Distribution of Enterotoxigenic Escherichia coli in Diarrheal Calves and Healthy Cattle

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ABSTRACT. Outbreaks of calf diarrhea occurred on three dairy farms in Shiga Prefecture in the spring in 1980. Escherichia coli strains producing heat-stable enterotoxin (ST) were isolated in pure culture and at high frequency from the intestinal contents and parenchymatous organs of dead calves. Most of these strains were typed as 0101-K99. All the strains were nontoxin and typed as biovar B. They were resistant to two drugs, tetracycline (Tc) and chloramphenicol (Gm) or six, Tc, Cm, streptomycin (Sm), sulfadimethoxine (Su), kanamycin (Km), and ampicillin (Ap), and contained R plasmids. With the properties of these ST-producing (ST+) strains as indicators, the ST+ strain contents of the intestines were compared between diarrheal calves and healthy cows kept in the same barn. The total E. coli count per gram of diarrheal feces of calves was 10^8-9, most of which were ST+ strains. The count in the rectal feces of healthy calves was 10^7-1, of which 10^6-6 were ST+, while that in the rectal feces of healthy cows was 10^8-3, of which 10^8-3 were ST+. These results suggest that ST+ strains with the same properties were distributed widely not only among diarrheal calves but also among healthy cattle kept in the same barns on the three farms where the outbreaks of calf diarrhea occurred.

Infection with enterotoxigenic Escherichia coli (ETEC) causes calf diarrhea which brings about a great economic loss in animal husbandry [13, 22]. Enterotoxins of two types are known; heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) [6, 21].

In epizootiological surveys, ST-producing (ST+) strains have been isolated at a higher frequency from diarrheal calves origin than LT-producing (LT+) strains [2, 10, 12, 15]. Moreover, most of the ST+ strains of diarrheal calf origin had the adhesive K99 antigen [10, 12, 15, 17]. From these findings, it is assumed that ST and K99 antigen possessed by E. coli may play an important role in causing calf diarrhea. Little information is available, however, on the relationship between such particular strains of E. coli and calf diarrhea in Japan.

During the period from April to June, 1980, the authors encountered outbreaks of calf diarrhea presumably caused by ST+ E. coli on three dairy farms in Shiga Prefecture. The present paper deals with the isolation and properties of ST+ strains of E. coli harbored in calves involved in the outbreaks of calf diarrhea and with the distribution of such strains among healthy cattle kept in the same barn with these diarrheal calves.

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<table>
<thead>
<tr>
<th>Dairy form</th>
<th>Origin</th>
<th>Number of examined</th>
<th>Number of ST⁻</th>
<th>Number of ST⁺ strains isolated on</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dead calves with diarrhea</td>
<td>2 14</td>
<td>2 11</td>
<td>11 0</td>
</tr>
<tr>
<td></td>
<td>The dams of above calves</td>
<td>2 4</td>
<td>1 1</td>
<td>0 1</td>
</tr>
<tr>
<td></td>
<td>Healthy cows</td>
<td>27 59</td>
<td>15 20</td>
<td>6 14</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A dead calf with diarrhea</td>
<td>1 7</td>
<td>1 6</td>
<td>6 0</td>
</tr>
<tr>
<td></td>
<td>The dam of the above calf</td>
<td>1 2</td>
<td>1 1</td>
<td>0 1</td>
</tr>
<tr>
<td></td>
<td>Healthy cows</td>
<td>9 33</td>
<td>6 9</td>
<td>1 8</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A dead calf with diarrhea</td>
<td>1 6</td>
<td>1 5</td>
<td>5 0</td>
</tr>
<tr>
<td></td>
<td>Diarrheal calves</td>
<td>5 20</td>
<td>3 4</td>
<td>1 3</td>
</tr>
<tr>
<td></td>
<td>Healthy calves</td>
<td>2 4</td>
<td>2 2</td>
<td>0 2</td>
</tr>
<tr>
<td></td>
<td>The dams of above calves*</td>
<td>8 16</td>
<td>6 6</td>
<td>0 6</td>
</tr>
<tr>
<td></td>
<td>Healthy cows</td>
<td>5 9</td>
<td>3 3</td>
<td>0 3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>63 174</td>
<td>41 68</td>
<td>30 38</td>
</tr>
</tbody>
</table>

ST⁺: Heat-stable enterotoxin-producing *E. coli* detected by the sucking mouse test.

