Yes, you can, in fact, reuse your leeches without the fear of nosocomial infections.

by

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Abstract

Introduction:

Leeches are used in human and veterinary medicine as a treatment for venous congestion. It is recommended to discard leeches after one use. A concern with reusing leeches is potential spread of bacterial infections. If a leech were to harbor bacteria from one patient in its gastrointestinal (GI) tract, it may transmit them to another patient, potentially, even serving as a vector for multidrug resistant (MDR) infection. We sought to determine the safety of reusing leeches inoculated with an MDR *Staphylococcus aureus*.

Study design:

Experimental, ex-vivo

Animals:

63 leeches were split into eight treatment groups and one control group.

Methods:

Treatment leeches were fed canine blood inoculated with an MDR strain of *Staphylococcus aureus* while control leeches were fed clean canine blood. Cultures were obtained at 1 day, 1 week, and 1-, 2-, 3- and 4-months post-inoculation. At each time point, cultures were taken of

aquarium water, GI contents, and blood that the leeches were allowed to feed on. Cultures were evaluated for the presence of *Staphylococcus aureus.*

Results:

All water samples were negative except for one tank at seven days. After two months and three months, *all* GI tracts and blood meal samples were negative, respectively.

Clinical significance:

Leeches will harbor MDR *Staphylococcus aureus* after inoculation with infected canine blood. This bacterium is not detectable in the water after 7 days and no longer detectable in the leech or blood meal after 3 months.

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Dedication

To dogs. Mine, yours, and no ones. We don’t deserve a single one of them, but somehow, someway, they chose us to evolve together with. We owe them better. We owe them everything. We owe them so much more. I hope this helps.

Chapter 1 - Who are the Leeches

The rise of the leeches: an origin story

The Latin word for "little ring" is annellus. It is a word used to describe the ringed physical appearance of the invertebrates belonging to the phylum of Annelida, the ringed worms. Of popular mention, this is the phylum to which the wiggly earth worms belong. Leeches themselves belong to a subphylum class named the Hirudinoidea. This class is further subdivided into a subclass of non-extinct members, Euhirudinea. And finally, the suborder Gnathobdellida, which describes their short muscular pharynx and toothed jaws. Since even leeches would consider the word Gnathobdellida a mouthful, Hirudiniformes is the most commonly used name for this suborder. The two species of leeches that will be discussed for the remainder of this literary masterpiece are *Hirudu medicinalis and H. verbana.* (Elliot, 2011).

The leech *H. medicinalis* was first described by Carl Linnaeus in 1758. While this may seem like a really long time ago, there are records of leech use as early as Ancient Egypt and their use recommended for medical treatment since the Greeks in 130 BC. In 1570, a medical exposition was written in France discussing their use as a treatment to help restore the balance of the four humours, also known as blood, phlegm, melancholy, and choler.

Leeches became very popular in France during the 17th and 18th centuries. The use of leeches in Paris, France in the early 1830's was reported to be almost 180,000 leeches per year; for just one hospital (Sawyer, 1986; Herter, 1968). The French government eventually imposed an import tax on the leeches. And of course, anything that is popular in Europe, becomes popular in America. Especially, if it isn't found here. Medicinal leech use became so popular that most wild sources

of *H. medicinalis* across several European countries were left bone-sucking-dry. To the point where many countries banned their export. At one lake in Kent, the leeches were hot branded so that they could estimate their populations. Eventually, this lake conducted a leech census every 2 years by pulling all of them out. Where there were once estimated to be over 10,000 leeches, there were now about 300 collected every second year (Wilkin, 1987/1989). They were endangered. To compensate for the void of these precious and high demand leeches, another innocent little creature became the target of exploitation. The related *H. verbana* had to be brought in from a producer in Turkey. Their exact ecologic history is very much unknown. This species is sometimes confused as the true medicinal leech, but unfortunately, it is the lesser of the two species and not the original.

Fortunately for human needs, their classically irresponsible attitudes allowed the leeches to thrive. Leeches were kept in jars in hospitals and pharmacies. Once used, they were tossed into a pond where they, fat and happy, were free to reproduce. Eventually, the Germans caught on and the first leech farm was created by the end of the 17th century. This farm in Hildesheim began producing between 3-4,000,000 leeches/year (Herter, 1968).

As you can now see, medicinal leeches very quickly spread all over the world, as is the case with all snake oil trends and propaganda. Over the years, however, we have found that leeches are, in fact, not snake oil (Zaidi, 2011). Perhaps for some of the things they were pretending to treat at the time, this was the case, but as will be discussed further on leeches do deserve their own doctorate for credibility.

The reproductive biology of leeches

Leeches are pretty good at multitasking, including when it comes to reproduction. At around 2 years of age, they are capable of presenting us with the gift of baby leeches. Leeches are hermaphroditic. Leeches have both male and female gonopores. The male gonopore contains a tube-like male copulatory organ that becomes exteriorized when mating. This setup not only allows them to receive genetics, but to also provide genetics. Once mating occurs, sperm can be stored for several months prior to cocoon deposition.

