Surface characters and extracellular toxins involved in the pathogenesis of 

*Aeromonas hydrophila*

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Received 15 May 2000; revised 12 June 2001

A small number of serotypically distinct strains of *A. hydrophila* obtained from diseased freshwater fish were examined for their pathogenic properties comprising of cell surface characteristics and extracellular toxins. Test strains exhibited homogeneity in their cell surface characteristics despite being serologically heterogeneous. Studies on extracellular biological activities revealed qualitative and quantitative differences in production of toxins, probably explaining their antigenic diversity. Three distinct proteases, namely heat stable metallo protease, heat labile serine protease and heat labile metallo protease were identified from the strains.

*Aeromonas hydrophila*, a potent bacterial fish pathogen is known to exhibit extreme antigenic heterogeneity, an attribute thwarting attempts to devise effective vaccines. The surface of *Aeromonas* bacteria as a complex antigenic entity has been known to be of key importance in pathogenicity. These protective surface characteristics of virulent strains contribute in overcoming the phagocytic barrier and the natural defence of the host permitting an easy establishment in the tissues. Besides, virulence has also been associated with the production of extracellular toxins, by these microorganisms such as protease, haemolysin, cytotoxin and enterotoxin. Despite numerous studies, the pathogenicity of *A. hydrophila* still remains a subject of speculation; the number and nature of factors involved have not been established. Owing to extreme serological heterogeneity of the organism, studies on the virulence properties of epidemiologically important strains are of interest to the production of vaccines. In the present investigation, selected serotypically distinct strains of *A. hydrophila* obtained from diseased freshwater fish of Tiruchirappalli region of Central Tamil Nadu were tested for certain cell surface properties and extracellular biological activities possibly involved in their pathogenesis. This work generating information about the pathogenic properties of the strains, has primarily been carried out to enable further studies on their immunogenicity in the Indian major carp, *Labeo rohita*.

**Materials and Methods**

**Bacterial strains**—*A. hydrophila* strains for the study were obtained from diseased freshwater fishes, *Channa, Mystus* and *Catla* spp. They were presumptively identified by the appearance of orange yellow colonies on Rimmier Shotts medium and confirmed using conventional biochemical tests as per the criteria of Austin and Austin. Despite numerous studies, the pathogenicity of *A. hydrophila* still remains a subject of speculation; the number and nature of factors involved have not been established. Owing to extreme serological heterogeneity of the organism, studies on the virulence properties of epidemiologically important strains are of interest to the production of vaccines. In the present investigation, selected serotypically distinct strains of *A. hydrophila* obtained from diseased freshwater fish of Tiruchirappalli region of Central Tamil Nadu were tested for certain cell surface properties and extracellular biological activities possibly involved in their pathogenesis. This work generating information about the pathogenic properties of the strains, has primarily been carried out to enable further studies on their immunogenicity in the Indian major carp, *Labeo rohita*.

**Fish pathogenicity (LD<sub>50</sub>)**—Duplicate groups of 8 *Labeo rohita* (10-12 cm; 18-20 g) (Indian major carp...
species) were injected, ip with 0.15 ml of different dilutions of the bacterial suspension ranging from 10-10⁶ cells/ml. Observations were made for 120 hr and 50% end point was calculated by the method of Reed and Muench.

Cell surface characteristics—Assays for surface properties were carried out on bacteria in logarithmic phase at 35°C for 18 hr in brain heart infusion agar or broth. Tests were carried out for self pelleting, precipitation of cells after boiling, haemagglutination, yeast cell coagglutination, aetiulin agglutination, congo red uptake, crystal violet binding, salt aggregation, binding of coomassie brilliant blue (CBB), suicide phenomenon and sensitivity to normal serum following the methods of previous workers.

Preparation of extracellular products—Extracellular products (ECP) were obtained using the cellophane plate technique of Liu. The protein concentration of ECP was estimated by the method of Bradford while LPS content was determined by the method of Keler and Nowotny.

