Enterocin Typing of Enterococci Isolated from Dried Infant Foods

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ABSTRACT
One hundred and fifty enterococcal isolates recovered from 16 market samples of infant foods and 35 from other sources were characterized and subjected to enterocin typing with 18 indicator strains. Among 150 enterococcal isolates, 114 (76%) were able to be typed by the indicator strains. Although 24 enterocin patterns were observed with these enterococci, the most prevalent types were X-9, 224, and 65-603. Occurrence of pattern X-9 either singly or in combination with many other types was most frequent. Many of the enterocin patterns in enterococcal isolates were recovered from samples of dairy water supply and hand washings of personnel working in a dairy plant that manufactured infant food; this suggests the possibility of these as sources of contamination. Enterocin typing of enterococci could prove useful in epidemiological studies.

INTRODUCTION
Of the types of microorganisms that gain access to milk and milk products as contaminants, the enterococcus group constitutes one of the most important (17, 26, 31). These organisms in such products is generally indicative of fecal contamination (2, 9, 21, 35). Frequently, these organisms are responsible for food spoilage (2, 17, 24, 36) and occasionally food poisoning (10, 14, 15, 16, 22, 32, 34, 38). Although their food poisoning potential still needs to be substantiated, there is sufficient evidence to show that some strains of enterococci are responsible for producing food poisoning syndromes in laboratory animals under certain conditions (3, 19). Enterococci also are involved in different human infections such as urinary tract infections (13, 18), subacute bacterial endocarditis (11), septicemia (4, 23), oral infections (30), and wound infections (25), as well as mastitis in animals (5).

In view of their relative resistance to a number of antimicrobial agents (20, 39, 40) and other adverse environmental conditions, enterococci are likely to pose serious health problems in humans as well as animals. To locate possible sources of such infections, epidemiological typing of enterococci is essential. Pleceas et al. (33) and Brandis (7) suggested that enterocin typing of enterococci may be of ecological and epidemiological value. Although there has been some work on enterocin typing of group D streptococci directly from human and animal sources, no attempt has been made to study the pattern of enterocin typing of these organisms isolated from milk, milk products, and other foods. Our study is, therefore, an attempt at enterocin typing of group D streptococci directly from market samples of milk-based and cereal-based dried infant foods available in India, as such foods are consumed by the most vulnerable section of the society.

MATERIALS AND METHODS
Analysis of Infant Foods
Sixteen tinned samples of dried infant foods, both milk-based and cereal-based and representing supplies from different manufacturers were procured from local markets and some dairy plants. Samples were analyzed for enterococci with modified citrate-azide agar (37), and total bacterial counts were determined on tryptone dextrose agar as per the method of American Public Health Association (1).

Analysis of Dairy Water Supply and Hand Washings of Dairy Workers
Five samples each of dairy water supply and handwashings of personnel working in a dairy plant engaged in the manufacture of an infant
food were collected aseptically. Samples were examined for enterococci and total bacterial counts by methods referred to earlier.

**Isolation and Characterization of Enterococci**

One hundred and ninety colonies suspected to be enterococci were recovered from 13 milk-based and 3 cereal-based infant food samples. All isolates were subjected to group D serology according to the procedure of Lancefield (28), Sherman's criteria (41), bile-aesculin-hydrolysis (12), and tyrosine-decarboxylase (29). On the basis of these and other biochemical and physiological characteristics, 150 isolates were designated as "true enterococci" and further characterized (6).

Similarly, 50 isolates also were recovered from samples of dairy water supply and hand washings of dairy personnel and subjected to all the tests mentioned above. Thirty five of them were characterized as "true enterococci".

All enterococcal isolates designated as "true enterococci" were subjected to enterocin typing procedure.

