Characterization of the Digestive Tract Microbiota of *Hirudo orientalis* (Medicinal Leech) and Antibiotic Resistance Profile

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Background: There are at least three distinct European leech species used medicinally: *Hirudo medicinalis*, *H. orientalis*, and *H. verbana*. Infection caused by leech microbiota is the most widely reported complication. Few studies have reported the culturable and unculturable bacteria and examined the antibiotic resistances in *H. orientalis*.
Methods: Following stratified random sampling from a major worldwide leech supplier, *Hirudo orientalis* leeches were identified by visual comparison and amplification and sequencing the *cox1* locus. Combined culture and culture-independent approaches were used to characterize the microbiota of the midgut, and bacterial *gyrB* sequences from distinct colonies were used to identify the *Aeromonas* isolates. Nonculturable studies involved clone libraries of 16S rRNA genes, and Etests were used to investigate antibiotic sensitivities.
Results: Analysis of 16S rRNA gene clone libraries revealed the presence of several species in the intraluminal fluid of the crop, including a new finding of *Morganella morganii*, with *Rikenella*-like (35 percent) and *Aeromonas veronii* (38 percent) dominant members. The intestinum contained bacteria not previously isolated from the leech: *Magnetospirillium* species and *Roseospira marina*. Etests showed all *A. veronii* isolates were sensitive to ciprofloxacin, with either a complete or intermediate resistance to Augmentin.
Conclusions: The authors show diverse microbiota in the leech digestive tract. The pathogenic potential of the additional gut symbionts isolated in this study is yet to be elucidated; however, *M. morganii*, which is a known human pathogen, is a new finding. In addition to adding to the knowledge base regarding antibiotic sensitivities, this article serves as an update to the reconstructive surgeon regarding leech therapy. (*Plast. Reconstr. Surg.* 133: 408e, 2014.)

Although blood letting and the use of leeches dates back to pharaonic Egypt,[1] the U.S. Food and Drug Administration only approved the use of *Hirudo medicinalis* as a medical device in 2004.[2] Leeches are used by plastic,[3–7] maxillofacial,[8–10] and other surgeons[11,12] to aid salvage of venously congested pedicled flaps,[13,14] free-tissue transfers,[3,15–17] replanted digits,[6,18–25] ears,[26–32] lips,[33–36] nasal tips, and the penis.[12] Nonsurgical uses include the treatment of chronic pain syndromes associated with degenerative diseases.[37–39]

From 1987, *H. medicinalis* was considered the sole European medicinal leech species in clinical use. In fact, there are at least three species: *H. medicinalis*, *H. orientalis*, and *H. verbana*.[40,41] Although most commercial suppliers sell medicinal leeches labeled as *H. medicinalis*, Siddall et al.[40] revealed that nearly all annelids were genetically distinct *H. verbana*. We have received shipments from commercial suppliers that contained both *H. verbana* and *H. orientalis*. It is likely that both are used in large quantities in North America and Northern Europe. Commercially used *H. verbana* mainly originate from Turkey, southeastern

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Europe, and the Krasnodar Territory in Russia. Isolation by distance has shaped the genetic setup of *H. orientalis*, hailing mainly from scattered inhospitable arid and alpine areas of Central Asia and Transcaucasia.[42] It is possible to identify leech species by visual comparison to published descriptions of external color patterns[41] (Fig. 1).

Infection is a widely reported complication of leech therapy, with an incidence between 4.1 and 36.2 percent (Table 1). The dermal incisions from the leech teeth allow entry of pathogenic microbes from the digestive tract by means of regurgitation or from the anterior sucker (Fig. 2). The common clinical presentation of infection is cellulitis,[43,44] often with a foul odor, which may be complicated by subcutaneous abscess formation. Extensive tissue loss and septicemia have been reported.[45–47] Several publications advocate prophylactic antibiotics to combat leech-borne infections.[5–7,48–54] It is noteworthy that recently ciprofloxacin-resistant infections have been reported.[55,56]

