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## Antibiotic susceptibility of body surface and gut micro flora of two aquatic leech species (*Hirudinaria manillensis* and *Hirudinaria javanica*) in Malaysia

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### PEER REVIEW

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#### Comments

This is a good study and the authors have studied the antibiotic susceptibility of body surface and gut micro flora of two aquatic leech species (*H. manillensis* and *H. javanica*). The article has scientific merit.

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### ABSTRACT

**Objective:** To elucidate the antibiotic susceptibility of body surface and gut associated microflora of two local aquatic leech species *Hirudinaria manillensis* and *Hirudinaria javanica*.

**Methods:** Four commercially available antibiotics (doxycycline, chloramphenicol, tetracycline and ciprofloxacin) were used in this study. A total of 13 isolated gut and two surface micro flora from *Hirudinaria manillensis* and two gut and two surface micro flora from *Hirudinaria javanica* were tested for their antibiotic susceptibility.

**Results:** Based on the susceptibility, it was observed that all the isolated bacteria were found to be susceptible to at least three of the antibiotics except *Microbacterium resistens*, *Serratia marcescens* and *Morganella morganii*. This study also found that the bacterial species *Bacillus fusiformis* has displayed resistance against tetracycline and *Tsukamurella inchonensis* against chloramphenicol.

**Conclusions:** Among all the antibiotics tested, ciprofloxacin was found to be the best bactericidal agent. The immersion of leeches in ciprofloxacin before the application to the patient may be beneficial to prevent invasive infection of the patient. Further study is needed to sterilize the live leech by immersion/oral mode of administration for the tested antibiotics.

### KEYWORDS

*Hirudinaria manillensis*, *Hirudinaria javanica*, Microflora, Antibiotic susceptibility

## 1. Introduction

The application and practice of using leeches in medicine is known as hirudotherapy. In medieval and early modern medicine leeches were used to remove blood from patients in an attempt to balance the biological humours[1]. Traditionally, leeches are widely used as a model animal in toxicological, physiological, neurobiological, biochemical,

histological and many other studies[2–10]. Recently, leeches has been used for several therapeutic applications in the medical field ranging from cardiology, gynecology, plastic and reconstructive surgeries. Leeches are widely used to treat venous congestion in microvascular replantation, free and conventional flap surgery and traumatology[1,4,11–12]. The leech is anatomically and behaviorally a very unique species of the phylum Annelida with a powerful clinging

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sucker to gorge the host's blood. The leech attaches and makes Y-type cuts into the skin until the superficial vessels are open and absorbs blood through its proboscis which is used to puncture the skin. During feeding, it secretes and releases a complex mixture of different biologically and pharmacologically active substances into the wound site. It then feeds for approximately 10–20 min. It can gorge itself on ten times as much blood as its body weight. This process drains venous blood from congested tissue<sup>[4,13,14]</sup>. This needs full contact with the wound sites and introduces gut flora by initial secretion into the wound site. Because of this close contact, the bacterial flora of the leech may contaminate the host. The rate of 20 % infectious complications in leech therapy has been reported due to the leech endosymbiotic bacteria. Several pathogenic bacterial flora have been isolated from the surface, mouth and gut of leech species. Endosymbiotic bacteria helps in digesting red blood cells, ingested in the gut lumen, because leeches lack the digestive enzymes required to break down blood cells<sup>[15–17]</sup>. Suppression of leech enteric bacteria and all other flora by antibiotic administration to the patient may be an effective strategy for preventing an invasive infection as well as bacterial colonization of devitalized tissue, since this could be a source of later infection. In order to prevent prophylactic antibiotic administration to the patient, decontamination of the leech bacterial flora might be also reasonable. Very limited attempts have been made on the eradication of gut and surface associated with bacteria from leech used for medical purposes. To our knowledge, no information is available for the body surface and gut micro flora and their antibiotic susceptibility of locally available leech species in Malaysia. Hence this study was conducted to assess the antibiotic susceptibility of body surface and gut microflora of two local aquatic leech species *Hirudinaria manillensis* (*H. manillensis*) and *Hirudinaria javanica* (*H. javanica*) against four commercially available antibiotics (doxycycline, chloramphenicol, tetracycline and ciprofloxacin). This study also aimed to identify the suitable antibiotic for sterilization of live leech used for medical interventions.