Spongy cocoons are laid in damp locations outside of the body of water. Each cocoon can contain up to 16 or more eggs with each mature leech laying up to 8 cocoons per season. This usually occurs in the warmer summer months in the wild. However, under human care conditions, this can occur year round with some animals producing cocoons up to twice per year with up to 30 eggs each (Zapkuviene, 1972). Hatching is temperature dependent and can take anywhere from 4-10 weeks. Once hatched, the babies can survive for at least 100 days without feeding, but like all creatures, they need food to grow.

The feeding biology of leeches

Leeches of the genus *Hirudo* are freshwater species that live in slow moving streams or ponds. These animals patiently lie in wait while being one with their environment in order to feel changes in temperature, chemicals, light, or motion that informs them of the presence of an ever-welcomed guest. Dickinson and Lent performed a feeding behavior study in medicinal leeches in 1984 which provided a lot of the foundation for understanding the feeding biology of these creatures. This information is summarized below. Several studies produced since then have expanded on this original research and have shown time and time again the wonder that is the hungry leech.

Mechanical and photo- sensitivity are provided by a collection of mechanosensory hair cells that are found in seven pairs of clusters in the mid-body of the leech. These are known as segmental sensillum which are simple sense organs that are found at the end of a nerve connection. Segmental sensillum can be found throughout the body of the leech including around their anterior and posterior suckers. When one of these stimuli is sensed, the leech is alerted and thus begins to swim in the direction of the signal. Studies have shown that mechanical stimulus is what they respond to the most. Once contact is made with a surface, the leech stops swimming and begins its exploration via crawling. This is known as thigmotaxis, in which the movement of the organism is directed by contact with a solid or rigid surface. This also allows the leeches to identify crevices on their host that would provide them with safety during their prolonged feedings. The driving factor at this point becomes temperature. With biting being triggered once a surface temperature of 37- 40º C, or a temperature warmer than its ambient water source, is identified.

Once a leech finds a suitable snack bar, it bites. The bite is made with hundreds of calcified teeth arranged in set of tri-radiate incisions. The leech then rhythmically contracts its muscular pharynx in peristaltic waves that pump blood from the buccal cavity into the crop. Additionally, the leeches then flex their bodies in a sinusoidal shape that closely resembles their swimming patterns. Interestingly enough, each leech dances to its own tune as it has its own characteristic rhythm which can be seen to be consistently displayed across multiple feedings. Initially, peristalsis of the anterior portion of the leech is the dominant motion while flexion takes over towards the end of the feeding. Leeches will feed for a period of 20-40 minutes and then they detach when satiated. Usually, this is when they have reached 9x their initial body weight. At this point, they are so heavy, they cannot move…talk about a food coma. But fortunately, for the leeches, this weight is lost within the first week and thus they are able to regain locomotor function.

Food is stored within the crop of the leech and enzymatic digestion of food takes place within their intestines. Once they have ingested their food, the leeches secrete a large amount of mucin and then proceed to compress the blood into a luminal fluid consisting of very tightly packed erythrocytes by excreting the water and solute components (Marden, 2016). This can then remain stored within the leech for up to one year. It is yet unclear how the blood meal remains stable within the crop, but it is suspected that proteolysis is inhibited (Lemke, 2020; Roters, 1992; Lent, 1988; Zerbst-Boroffka, 1973)

Leeches have been found to have a best friend, the symbiote *Aeromonas spp.*, which aids in digestion of their food via hemolysis (Lemke, 2020; Maltz, 2011/2014; Siddall, 2011; Bomar, 2011; Indergrand, 2000). It is also believed that the presence of *Aeromonas*, inhibits the growth of other species of bacteria within the crop of the leech.

Aeromonas spp., the bestest friend that anybody can have.

The health of an organism greatly depends on its gastrointestinal tract flora. This flora is subjected to many factors including circadian rhythms, mental health, diet, environment, etc. Medicinal leeches have been found to have a symbiotic relationship with a few different species of *Aeromonas* bacteria. The presence of this bacteria within the leech has been the center of various forms of research all of which have attempted to elucidate the importance of this relationship. It has been found that this relationship is so important to the leeches, that the offspring within cocoons are found to host the same strains as their parents. Here we will outline a few ways in which the leech and the bacteria co-exist.

The love language of a leech is food-based. They produce several factors that provide energy for their bacterial inhabitants. The mucin secreted to protect their epithelium after feeding, has been found to serve as a source of nutrients for the bacteria. Of importance, it has been found that the bacteria express mucin and glycan utilization genes that far exceed their ribosomal protein coding genes. This discovery has been incredibly important for research involving these two lovers because it has allowed scientists to develop culture media which can successfully maintain these bacteria.