Extracellular biological activities —LD₅₀ of ECP of all strains was determined in groups of 8 fish (L. rohita) injected ip (0.15 ml) with different serial dilutions of ECP up to a minimum concentration of 5μg protein/ml. LD₅₀ was expressed as μg ECP protein/g body weight of fish by the method of Reed and Muench. Proteolytic (caseinase, gelatinase and elastase), amylolytic and haemolytic activities were determined by radial diffusion method in their respective agar plates by the method of Santos et al. Enterotoxicity of ECP was determined in 2-4 day old mice and interpreted according to Dean et al.

Biological activity inhibition assays —The proteolytic and haemolytic activities of ECP were assayed again after heat treatment of the samples each at 56°C for 30 min and 80°C for 10 min. The effect of two major inhibitors on the proteolytic activities was also studied by adding the same volume of ECP to freshly prepared 50 mM ethylenediaminetetraacetic acid (EDTA) in PBS or to 100 mM phenylmethylsulphonylfluoride (PMSF) in 2-propanol and incubating at room temperature for 20 min (Santos et al.).

Statistical analysis

The correlation between pathogenicity of test strains and phenotypic characters were statistically analysed using Karl Pearson’s method of correlation coefficient.

Results

When tested for pathogenicity in fish (Table 1), the type strain exhibited no mortality (avirulent) at 10⁹ dilution (undiluted culture), while test strains showed mean LD₅₀ (ip) ranging from 4.35X10⁴ to 2.62X10⁵ cells/ml and belonged to the virulent category. AHO2 was the most lethal, recording LD₅₀ value of 4.35X10⁴ cells/ml. Table 2 gives the response of A. hydrophila to cell surface properties tested for and possibly involved in pathogenesis. All test strains demonstrated the ability to precipitate after boiling, uptake congo red and to bind crystal violet and CBB, but were negative for suicidal activity and sensitive to normal fish serum in contrast to the type strain. Self pelleting ability was observed only with AHO2. As low as 10³-10⁵ cells/ml were sufficient for haemagglutination of sheep erythrocytes and coagglutination of yeast cells. The molarity of ammonium sulfate required to field a strong aggregation of bacteria (hydrophobicity) ranged from 0.5-1.5 M.

The biological activities of extracellular products of A. hydrophila strains as determined by in vitro and in vivo assays are summarized in Table 3. Total ECP protein and lipopolysaccharide content ranged from 0.56 to 1.46 and 0.5 to 1.2 mg/ml, respectively, with AHO2 recording the highest. The lethal dose (LD₅₀) as a measure of ECP toxicity in fish ranged from 1.86 to 3.05 μg ECP protein/g fish for the test strains. Elastase, caseinase and gelatinase were detected in ECP samples of all strains except ATCC 7966, which showed no elastase activity. With decreasing values in LD₅₀ of the test strains a corresponding increase in elastase and gelatinase activities was observed. Thus, statistically a high degree of correlation was obtained between pathogenicity and the proteolytic activities of elastase (r = -0.87) and gelatinase (r = -0.98). In other

<table>
<thead>
<tr>
<th>Strain (Seroype)</th>
<th>LD₅₀ (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHO1 (021)</td>
<td>2.62X10⁵</td>
</tr>
<tr>
<td>AHO2 (024)</td>
<td>4.35X10⁴</td>
</tr>
<tr>
<td>AHO3 (027)</td>
<td>5.85X10⁴</td>
</tr>
<tr>
<td>AHO4 (028)</td>
<td>2 X 10⁵</td>
</tr>
<tr>
<td>ATCC 7966</td>
<td></td>
</tr>
</tbody>
</table>

* No mortality at 10⁹ dilution (undiluted culture)
Table 2—Possible cell surface properties involved in the pathogenesis of A. hydrophila strains

