Protection against Atypical Aeromonas salmonicida Infection in Carp (Cyprinus carpio L.) by Oral Administration of Humus Extract

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ABSTRACT. Humic substances are formed during the decomposition of organic matter in humus, and are found in many natural environments in which organic materials and microorganisms have been present. In the present study, oral administration of humus extract to common carp (Cyprinus carpio L.) induced effective protection against experimental atypical Aeromonas salmonicida infection. Mortality of fish and development of skin lesions such as hemorrhages and ulcers were significantly suppressed in carp treated with 10%, 5% or 1% humus extract adsorbed on dry feeding pellets. The median surviving days was also greater in fish treated with 10% or 5% humus extract than in untreated fish. Atypical A. salmonicida was isolated from ulcerative lesions of part of dead fish, but Aeromonas hydrophila and Flavobacterium sp. were also isolated from these fish, verifying bacterial population changes during the progression of skin lesions. These results clearly show that treatment of fish with humus extract is effective in preventing A. salmonicida disease.

KEY WORDS: atypical Aeromonas salmonicida, carp, humic substance, humus extract, ulcer.

Humic substances are formed during the decomposition of organic matter in humus. They can be found in many natural environments in which organic materials and microorganisms are or have been present [14]. The chemical composition, structure and functional groups can vary greatly, according to their origin and age and the conditions of the humification process (humidity, aeration, temperature, mineral microenvironment, etc.). Humic substances can be divided into humic acids, fulvic acids and hynathmolic acids according to their hydrophilic properties [7]. Natural humification products such as humus, peat, sapropel and mumie have been used to develop pharmacologic agents with diverse applications in medical practice [13, 15]. These have been successfully used as anti-inflammatory agents because they have local anti-inflammatory and analgesic properties [6, 11]. The use of coal-derived humic acid and fulvic acid as antimicrobials has also been investigated [10], and the anti-HIV activity of oxihumate [12] and synthetic humic acid analogues [8] has been reported. No measurable side effects have been observed [5, 11]. However, the potential of humic substances in the treatment of animal diseases has scarcely been investigated. Humic substances should be applicable to fish farming to reduce the incidence of various infectious diseases. Even when environmental conditions are favorable and the fish are healthy, mortality currently occurs if infectious agents are introduced into the farm.

Atypical Aeromonas salmonicida is a fish pathogen that causes several diseases in freshwater fish such as goldfish (Carassius auratus L.) [9] and eel (Anguilla japonica) [1], and also seawater fish [3]. Since 1996, A. salmonicida infection characterized by the formation of ulcers on the body surface and fins has become prevalent in colored carp (Cyprinus carpio koi L.) cultivation in Japan [2]. Vaccination is believed to be effective in principle against the disease, but no effective vaccine has yet been developed. We therefore investigated the effect of oral administration of humus extract on protection of carp from atypical A. salmonicida infection.

MATERIALS AND METHODS

Fish: Fry of the common carp (Cyprinus carpio L.) were kindly provided by the Agricultural, Food and Environmental Sciences Research Center of Osaka Prefecture, Neyagawa, Japan. They were grown in 170 l plastic aquaria filled with dechlorinated tap water (passing through once, at a flow-through rate of 40 l/hr; water temperature regulated to 20 to 25°C) and aerated. The fish were fed a commercial floating dry pellet twice daily. A daily regimen of 15 hr of light followed by 9 hr dark was maintained.

Humus extract: Humus extract was prepared from humus (collected in Nagasaki Prefecture, Kyushu, Japan) using water. The humus was added 6 volumes of dechlorinated water (v/w), and the mixture was agitated every day for 30 days, then left to stand at 25 to 28°C for 4 months. Supernatant was collected and filtered using a membrane filter (pore size: 25 μm). The resulting humus extract has pH 2.8 and contains various minerals including Al, Ca, Mg, Na and Si. The extract contained 1,500 ppm of sulfate. No culturable bacteria were found in the extract. There were small amounts of protein and carbohydrate (0.7% of the total weight).

Administration of humus extract: Fish were divided in each group and acclimatized in 40 l aquaria (flow-through rate 20 l/hr, water temperature 18–19°C) which were aerated. Fish weighing 26 ± 4 g (first trial) and 19 ± 2 g (second trial) were used for the experiments. Humus extract was sprinkled on the dry feeding pellets to provide final concentrations of 20 to 0.2% of the dry weight, and was
adsorbed into the pellets which were then dried in an incubator at 25°C. The fish were fed pellets containing humus extract twice daily (total 2% of the fish body weight per day) for 30 days prior to challenge by *A. salmonicida*, and for 22 consecutive days immediately after bacterial challenge. Control fish were fed the dry pellet without humus extract.

**Bacterial challenge**: The fish were challenged with virulent atypical non-pigment producing *A. salmonicida*. Strain T1031 donated by Niigata Prefectural Inland Water Fisheries Experimental Station, Nagaoka, Japan was cultured in heart infusion broth (Nissui Pharmaceutical Co., Tokyo, Japan) at 23°C for 5 days, with shaking. The fish were immersed at $1 \times 10^6$ cfu/ml for 60 min in both experiments, and were then observed for 22 days to determine survival, and any formation of ulcers and hemorrhagic lesions on the skin. Bacterial isolation was performed by cultivation from hemorrhagic and ulcerative lesions, and from visceral organs of dead fish. This was also done in all surviving fish.