Aeromonas spp. perform β-hemolysis in order to digest the erythrocytes and release the protein, lipid, and heme contained within. Symbiotes have also been found to contain genes encoding for proteins that aid in the catabolism of arginine, an energy poor source. In return, the bacteria are able to synthesize vitamins, such as B-vitamins, to supplement the leeches requirements. Additionally, the symbiote is capable of sequestering iron. This serves two functions, 1. It

protects the leeches against heme toxicity and 2. It restricts a nutrient required for other bacteria to grow.

Leeches and their symbiont, both produce antimicrobial peptides (AMP). The ones produced by leeches are produced in high concentrations in starved animals when the *Aeromonas* populations are low and vice versa. The presence of *Aeromonas* populations suppress the production of the leech AMP's. These bacteria, in turn produce their own AMP's against other bacterial species. (Tasiemski, 2015).

Several AMP's have been discovered in medical leeches with actions against either gram positive, gram negative, or both types of bacteria. One study looked at twelve isolates and found that eight of these possessed antimicrobial activity against *E. coli, B. subtilis, and C. thrachomatis.* Two of the AMP's exhibited inhibition of growth at an MIC of 10 μmol (Grafskaia, 2019). More and more information regarding these AMP's is being discovered daily as the potential for AMP's as a form of drugs to be used against resistant bacterial infections. All of these factors and team effort, allow the *Aeromonas* bacterial colonies to remain under control and without worry of over colonization of other potentially pathogenic species explaining the very rarely simple microbiota of the leech GI tract.

Magical properties of leech saliva

Science is magic that is real and leeches should be considered the true wizards of the scientific world. Their saliva has been found to contain numerous beneficial properties, highlighting their successful use in ancient and modern medicine. While leeches are known for their sanguivorous activity, this is not the sole benefit in medicinal use. Their bloodthirsty nature is the primary use, allowing for them to be used to improve congestion of wounds by draining stagnant blood. Their saliva contains hirudin, which has been found to be one of the most powerful anti-coagulants known to man and the original anticoagulant preceding the discovery of heparin.

To date, only 15% of the salivary proteins of medicinal leeches have been identified and characterized with 189 and 344 identified in *H. medicinalis and H. verbana*, respectively. I will direct you to Table 1 from Lemke, et al and the original article for discussions on individually isolated compounds. To limit the length of this riveting thesis, we will summarize the function of these proteins in a few short sentences.

The main physical action of leeches is to suck blood from host tissue. With the addition of the proteins found within leech saliva, this benefit is exponentiated as they create local changes to the host which include but are not limited to changes in local blood pressure and flow, anti-thrombosis, vasodilation, antimicrobial, anti-inflammatory, and analgesic properties. This allows blood to not only be removed, but to continue to flow in the area of feeding for up to several hours after feeding and thus improving congestion at sites of reconstructive surgery, skin flaps, hematomas, etc. Many of these proteins are currently being investigated as potential drug leads.

Acute venous obstruction of flaps is more likely to be damaging than acute injury where both arterial and venous supplies are damaged. For this reason, the use of leeches has been shown to time and time again provide an increased significance in flap survival rates. A recent systematic review into the use of leeches in plastic and reconstructive surgery found a success rate of 78% with only a 22% rate of complication (Whittaker, 2012). Due to their great success and popularity in human medicine, Hirudo therapy has slowly creepy crawled its way into veterinary medicine.

Salivary Protein	Mechanism of Action	Biological Significance
Hirustasin (Mass: 5.866 kDa)	Tissue kallikrein inhibitor and inhibitor of trypsin, chymotrypsin and neutrophil cathepsin G	Anti-inflammatory
Apyrase (Mass: 45 kDa)	Cleavage of adenosine 5′-diphosphate	Inhibitor of platelet aggregation
Bdellin B-3 (Mass: 6.141 kDa)	Inhibitor of plasmin, trypsin and sperm acrosin	Anti-inflammatory
Calin (Mass: 65 kDa)	Prevents the binding of von Willebrand factor to collagen	Inhibitor of platelet aggregation
Collagenase (Mass: 50 kDa)	Cleavage of collagen	Collagen digestion
Destabilase (Mass: 12.6–12.9 kDa)	Cleavage of fibrin clots, cleavage of peptidoglycans in bacterial walls	Anticoagulant/antimicrobial
Eglin C (Mass: 8.1 kDa)	Neutrophil elastase inhibitor, cathepsin G inhibitor	Anti-inflammatory
Hirudin (Mass: 7.1 kDa)	Thrombin inhibitor	Anticoagulant
Hirudin-like factors (Mass: 4.27–6.67; isoforms HLF1-HLF3)	Unknown for the three European species	
Hyaluronidase (Mass: 27.5 kDa)	Cleavage of hyaluronic acid	Extracellular matrix digestion
Leech-derived tryptase inhibitor (Mass: 4.7 kDa)	Mast cell tryptase inhibitor	Anti-inflammatory
Leech carboxypeptidase inhibitor (Mass: 7.2 kDa)	Carboxypeptidase B inhibitor	Unclear
Saratin (Mass: 12 kDa)	Inhibits the binding of von Willebrand factor to collagen	Inhibitor of platelet aggregation
Yagin (Mass: 15.4 kDa)	Factor Xa inhibitor	Anticoagulant

Reference 1: A summary table of some of the identified salivary proteins from the original article by Lemke, 2020.

