Aeromonas hydrophila in Wild-Caught Frogs and Tadpoles (Rana pipiens) in Minnesota¹,²,³

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Summary | Frogs and tadpoles were captured at 14 sites in and near Minnesota during 1978-79 and nearly all appeared healthy. Aeromonas hydrophila was isolated from 94 of 294 (32%) juvenile and adult frogs and from 96 of 104 (63%) tadpoles. Of the isolates from frogs and tadpoles respectively, 68% and 47% were from the intestine only, 12% and 32% were from the intestine and other sites, and 20% and 21% were from extraintestinal sites only. Isolations were more frequent from frogs collected in March-June than in August-November. Evidence was not found that disease due to Aeromonas hydrophila was a primary cause of declining Rana pipiens populations in Minnesota.

Key Words | Aeromonas — Amphibia — Ranidae

Nearly all Rana pipiens used in research are caught in the wild (1). Over the last two decades, however, there has been a dramatic decline in frog populations in the upper Midwest, other parts of the United States (1-5), and in Britain (6). Minnesota was a primary collecting site for R pipiens, but reduced populations resulted in the state being closed for sale of mature frogs in 1975 (2).

The reasons for the apparently diminished populations of R pipiens are not known, but loss of wetland habitat and toxicity due to pesticides or other chemicals have been suggested (1,6). Redleg disease due to Aeromonas hydrophila has also been proposed as a factor (3). In a 1974 Wisconsin study, many sick or dead R pipiens with signs of redleg disease (cutaneous hemorrhages, ulcerated skin on toes and feet) were observed in the wild, and A hydrophila was isolated (4). While disease apparently caused by A hydrophila is commonly reported in captive frogs in the laboratory (7-10), the prevalence and significance of A hydrophila in wild R pipiens populations is not well documented, although it has been reported to occur spontaneously among amphibians in their native habitat (11,12).

The objective of this study was to establish the patterns of occurrence of A hydrophila in wild populations of R pipiens in and near Minnesota as a preliminary step in the assessment of the importance of A hydrophila as a cause of reduced R pipiens populations.

Materials and Methods

Collection: Frogs were collected in the spring, summer, and fall of 1978 and 1979, beginning with emergence from overwintering in lakes (March, 1978 and April, 1979) and ending when frogs began to enter lakes for overwintering (November, 1978 and October, 1979). The collection sites chosen were areas where frogs were relatively abundant based on our experience and that of commercial frog-catchers.

Despite considerable searching, frog populations dense enough for appropriate sampling were found only in grassy habitats (ditches, lawns, pastures) adjacent to streams or lakes with sandy shores bearing sparse to moderate (never dense) emergent and submerged vegetation. All such habitats were on calcareous glacial drift, the waters of which were somewhat alkaline and strongly dominated by calcium and bicarbonate ions. All sites were adjacent to agricultural land. Breeding ponds were close to the overwinter sites, generally 0.5-1.5 m deep, with abundant aquatic macrophytes. Twenty-eight collecting trips were made to 14 sites: Sand Hill River near Fertile, Polk Co, Minnesota (MN) (3 sites); Block Lake, Otter Tail Co, MN; Intersection of Highways 12 and 78, east of Dalton, Otter Tail Co, MN; Westport Lake, Pope Co, MN; Cushing Pond, Morrison Co, MN; Long Lake, Hennepin Co, MN; White Bear Lake, Ramsey Co, MN; Diamond Lake, Kandiyohi Co, MN; Cedar Lake, Scott Co, MN; farm pond near Becker, Sherburne Co, MN; Shell Lake, Wisconsin; and an unnamed pothole lake in Burke Co, North Dakota.

Collectors walked the fields of each site; frogs that hopped were sighted and captured by hand. The collectors wore new disposable plastic gloves which were changed after each frog was captured. Tadpoles were captured by nets or seines. Each frog or tadpole was placed in a separate autoclaved plastic jar which contained carbon-filtered, autoclaved tap water 2-3 cm deep. Water samples were taken from overwintering lake sites and breeding ponds. The animals were then transported to the laboratory.

