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Characterization of *Escherichia coli* Isolated from the Tonsils of Cattle

G. H. FRANK,* R. E. BRIGGS, AND R. A. SCHNEIDER

USDA Agricultural Research Service, National Animal Disease Center, Ames, Iowa 50010

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During our studies on tonsillar colonization by *Pasteurella haemolytica*, we consistently found *Escherichia coli* to be one of the most prominent and prevalent bacterial species in the tonsils of healthy cattle. Since tonsillar isolates have not been characterized, we grouped 124 isolates from 87 healthy cattle from eight sources by hemolytic zone size and by carbon source utilization and probed them for selected virulence genes. They formed 3 groups by hemolytic zone size and 18 groups (of 2 to 31 isolates) by their metabolic patterns. Most groups included isolates from more than one source. Two isolates contained the Shiga-like toxin gene, and nine others contained the F41 accessory gene.

A total of 124 *Escherichia coli* isolates were collected from the tonsils of 87 healthy calves over 4 months of age. The calves originated from three sources in Tennessee and from five sources in Iowa (Table 1).

Tonsillar washings were collected by instilling 3 ml of Dulbecco's phosphate-buffered saline (pH 7.2) into each palatine tonsillar sinus and immediately aspirating the fluid with a modified pipette (3). The tonsillar wash specimens were sonicated briefly and then spread on blood agar base (Difco Laboratories, Detroit, Mich.) with 5% bovine blood. After overnight incubation at 37°C, colonies with *E. coli*-like morphology were selected and their hemolytic zone sizes were recorded.

Isolates were grouped according to their catabolic properties with the Biolog System (Biolog, Inc. Hayward, Calif.) by using Biolog ES MicroPlates in accordance with the directions supplied by the company. The plates were read on a Molecular Devices THERMOmax Microplate Reader (Molecular Devices Corp., Menlo Park, Calif.), and a data base was compiled by use of Biolog MicroLog 3N software. The reactions were analyzed by use of the Biolog program MLCLUST, which grouped the isolates by a multiplicative measure of probability procedure on the basis of the utilization pattern of 95 carbon sources. Representative isolates from each metabolic group (a total of 54) were identified as *E. coli* by use of the API 20E (Analytab Products, Plainview, N.Y.) system of enterobacter identification or by use of Biolog GN MicroPlates (Table 2). The remaining isolates were identical morphologically and had metabolic patterns that were closely similar to those of the positively identified members of the same metabolic group. Since they were both citric acid negative and lactose positive on the ES plates, they were presumptively identified as *E. coli* and further identification was not done.

Isolates were tested for the presence of β-glucuronidase by the inoculation of lauryl tryptose broth with MUG (4-methylumbelliferyl-β-D-glucuronide) (Difco). After 24 h of incubation at 37°C, tests were examined for fluorescence under long-wave UV light.

Isolates were probed for the Shiga-like toxin (SLT) genes SLT-I and SLT-II (11); the enterotoxin genes STb and STaP; the attaching and effacing gene *eae* (4); the adhesin genes F41 and CS31A; and the F41 accessory gene by methods described by Moon et al. (9). The F41 accessory gene probe was a fragment of pDGA9 (a 1.2-kb *Sal*I-*Eco*RI fragment, pSLM206) (5).

The bovine tonsillar *E. coli* isolates formed a heterologous group. They formed three groups by hemolytic zone size, which were none, small (hemolysis evident around the periphery of the colony), and large (hemolytic zone approximately three times the diameter of the colony). The carbon source utilization test with Biolog ES MicroPlates divided the isolates into 18 metabolic groups, which were grouped under two major divisions (Table 2). Group sizes ranged from 2 to 31 isolates. In metabolic groups containing four or more members, 74 to 100% of isolates had the same hemolytic zone size. Most metabolic groups included isolates from calves from two or more sources, although the majority of isolates within a group tended to be from calves from a single source.

One isolate was collected from each of 59 different calves, two isolates were collected from each of 22 calves, three isolates were collected from each of 5 calves, and five isolates were collected from a single calf. Calves harbored *E. coli* strains with different hemolytic zone sizes, and those selected with the same hemolytic zone size from the same calf often fell into different metabolic groups. Even those from the same calf and in the same metabolic group had some differences in their metabolic patterns.

Two tonsillar isolates (1.6%) hybridized with the SLT-I probe, and nine (7.2%) hybridized with the F41 accessory gene probe (Tables 1 and 2). None of the isolates hybridized with probes for the SLT-II, STb, STaP, *eae*, F41, and CS31A genes.

The SLTs are generally produced by enterohemorrhagic *E. coli*. There is a significant association between verocytotoxin (SLT)-positive *E. coli* and diarrhea in cattle and in buffalo calves (7). Most strains that cause edema disease in pigs produce high levels of SLT (11). Both SLT-I-positive isolates had S-hemolytic zones but were members of different metabolic groups and were from different sources. The *eae* gene, which is necessary for the attaching and effacing activity by enteropathogenic *E. coli*, was not found. This gene has been found in some enterohemorrhagic *E. coli* strains (4).

* Corresponding author. Mailing address: Respiratory Disease Research Unit, USDA, ARS, National Animal Disease Center, 2300 Dayton Road, Ames, IA 50010. Phone: (515) 239-8280. Fax: (515) 239-8458.