Leeches: the ultimate phlebotomist of veterinary medicine

Hirudo therapy has been incorporated in veterinary medicine for the same purposes as have been described in humans since ancient times. They are most commonly used for oncological patients following reconstructive skin flaps post mass removal. Other described uses for veterinary patients include osteoarthritis, neuropathies, eczema, polycythemia vera, and mastitis amongst many others (Net, 2001; Canoplat, 2004/2004; Buote, 2014; Kermanian, 2022).

Despite evidence of efficacy, leech therapy is still not common in veterinary medicine. One reason for their scarcity in veterinary medicine might include the recommendation to sacrifice medicinal leeches after a single use. In human medicine, re-using leeches is forbidden due to the concern of cross contamination of bacteria from infected patients. Unfortunately, while the leeches experience a forced relationship with medicine, the *Aeromonas* can elect to not participate in the same reciprocal relationship it shares with the leeches. *Aeromonas spp.* have been implicated in wound infections for patients treated with medicinal leech therapy. These infections can cause further damage and injury to the wound with very significant consequences.

As described previously, we know that leeches and their symbiont, *Aeromonas*, have exceptional antimicrobial properties. But if leeches can harbor a potentially infectious bacteria in their gastrointestinal (GI) tract and then transmit that to a patient, it is possible that if a leech were to feed on a patient with a multidrug resistant bacterial (MDR) infection, it could harbor that MDR bacterial species and, when placed on a different patient, could potentially transmit that infection. Additionally, it is unknown if they can transmit bacteria through the water column of their housing system. This is of importance because within veterinary medicine, many patients may

receive leech therapy as a last-ditch effort to save their skin flap and infection with an MDR bacterial species may be common in these animals.

Single use of leeches in veterinary medicine may not be practical due to the cost associated with purchasing new leeches for each patient and the time for leeches to be shipped from their source to the veterinary hospital. For example, without factoring in hospital markup, the cost of overnight shipping for five leeches for a patient was approximately $277.75 at the time this thesis was prepared (Leeches U.S.A. Ltd, Westbury, NY, USA). This has precluded many advancements in Hirudo therapy within veterinary medicine.

Conclusions

While often considered with disgust, leeches, like many other undervalued inhabitants of this earth, have a variety of fascinating properties. These properties allow them to have a close relationship with a bacteria and thus having one of the simplest gut microbial flora known to man. Additionally, the proteins produced by these animals go far beyond their blood sucking abilities and allow them to be heroes amongst bloodletters. As with all things, incorporation into veterinary medicine has been slow and largely due to associated costs. Hopefully, this report has demonstrated invaluable use of leeches across medical fields and will inspire the continued exploration and use of these creatures, both in vivo and via the reproduction of their plethora of medicinal compounds. Who knows, someday, you could be thanking a leech for saving your leg.

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Chapter 2 - Evaluation of Re-used Medicinal Leeches as a Potential Source for Nosocomial MDR Bacterial Infections

Abstract

Introduction:

Leeches are used in human and veterinary medicine as a treatment for venous congestion. It is recommended to discard leeches after one use. A concern with reusing leeches is potential spread of bacterial infections. If a leech were to harbor bacteria from one patient in its gastrointestinal (GI) tract, it may transmit them to another patient, potentially, even serving as a vector for multidrug resistant (MDR) infection. We sought to determine the safety of reusing leeches inoculated with an MDR *Staphylococcus aureus*.

Study design:

Experimental, ex-vivo

Animals:

63 leeches were split into eight treatment groups and one control group.

Methods:

Treatment leeches were fed canine blood inoculated with an MDR strain of *Staphylococcus aureus* while control leeches were fed clean canine blood. Cultures were obtained at 1 day, 1

week, and 1-, 2-, 3- and 4-months post-inoculation. At each time point, cultures were taken of aquarium water, GI contents, and blood that the leeches were allowed to feed on. Cultures were evaluated for the presence of *Staphylococcus aureus.*

Results:

All water samples were negative except for one tank at seven days. After two months and three months, *all* GI tracts and blood meal samples were negative, respectively.

Clinical significance:

Leeches will harbor MDR *Staphylococcus aureus* after inoculation with infected canine blood. This bacterium is not detectable in the water after 7 days and no longer detectable in the leech or blood meal after 3 months.