*: One was the dam of a dead calf, other five the dams of diarrheal calves, and the other two the dams of healthy calves.

**: DHL agar containing chloramphenicol.

**MATERIALS AND METHODS**

*Experimental animals:* As shown in Table 1, a total of 63 animals on dairy farms A, B, and C were examined. They consisted of four calves (two-five days of age) died of diarrhea, five calves (three-eight days of age) suffering from diarrhea, two healthy calves (seven-21 days of age) raised in the same barn with these diseased calves, and 52 cows (one-six years of age), including the dams of these dead and diarrheal calves.

*Bacteriological examination:* The intestinal contents (the duodenum or the jejunum), liver, spleen, lung, kidney, and some intestinal lymph nodes were collected from the four dead calves at necropsy on the three farms. Diarrheal feces were collected from the five diseased calves on farm C at severe diarrheal status. Rectal feces were harvested from the two healthy calves on farm C and 52 healthy cows on farms A, B, and C when the sampling was made from diseased calves. One-gram samples were taken from the intestinal contents, diarrheal feces, rectal feces, and homogenized organs. They were 10-fold serially diluted in sterilized physiological saline. Then, 0.1 ml of each dilution was streaked on both deoxycholate-hydrogen sulfide-lactose agar (DHL agar, Eiken Chemical Co., Tokyo) and on DHL agar containing 25 μg/ml of chloramphenicol (Cm) to isolate ST⁻ strain by incubation at 37°C for 18 hours. Samples from diseased calves were also cultured on DHL agar after enrichment in selenite broth (Eiken) and then examined for the presence of salmonellae. Two to five *E. coli*-like colonies grown on each agar plate were picked up and subjected to passage in stock culture medium (Difco Laboratories, Detroit, Mich.). The isolates were identified as *E. coli* by the IMViC system.

*Enterotoxin test:* To examine the production of enterotoxin, the medium prescribed by Evans et al. [6] was used. A cell-free culture supernatant was prepared in the same manner as mentioned in a previous report [28]. LT was examined by the CHO-K1 cell method of Kudo et al. [11] and ST by the sucking mouse test of Dean et al. [7].

*K99-antigen test:* The slide agglutination test was carried out by the method of Guinée et al. [7].

*O-antigen typing:* The method of Edwards and Ewing [5] was used.

*Biotyping:* The method of Braaten and Myers [3] was used. Biotyping was made on the basis the fermentation test on five sugars i.e., adonitol, dulcitol, salicin, sorbose, and sucrose.

*Drug resistance test:* The agar plate-dilution method was used to examine isolated strains for resistance to nine drugs, TC, CM, SM, SU, KM, AP, nalidixic acid (NA), rifampicin (Rf), and mercuric chloride (HgCl₂). Detection of R plasmids was made by the same manner as mentioned in a previous report [25].
RESULTS

Incidence of calf diarrhea: The numbers of Holstein dairy cows raised on farms A, B, and C were about 30, 40, and 45, respectively. These farms were segregated from one to another and there was no intercommunication. Calf diarrhea broke out simultaneously on the three farms, during the period from April to June, 1980. One each of farms A and B, nine newborn calves became diarrhea and seven of them died. One farm C, seven newborn calves all suffered from diarrhea and two of them succumbed. On the three farms, most calves began to excrete yellow watery diarrheal feces two or three days after birth. They began to suffer from anorexia, dysstasia, enophthalmos, and dehydration within two days after the onset of diarrhea and died eventually.

Bacteriological examinations:

E. coli organisms were isolated from all the materials collected from the nine diarrheal calves including four dead calves. No salmonellae were isolated from any sample of diseased calves. A total of 174 E. coli strains (106 on DHL agar and 68 on DHL agar containing Cm) were subjected to the enterotoxin test, test for K99-antigen, O-group typing, biotyping, and drug resistance. Their origins are listed in Table 1.

Enterotoxigenicity of E. coli isolates:

As shown in Table 1, ST+ E. coli strains were isolated from the small intestinal contents and organs of four dead calves from the three farms. Of 27 E. coli isolates from the four dead calves on farms A, B, and C, 22 (81%) were of ST+. ST+ strains were also isolated from the diarrheal feces of three calves (60%) of five affected calves on farm C. Of 20 E. coli strains isolated from the five diarrheal calves, four (20%) were of ST+. Of four E. coli strains isolated from the rectal feces of the two healthy calves on farm C, one strain from each was ST+. Of the 52 healthy cows, including the dams of the infected and healthy calves examined, 32 (62%) were found to harbor ST+ strains in their rectal feces. Of the 123 strains examined, 40 (33%) were of ST+. ST+ strains were detected from eight of 11 dams of four dead calves, five infected calves, and two healthy ones. ST+ strains were not detected in the remaining three dams. No LT-producing strains were found in this survey.