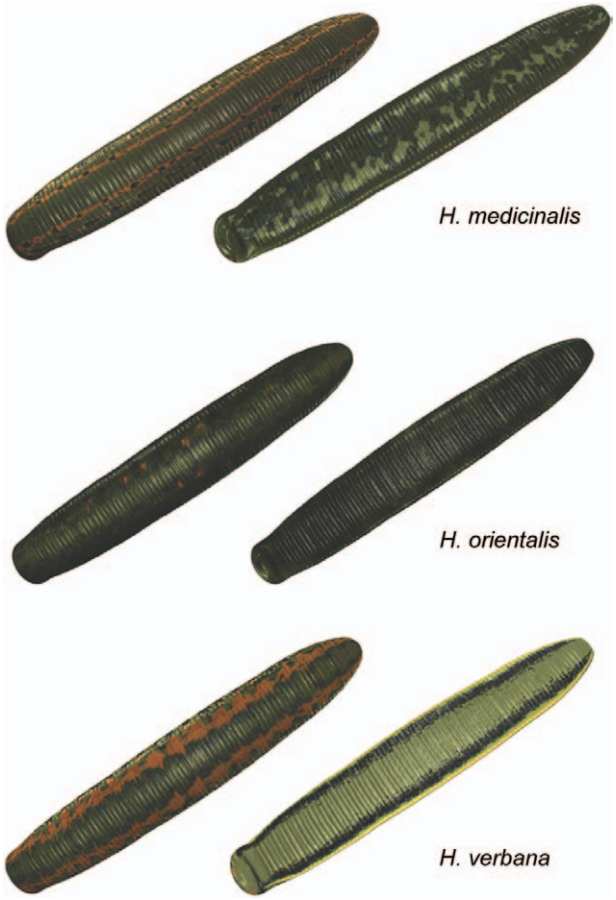

Fig. 1. The characteristic color patterns of the dorsal and ventral surfaces of *H. medicinalis* (*above*), *H. orientalis* (*center*), and *H. verbana* (*below*). [Computer-generated image courtesy of Stephen Atherton, B.Sc.(Hons.), M.A., R.M.I.P.]

Table 1. Published Case Series and Infection Rates following Leech Therapy

Authors, Year	No. of Patients	Period (yr)	Infection Rate (%)
Mercer et al., 1987	30	3	20
Lineaweaver et al., 1982	42	Not stated	7
De Chalain et al., 1996	18	5	11
Sartor et al., 2002	122	5	4.1
Bauters et al., 2007	47	2	36.2
Whitaker et al., 2011	35	4	20

The digestive tracts of animals, including humans, are colonized by complex microbial communities, which provide important functions to the host, including the synthesis of essential nutrients, stimulation of the immune system, and resistance against colonization of pathogens.[57,58] The digestive tract of the leech consists of the crop (the larger organ) in which the ingested blood meal is stored and where water and salts are absorbed,[59] and the intestinum, where further digestion of the blood meal is carried out (Fig. 3). In the crop, the symbionts form mixed-species microcolonies that either resemble polysaccharide-embedded biofilms or bacteria proliferating on host-produced mucin rafts.[60,61]

Fig. 2. The incisions into the dermis made by the leech jaws and teeth allowing the entry of pathogenic microbes from the leech digestive tract by means of regurgitation.

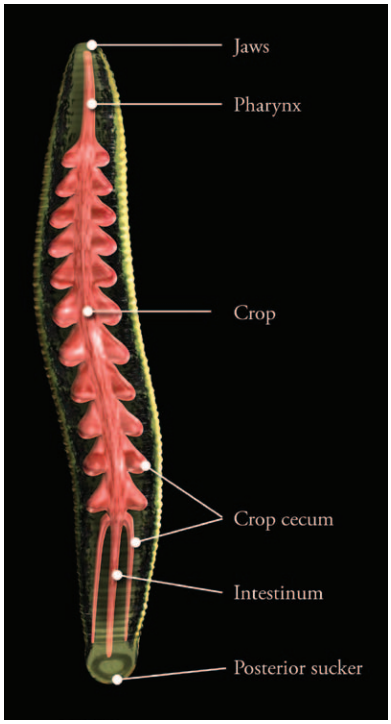

Fig. 3. A computer-generated image showing the basic anatomy of the leech.