Sampling: Specimens were sampled within 48 hours of capture, except those from distant collection sites such as North Dakota. Frogs were killed by cranial and
spinal pitting and tadpoles by decapitation. Snout-vent length was measured, and the sex of larger frogs was determined. Samples for bacteriologic examination were taken aseptically; instruments were cleaned, dipped in 95% ethyl alcohol, and flamed between each sample from each animal. Skin, muscle, heart blood, and intestine were sampled in that sequence from each frog, and skin, muscle, and intestine from each tadpole.

Skin samples of approximately 1 cm² were taken from the lateral side of frog thighs or from tails of tadpoles. Before removal, the area was swabbed with 10% formalin. From the underlying area, a muscle sample of approximately 0.75 cm³ was removed. Blood samples were taken by inserting a sterilized 18-gauge needle into the exposed heart, allowing passive filling of enough blood so that 3-4 drops could be used for inoculation of media. When this procedure was not successful, the entire heart was removed and several drops of blood were allowed to fall into the culture tube. Intact sections 1.5-2 cm long were taken from the midgut.

Bacteriologic examination: Each sample was placed in a nutrient broth of peptone meat extract immediately after removal from the animal and incubated for 24 hours at 30° C. After thorough mixing, approximately 3 ml from each water sample was inoculated into 10 ml of nutrient broth. Broth cultures were then streaked on MacConkey agar and incubated at 30° C for 24-36 hours after which opaque non-lactose fermenting colonies were picked, subcultured on nutrient slants, and incubated for 24 hours at 30° C. Isolates that were oxidase-positive were retained for further testing.

Further biochemical tests were performed by standard methods (13,14). Cultures were incubated for 24 hours at 30° C, except the lysine decarboxylase test which was incubated at 37° C. Cultures that were Gram-negative, motile, showed fermentation on triple sugar-iron-agar, and fermented mannitol, but were negative to lysine decarboxylase and hydrogen sulfide and failed to ferment malonate, dulcitol, and inositol were considered to be A. hydrophila (13-15). A known culture of A. hydrophila was used as a control for each test of all groups of isolates.

Results
Health of frogs and tadpoles: Dead or obviously ill frogs and tadpoles were rarely seen in the field. Dead frogs were occasionally observed on lake bottoms in the spring as the ice melted at overwintering sites. Nearly all specimens received at the laboratory seemed clinically healthy as judged by movements, posture, and skin coloration and luster.

Isolation by site and season: A. hydrophila was isolated from 94 of 294 (32%) frogs and from 66 of 104 (63%) tadpoles. The intestine was the most common site of isolation. In 75 of 94 (80%) frogs from which it was detected, A. hydrophila was found in the intestine. In 64 of 94 (68%), it was found only in the intestine. In tadpoles from which it was isolated, A. hydrophila was recovered from the intestine in 52 of 66 (79%). In 31 of 66 (47%) tadpoles, it was found only in the intestine. A. hydrophila was cultured from 34 of 104 (33%) samples of tadpole skin, but from only 18 of 294 (6%) samples of frog skin. Isolations were made from 5 of 294 (2%) samples of frog muscle and 6 of 104 (6%) samples of tadpole muscle. The organism was recovered from 12 of 294 (4%) samples of frog heart blood.

During the spring and early summer (March-June), the organism was isolated from 39 of 85 (46%) frogs; while in the late summer and early fall (August-November), it was isolated from 55 of 209 (26%) frogs. Frogs collected in August-November were in summer foraging areas or in congregations near overwintering sites. Frogs collected in March-June were just emerging from overwintering sites, or they were near breeding ponds. Both March-June collection sites had high recovery rates of A. hydrophila (overwintering sites, 30 of 67 (45%) frogs; breeding ponds, 9 of 18 (50%).

Isolation by sex: The difference in isolation of A. hydrophila between male and female frogs was small; 52 of 137 (38%) males and 27 of 79 (34%) females were infected (Table 1). Small frogs were not sexed.

Isolation by frog size: Seasonal variations in A. hydrophila isolation were seen in all sizes of frogs, but they were less pronounced in the largest frogs (Table 2). The organism was cultured less often from the smallest frogs in late summer and early fall than from larger frogs. Small frogs that overwintered successfully had a rate of infection approximating those of larger frogs.