TABLE 1. Distribution of *E. coli* according to source, hemolytic zone size, and virulence genes

Source[a]	No. of calves	No. of calves with indicated hemolytic zone size[b]			No. of *E. coli* isolates
		O	S	L	
TN 1	45	6	26 (1 T)[c]	13 (4 F)[d]	45
TN 2	15	7 (1 F)	10	9 (1 F)	26
TN 3	8	2 (1 F)	6	8 (1 F)	16
IA 1	8	3	7	6	16
IA 2	2	1	2	0	3
IA 3	6	2 (1 F)	8	2	12
IA 4	2	2	3 (1 T)	0	5
IA 5	1	1	0	0	1

[a] TN 1, beef calves from a farm in Tennessee; TN 2 and TN 3, beef calves from an order buyer barn in Tennessee; IA 1 to IA 5, dairy calves from five sources in Iowa.
[b] Hemolytic zone sizes: O, none; S, small (easily visible around the periphery of the colony); L, large (approximately 3 times the diameter of the colony).
[c] Number of SLT-I probe-positive isolates in the group.
[d] Number of F41 accessory gene probe-positive isolates in the group.

Since none of the tonsillar isolates were STb or STaP probe positive, they were not considered to be enterotoxigenic *E. coli* (ETEC) (8). Because the adhesion genes K88, K99, and 987p are not known to occur in isolates that are not ETEC (10), the probes were not included in the search for virulence genes. Mainil et al. (6) probed 424 *E. coli* isolates from calves that were less than 1 month old with enteric or systemic disease thought to be caused by *E. coli* with four enterotoxin genes and the K99 adhesion factor gene. Of those, 22% hybridized with at least one enterotoxin probe and 19% hybridized with the K99 probe. Most of the ETEC isolates found were $K99^+$ $STaP^+$, and the authors considered it reasonable to use STaP and K99 gene probes diagnostically for ETEC infections in calves.

Although the K88, K99, and 987p adhesin genes were not likely to be found and F41 and CS31A were not found in the tonsillar isolates, nine isolates (6.9%) were positive for the F41 accessory gene. The F41 accessory gene comprises accessory genes to the bovine-porcine ETEC adhesin F41 (2). These genes are homologous to both the K88 (porcine adhesin) (1) and CS31A (bovine septicemia associated pilus) (5) accessory genes. The known F41 accessory gene homology has led to the suggestion that there are other adhesins, distinct from the above, which are using the accessory genes. For example, the strain from which the CS31AS gene was cloned was noticed because it is a virulent strain which hybridizes to the F41 accessory gene probe but not to the K88 or F41 adhesin subunit probes (5). The F41 accessory gene-positive isolates varied in their hemolytic zone sizes and metabolic groups and were present in calves from four sources.

Five isolates were negative for β-glucuronidase, but none of them were probe positive for the virulence genes. These five isolates and the two SLT-I probe-positive isolates were negative in tests with O157 antiserum (*E. coli* O157 Latex Test, Oxoid, Hampshire, United Kingdom). While most O157 isolates are negative for β-glucuronidase (13, 15), many β-glucuronidase-negative isolates from cattle are not of that serotype. In a recent survey, approximately 80% of β-glucuronidase-negative *E. coli* isolates from bovine fecal specimens were non-O157 (14).

The bovine palatine tonsils are completely embedded in the soft tissue of the pharynx. The palatine tonsillar sinuses begin as single openings on each side of the pharynx. The sinuses immediately divide into two channels that subdivide into many blind subbranches within the tonsil (12). Because

TABLE 2. Divisions and metabolic groups of *E. coli*, their hemolytic zone sizes, numbers of sources, and distributions of virulence genes

Division and metabolic group	No. of isolates[a]	No. of isolates with indicated hemolytic zone size[b]			No. of sources
		O	S	L	
Division A					
A	2 (1)	0	0	2	1
B	2 (1)	2	0	0	1
D	7 (5)	1	0	6	4
E	4 (2)	4 (2 F; TN 2, IA 3)[c]	0	0	4
F	3 (1)	2	0	1	3
G	20 (3)	0	19 (1 T; TN 1)[d]	1	1
H	31 (11)	8	0	23 (5 F; 4 TN 1, 1 TN 2)	7
I	5 (3)	1	4 (1 T; IA 4)	0	3
J	3 (1)	0	1	2	3
K	6 (3)	5	1	0	3
L	11 (9)	0	11	0	3
M	2 (1)	0	2	0	1
Division B					
N	9 (5)	0	8	1	4
O	2 (1)	0	2	0	1
P	5 (1)	0	5	0	3
Q	8 (2)	0	7	1	4
R	2 (1)	0	2	0	2
S	2 (1)	1 (1 F; IA 5)	0	1 (1 F; TN 3)	1

[a] The value in parentheses is the number of isolates in the metabolic group which were identified as *E. coli* by the API 20E system of *Enterobacter* identification or by use of Biolog GN MicroPlates. Other members of the group were presumptively identified as *E. coli*.
[b] Hemolytic zone size: O, none; S, small (visible around the periphery of the colony); L, large (approximately 3 times the diameter of the colony).
[c] Number of F41 accessory gene probe-positive isolates in the group and their source(s) as defined in Table 1, footnote *a*.
[d] No. of SLT-I probe-positive isolates in the group and their source(s) as defined in Table 1, footnote *a*.

of the structure of the bovine tonsil, there may be no need for bacterial attachment for colonization. This lack of the requirement to attach would allow an even greater array of *E. coli* and other pathogens to colonize cattle in this location.

The tonsil is a reservoir for *E. coli* in healthy cattle. It is likely that virulent *E. coli* can be present and can be transferred to calves when they are licked by their dams at birth. The SLT-positive *E. coli* have important implications for animal health (diarrhea) and as food-borne pathogens for humans.

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