To understand the interaction of the complex microbial communities with hosts, some easily characterized "model systems" have been used. The medicinal leech is an example of such a model system.[62,63] Early descriptions of the digestive tract microbiota of medicinal leeches reported an unusual simplicity; only one bacterium was culturable.[64] Subsequent studies supported the presence of one dominant microbe, an *Aeromonas* species, but disagreed on two—the identity of the *Aeromonas* species and the presence of other microbes in the leech midgut.[43,65–68] Early case reports identifying *Aeromonas hydrophila* infections used commercial chemotaxonomy phenotypic tests, which may not accurately identify environmental *A. hydrophila* to the species level.[68–71] The second controversy is the complexity of the midgut microbiota. A few studies implementing nonquantitative approaches reported the presence of additional bacteria within the midgut,[65] whereas other studies reported only *Aeromonas* species.[66] Studies of invertebrate midguts using culture-independent methods to detect diverse gut microbial communities in a wide range of hosts reported more complex microbial communities and were consistent with the observation that 95 percent of microbes in any environment cannot be cultured in a laboratory setting.[72]

There have been attempts to characterize the culturable and unculturable microbiota of clinically used medicinal leeches using a combination of molecular and biochemical studies. Only one publication has previously investigated *H. orientalis*, which is used across Western Europe and North America. It has been demonstrated previously that the digestive tracts of *H. verbana, H. medicinalis,* and *H. orientalis* have different microbiota. *Hirudo verbana* contained *A. veronii*[66,73] and *Rikenella*-like species, whereas *H. orientalis* was colonized with *A. veronii* or *A. jandaei*. From *H. medicinalis*, one polymerase chain reaction–based study detected *A. hydrophila*.[74]

In view of the use of leeches worldwide by reconstructive surgeons, and the potential for adverse effects associated with infections such as reduction in salvage rates, prolonged antibiotic therapy, inpatient stay, and additional procedures, we investigated the culturable and unculturable microbiota of *H. orientalis* using contemporary biochemical and molecular genetic techniques. In addition to investigating the microbiota, we also assessed, for the first time using highly sensitive methods, the resistance profiles to antibiotics commonly used by the surgical community.[7]

MATERIALS AND METHODS

Animal Identification

Leeches were identified as *H. orientalis* by visual comparison to published external color patterns[41] and DNA barcoding. DNA barcoding involved amplification and sequencing of 630 bp from the eukaryotic mitochondrial *cox1* locus and comparison to previously sequenced European medicinal leeches and other species of Hirudinidae.[40] A combined culture and culture-independent approach was used to characterize the microbiota in the midgut (crop and intestinum)[66,73] of *H. orientalis*.

Culture-Based Studies

Five starved leeches were selected by stratified random sampling from a major worldwide leech supplier. Following dissection, 100 μl of intraluminal fluid was extracted from the crop (Fig. 3). The intraluminal fluid was diluted serially and plated onto blood agar under aerobic conditions. Plates were incubated at 30°C until growth was observed.

Morphologically distinct colonies were secondarily streaked onto blood agar, and incubated overnight at 30°C. Colony polymerase chain reaction was performed on individual colonies using GoTaq. Universal 16S rRNA gene

and *Aeromonas*-specific DNA Gyrase *gyrB* primers[73,75] were used both for amplification and for sequencing [16S primers, 1492R (5′-TACGGYTACCTTGTTAGGACTT-3′) and 27F (5′-AGAGTTTGATCMTGGCTCAG-3′); *gyrB* primers, *gyrB3F* (TCCGGCGGTCTGCACGGCGT) and *gyrB14R* (TTGTCCGGGTTGTACTCGTC)].

Total reaction volume was 100 µl: 50 µl of GoTaq (either 1 µl of primer 1492R and 1 µl primer 27F or 1 µl of gyrase B 3F and 1 µl of gyrase B 14 R, 1 µl of resuspended DNA template; the colony was resuspended in 20 µl of nanopure water) and 47 µl of nanopure water. The thermal profile used was as follows: cycle 1, 95°C for 5 minutes; cycle 2 (×34), 95°C for 30 seconds (amplification), 55°C for 30 seconds, and 72°C for 1 minute 30 seconds; and cycle 3, 72°C for 10 minutes.