Isolation by site of collection: Infection rates for frogs and tadpoles varied considerably according to collection site (Table 3) of which only the principal ones are

Table 1
Isolation of A. hydrophila from Rana pipiens by season and sex

<table>
<thead>
<tr>
<th>Collection season</th>
<th>Male Frogs</th>
<th>Female Frogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>collected</td>
<td>Number positive (1%)</td>
</tr>
<tr>
<td>March-June</td>
<td>39</td>
<td>19 (48.7%)</td>
</tr>
<tr>
<td>August-November</td>
<td>98</td>
<td>33 (33.7%)</td>
</tr>
<tr>
<td>Total (March-November)</td>
<td>137</td>
<td>52 (38.0%)</td>
</tr>
</tbody>
</table>

(1) Number of frogs from which A. hydrophila was cultured from any site.

ever, with disease in frogs (9,16-18). The disease has been called "red-leg" (17), but the signs are not sufficiently consistent or specific to justify the term (9). In the present study, dead or obviously ill frogs and tadpoles were rarely seen in the field, yet A hydrophila was often isolated. The organism has been isolated from apparently healthy R pipiens (19,20) and from apparently healthy members of other species of frogs such as bullfrogs (Rana catesbeiana) (10,21). It is difficult to judge the health of a frog by its appearance (1,7), and survivability for long periods in the laboratory has been suggested as the principal criterion for frog health (1). In recent years, apparently healthy frogs have died in much greater numbers than formerly following commercial shipment (1,3). It is not known, however, whether decreased survival of frogs in the laboratory signifies decreased survivability if these frogs had been left in their native habitat.

Collection sites in this study were selected because of the presence of frogs in relatively large numbers, so that adequate numbers could be collected. It is possible, therefore, that the frogs collected were the survivors of unknown factors responsible for decreases in other frog populations, and as such may not truly represent the entire frog population before its apparent decline. The collection method may have resulted in a non-representative sample of the frog population at each site, as the frogs collected were those that hopped when disturbed. These frogs may differ in unknown ways from non-hopping frogs.

In this study, A hydrophila was isolated most frequently from frogs in the spring months and from tadpoles. In Minnesota, R pipiens overwinters in water deep enough not to freeze completely, and as the surface ice melts during spring thaw, frogs emerge and migrate to shallow breeding ponds (22). Adults leave the breeding ponds soon after egg laying, and spend the summer foraging in fields and meadows adjacent to marshes, ponds, lakes, and streams (22). Young frogs begin to forage outside the breeding ponds soon after metamorphosis. In September, the frogs start to migrate toward overwintering sites in lakes and streams (22). Aeromonas hydrophila, which is considered to be a resident aquatic microorganism (15,23), was isolated most frequently from the life cycle stages of R pipiens most closely associated with water. In temperate zones, A hydrophila populations appear to increase with warmer water temperatures in the spring and summer, and to decrease in the fall and winter (24-26). The pattern of isolation from water in this study was consistent with previous findings.

### Table 2

<table>
<thead>
<tr>
<th>Collection season</th>
<th>&lt;60</th>
<th>61-69</th>
<th>&gt;70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frogs collected</td>
<td>Number positive (%)</td>
<td>Frogs collected</td>
<td>Number positive (%)</td>
</tr>
<tr>
<td>March-June</td>
<td>21</td>
<td>10(47.6)</td>
<td>29</td>
</tr>
<tr>
<td>August-November</td>
<td>93</td>
<td>16(17.2)</td>
<td>46</td>
</tr>
<tr>
<td>Total (March-November)</td>
<td>114</td>
<td>26(22.8)</td>
<td>75</td>
</tr>
</tbody>
</table>

*Number of frogs from which A hydrophila was cultured from any site

### Table 3

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Frogs</th>
<th>Tadpoles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number collected</td>
<td>Number positive (%)</td>
</tr>
<tr>
<td>Westport Lake, MN</td>
<td>38</td>
<td>14(36.8)</td>
</tr>
<tr>
<td>Fertile, MN</td>
<td>44</td>
<td>21(47.7)</td>
</tr>
<tr>
<td>Dalton, MN</td>
<td>7</td>
<td>0(0.0)</td>
</tr>
<tr>
<td>Diamond Lake, MN</td>
<td>50</td>
<td>19(38.0)</td>
</tr>
<tr>
<td>Block Lake, MN</td>
<td>49</td>
<td>9(18.4)</td>
</tr>
<tr>
<td>Cedar Lake, MN</td>
<td>57</td>
<td>19(33.8)</td>
</tr>
<tr>
<td>Sherburn Co Pond, MN</td>
<td>17</td>
<td>10(58.8)</td>
</tr>
<tr>
<td>Cushing Pond, MN</td>
<td>24</td>
<td>7(29.2)</td>
</tr>
</tbody>
</table>

