Intestinal Microflora of Coastal Puffer Fishes

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Three coastal species of puffer Fugu vermicularis vermicularis F. niphobles and Canthigaster rivulata, were examined for intestinal microflora. Six groups of genus Vibrio, along with genus Pseudomonas, were found to be predominantly detected in most specimens. It was noted that the viable counts of a Vibrio group, tentatively identified as V. alginolyticus, and the total viable counts showed a positive correlation (r=0.92).

Tetrodotoxin (TTX) was known as a potent neurotoxin specific to puffer fish. In 1964, however, this toxin was also found in the California newt Taricha torosa.13) Since then, it has been detected in various vertebrates and invertebrates.1-7) Recently, we9) have found the production of TTX and anhydroTTX by a strain of Vibrio sp. which was isolated from the intestines of a xanthid crab Atergatis floridus. TTX-producing bacteria of the genus Vibrio have also been isolated from the vermiculated puffer Fugu vermicularis vermicularis,9) and a starfish Astropecten polyacanthus,10) whereas a strain of the genus Pseudomonas from a calcareous alga Jania sp. was also found to produce TTX.11 In this situation, ecological studies on TTX-producing bacteria seemed to be essential. This paper deals with the intestinal microflora of three species of puffer fishes which are popular in coastal waters of Japan.

Materials and Methods

Fish

Five specimens of the vermiculated puffer F. vermicularis vermicularis (52–123 g in body weight) were caught off Ohara, Chiba Prefecture, in November 1985 when the water temperature was 21.7°C. Eleven specimens of the grass puffer F. niphobles were collected off Shimoda, Shizuoka Prefecture, one (74 g) in March (13.1°C), four (55–80 g) in April (14.4°C), and six (35–73 g) in July (24.3°C). Eleven specimens of the scribbled toby Canthigaster rivulata were also caught off Shimoda, two (20–24 g) in March (13.1°C), five (12–25 g) in April (14.4°C) and four (25–49 g) in July (24.3°C). All those live specimens were used for both bacteriological examination and lethal potency assay. In addition, seawater samples were also collected in April and July when the grass puffer and scribbled toby specimens were caught.

Assay of Lethal Potency

Most specimens of those puffers were dissected into various tissue, and assayed for lethal potency by the official method for TTX.12)

Bacteriological Sampling

Intestinal contents of each live puffer specimen were squeezed out into a test tube, and to the contents was added nine volumes of the diluent reported previously.13) The tenfold dilution thus prepared was further diluted serially. Each sample was inoculated onto PYBG agar,13) 1/20PYBG agar,13) N-PYBG agar,13) BTB-Teepol agar (Eiken), MPEA agar13) and MAGPC agar.14) The PYBG, 1/20PYBG, BTB-Teepol and MPEA plates were aerobically, and the PYBG, N-PYBG and MAGPC plates anaerobically incubated, both at 25°C for 7 to 8 days. Anaerobiosis was established, by a GasPak anaerobic system (BBL).

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Identification of Bacteria

After incubation, bacterial colonies were counted, and about 20 colonies were isolated at random from each plate. The bacteria isolated aerobically were identified at generic level according to Shewan et al., Cowan, Sugita et al., and Starr et al. Vibrio strains were classified into nine groups by abilities: to swarm on solid agar; form a yellow colony on TCBS agar (Eiken); produce indole; and produce acids from arabinose. Some Vibrio strains were further examined for abilities: to grow in 8% NaCl; produce H2S; and grow at 40°C.

On the other hand, anaerobic bacteria were classified on the basis of Gram reaction, cellular morphology, spore formation and the ability to grow aerobically.

On suitably diluted bacterial samples, colonies of each bacterial group were counted and expressed in number per gram or ml material. The maximum count of each bacterial group through the six media was regarded as viable count. Total viable count (TVC) was obtained by summation of viable counts of bacterial groups.

Results

Lethal Potency of Puffer Fishes

All the specimens of the vermiculated puffer, grass puffer and scribbled toby were found to be toxic as shown in Table 1.

Viable Counts of Bacteria

The number of colonies that formed on a plate widely varied according to the type of agar medium, and also between aerobic and anaerobic conditions: On PYBG agar medium, 10^3 to 10^8 g^-1; 1/20PYBG, 10^2 to 10^8 g^-1; BTB-Teepol, 10^6 to 10^8 g^-1; MPEA, <2 x 10^2 to 10^4 g^-1; N-PYBG, <2 x 10^5 to 10^6 g^-1; and MAGPC, <2 x 10^5 to 10^6 g^-1.

The seawater sample showed similar variation, with colony counts ranging from <2 x 10^1 to 10^6 ml^-1.