Gel electrophoresis was performed on polymerase chain reaction products using a 0.7% agarose gel. The polymerase chain reaction products were purified using Qiagen kit, and DNA was quantified using a spectrophotometer. Polymerase chain reaction products were sequenced using primers 27F and 1492R (16S) and gyrase B 3F and 14R.[60] The reactions were run on an ABI PRISM 3100 (Applied Biosystems, Foster City, Calif.) capillary DNA sequencer. Sequences were aligned using ContigExpress (ContigExpress, New York, N.Y.) and analyzed using VectorNTI 7 (Life Technologies, Grand Island, N.Y.) or Geneious 6.06 (BioMatters, Ltd., Aukland, New Zealand) and deposited in GenBank. The sequences were then compared with the National Center for Biotechnology Information database using BLASTX and BLASTN.[66,76] The sequences were aligned using MUSCLE and the phylogeny reconstructed using PHYML with HKY85 substitution model and 1000 bootstraps.

Nonculturable Studies

Two *H. orientalis* selected by stratified random sampling were fed a sterile blood meal at room temperature (21°C). After 42 hours, the crop was dissected and intraluminal fluid extracted. The intestinum was removed intact. Then, 200 µl of intraluminal fluid was placed into a sterile microcentrifuge tube. The intestinum sample was suspended in 500 µl of 0.85% sodium chloride and vortexed in a microcentrifuge tube. DNA was extracted from both tubes using a MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, Wis.). DNA was quantified by spectrophotometry. Polymerase chain reaction was subsequently performed on 100 µg of DNA using 16S primers (27F and 1492R) as described above.

The polymerase chain reaction amplicon was quantified using gel electrophoresis. A 16S rRNA gene clone library was constructed by cloning 16S rRNA gene amplicons into pCR2.1 and transformed into *Escherichia coli* TOP10 cells using a TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. (The accession numbers for Ho_clone1-9, Ho1151, Ho1154, Ho11531, Ho11482, and Ho1149 are KC417278-86, KC702159, KC702160, KC70216, KC70216, and KC702163, respectively.)

The cloned DNA was amplified by colony polymerase chain reaction of 48 white colonies from intraluminal fluid and 48 white colonies from intestinum using M13 reverse primer and M13 forward primer. Thermocycling was similar to that previously described but with an annealing temperature of 48°C. Amplicons from individual clones were digested separately using the restriction endonucleases *Hae*III and *Taq*αI (New England Biolabs, Ipswich, Mass.) to generate restriction profiles, which were visualized in a 2.0% Metaphor agarose gel (Cambrex, East Rutherford, N.J.) stained with ethidium bromide. The digest setup included two total reaction volumes of 20 µl. The *Taq*αI digests were incubated at 65°C overnight (12 µl of polymerase chain reaction product, 0.2 µl of bovine serum albumin, 2 µl of Buffer 4, 2 µl of *Taq*αI, and 3.8 µl of nanopure water). The *Hae*III digests were incubated at 37°C overnight (12 µl of polymerase chain reaction product, 0.2 µl of bovine serum albumin, 2 µl of Buffer 4, 2 µl of *Hae*III, and 3.8 µl of nanopure water). Representative plasmids were sequenced using BigDye version 1.1. Sequences were assembled using ContigExpress and compared to known 16S rRNA gene sequences using the Ribosomal Database Project classifier.

Generation of Spontaneous Rifampicin-Resistant Mutants

A. veronii spontaneous rifampicin-resistant mutants were generated by plating 100 µl of an overnight culture on Luria-Bertani rifampicin 100 µg/ml, yielding 1106Rf and 1107Rf. Plates were incubated overnight at 30°C.

Growth Curve

Cultures 1106Rf, 1107Rf, and HM21S were grown overnight in 5 ml of Luria-Bertani broth containing antibiotics under aerobic conditions in a shaker at 30°C. The optical density at 600 nm was measured using a spectrophotometer and the cultures were diluted to an optical density of 0.1.

Ten microliters of 0.1 optical density of overnight cultures was added to 200 μl of Luria-Bertani broth in a 48-well plate. The growth was monitored by measuring the optical density at 600 nm every 10 minutes for 24 hours using a spectrophotometer. This growth curve of the rifampicin-resistant mutants was compared to HM21S, an *A. veronii* isolate spontaneously resistant to streptomycin, cultured from the crop of *H. verbana*.

