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.

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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 bloodletting, 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].

In recent years, recognition of a number of antithrombotic substances and a vasodilator in the saliva of the medicinal leech [4,5] has generated renewed interest in the use of the leech itself to promote venous drainage in tissues whose vitality is threatened by venous congestion and obstruction, especially after plastic and reconstructive surgery. In 2004, this treatment modality received the approval of the Food and Drug Administration in the USA.

Hirudotherapy, as it is termed, has been complicated by infections caused by *Aeromonas* spp. which are considered to be obligatory endosymbionts of the leech [4]. Infections with aeromonads have been observed in 7–20% of patients treated with leeches after reconstructive surgery; in these cases the chances of successful re-implantation or flap survival dropped by 30%. Therefore, antibiotics such as cephalosporins or fluoroquinolones are given prophylactically for the duration of the treatment [1,6].

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 × 60 × 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

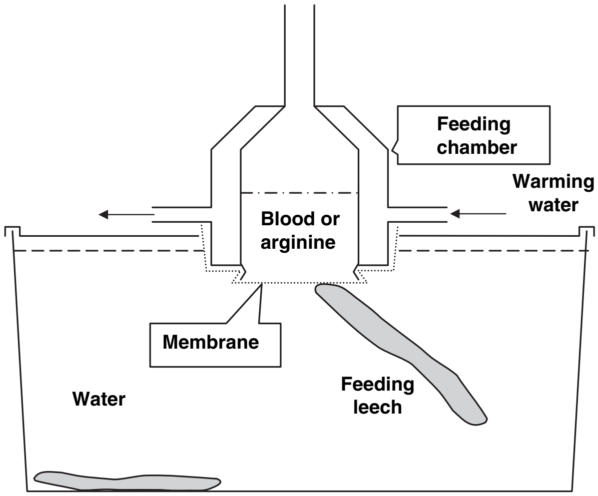

FIG. 1. Schematic representation of the artificial feeding chamber for leeches.

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