A modified plate assay technique of Tomura et al. (43) was used for enterocin typing of all the strains of enterococci isolated from infant foods and other sources. A total of 18 indicator strains were used in our study. Each indicator strain was grown in 10-ml glucose-broth and incubated at 37°C for 8 h. Samples of .1 ml (containing approximately 50 to 100 × 10^6 cells were spread on the surface of serum agar plates. The inoculated plates were dried in the incubator at 37°C for 30 min. Each petri plate was divided into eight equal parts, and each part was spot inoculated with an 18-h old brain-heart infusion broth culture of the organism. Sterile brain-heart infusion broth similarly was spot inoculated in the center of the agar plate to serve as a control. The inoculated plates were incubated at 37°C for 18 h, and zones of inhibition were measured in millimeters. An average zonal diameter of 2.0 mm or more (excluding spot inoculum) was taken as positive indication of enterocin production (8) after replicate trials.

**Indicator Strains:**

The following 18 indicator strains for enterocin typing were obtained through the courtesy of Takako Ito, Department of Microbiology, Tokyo Women's Medical College, Tokyo, Japan:

- Liq. A: Group D Streptococcus faecalis var. liquefaciens strain A.
- Liq. B: Group D. S. faecalis var. liquefaciens strain B.
- 2025: Group D. S. faecalis var. liquefaciens 2025.
- 10541: Group D S. faecalis ATCC 10541.
- 9790: Group D S. faecalis NCT 9790.

\[
\begin{align*}
7073 & \\
X-9 & \\
65-609 & \\
815-2 & \\
1151 & \\
1081 & \\
927 & \\
244 & \\
697 & \\
739 & \\
\end{align*}
\]

Group D strain isolated from patients.

- T3: Group A, type 3 strain R1-T3.
- T12: Group A, type 12 strain R1-T12.
- Pc-T12: Group A, type 12 strain Pct-T12.

**RESULTS AND DISCUSSION**

Upon bacteriological examination of the market samples of tinned dried infant foods, the average total and enterococcal counts were higher (13 × 10^4 and 48 × 10^2 cfu/g) in cereal-based than in milk-based infant foods (43 × 10^2 and 19 × 10^2 cfu/g). This may have been because the cereal was a source of contamination as higher bacterial counts in cereal-based infant foods were reported (42).

Distribution of different types of enterococci isolated from samples of infant foods is in Table 1. Of a total of 150 isolates of true enterococci examined for enterocin typing, 127 were recovered from milk-based infant foods and 23 were from cereal-based infant food samples. The most predominant types of enterococci were S. faecalis var. faecalis, which constituted as much as 28.7%, followed by S. faecium (26%) while S. faecalis var. zymogenes represented only 12% of the total number of isolates tested. In the same table is distribution
of individual strains, both enterocin typeable
and nontypeable. Among 150 enterococcal
isolates tested, 114 (76%) produced enterocin
and, hence, could be typed by the indicator
strains. Although Brock et al. (8) reported
enterocin typeability of 50% among enterococci,
Sharma et al. (40) observed 75.4% enterocin
typability among group D streptococcal isolates
from hospital sources. Pleceas et al. (33),
however, while testing the sensitivity of 471
strains of group D streptococci against a set of
12 known D streptogenic bacteriocin producer
strains, reported that 92.2% of the strains were
sensitive to one or several D Streptocins.
However, in a number of cases the occurrence
of small inhibition zones makes clear dif-
ferentiation between positive and negative
reactions difficult (Brandis and Pleceas, un-
published). Thus, our findings agree with above
reports, although any direct reference to
enterocin typeability of enterococci from food
sources is lacking.

Patterns of enterocin typing (singly or in
combination) of the enterococcal isolates
recovered from infant food samples and ex-
amined in this study are in Table 2. Twenty-four
patterns were observed. Pleceas et al. (33)
classified sensitive strains of group D strep-
tococci using a set of 12 known D streptogenic
bacteriocin-producing strains into 25 types.
Similarly, Kekessy and Piquet (27) were also
able to observe 25 types among 130 strains of
S. faecalis.

The most prevalent enterocin type in the
present investigation (Table 2) among S.
faecium and S. durans was 65-603. The latter
type was observed in 8.6% of typeable strains
of S. faecium. However, types X-9 and 244 also
were encountered commonly in S. faecium.
Enterocin type 2025 was the most prevalent
type (27.3%) in typeable strains of S. durans.
All S. faecium strains that exhibited enterocin
pattern 65-603 were recovered from cereal-based
baby foods. Although our findings are not
consistent with observations of Sharma et al.
(40), who reported enterocin types X-9 and
244 among the strains of S. faecium and S.
durans, the presence of enterocin type 65-603
in our study indicates the predominance of this
type in the strains of S. faecium and S. durans
isolated from infant foods.