Competition Assay

Using previously established tests,[60,66,77] the colonization ability of 1106Rf and 1107Rf was tested against a competitor strain, HM21S, by inoculating a blood meal with 250 colony-forming units/ml of each strain. Then, 500 μl of blood was removed from each blood meal before feeding to verify input.[75] Six *H. verbana* and seven *H. orientalis* were fed 5 ml of heat-inactivated inoculated defibrinated sheep blood. Blood was placed in a 15-ml Falcon tube (Becton Dickinson, Franklin Lakes, N.J.) covered with Parafilm (Pechiney Plastic Packaging, Inc., Chicago, Ill.). A sterile needle pierced the Parafilm, releasing negative pressure as the leech fed. After 42 hours at room temperature, leeches were killed and intraluminal fluid was removed aseptically. Serial dilutions were plated as before on Luria-Bertani Rf 20 μg/ml and Luria-Bertani Sm 100 μg/ml. Plates were incubated overnight at 30°C and the number of colony-forming units was counted. The competitive index value was calculated using the following equation: *competitive index value* = (*mutant output/competitor output*)/(*mutant input/competitor input*). A competitive index of 1 indicated the mutant colonized the same level as the competitor strain and competitive index less than 1.0 indicated that the mutant had a colonization defect.

Antibiotic Sensitivity Testing

A lawn of *A. veronii* strains isolated from three *H. orientalis* crops was plated onto Mueller-Hinton agar plates. An Etest (AB bioMérieux, Marcy l'Etoile, France) comprising a predefined gradient of antibiotic concentrations on a thin, inert, nonporous plastic strip was placed on each plate, to determine the on-scale minimum inhibitory concentration of ciprofloxacin, ampicillin, and Augmentin (GlaxoSmithKline, London, United Kingdom) according to the AB bioMérieux protocol. One side of the strip carried the minimum inhibitory concentration reading scale in micrograms per milliliter and a two- or three-letter code to identify the antibiotic (i.e., CL, ciprofloxacin; XL, Augmentin/Co-amoxiclav; AM, amoxicillin). A predefined exponential gradient of antibiotic, dried and stabilized, was on the other side. Clinical and Laboratory Standards Institute interpretative standards (in micrograms per milliliter) are shown in Table 2.

RESULTS

Identification of Culturable Bacteria

Only one colony morphology was detected on Luria-Bertani agar from *H. orientalis*. Five strains, obtained from four annelids, were identified with the API 20E test as *A. hydrophila*. As the 16S rRNA gene sequence is problematic for identifying *Aeromonas* to the species level,[78] we polymerase chain reaction–amplified and sequenced *gyrB* to successfully identify *Aeromonas* species.[79] Phylogenetic analysis of the *gyrB* sequence results identified all five strains in a clade as *A. veronii* (Fig. 4). This clade had greater than 99 percent bootstrap support.

Culture-Independent Identification of Bacteria

Figure 5 illustrates the different restriction patterns of amplicons that were obtained from individual clones following digestion with the restriction endonucleases *Hae*III and *Taq*αI (New England Biolabs), visualized in a 2.0% Metaphor agarose gel (Cambrex, East Rutherford, N.J.) stained with ethidium bromide. Multiple clones for each representative restriction profile were sequenced using M13R and M13F primers. Table 3 shows a clone library containing a detailed description of the microbiota (division, genus, and species) of both the crop (intraluminal fluid) and the intestinum from two *H. orientalis* specimen studied.

Competition Assay

Spontaneous antibiotic-resistant mutants were isolated, and no difference in the growth rates of 1106Rf, 1107Rf, and Hm21S were observed when tested in a rich, liquid medium at 28°C (data not shown). When equal amounts of the *H. orientalis* isolates were computed against the

Table 2. Clinical and Laboratory Standards Institute Interpretative Standards of the Etest

	Susceptible (μg/ml)	Intermediate (μg/ml)	Resistant (μg/ml)
Ampicillin (AM)	≤8	16	≥32
Augmentin (XL)	≤4	8	≥16
Ciprofloxacin (CL)	≤4	8	≥16

