Elimination of symbiotic *Aeromonas* spp. from the intestinal tract of the medicinal leech, *Hirudo medicinalis*, using ciprofloxacin feeding

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Abstract

The use of the medicinal leech (*Hirudo medicinalis*) in promoting venous drainage in tissues whose vitality is threatened by venous congestion and obstruction, especially in plastic and reconstructive surgery, has been complicated by infections caused by *Aeromonas* spp. These are leech endosymbionts for which patients undergoing hirudotherapy frequently receive systemic chemoprophylaxis. In order to evaluate the possibility of rendering leeches safe for use on patients, *H. medicinalis* were fed artificially with a 2 g/L arginine solution (used as a phagostimulant) supplemented with ciprofloxacin (100 mg/L). Aeromonads were detected in 57 out of 80 control leeches (71.3%), but in none of the 56 leeches treated with ciprofloxacin (p <0.001). Treated leeches survived for up to 4 months. Tested weekly, 61% of these leeches took human blood for at least 4 weeks after treatment and all remained negative for aeromonads. All water samples in which leeches were kept before treatment were contaminated with *Aeromonas* spp.; none were detected in any of the NaCl/arginine solutions with which treated animals were fed. Molecular characterization of two phenotypically distinct isolates using *gyrB* sequencing showed that one clustered tightly with *A. veronii* and the other was closely related to *A. media*. Other environmental bacteria and fungi were isolated from 26.5% of treated leeches that had taken a blood meal 1–4 weeks after treatment. Ciprofloxacin reduced the number of leech-associated aeromonads to undetectable levels for extended periods. Most treated leeches were ready to take a blood meal after treatment, suggesting the possibility of using ciprofloxacin-treated leeches instead of chemoprophylaxis in patients undergoing hirudotherapy.

Keywords: *Aeromonas*, artificial feeding, *Hirudo medicinalis*, medicinal leech, symbiont

Introduction

The use of the medicinal leech (*Hirudo medicinalis*) for the treatment of human diseases has been known since ancient Egyptian times. Galen (130–201 AD) used leeches for blood-letting, which was based on the belief that removal of the patient’s blood would correct the humoral imbalance and restore good health [1]. In the 18th and 19th centuries, the popularity of leeching reached its height in Europe. In 1884, Haycraft discovered hirudin, an anti-coagulant substance in leech saliva. Markwardt [2] isolated and characterized this molecule and in 1986 it was produced by genetic engineering [3].

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In order to reduce the risk of infection, attempts have been made to remove or decrease the number of aeromonads inside and outside leeches, by frequent changes of the water in leech containers, and by submerging leeches in hypochloric acid and antibiotics, however, these treatment modalities were not sufficient to destroy the internal bacterial flora of the leech [7,8]. Antimicrobial prophylaxis for bacteria remaining in the digestive tract is thus necessary to make the leeches suitable for the treatment of patients, preventing their internal bacteria from constituting a source of infection.

The purpose of this study was to attempt to suppress the endosymbiotic aeromonads by feeding leeches artificially with a blood-free antibiotic solution, with the ultimate aim of eliminating the need for chemoprophylaxis in the patients. Ciprofloxacin, which is an agent of choice in the therapy and prophylaxis of infection as a result of these organisms, was chosen after preliminary testing showed leech-associated Aeromonas spp. to be fully susceptible.

Materials and Methods

Leeches
Leeches (Hirudo medicinalis) collected in the wild in Turkey (Ergene Ltd, Tekirdag) were kept in holding vessels (40 x 60 x 40-cm plastic containers) in chlorine-free tap water that was changed weekly. The authors are familiar with recent changes in the taxonomy of Hirudo species in which many medicinally applied leeches have been shown to be H. verbana rather than H. medicinalis. As the leeches used in this study were not formally identified, the latter term was kept for convenience [9]).

Artificial feeding
The artificial feeding device comprised a glass feeding chamber resembling an inverted thistle funnel with a jacket added for temperature control (Fig. 1). The chamber was pre-warmed to 38–40°C by running warm water through its outer jacket. This apparatus was inserted into a 250-mL plastic container, to which 100 mL of dechlorinated water had been added, through a close-fitting hole cut into its lid. A Parafilm membrane (American National Can, Chicago, IL, USA) was stretched tightly across the lower part of the inverted funnel. The feeding solution was added through the tube of the funnel. This was a 2 g/L arginine solution in 6.4 g/L NaCl, which is a known phagostimulant for leeches [10,11]. For the decontamination experiments the same solution was supplemented with 100 mg/L ciprofloxacin. Outdated transfusion units of human blood replaced the artificial phagostimulant to assess the readiness of leeches to take a blood meal after treatment. After feeding, leeches were maintained individually in sterilized chlorine-free tap water containing ciprofloxacin at a concentration of 20 mg/L and examined periodically for bacterial contamination.

