ISOLATION OF MOTILE AEROMONAS SPP. FROM FISH AND THEIR CYTOTOXIC EFFECT ON VERO CELL CULTURES

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The presence of motile Aeromonas spp. in fish and other sea food on the Belgrade retail market was investigated with the aim of determining the ability of these bacteria to produce and secrete toxins. Nine strains of motile Aeromonas spp. were isolated from seventy-eight food samples. Aer. sobria was identified in three cases, while six of the obtained strains were identified as Aer. hydrophila. Strains of motile Aeromonas spp. from different sources were analysed for cytotoxicity on Vero cell cultures. A cytotoxic effect was detected for all tested strains, but of different intensity.

Key words: cytotoxicity, isolation, motile Aeromonas spp., Vero cells.

INTRODUCTION

The genus Aeromonas, in the family Vibrionaceae, comprises non-motile psychrophilic and motile mesophilic aeromonads. The psychrophils are well known as pathogens for fish but not humans. Mesophilic Aeromonas spp. occasionally cause wound infections, septicaemia, and gastrointestinal infections in humans. Aeromonas spp. are environmental bacteria, widespread in water, sewage and soil, and well recognized as pathogens of fish, reptiles and amphibians. Animals can be fecal carriers of Aeromonas spp. (Panin, 1993).

The main source of aeromonas infection is water, even chlorinated water as well as sea food (Burke et al., 1984 a,b; Haninen and Siitonen, 1995). Food products like poultry, raw meat, and vegetables also may contain enteropathogenic Aeromonas spp., (Burke et al., 1984a,b). The incidence of Aeromonas spp. in food is high but the majority of Aeromonas spp. isolates from food are not enteropathogenic strains (e. g. Aeromonas caviae), (Merino et al., 1995). The enteropathogenic strains are mainly represented by Aeromonas veronii sobria and Aeromonas hydrophila. Food-borne gastroenteritis associated with Aeromonas spp. has been reported in humans from all age groups and is particularly severe in risk populations like very young children and old immunocompromised patients. It is important that Aeromonas spp. found in food are able to produce different exotoxins, some of which are clearly enterotoxins.

A correlation at a highly significant level between cytotoxicity and virulence in suckling mice was observed and it is of interest that the production of cytotoxin
has also been correlated with gastroenteritis (Millership et al., 1986; Wong et al., 1996). Vero cells are reported to be the most sensitive cell line for the detection of aeromonas cytotoxicity (Barer et al., 1986). Toxin production appears to contribute to virulence but is not the only factor that defines virulence. The main additional virulence factors of Aeromonas spp. that can be associated with gastroenteritis are: endotoxin (LPS-lipopolysaccharide), S-layers, and fimbriae (adesins). (Merino et al., 1995).

This study was carried out to investigate the presence of motile Aeromonas spp. mainly in fish on sale in Belgrade examine the ability of these bacteria to produce and secrete factors/toxins and to determine their cytotoxic effects on cultured cells.

MATERIALS AND METHODS

Sample collection

Samples were collected from January through June 1998 from the retail market in Belgrade. Samples were: freshwater fish (14 samples), saltwater fish (56 samples) and sea-food (8 samples). The freshwater samples were mainly salmon and trout, the saltwater mackerel, and the sea-food squid. Most of the saltwater fish samples were frozen, while freshwater fish were mainly fresh or just cooled. After the skin surface had been removed 25g of meat was taken aseptically and pooled in sterile Stomacher bags for bacteriological analyses.

Media

Selective enrichment broth: alkaline peptone water, selective agar, starch ampicillin agar. Confirmatory test media: Aeromonas hydrophila medium, Vibriostatic test agar (O/129).

Microorganisms

The microorganism collection came from different sources: fish meat-6 strains of Aer. hydrophila, 3 strains of Aer. sobria (Faculty of Veterinary Medicine, Belgrade); poultry meat- 2 strains of Aer. hydrophila (Institute for Meat Hygiene and Technology, Belgrade); water- 1 strain of Aer. hydrophila (Serbian Public Health Institute) and from a human/clinical source -1 strain of Aer. hydrophila CCM 4528 (Czech Collection of Microorganisms).