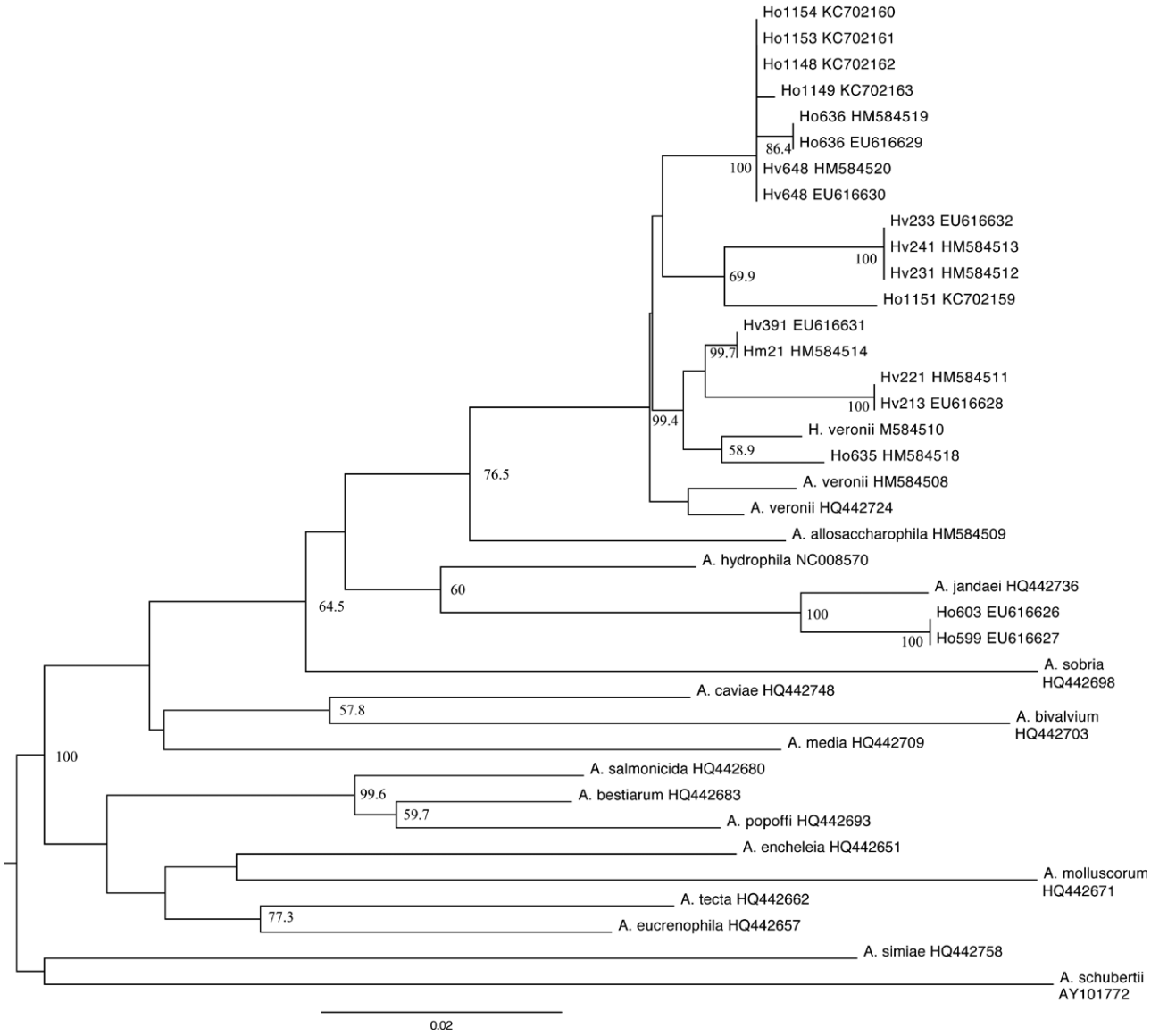

Fig. 4. Phylogenetic tree of *Aeromonas* strains using *gyrB*. A maximum likelihood tree was generated from the DNA sequence, and bootstrap support values from 1000 replicates are shown.

H. verbana isolate Hm21S, no colonization defect was detected (Fig. 6) in either host *H. orientalis* or *H. verbana*. These data indicated that the symbionts can proliferate equally well in either host.

Etest Antibiotic Sensitivity Testing Results

All strains were resistant to amoxicillin and two of three were resistant to Augmentin, with the remainder showing intermediate resistance. All

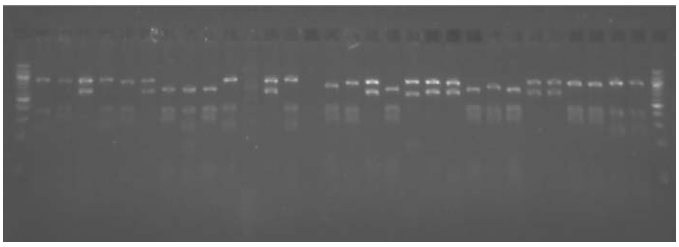

Fig. 5. Metaphor agarose gels (2.0%) stained with ethidium bromide illustrating the amplicons from individual clones following digestion with the restriction endonucleases *Hae*III and *Taq*αI.