<table>
<thead>
<tr>
<th>Assay</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AH01</td>
</tr>
<tr>
<td>Self pelleting</td>
<td>-</td>
</tr>
<tr>
<td>Precipitation of cells after boiling</td>
<td>+</td>
</tr>
<tr>
<td>Haemagglutinating power (cells/ml)</td>
<td>2\times10^4</td>
</tr>
<tr>
<td>Yeast cell coagglutinating power (cells/ml)</td>
<td>8\times10^3</td>
</tr>
<tr>
<td>Acriflavine agglutination</td>
<td>+</td>
</tr>
<tr>
<td>Congo red uptake</td>
<td>++</td>
</tr>
<tr>
<td>Crystal violet binding</td>
<td>+</td>
</tr>
<tr>
<td>Hydrophobicity 2M</td>
<td>+</td>
</tr>
<tr>
<td>1.5M</td>
<td>+</td>
</tr>
<tr>
<td>1M</td>
<td>+</td>
</tr>
<tr>
<td>0.5M</td>
<td>+</td>
</tr>
<tr>
<td>Binding of coomassie brilliant blue</td>
<td>++</td>
</tr>
<tr>
<td>Suicide phenomenon</td>
<td>-</td>
</tr>
<tr>
<td>Sensitivity to normal fish serum</td>
<td>-</td>
</tr>
</tbody>
</table>

- negative; + positive; +++ uptake of dye within 15 sec, ++ within 30 sec, + within 60 sec

Table 3—Biological properties of crude extracellular products of A. hydrophila strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total ECP protein (mg/ml)</th>
<th>LPS content (mg/ml)</th>
<th>LD50 (µg protein/g body wt. of fish)</th>
<th>Elastase (units/ml)</th>
<th>Caseinase (units/ml)</th>
<th>Gelatinase (units/ml)</th>
<th>Amylase activity (units/ml)</th>
<th>Haemolytic activity (units/ml)</th>
<th>Enterotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH01</td>
<td>0.98</td>
<td>0.50</td>
<td>2.14</td>
<td>60±4.358</td>
<td>98±1.000</td>
<td>35±2.886</td>
<td>76.67±2.886</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td>AH02</td>
<td>1.46</td>
<td>1.20</td>
<td>1.86</td>
<td>80±5.000</td>
<td>73±2.645</td>
<td>126±5.196</td>
<td>40±0.000</td>
<td>75±5.000</td>
<td>0.081</td>
</tr>
<tr>
<td>AH03</td>
<td>0.75</td>
<td>0.80</td>
<td>2.61</td>
<td>38±2.886</td>
<td>92±1.732</td>
<td>94±4.582</td>
<td>25±5.000</td>
<td>86.67±2.886</td>
<td>0.075</td>
</tr>
<tr>
<td>AH04</td>
<td>0.66</td>
<td>0.80</td>
<td>3.05</td>
<td>20±0.000</td>
<td>81±5.567</td>
<td>92±1.732</td>
<td>41.67±2.886</td>
<td>76.67±2.886</td>
<td>0.084</td>
</tr>
<tr>
<td>ATCC 7966</td>
<td>0.56</td>
<td>0.90</td>
<td>4.42</td>
<td>84±4.582</td>
<td>93±5.196</td>
<td>46.67±2.886</td>
<td>50±0.000</td>
<td>0.072</td>
<td></td>
</tr>
</tbody>
</table>

*Values are the mean ± SD from 3 cultures of each strain

Words the greater the virulence the higher were the proteolytic activities (elastase and gelatinase) of the test strains. Such a correlation does not exist between pathogenicity and caseinase or amylytic activity. Although haemolytic activity for the test strains was well pronounced it showed no correlation with pathogenicity. Negative results with respect to fluid accumulation ratio (<0.09) were recorded for enterotoxicity in mice.

Inhibition assays were carried out (Table 4) for the purpose of briefly characterizing the protease and haemolysin. At 56°C, caseinase and gelatinase of strains AH01, AH02 and AH04 were more stable than the elastase enzyme; activity ranged from 64-80% for caseinase, 53-92% for gelatinase and only 23-33% for elastase. AH03 on the other hand was found to be heat labile, with residual caseinolytic and gelatinolytic activities of only 10 and 13%
Table 4—Inhibitory assays of biological activities of crude ECP of A. hydrophila

<table>
<thead>
<tr>
<th>Strain</th>
<th>Residual Proteolytic Activity (%)</th>
<th>Residual HAC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caseinase</td>
<td>Gelatinase</td>
</tr>
<tr>
<td></td>
<td>56°C</td>
<td>80°C</td>
</tr>
<tr>
<td>AHO1</td>
<td>74± 1.000</td>
<td>30± 2.645</td>
</tr>
<tr>
<td>AHO2</td>
<td>75± 5.196</td>
<td>30± 2.645</td>
</tr>
<tr>
<td>AHO3</td>
<td>75± 5.196</td>
<td>30± 2.645</td>
</tr>
<tr>
<td>ATCC 7966</td>
<td>75± 5.196</td>
<td>30± 2.645</td>
</tr>
</tbody>
</table>

*Values are the mean ± SD from 3 cultures of each strain
Not applicable
HA—Haemolytic activity

respectively; complete loss of elastase activity was noted. Protease of type strain too was unstable after heat treatment at 56°C.