Bacteriological examination
Samples were taken from the water in the holding vessels, from the exterior surface of the animals as well as from their alimentary tract and from the feeding solution. Leeches were disinfected externally by immersion in 70% ethyl alcohol for 1 min, followed by transfer to sterile distilled water. The purpose of this was to ensure that external contaminants did not interfere with cultures from the intestinal tract.

In order to prevent the entry of alcohol to the digestive tract and to avoid the discharge of gut contents through the mouth or anus, a ligature was applied to the proximal and distal ends (c. 2–3 mm) of the leech. Swabs were taken from the outer surface of the animals, which were then transected c. 10 mm distal to the anterior sucker, and crop fluid was collected using a 10-μL disposable bacteriological loop (Quadloop; Miniplast, Ein Shemer, Israel) inserted into the crop. A second incision about 2 mm proximal to the posterior sucker was made and the intestinal contents were sampled with a loop. Samples were inoculated on 5% sheep blood agar, incubated at 37°C and examined after 24 and 48 h. Oxidase positive organisms that grew on MacConkey agar and fermented glucose were identified using API 20NE panels (bioMerieux, Marcy-l’Etoile, France).

Molecular identification of Aeromonas species
DNA was extracted using a commercial kit (Wizard Genomic DNA Purification Kit; Promega, Inc., Madison, WI, USA). Amplification of the gyrB gene was performed using primers
were negative (Table 1). Aeromonads were also not detected in any of the 15 arginine/NaCl samples from feeding chambers that were used to feed antibiotic-treated leeches, whereas other environmental microorganisms were found in 16% (9 out of 56) of these leeches.

Environmental bacteria such as Ochrobactrum spp. and some filamentous fungi were also isolated from 26.5% of treated leeches that had taken a blood meal 1–4 weeks after treatment (data not shown). All 106 water samples in which leeches were kept before treatment were contaminated with Aeromonas spp.

The dominant species was identified by the API 20NE kit as A. sobria. Two different phenotypes within this taxon were studied further: isolates designated 1 and 3. The API 20 NE profile for isolate 1 was 7176755 (99.2%, T 1.0). Additional conventional tests showed the isolate to be Voges-Proskauer positive, DNAse positive and ornithine decarboxylase negative, all properties consistent with an identification of A. veronii biovar sobria [14]. The profile for isolate 3 was 7176744 (84.8%, T 0.72) being much less compatible with the identification of A. sobria. This organism was Voges-Proskauer negative, DNAse negative, ornithine decarboxylase negative and confirmed negative for citrate, a combination much less consistent with an identification of A. veronii biovar sobria.

Clinical diagnostic laboratory methods for identification of Aeromonas spp. are known to be inconclusive [14–16], so molecular characterization was undertaken to clarify the taxonomic status of these isolates. Indeed, the gyrB sequences revealed that isolate 1 was very closely matched with A. veronii and that isolate 3 bore most similarity to A. media (Fig. 2).

The API reactions and the additional conventional testing of this isolate were consistent with this taxon [14].

### Discussion

It is generally accepted that the medicinal leech has an obligatory symbiotic relationship with the bacteria of the genus Aeromonas, which live in its intestinal tract. The symbiotic bacteria: (i) contribute enzymes such as proteases, lipases and amylases, which play a major role in digestion; (ii) provide vitamins of the B-complex, which are sparse in blood; and (iii) secrete antimicrobial substances, which prevent the growth of other bacteria and accordingly retard putrefaction so that the leech can store blood for long periods [4,17].

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### Results

Approximately 80% of the leeches engorged fully when fed artificially either on human blood or on the arginine/NaCl solution with and without antibiotics. In 94.4% of the 106 unfed leeches treated with 70% ethyl alcohol no bacteria were grown from exterior surface samples. However, this treatment caused the death of the leeches as expected. Aeromonas spp. were detected in 57 out of 80 control leeches fed only with arginine/NaCl solution (71.3%), but in none of the 56 leeches treated with ciprofloxacin (p <0.001 using Fisher’s exact test). In the untreated control leeches, bacteria were found in the intestine, but not in the crop, in 35 animals (61.4%); they were confined to the crop in nine animals (15.8%), whereas in 13 (22.8%) they were present both in the crop and intestine.

A blood-meal, through artificial feeding, was offered to 56 leeches treated with antibiotics. Thirty-four of them took a blood meal 1–4 weeks after treatment, whereas 22 leeches refused to feed, all of them in the first 2 weeks. All 56 leeches were dissected and examined for Aeromonas spp. All were negative (Table 1). Aeromonads were also not detected in the water in which the leeches were kept during the experimental period.

**Table 1. Presence of aeromonads in leeches treated with ciprofloxacin (100 mg/L)**

<table>
<thead>
<tr>
<th>Blood meal offered</th>
<th>n</th>
<th>Blood meal taken</th>
<th>Aeromonas spp.</th>
<th>Other environmental organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 1 week</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>At 2 weeks</td>
<td>31</td>
<td>14</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>At 3 weeks</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>At 4 weeks</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>34 (61%)</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

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### Statistical methods

Fisher’s exact test was used to compare treated with control leeches.