Table 3. 16S Clone Libraries from the Intraluminal Fluid and Intestinum of *H. orientalis*

Source	Bacterial Division	Organism	Animal 1	Animal 2	Laufer et al., 2008[80]
ILF	α-Proteobacteria	*Anaplasma marginale*	ND	7 (26)	
	α-Proteobacteria	*Ochrobactrum*	ND	2 (7.7)	
	γ-Proteobacteria	*Aeromonas veronii*	11 (38)	6 (23)	
	γ-Proteobacteria	*Morganella morganii*	8 (27)	4 (15)	
	Bacteroidetes	*Rikenella*	10 (35)	4 (15)	65 (98)
	Clostridia	*Clostridium*	ND	3 (26)	
Intestinum	α-Proteobacteria	*Magnetospirillum*	2 (7)	5 (17)	
	α-Proteobacteria	*Roseospira marina*	5 (18)	8 (28)	
	γ-Proteobacteria	*Aeromonas veronii*	5 (18)	2 (6.9)	3 (6)
	γ-Proteobacteria	*Morganella morganii*	3 (10)	8 (28)	
	δ-Proteobacteria	*Desulfovibrio*	4 (14)	ND	11 (20.7)
	Bacteroidetes	*Rikenella*	9 (32)	6 (21)	23 (46)

ILF, intraluminal fluid; ND, not determined.

strains were sensitive to ciprofloxacin (Table 4). Figure 7 clearly shows *Aeromonas* strain 1148 exhibiting sensitivity to ciprofloxacin.

DISCUSSION

This study reveals that the microbiota of the medicinal leech is not as simple as many believe, and the microbiota of *H. orientalis* is more diverse than described in the only previous study.[80] Culture-dependent studies confirmed *A. veronii* in all four leeches, which concurs with previous studies,[66,73] but we did not detect *A. jandaei*. We confirm that the API20E tests fail to accurately identify *A. hydrophila* to the species level,[7] and highlight that care has to be taken when identifying *Aeromonas* species without complementary molecular approaches.

Our culture-independent studies were more revealing. Our 16S rRNA gene clone libraries revealed several species in addition to *A. veronii* in the intraluminal fluid of the crop, including α-Proteobacteria (*Anaplasma marginale* and *Ochrobactrum anthropii*), γ-Proteobacteria (*M. morganii*), a *Rikenella*-like Bacteroidetes, and a *Clostridium*-like Clostridiales. The relative abundance of the Bacteroidetes (35 percent) and *A. veronii* (38 percent) suggests that they are the dominant members of the crop community, as reported in *H. verbana*.[66] Bomar et al. and Kikuchi and Graf showed that these symbionts form polysaccharide-embedded, mixed-species microcolonies resembling floating granular biofilms[81] or mucous rafts.[61]

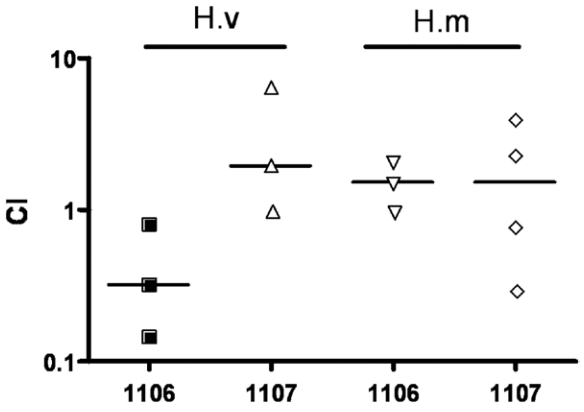

Fig. 6. A scatterplot showing a competition assay suggesting that there is no colonization defect of 1106Rf or 1107Rf in the digestive tract of *H. orientalis* or *H. verbena*.