The data recorded in Table 2 further reveal
that the most prevalent enterocin pattern among

<table>
<thead>
<tr>
<th>TABLE 2. Percent distribution of types of enterococcal among 150 isolates recovered from infant foods.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types of sample</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>S. faecalis</td>
</tr>
<tr>
<td>Milk-based</td>
</tr>
<tr>
<td>Cereal-based</td>
</tr>
<tr>
<td>Infant food</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Number typeable</td>
</tr>
<tr>
<td>Percent typeable</td>
</tr>
</tbody>
</table>

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TABLE 2. Number and percent distribution of enterocin types occurring singly or in combination in enterococci isolated from different market samples of infant foods.

<table>
<thead>
<tr>
<th>Enterocin type, single combination</th>
<th>Streptococcus faecalis var. faecalis</th>
<th>S. faecalis var. liquefaciens</th>
<th>S. faecalis var. zymogenes</th>
<th>S. faecium</th>
<th>S. durans</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-9</td>
<td>6 (17.6%)</td>
<td>1 (4.8%)</td>
<td>1 (7.7%)</td>
<td>7 (20.0%)</td>
<td>0</td>
</tr>
<tr>
<td>244</td>
<td>3 (8.8%)</td>
<td>1 (4.8%)</td>
<td>2 (15.4%)</td>
<td>7 (20.0%)</td>
<td>2 (18.2%)</td>
</tr>
<tr>
<td>X-9, 244</td>
<td>2 (5.9%)</td>
<td>2 (9.5%)</td>
<td>...</td>
<td>1 (2.9%)</td>
<td>...</td>
</tr>
<tr>
<td>9790</td>
<td>2 (5.9%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>X-9, 9790</td>
<td>1 (2.9%)</td>
<td>1 (4.8%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1151</td>
<td>1 (2.9%)</td>
<td>...</td>
<td>...</td>
<td>1 (2.9%)</td>
<td>...</td>
</tr>
<tr>
<td>1081</td>
<td>2 (5.9%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>X-9, 1081</td>
<td>7 (20.6%)</td>
<td>...</td>
<td>...</td>
<td>1 (2.9%)</td>
<td>...</td>
</tr>
<tr>
<td>10541, 65-603</td>
<td>...</td>
<td>...</td>
<td>6 (46.2%)</td>
<td>3 (8.6%)</td>
<td>...</td>
</tr>
<tr>
<td>10541, 7073</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2025</td>
<td>1 (2.9%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>244, 7073</td>
<td>4 (11.8%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2025, 9790</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>1 (9.1%)</td>
<td>...</td>
</tr>
<tr>
<td>2025, 7073</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>815-2</td>
<td>...</td>
<td>1 (4.8%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>7073</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>244, Liq. A</td>
<td>3 (8.8%)</td>
<td>9 (42.8%)</td>
<td>1 (7.7%)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Liq. B</td>
<td>...</td>
<td>9 (42.8%)</td>
<td>1 (7.7%)</td>
<td>1 (2.9%)</td>
<td>...</td>
</tr>
<tr>
<td>244, Liq. A, Liq. B</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>1 (2.9%)</td>
<td>...</td>
</tr>
<tr>
<td>697</td>
<td>1 (2.9%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>X-9, 244, 1081, 10541, Liq. A, Liq. B</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>21</td>
<td>13</td>
<td>35</td>
<td>11</td>
</tr>
</tbody>
</table>