Bacteria Isolated

A total of 2147 strains of aerobic and facultatively anaerobic bacteria were isolated aerobically from the intestinal contents of the three species of

<p>| Table 1. Lethal potency of vermiculated puffer, grass puffer and scribbled toby specimens |
|----------------------------------------|-----------------|-----------------|-----------------|
| Species                  | Date of collection | Lethal potency (MU/g) |   |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Muscle</th>
<th>Skin</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vermiculated puffer</td>
<td>Nov. 17, 1985</td>
<td>&lt; 5</td>
<td>100±66</td>
<td>17±24</td>
</tr>
<tr>
<td>Grass puffer</td>
<td>Apr. 3, 1986</td>
<td>8±8*</td>
<td>134±112</td>
<td>96±95</td>
</tr>
<tr>
<td></td>
<td>July 30, 1986</td>
<td>22±17</td>
<td>62±62</td>
<td>35±27</td>
</tr>
<tr>
<td>Scribbled toby</td>
<td>Apr. 3, 1986</td>
<td>15±11</td>
<td>71±45</td>
<td>23±20</td>
</tr>
<tr>
<td></td>
<td>July 30, 1986</td>
<td>8±12</td>
<td>35±30</td>
<td>65±55</td>
</tr>
</tbody>
</table>

* Mean±standard deviation.

| Table 2. Characters and grouping of the Vibrio strains isolated from intestines of three puffer fishes |
|----------------------------------------|-----------------|-----------------|-----------------|
| Groups                  |                | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Characters*1:          |                | + | + | + | - | - | - | - | - | - |
| Swarming               |                | + | - | - | - | + | + | + | + | - |
| Yellow colony on TCBS  |                | - | + | + | - | - | - | - | - | - |
| Indole production      |                | + | + | + | + | + | - | + | + | - |
| Acids from arabinose   |                | - | - | - | - | + | - | - | - | - |
| Grouping*2:            |                | Seawater | 12 | 0 | 2 | 1 | 8 | 11 | 0 | 7 | 32 |
|                        | Vermiculated puffer | 38 | 0 | 0 | 0 | 102 | 41 | 0 | 122 | 55 |
|                        | Grass puffer     | 173 | 19 | 33 | 115 | 39 | 30 | 43 | 29 | 66 |
|                        | Scribbled toby   | 110 | 1 | 31 | 35 | 106 | 153 | 7 | 45 | 150 |
| Total                  |                | 333 | 20 | 66 | 151 | 255 | 235 | 50 | 203 | 303 |

*1 +, Positive reaction; -, negative reaction.

*2 Number of strains.
puffer fish and the two water samples. The strains were composed of Vibrio (1616 strains), Pseudomonas (268), Flavobacterium (19), Moraxella (8), Staphylococcus (2), Acinetobacter (1) and others (233) which lost the viability after transfer. The Vibrio strains were divided into nine groups on the basis of four characters, as shown in Table 2. All strains of Vibrio group 1 grew in 8% NaCl and at 40°C, and did not produce H₂S. It strongly suggested that Vibrio group 1 was V. alginolyticus.

On the other hand, a total of 1202 strains of anaerobic bacteria were isolated anaerobically. The strains were obligate anaerobes (82 strains) and facultative anaerobes (1120). The former consisted of Bacteroidaceae (77), Gram-positive cocci (4) and Clostridium (1), while the latter of Gram-negative rods (981), Gram-positive cocci (85), Gram-positive rods (52) and yeasts (2).

Microflora of Seawater

The generic composition of the seawater samples are shown in Table 3. Four groups of Vibrio, along with each genus of Pseudomonas, Moraxella and Flavobacterium, were detected in the April sample with viable counts ranging from 10² to 10³ ml⁻¹, and a TVC of 10⁴ ml⁻¹. In the July sample, seven groups of Vibrio, Pseudomonas and Flavobacterium occurred with viable counts ranging from 10¹ to 10² ml⁻¹ and a TVC of 10³ ml⁻¹.

Microfloras in Intestinal Contents of Puffers

Tables 4 through 6 show the microfloras of intestinal contents of the three puffers, with TVC values ranging from 10⁴ to 10⁹ g⁻¹.

A total of ten bacterial genera and/or groups were detected in the vermiculated puffer (Table 4). Vibrio groups 5, 8 and 9 were predominantly detected in all the five specimens. Vibrio group 6, Pseudomonas, Flavobacterium and Bacteroidaceae were highly counted (10³ to 10⁶ g⁻¹) in more than three specimens.