*Number of frogs or tadpoles from which A hydrophila was cultured

listed. These data are difficult to interpret for frogs, however, due to the confounding effects of season and frog size. Certain sites were only suitable for collection of frogs emerging from overwintering. Other sites were suitable only for collection of foraging frogs in late summer so that a relatively greater proportion of small frogs was collected.

**Isolation from water: Aeromonas hydrophila**

A hydrophila was isolated from 1 of 8 (12.5%) water samples taken from breeding ponds. Other sites were suitable only for collection of foraging frogs in late summer so that adequate numbers could be collected. It is possible, therefore, that the frogs collected were the survivors of unknown factors responsible for decreases in other frog populations, and as such may not truly represent the entire frog population before its apparent decline. The collection method may have resulted in a non-representative sample of the frog population at each site, as the frogs collected were those that hopped when disturbed. These frogs may differ in unknown ways from non-hopping frogs.

### Discussion

Aeromonas hydrophila has long been associated with disease in frogs (9,16-18). The disease has been called "red-leg" (17), but the signs are not sufficiently consistent or specific to justify the term (9). In the present study, dead or obviously ill frogs and tadpoles were rarely seen in the field, yet A hydrophila was often isolated. The organism has been isolated from apparently healthy R pipiens (19,20) and from apparently healthy members of other species of frogs such as bullfrogs (Rana catesbeiana) (10,21). It is difficult to judge the health of a frog by its appearance (1,7), and survivability for long periods in the laboratory has been suggested as the principal criterion for frog health (1). In recent years, apparently healthy frogs have died in much greater numbers than formerly following commercial shipment (1,3). It is not known, however, whether decreased survival of frogs in the laboratory signifies decreased survivability if these frogs had been left in their native habitat.
It is interesting that whereas tadpoles had the highest rate of infection, small frogs collected in the late summer (and which had recently undergone metamorphosis) had the lowest rate of infection. Apparently the organism did not persist in R piperi after the change from tadpole to frog.

Conditions during the overwintering period may also contribute to the higher infection rate observed in frogs emerging from overwintering sites. During overwintering, frogs do not feed and are exposed to low temperatures. Laboratory experiments simulating overwintering conditions for R pipiens (19) and R catesbeiana (21) have shown that the numbers and variety of types of intestinal microflora are greatly reduced. Some strains of A hydrophila are considered facultative psychrophiles (27), and lower temperatures may give A hydrophila and other potential pathogens a selective advantage for maintenance and survival during overwintering (21).

Aeromonas hydrophila is often considered to be an opportunistic pathogen in frogs and other animals including man; such stresses as intercurrent disease, spawning, or shipment may precipitate disease (20,28,23,29-31). Human septicemia has been associated with the combined presence of A hydrophila in the intestine and lesions of the gastrointestinal tract (30,31). Frogs emerging from overwintering sites appear to be especially vulnerable due to the stress associated with overwintering and the apparent selection for potentially pathogenic gut microflora discussed previously (21,28).

The results of this study neither refute nor confirm the speculation that disease due to Aeromonas hydrophila is responsible for reduced frog populations in Minnesota. It has been demonstrated, however, that A hydrophila can be isolated from many apparently healthy frogs and tadpoles in their natural environment. Others have correctly observed that the mere isolation of A hydrophila from a dead or dying frog is of questionable diagnostic value (10). We speculate that if A hydrophila is associated with declining R pipiens populations in Minnesota, its importance may be secondary to as yet unidentified primary factors.

References
6. Cooke AS. Indications of recent changes in status in the British Isles of the frog (Rana temporaria) and the toad (Bufo bufo). J Zool Lond 1972; 167:161-78.