Introduction

Medicinal leeches (*Hirudo medicinalis* and *verbena*) have been used for thousands of years. They are considered one of the oldest medical practices with continued studies detailing their function and identifying new uses. In modern medicine, they are used to help improve the venous flow of congested skin or muscle flaps or wounds following a surgical reconstruction or repair. Leeches have also crawled their way into veterinary medicine for the same purposes, most commonly for oncological patients following reconstructive skin flaps post mass removal. Other described uses for veterinary patients include osteoarthritis, neuropathies, eczema, polycythemia vera, and mastitis amongst many others[1-4].

Starting with hirudin, an anticoagulant, scientists have discovered over 100 different compounds within the leech saliva. These compounds work together and with the host to create an anti-inflammatory and anti-coagulant environment, as well as exerting anesthetic and analgesic effects. Hirudin, antistastin, and ghilanten produce a synergistic inhibitory effect on factor Xa[5]. These effects remain for up to several hours after the leech has finished feeding. While the blood meal consumed by a leech actively aids in the improvement of venous stasis, the persistence of the effect of these compounds allows for continued bleeding from the leech bite, further decreasing congestion and improving microvascular flow[6-9]. A recent study found that medicinal leech therapy resolved 75% of venous congestion lesions in small animal patients[10].

While the benefits of medicinal leech therapy have been proven, their use is still not common in veterinary medicine. One reason for their scarcity in veterinary medicine might include the recommendation to sacrifice medicinal leeches after one a single use. In veterinary medicine this

is not always practical due to the cost associated with purchasing new leeches for each patient and the time for leeches to be shipped from their source to the veterinary hospital. Since leeches only feed once every three to six months, if a veterinary hospital could set up a leech colony and reuse each leech this may increase the attractiveness of this therapeutic modality.

An obvious concern with the use of, and therefore the reusing, of leeches is cross contamination of bacteria from infected patients. Leeches have a symbiote bacteria, *Aeromonas hydrophila,* which has been reported to be transmitted to ~18% of human patients undergoing leech therapy[11]. For this reason, human patients are prophylactically treated with antibiotics to prevent such infection[12]. If leeches can harbor a potentially infectious bacteria in their gastrointestinal tract and then transmit that to a patient, it is possible that if a leech were to feed on a patient with a multidrug resistant bacterial (MDR) infection, it could harbor that MDR bacterial species and, when placed on a different patient, could potentially transmit that infection. Additionally, it could be possible for them to transmit bacteria through the water column of their housing system. Depending on the bacterial species, there may be zoonotic implications for the hospital staff. To the authors' knowledge, there are currently no studies evaluating the harboring or transmission of MDR bacteria in leeches.

Recent data has shown that leeches have antimicrobial properties in their saliva. A study conducted by Abdualkader et al. (2011). showed that leech saliva extract has antibacterial activity against *Staphylococcus aureus, Salmonella* typhi, and *Escherichia coli.* This research proposes that the antibacterial properties of saliva extracted from Malaysian leeches (a non-medicinal leech species) are novel, but could vary from species to species. Furthermore, it has

been shown that Gram-negative and Gram-positive inhibitory activity may vary from species to species of leech. Other research shows that the peptides theromacin and theromyzin, from the coelomic liquid of leeches, have antibacterial activity against Gram-positive bacteria rather than Gram-negative bacteria[14]. This suggests that future studies should further evaluate the antibacterial activity in the saliva and gastrointestinal extracts from leeches of various origins, including those typically used for medicinal purposes.

Of particular interest is studies involving MDR *Staphylococcus aureus*. This bacterium is a Gram-positive organism that has major implications for zoonosis in humans. This bacterium is one of the leading nosocomial pathogens with global public health significance (CDC, 2019). It is known that 94% of these strains are resistant to penicillin via the production of penicillinase, an enzyme that breaks down the antimicrobial agent[15]. Other strains have been found to be resistant to different antimicrobials based on specific antimicrobial resistance genes. For example, the methicillin resistant *S. aureus* strains (MRSA) express the *mecA* gene which encodes for penicillin-binding protein. For this reason, MRSA shows a very virulent and a multi-drug resistant pattern. Emergence of this organism is considered a major public health problem[16-19]. Because this bacterium is frequently isolated in veterinary medicine patients, we chose to use this organism as our test species.

The objectives of this study were to determine if MDR *Staphylococcus aureus* can be transmitted from inoculated blood into a leech, how long it can persist within the leech, and how long it can persist within its environment. We also sought to determine if leeches could transmit the bacteria during refeeding. We hypothesized that leeches inoculated with *Staphylococcus aureus*

would not harbor or transmit the MDR bacterium after 6 months from inoculation. Additionally, we hypothesized that *Staphylococcus aureus* would not be isolated from the water of the aquaria.

Materials/Methods

Study Population:

From the power analysis, it was determined that 8 replicates for each time point would provide statistically significant results. Nine, 5-gallon aquariums were set up to house seven leeches each. Eight aquaria served as the inoculated study population and one aquarium served as the sterile control population. The aquariums were maintained at above husbandry standards for these animals since they were maintained in filtered aquaria. Leeches were purchased from a medical supplier (Leeches U.S.A. Ltd, Westbury, NY, USA) and were shipped overnight to our facility. The leeches were given a two-week acclimation period following arrival.