Heat labile haemolysins had a residual activity range of only 12-24%. Assays with metallo and serine protease inhibitors revealed that gelatinase and caseinase activity of all strains except AHO3 were largely inhibited by EDTA (residual activity, 11-69%) and less so with PMSF (70-100%). Elastase activity of the test strains on the contrary was inhibited more by PMSF (residual activity, 10-35%) than by EDTA (20-100%). A different pattern of inhibition was observed with AHO3, no activity was lost upon treatment with EDTA while with PMSF, residual activities of 68, 16 and 35% were recorded for caseinase, gelatinase and elastase, respectively. Proteases of ATCC 7966 were inhibited more by EDTA (65, 11 and 12 for caseinase, elastase and gelatinase, respectively) than by PMSF (85, 70 and 65).

Discussion

Test strains exhibited the same degree of virulence, when examined for their pathogenicity in fish; they belonged to the virulent category (<10⁷ cells/ml) as described by Mittal et al 10. The remarkable surface characteristics of test strains is a reflection of the antigenic entity of the bacteria. Despite being serologically heterogeneous, strains exhibited homogeneity in most of their cell surface characteristics. Most important, the self pelleting ability, binding of CBB and the ability for Congo red uptake have been attributed to the presence of a surface protein array 3-5 which is extractable with glycine as reported first by Dooley and Trust 17. Further, the surface array protein (S-layer) is considered as one of the major virulent elements of the bacterium 18-19. The high hydrophobicity as determined by salt aggregation test (0.5 M) has been reported to be conferred by fimbriae that facilitate the adhesive activity of the bacterium 20. Several workers 15,21,3-5 documented that certain other cell surface characteristics were useful in differentiating virulent A. hydrophila from the less virulent ones. In general, virulent strains tested by them did not agglutinate in acriflavin, were resistant to the bactericidal activity of fresh serum and were unstable after boiling (autoagglutination). Lallier and Daigernault 22 also reported that lack of agglutination in acriflavin had highly correlated with fish pathogenicity. Also in support of findings here, is the study by Sakai and Kimura 23 whose analogous reports with the non-motile A. salmonicida revealed that autoagglutinating strains possess the capacity to
escape the defence mechanism of the host by resisting the bactericidal activity of serum. The yeast
conglutination test was employed as a means of assessing the presence of mannosine sensitive fimbrial
adhesins on the surface of virulent bacteria. Further,
the pathogenicity of A. hydrophila has been
associated with non-suicidal activity of the strains.25
The suicidal phenomenon in Aeromonas species en- 
tues when certain strains are inoculated in broth
media containing 0.5% glucose. Accumulation of
acetic acid and other short chain fatty acids as result
of suppression of the tricarboxylic acid, reduces the
pH of the medium thereby inactivating the electron
transport chain, resulting in cessation of growth and
death of the organism.25,30 Supported by earlier
investigations, the present findings reveal the
virulence of the A. hydrophila strains of this study as
being related to multiple physiological processes of
the cell surface such as the presence of surface array
protein, LPS and fimbriae.