Cell Culture

The assay for cytotoxicity was performed using Buffalo Green Monkey (Vero) cells.

Bacteriological Analyses

The initial sample (25g) was homogenised in alkaline peptone water (225ml), and incubated aerobically at 28±1°C for 24h. 10µl loopfuls of enrichment broth were streaked on to dried starch ampicillin agar plates to obtain isolated colonies and incubated aerobically at 28±1°C for 24h. Yellow to honey-coloured colonies, surrounded by a cloudy zone of starch hydrolysis were recorded as presumptive positive Aeromonas spp. Presumptive positive colonies were confirmed as motile aeromonads with confirmatory tests: Aeromonas hydrophila medium. Vibriostatic test agar (resistance to O/129). Gram stain (Gram-negative, rods). oxidase (+), catalase (+). To further classify the colonies at the species
level, the following were used: ATB system (miniAPI instrument) with ID 32 GN strips (BioMerieux).

**Assay for cytotoxicity (CTE)**

Twelve *Aeromonas* spp. strains from different sources were assessed for cytotoxic activity using Buffalo Green Monkey (Vero) cells. The bacteria were subcultured into 10 ml of brain-heart infusion broth and incubated for 18h at 28 ± 1°C on a horizontal shaker at 150 rpm. The cell suspension was then harvested by centrifugation at 10,000 x g for 10 minutes and the supernatant was sterilised by filtration through a 0.45µm filter. Filtrates added to Vero cell monolayers and cytotoxicity (CTE) estimated after 45 minutes to 24h at 37 ± 1°C. The appearance of vacuoles, cell pigmentation, intensive light refraction, cell rounding and detachment of Vero cells under the light microscope were interpreted as evidence for cytotoxic activity.

**RESULTS AND DISCUSSION**

**Detection of motile Aeromonas spp. in food samples**

Nine strains of motile *Aeromonas* spp. were isolated and identified from seventy-eight samples of food. The most common species of motile aeromonads found was *Aer. hydrophila* which was represented with 6 strains. The remaining three isolated strains were identified as *Aer. sobria*. As shown in Table 1, most isolates were obtained from freshwater fish and only one from saltwater fish. No isolates were recovered from squid sea-food.

**Table 1. Aeromonas spp. isolated from the examined food samples**

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No. of samples tested</th>
<th>Species isolated</th>
<th>No. of strains isolated</th>
<th>Label of obtained isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshwater fish</td>
<td>14</td>
<td><em>Aer. hydrophila</em></td>
<td>6</td>
<td>1/3/11/15/19/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aer. sobria</em></td>
<td>2</td>
<td>7/17</td>
</tr>
<tr>
<td>Saltwater fish</td>
<td>56</td>
<td><em>Aer. sobria</em></td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Sea-food</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>78</strong></td>
<td><strong>Total</strong></td>
<td><strong>9</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Aeromonas cytotoxicity**

Twelve strains of motile *Aeromonas* spp. (3 strains of *Aer. sobria* and 9 strains of *Aer. hydrophila*) from different sources were analysed. The morphological changes in Vero cells caused by supernatant fluids from the examined motile *Aeromonas* spp. were very similar and toxigenic. The cytotoxic activity of *Aer. hydrophila* strain on Vero cells is shown Figure 1. CTE was evident 45 minutes after inoculation of the supernatant. Supernatants of two strains induced complete CTE (++++) of Vero cell monolayers 4-5h and one more strain 24h after inoculation. Very strong CTE after 24h of incubation was induced by seven
Figure 1. Cytotoxic activity of Aer. hydrophila strain on Vero cells. A:B/C/D/E - cells treated with supernatant of strain 3
supernatants and only two supernatants induced slight changes (+) in the
cultured cells (Table 2). Thus, most supernatants induced very strong CTE (+++) on
Vero cells during the incubation period.