Staphylococcus aureus preparation:

Selection and preparation of *Staphylococcus aureus* culture for inoculation:

A *Staphylococcus aureus* isolate (2022-5#3SA) was selected owing to its niche as a commensal and known to reside in skin, soft tissues, and/or superficial infections. The isolate, 2022-5#3SA, is multidrug resistant with resistance determinants to more than three classes of antimicrobials. A single isolated pure colony of *Staphylococcus aureus* from a blood agar plate was inoculated into 10 ml of Mueller-Hinton broth and incubated overnight at 37°C. One hundred microliters of the overnight broth culture were inoculated into a fresh 10 ml Mueller-Hinton broth and incubated for 3-4 h at 37° C to reach an absorbance of 0.4 at 600 nm [~1 x 10^8 colony forming unit

(CFU)/ml]. Serial dilution and plate counting were done to enumerate the bacterial concentration present in the inoculum.

Meal preparation and leech inoculation:

Fresh whole blood from a canine was obtained from the hospital's blood donor program at the start of the project and at the 3-month time point. Discussion with leech keepers recommended using blood sausages as feeding vessels for the leeches. A commercially available sausage casing was purchased. Ten grams of blood were weighed out and nine individual blood sausages were prepared. Eight of these were then spiked with 1 x 10^8 CFU/ml culture of *S. aureus* and used for feeding leeches from the treatment groups. A ninth sausage was prepared in the same manner but not inoculated and this was fed to the control group.

Feeding:

For inoculation, all members of each group were placed in a dry container containing a blood sausage. The leeches were allowed to feed until satiated. Some leeches did not appear interested in the sausage and so the sausage contents were poured onto a sterile sample cup lid and placed in the dry container. The leeches fed more consistently using this method and so this is the feeding method used for the duration of the study.

Sampling:

At 1 day, 7 days, 1 month, and 2 months following inoculation, aerobic cultures, and susceptibilities were performed for one leech of each setup. Three samples were obtained per set up per time point: water from the aquarium, leech GI contents, and newly fed blood. Once a

leech was sampled, it was placed in a separate holding unit within their current aquarium to keep them separate from the unsampled leeches.

Water sampling:

A sterile culture tube was dipped into the aquarium to obtain a water sample from each aquaria. A sample from each aquarium was taken as a control the day prior to feeding.

Leech gastrointestinal (GI) tract sampling:

The leech's GI tract was sampled by gently massaging the animal from caudal to cranial to collect the contents of its crop from its mouth using sterile swabs.

Patient sample:

To determine if leeches could transmit bacteria to a new patient, each leech that was being sampled was allowed to feed from ~2 mL of non-inoculated blood that was placed in a sterile sample cup lid. The leech was allowed to feed until satiated. Once satiated, the remnant blood in the feeding container was swabbed and collected for culture using sterile swabs.

At the two-month time point, it was noted that two tanks that had a leech that tested negative for MDR *Staphylococcus aureus* a month earlier now had a positive leech. We elected to sample every leech from each aquaria at three and four months post-inoculation. Each leech was housed in a separate holding container labeled with their personal identification number and the number of the group that they belonged to from this point on.

Isolation and Identification of *Staphylococcus aureus*:

The leech gut contents, water, and the patient sample were enriched in Mueller-Hinton broth (Becton and Dickson, Sparks, MD) with 6.5% sodium chloride (Sigma-Aldrich, St. Louis, MO) at 37°C for 24 hours. The enriched suspension was then inoculated onto Mueller-Hinton and blood agar plates and incubated at 37°C for 24 hours (Amachawadi et al., 2015). Putative *Staphylococcus aureus* colonies were selected from the agar plates and subjected to catalase and coagulase tests. The species confirmation were done by PCR detection of *staph* (756 bp) and *nuc* (279 bp) genes. The confirmed *Staphylococcus aureus* isolates were stored in Protect beads (Cryo-Vac®, Round Rock, TX) at -80oC for further use.

Antibiotic susceptibility determinations:

Minimum inhibitory concentrations (MICs) were determined by broth-microdilution method as per CLSI guidelines (2023). The MIC for *S. aureus* isolates were determined by the broth micro-dilution method using the Sensititre® automated antimicrobial system (Trek Diagnostics Systems, Cleveland, OH). National Antimicrobial Resistance Monitoring System Gram-positive panel plates (CMV3AGPF) were used with the aid of the Sensititre® automated inoculation delivery system (Trek Diagnostics Systems, Cleveland, OH). Appropriate ATCC (American Type Culture Collection, Manassas, VA) quality control strains; *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213 were used as reference standards for susceptibility testing. The MIC for each isolate were recorded and classified as resistant, intermediate, or sensitive based on the Clinical Laboratory Standards Institute (CLSI, 2023) guidelines.