Additional characters of ST+ E. coli:

Additional characters of ST- E. coli strains are shown in Table 2. Of 174 strains of E. coli examined, 59 (34%) had K99-antigen. The rate of possession of this antigen among strains of each origin was follows: 20/27 (70%) of the strains originating from four dead calves, 4/20 (20%) of those from the diarrheal calves, 2/4 (50%) of those from the two healthy calves, and 33/123 (27%) of those from the 52 healthy cows. All the strains possessing K99-antigen (K99+) were ST+. No strains isolated in this survey were ST−K99+

<table>
<thead>
<tr>
<th>Character</th>
<th>ST+ strain (68 strains)</th>
<th>ST− strain (106 strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K99+</td>
<td>59 (87%)</td>
<td>0</td>
</tr>
<tr>
<td>0101</td>
<td>41 (60%)</td>
<td>Not tested</td>
</tr>
<tr>
<td>Non-motile</td>
<td>68 (100%)</td>
<td>25 (24%)</td>
</tr>
<tr>
<td>Biovar B</td>
<td>68 (100%)</td>
<td>5 (5%)</td>
</tr>
<tr>
<td>Drug resistance*</td>
<td>68 (100%)</td>
<td>41 (39%)</td>
</tr>
<tr>
<td>Tc and Cm</td>
<td>48</td>
<td>21</td>
</tr>
<tr>
<td>six drugs**</td>
<td>30</td>
<td>20</td>
</tr>
</tbody>
</table>

ST+: Heat-stable enterotoxin-producing E. coli detected by the suckling mouse test.
Tc: Tetracycline.
Cm: Chloramphenicol.
*: All of 68 ST+ strains had R plasmid.
**: Tc, Cm, streptomycin, sulfadimethoxine, kanamycin, and ampicillin.
Table 3. Proportion of ST* E. coli in calves and dairy cows during the outbreaks of neonatal calf diarrhea

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals tested</th>
<th>Sample tested</th>
<th>Log number of E. coli organisms/g (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Organs**</td>
<td>3.9–6.0 [4.7]</td>
</tr>
<tr>
<td>Diarrheal calves</td>
<td>3</td>
<td>Diarrheal feces</td>
<td>7.7–9.7 [8.9]</td>
</tr>
<tr>
<td>Healthy calves</td>
<td>2</td>
<td>Rectal feces</td>
<td>6.4–7.8 [7.1]</td>
</tr>
<tr>
<td>Healthy cows</td>
<td>32</td>
<td>Rectal feces</td>
<td>4.4–8.3 [6.2]</td>
</tr>
</tbody>
</table>

ST* E. coli: Heat-stable enterotoxin-producing E. coli was presumably counted from the number of E. coli organisms grown on DHL agar containing chloramphenicol.

*: Two duodenum and two jejenum contents.

**: Liver, spleen, kidney, and intestinal lymph nodes.

In O-group typing, 41 (60%) of a total of 68 ST* strains were typed as O101 and the other 27 strains (40%) untypable (Table 2). All the untypable strains were originated from rectal feces of healthy cows.

All 68 ST* strains and 25 of 106 ST– strains were non-motile in SIM medium (Table 2).

All 68 ST* strains and only five of 106 ST– strains were classified into biovar B of Braaten and Myers (Table 2).

Frequency of drug resistance was much higher in ST* strains than in ST– strains. There was a difference in the pattern of drug resistance between the ST* strains originating from farms A and B and those from farm C. The former exhibited resistance to Tc and Cm, and the latter to Tc, Cm, Sm, Su, Km, and Ap. All of these resistance were mediated by conjugative R plasmids.