**RESULTS**

**Fish mortality after *A. salmonicida* challenge**: Challenge tests were performed two times individually under similar experimental conditions. Figure 1A shows that the administration to carp of humus extract (10% and 5%) induced effective protection against experimental *A. salmonicida* infection in the first experiment (10 fish in each group). Of the non-treated carp in the control group, following challenge with *A. salmonicida*, 8 carp died within 11 days. In contrast, the survival rates of fish treated with 10% and 5% of humus extract were 70% ($P<0.05$ compared to control fish by $\chi^2$ test) and 90% ($P<0.01$), respectively at 22 days after challenge. However, only 30% of the carp administered 20% extract survived (not significant). In the second experiment (8 fish in each group), 63% ($P<0.01$) and 50% ($P<0.05$) of fish administered 5% and 1% humus extract, respectively, survived at 22 days after challenge, whereas all control fish died within 15 days.

**Development of skin lesions**: Skin lesions were significantly suppressed in humus-treated carps (see Table 1). The control fish showed skin hemorrhages 4 days after challenge, and skin ulcers developed 5 days after challenge; Figure 2 shows fish died 8 days (note hemorrhage on the peduncle and abdomen, Fig. 2A) and 11 days (ulcer formation and hemorrhage, Fig. 2B) after challenge, respectively. Gross lesions observed in control fish group were much more severe than in 10%, 5% or 1% humus-treated fish. The median surviving days is also greater in fish treated with 10% or 5% humus extract ($P<0.001$, Student’s $t$-test) than in untreated fish.

**Bacterial reisolation**: Atypical *A. salmonicida* was isolated from hemorrhagic and ulcerative lesions of part of dead fish, but *Aeromonas hydrophila* and *Flavobacterium* sp. were also isolated from these fish, verifying bacterial population changes during the progression of skin lesions. No *A. salmonicida*, *A. hydrophila* or *Flavobacterium* was isolated from survived fish treated with 10, 5 and 1% humus extract. The results of the present study clearly show that treatment of fish with humus extract is effective in preventing *A. salmonicida* disease.

**DISCUSSION**

We infer that humus extract protected carp against *A. salmonicida* challenge as shown by reduced mortality, extended survival, and only mild skin lesion formation. To investigate the mechanism of protection by the humus extract we tested lysozyme activity, which exhibits antibacterial activity against gram-positive bacteria and is an indicator of fish health condition, both in skin mucus and serum collected from humus-treated and control carp. No measurable increase in activity was detected when *Micrococcus*
**Table 1. Assessment of skin lesions of carp 22 days after atypical *Aeromonas salmonicida* challenge**

<table>
<thead>
<tr>
<th>Humus extract</th>
<th>No lesion</th>
<th>Slight (hemorrhage)</th>
<th>Weak (ulcer)</th>
<th>Severe (severe ulcer)</th>
<th>Dead</th>
<th>Median surviving days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>20%</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>70 (NS)</td>
<td>9 (NS)</td>
</tr>
<tr>
<td>10%</td>
<td>20</td>
<td>40</td>
<td>10</td>
<td>0</td>
<td>30 (P&lt;0.001)*</td>
<td>45 (P&lt;0.001)**</td>
</tr>
<tr>
<td>5%</td>
<td>22</td>
<td>28</td>
<td>17</td>
<td>11</td>
<td>22 (P&lt;0.001)</td>
<td>57 (P&lt;0.001)</td>
</tr>
<tr>
<td>1%</td>
<td>0</td>
<td>38</td>
<td>13</td>
<td>0</td>
<td>50 (P&lt;0.05)</td>
<td>16 (NS)</td>
</tr>
<tr>
<td>0.2%</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>88 (NS)</td>
<td>9 (NS)</td>
</tr>
<tr>
<td>Non-treated</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>89</td>
<td>8</td>
</tr>
</tbody>
</table>

Combined data from two individual challenge tests.

* Significant difference in mortalities between group receiving humus extract and non-treated group estimated by χ² test.

** Significant difference between group receiving humus extract and non-treated group estimated by Student’s *t*-test.

NS: not significant.

**Fig. 2. Gross regions in fish died after atypical *A. salmonicida* challenge, showing hemorrhage on the peduncle and abdomen (fish in the control group died 8 days after the challenge; A) and ulcer formation (control fish died 11 days after the challenge; B).**
Van Rensburg et al. [10] reported in vitro antimicrobial activity of fulvic acid. Seven bacterial strains from the Genus Streptococcus, Pseudomonas, Escherichia, Klebsiella and Proteus were sensitive to fulvic acid at high concentration (eg; 15 mg/ml). It appears that the antibacterial activity was due to the low pH of the reaction mixture given the high concentration of fulvic acid. It is reasonable to assume that humic substances absorbed via the intestinal tract of the carp affect host physiological conditions, such as innate immune responses, conferring protection against microbial infection.

No toxicity has been reported in humic substances [5, 11], since these are mostly carboxylic acids and ordinary physiological metabolites. Humic substances and/or humus extract can therefore be used in fish as food additives and for immunopotentiating materials in aquaculture. Further analysis of protective mechanisms activated by humus extract, and separation of biologically active components in humus, are now in progress.

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REFERENCES