Statistical Analysis:

The data was analyzed for descriptive statistics using Excel (Microsoft Corp. 2018).

Results

Water samples:

There were a total of 36 samples, which included one sample from each aquaria prior to inoculation as a baseline. All aquaria tested negative for *Staphylococcus aureus* at baseline. All aquaria tested negative at all time points tested except for one of the treatment aquariums at seven days post-leech feeding. Sampling was discontinued after the two-month samples were obtained due to persistently negative results across all groups **(Figure 1 and table 1)**.

GI content and "blood meal" samples:

There were a total of 67 samples. Nine samples, one from each system, were obtained at one day, one week, and one-month post-inoculation. GI samples from one aquarium tested positive at day one, negative at 1 week, and then positive again at 1 month. This also occurred for the blood meal sample from a different aquarium. For the remainder of the blood meal samples, there were no positive samples until the 2-month time point. For these reasons, at the three month and four-month sample dates, all leeches from each system were sampled for a total of 67 leeches from the treatment group and two from the control group. The results from these samples are summarized in **(Figure 1 and table 2)**.

For the GI and blood meal groups, there were no positive samples starting at 2 and 3 months, respectively. There was one positive control for the blood meal and GI samples at one day and one-week post-inoculation, respectively **(Figure 1 and table 1)**.

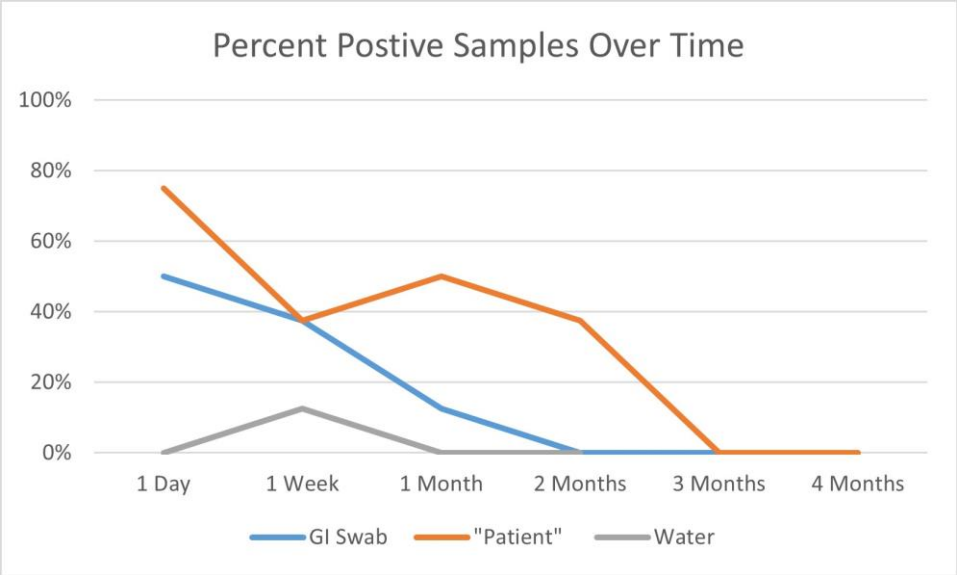
Sample	1 Day	1 Week	1 Month	2 Months	3 Months	4 Months
GI Swab	50%	*37.5%	12.5%	0%	0%	0%
Blood meal	*75%	37.5%	50%	37.5%	0%	0%
Water	0%	13%	0%	0%	-	-

Table 1 Percentage of positive samples by time point and sample type. Asterisks indicate a positive control sample at that time point**.**

Time Point	Samples	GI Positives	Blood meal Positives
1 Day	8	4	6
1 Week	8	3	3
1 Month	8	1	4
2 Months	8	0	3
3 Months	19	0	0
4 Months	16	0	0

Table 2 Total sample quantities for both the GI and blood meal sample groups and the total positive samples for the GI and blood meal sample groups by time point. Sample quantities increased at time points 3 and 4 months since the protocol was changed to sample every individual animal, three animals died between time points 3 and 4 months**.**

Figure 1 A simple line graph demonstrating the percentage of positive results by sample type over the time points. The orange asterisk and the blue asterisk represent a time point with a positive control sample.



Discussion

As discussed, the concern for reusing leeches is transmission of bacterial infections. Often times, the wounds for which leeches are used in veterinary medicine have an MDR bacterial infection. This could be detrimental to another patient if a leech were to become contaminated and then spread that infection. In our study, we found that when inoculated with MDR resistant *Staphylococcus aureus,* a positive culture was only obtained from one aquarium at one-week post-inoculation yielding an overall 2% positive rate. This makes it extremely unlikely that the bacteria are being transmitted to other leeches or humans via the water. It is important to note, however, that all leeches were inoculated and so it was not possible to detect leech to leech transmission via the water. It is also possible that this sample was positive due to a sampling error.