Nine bacterial genera and/or groups were detected at densities ranging from 10² to 10⁶ g⁻¹ in the grass puffer specimen collected in March while a total of nine genera/or groups occurred with viable counts from 10³ to 10⁶ g⁻¹ in April (Table 5). Vibrio groups 1, 4, 6 and 9 and Pseudomonas occurred predominantly in more than 60% of the March and April specimens. On the other hand,
Table 5. Generic composition of intestinal bacteria from eleven grass puffer specimens

Viable counts (log No. g⁻¹)

| Genera          | March | April | July | Specimen No. |        |        |        |        |        |        |        |        |        |        |        |        |
|-----------------|-------|-------|------|--------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|                 | 1     | 2     | 3    | 4            | 5     | 6     | 7     | 8     | 9     | 10    | 11    | Mean ± S.D.*¹ |
| *Vibrio group*  |       |       |      |              |       |       |       |       |       |       |       |       |       |       |       |       |
| 1               | 6.30  | 2.94  | 5.94 | 4.68         | 2.70  | 6.32  | 7.83  | 5.11  | 7.48  | 8.41  | 7.92  | 5.97 ± 1.95 |
| 2               | nd*²  | nd    | nd   | nd           | nd    | nd    | 3.58  | 5.92  | 8.04  | nd    | 5.85 ± 2.23 |
| 3               | nd    | nd    | nd   | nd           | nd    | 5.57  | 3.58  | 5.20  | 8.60  | 6.81  | 5.95 ± 1.88 |
| 4               | 6.63  | 2.76  | 5.64 | nd           | nd    | 6.45  | 3.58  | 5.23  | 7.59  | 7.34  | 7.66  | 5.88 ± 1.75 |
| 5               | 5.61  | nd    | 4.00 | nd           | nd    | 5.85  | 7.58  | 4.62  | 7.23  | 7.59  | 7.52  | 6.25 ± 1.44 |
| 6               | 5.60  | 2.46  | 5.58 | 4.38         | nd    | 6.04  | 7.68  | nd    | 7.20  | 7.34  | 6.81  | 5.90 ± 1.66 |
| 7               | 5.60  | nd    | nd   | nd           | nd    | nd    | 5.59  | 7.23  | 7.34  | 7.72  | 6.70 ± 1.02 |
| 8               | 6.20  | 2.30  | nd   | nd           | nd    | nd    | 5.85  | 4.04  | nd    | 7.34  | 6.49  | 5.37 ± 1.86 |
| 9               | 5.61  | 2.30  | 5.26 | 5.70         | 2.78  | nd    | nd    | 5.52  | 6.38  | 7.34  | nd    | 5.11 ± 1.72 |
| *Pseudomonas*   |       |       |      |              |       |       |       |       |       |       |       |       |       |       |       |       |
| 1               | 5.61  | 2.78  | 6.18 | nd           | 3.98  | 6.92  | 8.15  | 4.92  | 6.59  | 7.34  | 6.81  | 5.93 ± 1.63 |
| *Moraxella*     |       | 2.78  | 5.18 | nd           | nd    | nd    | nd    | nd    | nd    | nd    | nd    | 3.98 ± 1.70 |
| *Bacteroidaceae*| 5.51  | nd    | 4.15 | nd           | nd    | 3.74  | 2.30  | nd    | nd    | nd    | 2.67  | 3.67 ± 1.27 |
| *Yeasts*        | nd    | nd    | nd   | nd           | nd    | nd    | nd    | nd    | nd    | nd    | nd    | 2.97  |
| TVC             | 7.00  | 3.52  | 6.54 | 5.76         | 4.03  | 7.21  | 8.67  | 6.33  | 8.10  | 8.97  | 8.38  | 6.77 ± 1.80 |

*¹, *² Refer to the footnotes in Table 4.
Table 6. Generic composition of intestinal bacteria from eleven scribbled toby specimens

<table>
<thead>
<tr>
<th>Genera</th>
<th>March</th>
<th>April</th>
<th>July</th>
<th>Mean ± S.D.*1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td><em>Vibrio group</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.40</td>
<td>6.30</td>
<td>8.41</td>
<td>7.11</td>
</tr>
<tr>
<td>2 nd*2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>3 nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>4 nd</td>
<td>5.11</td>
<td>8.15</td>
<td>7.41</td>
<td>6.90</td>
</tr>
<tr>
<td>5 6.56</td>
<td>5.11</td>
<td>6.78</td>
<td>7.11</td>
<td>6.30</td>
</tr>
<tr>
<td>6 7.04</td>
<td>nd</td>
<td>6.88</td>
<td>7.51</td>
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</tr>
<tr>
<td>7 nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>8 nd</td>
<td>5.11</td>
<td>7.97</td>
<td>6.96</td>
<td>7.00</td>
</tr>
<tr>
<td>9 7.40</td>
<td>5.41</td>
<td>8.72</td>
<td>7.15</td>
<td>6.98</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.36</td>
<td>4.58</td>
<td>8.15</td>
<td>7.52</td>
<td>7.30</td>
</tr>
<tr>
<td><em>Moraxella</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.26</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>Flavobacterium</em></td>
<td>nd</td>
<td>4.28</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>Bacteroidaceae</em></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>5.83</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>Anaerobic cocci</em></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TVC</td>
<td>7.92</td>
<td>6.43</td>
<td>9.07</td>
<td>8.15</td>
</tr>
</tbody>
</table>