ST* E. coli counts in rectal feces:

All ST* E. coli strains isolated in the present survey were K99*, non-motile, biovar B, and resistant to Tc and Cm. Then, on the assumption that those with these characters are ST*, ST* E. coli were counted with each group. Table 3 shows the relationship between the total E. coli and the ST* E. coli counts. In diarrheal calves, both counts were 10^9.2–10.2 in the intestinal contents, indicating that most organisms isolated were ST*. They were equally 10^3.9–6.0 in the parenchymal organs. In the diarrheal feces of the infected calves, the total count was 10^7.7–9.7, of which 10^7.5–8.7 were ST*. In the rectal feces of the healthy calves, the total count was 10^6.4–7.8, of which 10^5.2–6.3 were ST*. In the rectal feces of the healthy cows, the total count was 10^4.4–8.3, of which 10^2.5–5.9 were ST*. ST* E. coli organisms were isolated less frequently from cows than from calves.

DISCUSSION

Calf diarrhea broke out concurrently on three dairy farms. Bacteriological examinations were carried out on the small intestinal contents and parenchymal organs of the dead calves and the diarrheal feces of the infected calves. Only E. coli organisms were isolated from all the materials examined. Especially in the dead calves, the average number of E. coli organisms isolated was 10^6.6/g in the small intestinal contents, and 10^4.7/g in the parenchymal organs; most organisms isolated were of ST–K99*. From these results, it was concluded that the cause of calf diarrhea in the present outbreaks was ST–K99* E. coli as reported by other workers [10, 12, 15, 17]. Infection with rota or corona virus is
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known to cause diarrhea in newborn calves. Recently, many fatal cases of mixed infection with rota virus and E. coli have been reported [2, 8, 9, 14]. However, no virological examination was performed in the present survey. Virological examinations should be carried out in further studies.

Larivière et al. [12], Moon et al. [15], Myers and Guinée [17], and Sivaswamy and Gyles [10] reported that E. coli of diarrheal calf origin belonged mostly to such particular serovars as O8, O9, O20, and O101. In Japan, previous studies also revealed the presence of E. coli of particular serovars among diarrheal calves. Nakazawa et al. [18] and Ueda et al. [26] pointed out an especially high incidence of O101 strains. In the present survey, all E. coli strains originating from dead and diarrheal calves on all farms were typed as O101.

On the other hand, it is known that ST gene as well as K99 antigen gene is controlled by transmissible plasmids [19, 24]. From the characteristics of these plasmids, a close correlation may not always be present between the possession of these genes and the serovar. As mentioned above, however, the ST−K99 E. coli strains originating from the diarrheal calves in the present outbreak tended to belong to some particular O groups, such as O101. It seems that the plasmids mentioned above may be transmitted selectively to and persist in E. coli strains of such particular serovars as O101.

The present survey found that ST−K99 E. coli organisms were distributed extensively not only in diarrheal calves but also in healthy calves and other cows kept in the same barn with the infected calves. It should be noted that small quantities, or 10^2.5/g on the average, of ST+ E. coli organisms were isolated from the rectal feces of 32 (62%) of healthy cows, including the dams of the diarrheal calves examined. Therefore, at least calves born from the cows carrying these particular E. coli organisms are always exposed to the infection with such organisms. On the other hand, over 10^5/g of ST− strains were also isolated from fecal feces of some of the healthy calves. It seems likely that such factors as mixed infection with other causative agents or levels of maternal antibody against ST− organisms may be related to the occurrence of diarrhea caused by ST− E. coli organisms.

Enterotoxigenic E. coli (ETEC) is settled and multiply in the upper part of the small intestine of the calf [13, 22]. Isaacson et al. [10] and Larivière et al. [12] counted ST+ E. coli organisms in the jejunum of diarrheal calves and found more than 10^7/g in most calves. In the present survey, ST− E. coli count was also more than 10^7/g in the duodenum or jejunum contents of dead calves and diarrheal feces of the infected calves. On the other hand, it was 10^2.5/g on the average in the rectal feces of the healthy calves housed in the same barn with the infected calves. Therefore, it seems impossible to diagnose of diarrhea caused by E. coli simply from the ST− E. coli count in the rectal feces. Further studies must be performed on large numbers of cases.

Various antibiotics have been used for prevention and treatment of calf diarrhea. Of E. coli strains isolated in the present survey, the ST−K99+ strains of farm C origin were resistant to Tc, Cm, Sm, Su, Km, and Ap due to the presence of transmissible R plasmids. Therefore, if such drugs are administered to healthy cows and calves, ST+ E. coli organisms will multiply selectively in the intestine. As a result, diarrhea will be induced in calves, which will make themselves persistent sources of environmental contamination on the farm. It should be very careful, therefore, to treat calf diarrhea with antibiotics because of the
possible acquirement of drug resistance by the organisms.

REFERENCES


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