Table 2. The relative intensity of cytotoxic changes (CTE) in Vero cells induced by
supernatants of Aeromonas spp.

<table>
<thead>
<tr>
<th>Intensity of CTE</th>
<th>CTE on Vero cell monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45'-2h</td>
</tr>
<tr>
<td></td>
<td>No of strains %</td>
</tr>
<tr>
<td>-</td>
<td>0 0</td>
</tr>
<tr>
<td>+</td>
<td>1 8.33</td>
</tr>
<tr>
<td>++</td>
<td>4 33.33</td>
</tr>
<tr>
<td>+++</td>
<td>7 56.33</td>
</tr>
<tr>
<td>++++</td>
<td>0 0</td>
</tr>
<tr>
<td>In total</td>
<td>12 99.99</td>
</tr>
</tbody>
</table>

- without CTE
+ slight CTE
++ strong CTE
+++ very strong CTE
++++ complete CTE

Table 3. CTE of Aer. sobria supernatants

<table>
<thead>
<tr>
<th>Label of strain</th>
<th>Source</th>
<th>CTE on Vero cell monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>45–2h</td>
</tr>
<tr>
<td>7</td>
<td>Freshwater fish</td>
<td>++++</td>
</tr>
<tr>
<td>17</td>
<td>Saltwater fish</td>
<td>++</td>
</tr>
<tr>
<td>25</td>
<td>Broth control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vero cell control</td>
<td></td>
</tr>
</tbody>
</table>

- without changes
+ slight CTE
++ strong CTE
+++ very strong CTE
++++ complete CTE
The cytotoxic effects on Vero cells are presented separately in Table 3 for *Aer. sobria* and in Table 4 for *Aer. hydrophila* strains, including positive and negative controls.

**Table 4. CTE of *Aer. hydrophila* supernatants**

<table>
<thead>
<tr>
<th>Label of strain</th>
<th>Source</th>
<th>CTE on Vero cell monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td></td>
<td>45–2h</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>15</td>
<td>Freshwater fish</td>
<td>+++</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>101</td>
<td>Poultry</td>
<td>++</td>
</tr>
<tr>
<td>102</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>1019</td>
<td>Water</td>
<td>+++</td>
</tr>
<tr>
<td>CCM 4528</td>
<td>Human/clinical</td>
<td>+++</td>
</tr>
<tr>
<td>Broth control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vero cell control</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- without changes +++ very strong CTE
- slight CTE ++++ complete CTE
- strong CTE

Many Gram-negative bacterial pathogens synthesize cytolytic toxins as virulence factors. *Aer. hydrophila* and *Aer. sobria* have received increasing attention because of their frequent association with human diseases including diarrhea, wound infections, septicemia etc. (Albrecht, 1996). Both species secrete a cytotoxic, poreforming haemolysin called aerolysin which appears to be largely responsible for the virulence of these bacteria. Most Aeromonas toxins are thought to be involved in diarrheal diseases (Krovaceck et al. 1994) and the effect of diarrhogenic toxins is alteration of intestinal function. Wong et al. (1996) observed a correlation at a highly significant level between cytotoxicity and virulence in suckling mice but other proposed virulence factors, such as LPS and β-haemolysin were found not to be predictors of virulence of aeromonads in the model they used. Vero-cytotoxic activity was detected in all virulent aeromonas isolates tested, and at a low level in one of six avirulent strains (Wong et al., 1996).

Out of the total of seventy-eight samples of freshwater and saltwater fish and cephalopodas examined, motile *Aeromonas spp.*, were found out in nine samples. *Aer. sobria* was identified in three out of nine isolates, while six were identified as
Aer. hydrophila. All the tested filtrates of Aer. sobria and Aer. hydrophila caused cytotoxic effects in Vero cells, but the intensity of changes differed between filtrates.

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SADRŽAJ