The range of lethal doses of ECP was comparable
to that reported previously for Aeromonas by Santos
et al.6 On the contrary, lethal doses as high as 18-35
μg protein/g body weight of fish have also been
reported by Santos et al.6 in support of earlier
findings.26,27 Strain AH02 that was the most lethal to
L. rohita recorded the highest elastase and gelatinase
activity. Wakabayashi et al.28 and Hsu et al.29 stated
that there was a correlation between virulence and
quantitative elastase production. A similar hypothesis
may be made with the findings of this study. Chabot
and Thune30 too while attempting to link protease
production to pathogenicity and virulence showed that
most of their 11 strong elastase producing strains
were more virulent to channel catfish than their 9
weak elastase producing strains. However, the
estase and gelatinase activities of the present strains
were much less than those recorded in virulent
strains by Santos et al.7 even though the toxicity of
ECP in fish (LD50) in the two studies were comparable.
Such contradictory evidences indicate that
quantitative and qualitative differences exist in the
production of toxins in vitro and in vivo and that
both qualitative and quantitative production of the
toxins are important in establishing the virulence of a
particular strain. Amylase activity of the test strains is
not well pronounced and seems unlikely to be a
contributing factor in the pathogenicity of A.
hydrophila. Their involvement had not been stressed
even in earlier studies on the virulence factors of the
pathogen.8,9 Since haemorrhage is a conspicuous
feature of A. hydrophila infections in poikilo- 
thermic animals and because these haemorrhagic effects are
evident in vitro and in vivo, haemolysins definitively
contribute to the pathogenicity of A. hydrophila. The
haemolytic activity of ECP of the virulent test strains
as expected were as high as 75-87.5 units/ml. It may
therefore be agreed with earlier reports that a
relationship does exist between virulence of A.
hydrophila and extracellular proteases and
haemolysins.3,12

The enterotoxicity of A. hydrophila is considered
significant from the point of view of public health
concern. The A. hydrophila strains of this study
recorded fluid accumulation ratios of <0.09 in mice
and hence are not potentially enterotoxigenic.
Nevertheless, they may be responsible for mild
diarrhoeal outbreaks if improperly cooked food is
consumed. Partially enterotoxigenic A. hydrophila
were documented by previous workers when ratios
between 0.09 and 0.1 were recorded.3,32,33

Proteolytic inhibition assays in the present study
have recorded the secretion of both serine (inhibited
by PMSF) and metallo (inhibited by EDTA)
proteases. A predominance of heat stable caseinolytic
and gelatinolytic metallo protease has been recorded
with AH01, AH02 and AH04. AH03, on the other
hand, expressed only a heat labile serine protease; and
protease activity uninhibited by EDTA showed the
total absence of metallo protease. ATCC 7966
demonstrated the predominance of heat labile metallo
protease. Thus, in the present investigations 3
different extracellular proteases have been identified
and characterized as heat stable metallo protease, heat
labile serine protease and heat labile metallo protease.
There is controversy in literature concerning the
number and nature of proteases in the extracellular
products of A. hydrophila. Evidence exists for the
presence of two proteases34, one being heat labile and
the other heat stable.35,26 Kanai and Wakabayashi36 on
the other hand, found the presence of only a single
"type of protease which was heat stable and sensitive
to EDTA (heat stable metallo protease). Nieto and
Ellis36 found only heat stable proteases and inhibitor
studies showed the presence of both serine and
metallo proteases which were partially characterized
using isoelectric focussing (IEF) techniques resulting
in the separation of 4 to 6 protease fractions. Leung
and Stevenson37 while assessing the extent of
differences in the protease produced by a large
number of strains of A. hydrophila found the
predominance of thermostable metallo and
thermolabile serine proteases. With traditional biochemical techniques, Chabota and Thune\textsuperscript{39} identified a heat labile serine protease, a heat stable metallo protease and a moderately heat stable metallo protease. Thus a perusal of the above reports reveal that Aeromonas isolates secrete at least four or five different proteases. It is possible that some of the proteases described above may be similar to those identified in the present study. In the light of serotypic diversity of strains, it is not surprising that at least 3 different proteases have been identified in 5 strains in this study (AHO1, AHO2, AHO3, AHO4 and ATCC 7966) - heat stable metallo protease, heat labile serine protease and heat labile metallo protease. A. hydrophila of the present study were typically heat labile\textsuperscript{38,39} and demonstrated only a small percentage of residual activity (12-24%). Further, by examining a large number of isolates, the homogeneity of the toxins in diverse strains may be established to overcome the problem of extreme serological heterogeneity exhibited by the organism, in developing effective vaccines.

Acknowledgement
The authors are grateful to the Department of Biotechnology, New Delhi for financial support.

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