1 Not detected.

S. faecalis and its varieties is X-9, either singly or in combination with other types. Nearly half of the 34 strains of S. faecalis var. faecalis showed that pattern. This enterocin type, X-9, mainly was associated with other types, i.e., 244, 1081, 9780, etc. In one strain of S. faecalis var. faecalis recovered from a cereal-based brand of infant food, X-9 was associated with five other types simultaneously, and hence, was responsible for a new pattern, i.e., X-9, 244, 1081, Liq. A, Liq. B, 10541. The next most prevalent enterocin type was liq. A, which was exhibited by 20 strains (29.4%). The liq. A was also more common among strains of S. faecalis var. liquefaciens (42.8%). The enterocin type 10541 frequently (46.2%) was associated with strains of S. faecalis var. zymogenes. Most S. faecalis strains recovered from cereal-based infant foods showed patterns X-9 and 244, thereby indicating the prevalence of these types in cereal-based infant foods manufactured in India. Sharma et al. (40) also demonstrated the predominance of patterns X-9 and 1087 among group D streptococcal isolates from clinical cases.

Because many of the enterococci showed varied enterocin patterns, autoinfection with the same organisms does not appear to play an important role of S. faecalis in their enterocin typing. Possible sources of infection appear to be from dairy water supply and personnel (carriers) handling these products, because many of the common enterocin patterns were observed also in enterococci isolated from these sources (Table 3 and 4). Data in the two tables indicate the preponderance of type X-9 in enterococci recovered from both dairy water supply samples and hand washings of personnel handling milk during the manufacture of an infant food. Out of 25 typeable strains of
TABLE 3. Enterocin patterns of enterococci recovered from samples of dairy water supply.

<table>
<thead>
<tr>
<th>Enterocin pattern (single/combination)</th>
<th>Streptococcus faecalis var. faecalis</th>
<th>S. faecalis var. zymogenes</th>
<th>S. faecalis var. liquefaciens</th>
<th>S. faecium</th>
<th>S. durans</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-9</td>
<td>1</td>
<td></td>
<td>3</td>
<td>4</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>10541</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>65-603</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>10541, 65-603</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Liq. A</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Untypeable</td>
<td>1</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td></td>
<td>10</td>
<td>5</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

1 Not detected.

Enterococci recovered from both sources, at least 14 had the enterocin pattern X-9, followed by patterns 65-603 and 10541. Hence, dairy water could be the source of these enterocin types in infant foods. The pattern X-9, 244, 1081, Liq. A, Liq. B, 10541 was observed with the lone S. faecalis var. faecalis strain that was isolated from the hand washing of personnel engaged in manufacture of infant food from where enterococci showing the same enterocin pattern also was recovered. Because S. faecalis var. faecalis strains showing the enterocin pattern X-9, 1081 were not recovered from any samples of dairy water supply and hand washings of the dairy personnel, the possibility of the latter two sources for contributing X-9, 1081 types in the infant foods is ruled out. However, because the paucity of literature pertaining to sources of the common enterocin types in dairy products and other foods, this contention cannot be substantiated. Moreover, from the present study, a variety of species, strains, and enterocin types is obtained from infant foods. Hence, the possibility of some other sources contributing these types in infant foods cannot be ruled out. It may be possible that because of insufficient number of isolates recovered from the dairy water supply samples

TABLE 4. Enterocin patterns of enterococci recovered from hand washings of milk handlers.

<table>
<thead>
<tr>
<th>Enterocin pattern (single/combination)</th>
<th>Streptococcus faecalis var. faecalis</th>
<th>S. faecalis var. zymogenes</th>
<th>S. faecalis var. liquefaciens</th>
<th>S. faecium</th>
<th>S. durans</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-9</td>
<td>1</td>
<td></td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>X-9, 1081</td>
<td>1</td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>244, 7073</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Liq. A</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>244</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>X-9, 244, 1081, Liq. A, Liq. B, 10541</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Untypeable</td>
<td>3</td>
<td></td>
<td>2</td>
<td>1</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td></td>
<td>2</td>
<td>6</td>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

1 Not detected.
and hand washings of personnel subjected to enterocin typing, many enterocin types might have been missed in our investigation.

The enterocin typing method could be useful for epidemiological purposes as has been pointed out by Kekessy and Piquet (27). Distribution of common types, especially in food poisoning, also would be extremely informative. Therefore, efforts to develop an enterocin typing scheme of enterococci on routine basis for epidemiological purposes are promising and should be continued.

REFERENCES


34 Posztai, S., and F. Vetesi. 1972. Study on mouse