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Ten antibiotic-treated leeches were maintained individually in sterilized chlorine-free tap water containing ciprofloxacin at a concentration of 20 mg/L. Three died after 2 months, three after 3 months and four of them survived up to 4 months.

Aeromonads were not detected in any of the 15 arginine/NaCl samples from feeding chambers that were used to feed antibiotic-treated leeches, whereas other environmental microorganisms were found in 16% (9 out of 56) of these leeches.

Environmental bacteria such as Ochrobactrum spp. and some filamentous fungi were also isolated from 26.5% of treated leeches that had taken a blood meal 1–4 weeks after treatment (data not shown). All 106 water samples in which leeches were kept before treatment were contaminated with Aeromonas spp.

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**TABLE 1. Presence of aeromonads in leeches treated with ciprofloxacin (100 mg/L)**
In earlier studies, phenotyping of colonizing bacteria classified all aeromonad isolates from *H. medicinalis* as *A. hydrophila*. As identification techniques and classification of these organisms evolved, Graf [18] found *A. veronii* biovar sobria as a single species in the crop and intestine of this leech. In contrast, Mackay et al. [7] found *A. caviae* to be the most common species encountered. Using molecular taxonomic techniques, we identified two species among aeromonads colonizing the intestinal tract of a single population of leeches; *A. veronii*, phenotypically consistent with *A. veronii* var sobria, and *A. media*.

Recent studies utilizing DNA sequence-based analyses have thrown new light on the complex biology of the bacterial populations of the leech intestine; apart from *A. veronii*, additional taxa were identified, including genera in the α-, γ-, and δ-Proteobacteria, Fusobacteria, Firmicutes, and Bacteroidetes. The clearly dominant organisms were *A. veronii* and a novel clone of a *Rikenella*-like bacterium of the *Bacteroidetes* group, designated PW3, whereas the other clones, considered by the investigators to represent transient organisms, were typically present only in low numbers [19–21]. In addition, *A. veronii* and *A. jandaei* have been cultured from *Hirudo orientalis* [22], whereas *A. jandaei* has been also isolated from *Macrobdella decora* [23].

In the present study, aeromonads were reduced to levels undetectable by our methods for up to 4 weeks in all leeches treated with ciprofloxacin and subsequently fed with human blood. A blood meal is normally followed by proliferation of aeromonads in the gut. Furthermore these bacteria were neither found in the water in which the leeches were kept nor in the blood or arginine solution within the feeding chamber; the latter observation suggests that aeromonads were not present in detectable numbers in the mouthparts or saliva of the leeches.

Several groups have attempted to eliminate the bacterial load inside and outside leeches by treating them exteriorly with antibiotics and antiseptics, succeeding only in eliminating bacteria from the surface of the leech [7,8,18,19].

In this study, the artificial feeding of leeches was conducted under non-sterile conditions. It is possible that the bacteria and fungi found in the intestinal tract of the leeches after antibiotic treatment might have had their origin in the environment. However, it is equally conceivable that they were microorganisms that normally exist in very low quantities in the leech intestine as a result of their sensitivity to blood components or antimicrobial substances excreted by the dominant aeromonad, and that flourished after suppression of the aeromonad by ciprofloxacin.

Whitlock et al. [24] predicted that aeromonads would be an infection risk in hirudotherapy, and Dickson et al. [25]...
described a case after breast reconstruction. Since then, Aeromonas infections after leech application have become well known, including both soft tissue infections and other severe infections such as septicemia and even meningitis [26–29]. Infections have been observed in 7–20% of patients treated with leeches, reducing the chances of successful re-implantations or flaps by 30% [30]. Therefore, during treatment antibiotics such as cephalosporins or fluoroquinolones are commonly given prophylactically [31].

In the present work, our efforts were directed at detecting an antibiotic effect in treated leeches. It was shown that either Aeromonas spp. were reduced to undetectable levels in our system and possibly eliminated, or that surviving bacteria lost their ability to multiply. As a result of resource limitations at this stage, it was not possible to maximize the sensitivity of detection or to assess whether survivors had entered a ‘viable but not culturable’ state. The dramatic reduction in aeromonad densities was a useful endpoint for the study. It is highly likely that viable but not cultivable organisms might be of no clinical consequence in the clinical settings in which hirudotherapy is conducted.

In conclusion, the results of the present study suggest that the strategy of feeding leeches artificially with antibiotics reduces the number of aeromonads to undetectable levels for extended periods, and that treated leeches are likely to take a blood meal from patients undergoing hirudotherapy.

Transparency Declaration

None of the authors has any relationship (commercial or otherwise) that may constitute a dual or conflicting interest.

References