Clone libraries from the intestinum suggest a similarly diverse bacterial community, including bacteria not previously isolated from the leech such as α-Proteobacteria *Magnetospirillum* species and *Roseospira marina*. *Aeromonas veronii*, *Rikenella* species, and *Morganella morganii* were predominant in the intestinal flora. Like *Aeromonas* species and Bacteroidetes, *M. morganii* has also been implicated as a human pathogen. No clinical cases have been published in the literature; however, we have experienced a *Morganella* species causing a clinically significant soft-tissue infection in our unit. *Aeromonas veronii* isolates from *H. orientalis* were able to colonize both *H. verbana* and *H. orientalis* species equally.

Experimentally, the ability of *A. veronii* strains to cause septicemia has been assessed using intraperitoneal injections in mice.[82] Studies searching for virulence factors in *Aeromonas* species have demonstrated the importance of type III secretion systems in causing disease.[60,83] Type III secretion systems act as molecular syringes, penetrating the membrane of eukaryotic cells and injecting bacterial proteins into the cytosol. These proteins inhibit or stimulate phagocytosis, confer cytotoxicity, and induce apoptosis.[60,84] The importance of *Aeromonas* species as emerging pathogens is highlighted as the leading cause of wound infections in victims of the tsunami in Thailand[85] and causing necrotizing fasciitis in patients after liposuction.[86] It is postulated that the virulence factors

Table 4. Etest Results

Aeromonas Strain	Ciprofloxacin (μg/ml)	Augmentin (μg/ml)	Amoxicillin (μg/ml)
1147	0.004, sensitive	48, resistant	>256, resistant
1148	0.004, sensitive	12, intermediate resistance	>256, resistant
1151	0.003, sensitive	48, resistant	>256, resistant

associated with *Aeromonas* species are potentiated by venous congestion, leading to a localized area of immunocompromise that is more susceptible to infection. The most common clinical presentation of aeromonad infection is cellulitis[43,44]; in severe cases, extensive tissue loss and septicemia have been reported.[45–47,87] *Aeromonas* species seem to have a particular affinity for muscle tissue, and are capable of producing extensive proteolytic enzymes, leading to a clinical picture resembling clostridial myonecrosis with gas production.[88] The ability of *Aeromonas* species to invade the walls of blood vessels with resultant vasculitis, thrombosis, and hemorrhagic necrosis is of concern to microsurgeons.

Etest results suggest that quinolones are the most appropriate antibiotic therapy to help protect against infective complications. All three isolates were sensitive to ciprofloxacin, with the isolates showing either a complete or intermediate resistance to Augmentin. The plastic surgery community has long been aware of the infection risk associated with leech application[89,90]; however, despite the emerging evidence base that Augmentin can be ineffective,[91–93] with fluoroquinolones seeming to be consistently active,[50,51,65,91,92] even new and widely used textbooks and Web sites continue to advocate the use of amoxicillin and clavulanic acid preparations. Recently, the first cases to our knowledge of ciprofloxacin-resistant leech–transmitted *Aeromonas* were reported.[55,94]

The alternative to antibiotic prophylaxis suggested by Mumcuoglu et al.,[95] feeding leeches with ciprofloxacin, is not advisable. Prolonged exposure of leeches to ciprofloxacin would inevitably encourage *Aeromonas* species to develop resistance to ciprofloxacin, adding to the dramatic increase in multiresistant strains of bacteria, including ciprofloxacin.[96,97] If *Aeromonas* species in leeches were resistant to ciprofloxacin at a clinical distribution facility, it would make such infections much harder to treat. In addition, if *Aeromonas* species were removed from the symbiotic natural flora of the leech digestive system, a possibly more virulent bacteria would fill the resultant vacuum. Interestingly, it has been postulated that switching from cow blood to chicken blood for raising medicinal leeches may have led to the appearance of ciprofloxacin-resistant *Aeromonas* strains.[55]

The lack of clarity in clinical practice is borne out in previous findings that less effective and superseded antibiotics are often used.[7] In light of the recent reports from the United States and France highlighting new resistance patterns, it is prudent to take care in the prescription of leeches, being alert to standard infectious agents and the need to send swabs to accurately guide antibiotic therapy.

Fig. 7. The plate of *Aeromonas* strain 1148 exhibiting sensitivity to ciprofloxacin, intermediate resistance to Augmentin, and resistance to amoxicillin.

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