be because many isolates of *E. coli* from cases of diarrhea in young animals are untypable. In our experience, over 80% of the *E. coli* isolated from calves with diarrhea are not enterotoxigenic as indicated by inability to cause distention of the ligated intestine of the newborn calf. It has been shown that enterotoxigenicity as determined in the calf system correlates well with enteropathogenicity and that NETEC are also non-enteropathogenic (14).

There was not a good correlation between color or morphologic characteristics and enterotoxigenicity although there was a tendency for isolates with smooth and mucoid colonies to be enterotoxigenic. Information concerning colony type can be correlated with presence of antigens 1, 2, or 3 to aid in identification of ETEC.

We were unable to correlate the characteristics of clinical signs of disease, age of affected calves, etc., with successful isolation of ETEC. Typically, isolation of ETEC was made in calves less than 2 weeks of age that had a profuse, watery diarrhea. There are a number of possible reasons why ETEC may have been missed in calves with diarrhea. Calves often were treated with antibiotics prior to sampling, and samples may have been taken after the acute stage of the disease had passed. Often only one or two fecal specimens were obtained from a herd. Contents of the small intestine (where ETEC may be present in large numbers) were usually not available for culturing. Also, colonies of ETEC may have been present on T-7 agar plates but by chance not selected for determination of enterotoxigenicity since color and morphologic characteristics of ETEC and NETEC cannot be differentiated on T-7 plates.

The good correlation between presence of antigens 1, 2, or 3 and enterotoxigenicity, and the fact that the majority of bovine ETEC have

one of these three antigens, may facilitate more rapid identification of the presence of ETEC.

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