In this study, 12% of the GI content samples from the leeches obtained by stripping of the crop were positive for MDR *Staphylococcus aureus.* No GI samples tested positive after one-month post inoculation. However, 23% of the blood meal samples tested positive and it was not until the three-month time point that there were no more positive blood meal samples. These samples were important to obtain because while we could did not obtain a positive culture for the bacterium in the GI contents of the leech, it could be obtained after they were fed a second time indicating transmission to a new and clean blood meal. We speculate that this occurred because the leeches were fed the new blood meal first and then were stripped. In doing this, they may have deposited their GI bacteria into the meal (causing them to decrease their load) and then when they consumed more sterile food, they further diluted their load.

Throughout the sampling period, there were a total of two positive control samples, one GI sample and one blood meal sample. These results are believed to be due to contamination of samples, given that the control leeches were never fed prior to being obtained and did not feed on inoculated blood. Since there were a few other positive samples believed to be erroneous at these time points, these positive control samples could also have been due to a sampling error.

Initially, the protocol was to discontinue sampling when two negative time points were achieved for each group. It was, however, noted that two treatment groups tested negative and then subsequently tested positive. This was attributed to the fact that a different leech from each group was sampled each time between the time points one day through two months. We hypothesize that perhaps not every leech became inoculated at the inoculation feeding and thus an individual could have sampled negative while others in the same aquarium were positive. Future studies should consider weighing each individual leech before and after feeding to ensure that all leeches consumed enough for inoculation. Because this difference in results across time points was noted, it was elected to then begin to sample each individual leech from each system to obtain serial negative results for each animal **(table 2)**. Future studies could also consider maintaining all leeches in individual containers and testing each leech at each time point until there are two negative samples for that individual.

Unfortunately, several leeches escaped over the course of the study and so we had fewer treatment and control individuals at the three- and four-month time points. Additionally, while sampled leeches should be housed in a way that allows them to be identified from the unsampled individuals, it is not recommended to maintain them in smaller containers within their system.

We found that doing so meant that if a leech was to vomit post-feeding, the water within the container was not filtered as easily as the remainder of the system due to the smaller container having to be made secure with smaller holes to prevent the leeches from escaping. This led to the death of several of our sample leeches and decreased numbers at the three- and four-month time points.

Staphylococcus aureus was used for this study due to its zoonotic potential. While we have demonstrated that this particular species is no longer detectable in the blood meal after 3 months post-inoculation, there are many other harmful bacterial species that could be found on a small animal patient and potentially spread. Research has discovered anti-microbial compounds within leech saliva. One study found that the saliva of Malaysian leeches had inhibitory activity against only three bacteria (including *Staphylococcus aureus* and *E. coli*) of the two fungi and five bacterium (including *Pseudomonas aeruginosa,* another commonly seen infectious agent in small animal patient wounds) that were tested[13]. Further studies with different bacterial strains need to be performed to further assess the safety of reusing medicinal leeches in small animal patients or if additional prophylactic antibiotic measures should be taken. Additionally, it should be evaluated if a patient with an infected wound that is receiving Hirudu therapy experiences anti-microbial benefits from the saliva of healthy leeches.

One proposed way to make the cost of Hirudu therapy more accessible to pet owners and to have them available and at the ready for patients is for each veterinary hospital to maintain their own colony of leeches. This colony can be kept with as simple of a setup of two aquaria with filtration. They do not need lighting, water additives, or special care as long as they are kept in a

dark /cold room and allowed to feed every three to six months. In one aquaria, hungry leeches are housed. When these leeches are used, they are moved to a separate system for satiated leeches. This will ensure that 1. there are always leeches available at the hospital and 2.the hospital can set a more affordable fee to “rent out” the leeches per treatment. If there is a low number of cases requiring leeches, the hungry leeches can be fed portions of beef liver or other types of intermediate snacks.

With the information from this study, we can say that it is more than likely that the leeches can be safely reused after 3 months of fasting. Leeches consume 5-15 mL of blood per meal. Our study leeches were not allowed to feed to satiation since we needed them to be hungry again for sampling. It is possible that the reduced food intake used for our leeches could have affected the bacterial load they consumed as compared to clinical patients. However, in a clinical setting, if a leech is allowed to feed until satiation when being used for patient therapy, it will likely not want to eat for the next six months as noted by the author’s experience. This means that by the time the leech is ready to go to work again, the risk of MDR *Staphylococcus aureus* transmission is minimal.

Medicinal leeches inoculated with MDR *Staphylococcus aureus* sampled at various time points cease to yield positive culture and PCR results at 3 months post feeding. Veterinary hospitals can use this information to start a leech colony, with the size of the colony based on the demand of that hospital such that leeches can be reused only after every 3 months. This will allow for a more cost-effective way to provide this treatment option and thus enable greater opportunity to provide treatment to more patients.

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