*1, *2 Refer to the footnotes in Table 4.
the microflora of the July specimens widely differed from the March and April specimens (Table 5). *Vibrio* groups 1, 3–6 and *Pseudomonas* predominated in more than 80% of the specimens. *Bacteroidaceae*, obligate anaerobes, were isolated from 40 to 50% of grass puffer specimens.

Ten bacterial genera and/or groups were isolated from the scribbled toby specimens collected in March and April (Table 6). *Vibrio* groups 1, 4–6 and 9, and *Pseudomonas* were frequently predominant, while *Moraxella*, *Flavobacterium* and *Bacteroidaceae* occurred at a much lower density. In the specimens collected in July, 13 bacterial genera and/or groups were detected (Table 6). *Vibrio* groups 1, 4–9 and *Bacteroidaceae* occurred predominantly in 75 to 100% of the specimens. Other anaerobic forms, *Clostridium* and Gram-positive cocci, were occasionally isolated at densities ranging from $10^3$ to $10^4$ g$^{-1}$.

**Discussion**

As generally accepted, the intestinal tract of marine fish is colonized mainly by the genus *Vibrio*, which is classified into twenty species.\(^1\) In spite of this, most microbiologists\(^19\)–\(^21\) have been discussing the bacteria belonging to this genus in intestinal microflora of marine organisms at the generic, not species, level, since it is much laborious and time-consuming. Although the microflora of puffer intestines has not yet been reported, the present results showed that the genus *Vibrio* predominated in the intestines of the three puffers. The isolates of the genus *Vibrio* were further grouped based on the four characters (Table 2). As shown in Tables 3–6, *Vibrio* groups 1, 4–6, 8 and 9, along with *Pseudomonas*, occurred predominantly in the intestines of those puffers. The grass puffer specimens collected in two seasons showed similar microflora patterns, though the number of bacterial genera and/or groups was somewhat less in March and April specimens (Table 5). It was also true for the scribbled toby (Table 6). The predominance of these components seems to be invariable during all seasons because they were predominantly detected in the grass puffer and scribbled toby specimens, collected when the coastal waters of Shimoda were the lowest and highest temperatures in 1986. Twelve to thirteen bacterial genera and/or groups were detected in the grass puffer and scribbled toby specimens collected in July, whereas 10 bacterial genera and/or groups detected in the March and April specimens. This result showed that minor components of bacteria fluctuated. The seawater showed a similar variation in microflora (Table 3).

Sera et al.\(^22\) demonstrated that marine bacteria invading into the gastrointestinal tract of fish were partly screened by the action of gastric and bile acids excreted, resulting in the intestinal microflora specific to each fish. Sugita et al.\(^19\) suggested that the ability to attach on the inside wall of intestinal tracts is also important for indigenous bacteria. By those causes, bacteria of the 6 groups of genus *Vibrio*, along with the genus *Pseudomonas*, could have inhabited puffer intestines.

As described in **Results**, TVC in puffer intestines showed wide individual and seasonal variations. Similar phenomena have been observed in some freshwater fishes.\(^4\) Such variations in TVC could be associated with diets, species of host animals, and their physiological conditions, etc. The bacterium of *Vibrio* group 1 was tentatively identified as *V. alginolyticus*, and its population showed a close correlation ($r=0.92$) with the intestinal TVC, in the three puffers.

Recently, two TTX-producing bacteria of the genus *Vibrio* were isolated from the intestines of marine animals: a *Vibrio fischeri*-like bacterium\(^8\) from the xanthid crab *Atergatis floridus* and *V. alginolyticus*\(^9,10\) from the starfish *A. polyacanthus* and the vermiculated puffer. *V. alginolyticus* is widely distributed in marine environments.\(^16,26\) This, along with the general abundance of "*V. alginolyticus*" in puffer intestines as described above, suggests a close involvement of this bacterium in the toxification of puffers. Studies along those lines are now in progress.

**References**

6) T. Noguchi, H. Narita, J. Maruyama, and K.
Microflora of Puffer Fishes