## 2. Materials and methods

### 2.1. Collection of leech and isolation of surface and gut micro flora

Live leeches, *H. manillensis* and *H. javanica* were obtained from a private leech farm, Pulau Pinang, Malaysia. The leech species were segregated based on the morphological

features and acclimatized for one week separately under laboratory conditions. Leeches were not fed during this period until sampling. The isolation of leech body surface and gut micro flora was followed based on the method of Nonomura *et al.*<sup>[15]</sup>. Thirteen gut and two surface micro flora from *H. manillensis* and two gut and two surface micro flora from *H. javanica* were isolated and identified by 16s rRNA molecular technique in the earlier study (unpublished). The lists of isolated micro flora from two leech species which were subjected to antibiotic susceptibility study is given in Table 1.

**Table 1**

Lists of leech surface and gut bacterial flora used for the antibiotic susceptibility study.

Leech species	Source of isolation	Bacterial species	Gram (+/-)
<i>H. manillensis</i>	surface	<i>Serratia marcescens</i>	-
<i>H. javanica</i>	surface	<i>Serratia marcescens</i>	-
<i>H. javanica</i>	surface	<i>Staphylococcus saprophyticus</i>	+
<i>H. manillensis</i>	surface	<i>Acinetobacter venetianus</i>	-
<i>H. manillensis</i>	gut	<i>Moraxella</i> sp.	-
<i>H. manillensis</i>	gut	<i>Bacillus licheniformis</i>	+
<i>H. manillensis</i>	gut	<i>Staphylococcus sciuri</i>	+
<i>H. manillensis</i>	gut	<i>Microbacterium resistens</i>	+
<i>H. manillensis</i>	gut	<i>Ochrobactrum intermedium</i>	-
<i>H. manillensis</i>	gut	<i>Stenotrophomonas maltophilia</i>	-
<i>H. manillensis</i>	gut	<i>Bacillus fusiformis</i>	+
<i>H. manillensis</i>	gut	<i>Bacillus</i> sp.	+
<i>H. manillensis</i>	gut	<i>Bacillus cereus</i>	+
<i>H. manillensis</i>	gut	<i>Tsukamurella inchonensis</i>	+
<i>H. manillensis</i>	gut	<i>Staphylococcus saprophyticus</i>	+
<i>H. manillensis</i>	gut	<i>Microbacterium hydrocarbonoxydans</i>	+
<i>H. manillensis</i>	gut	<i>Brachy bacterium</i> sp.	+
<i>H. javanica</i>	gut	<i>Morganella morganii</i>	-
<i>H. javanica</i>	gut	<i>Bacillus</i> sp.	+

### 2.2. Antibiotic susceptibility test

Four commercially available antibiotics [doxycycline (30 µg), chloramphenicol (30 µg), tetracycline (30 µg) and ciprofloxacin (5 µg)] were tested in this study. Antibiotic susceptibility test was done based on the disc diffusion method followed by the guidelines of National Committee for Clinical Laboratory Standards (NCCLS). All the antibiotic disc were obtained from BBL, Sensi-Disc; Becton Dickinson and Company, Sparks, MD. Pure culture colonies of bacterial isolates were inoculated in Mueller Hinton agar broth at 37 °C for 24 h. A sterile cotton swab dipped into the bacterial suspension was spread evenly on the surface of the Mueller Hinton agar plate. The inoculated plate was allowed to dry before placing the diffusion discs containing antibiotics. Antibiotic disc were placed on the surface of the agar plates. Precaution was taken to ensure that there

was uniform contact between the antibiotic disc and agar plate. The plates were then incubated at 37 °C for 24 h. The inhibition zones were measured inclusive of the diameter of the discs. Results were expressed as sensitive, S ( $\geq 21$  mm); intermediate, I (16–20 mm) and resistant, R ( $\leq 15$  mm), respectively according to Vlkova *et al*[18].

### 3. Results

A total of 13 gut and two surface micro flora from *Hirudinaria manillensis* and two gut and two surface micro flora from *H. javanica* were studied for the antibiotic susceptibility testing in this study. It has been found that the majority of the isolated bacteria from the leech species are either pathogenic or opportunistic pathogens which can cause infectious diseases in humans. The susceptibilities of 19 bacterial isolates to four antimicrobial agents are given in Table 2.

**Table 2**

Antibiotic susceptibility of bacterial isolates from two aquatic leech species.

Bacterial species	Antibiotics			
	Doxycycline	Chloramphenicol	Tetracycline	Ciprofloxacin
<i>S. marcescens</i>	S	S	I	S
<i>Moraxella sp.</i>	S	S	S	S
<i>Bacillus licheniformis</i>	S	S	S	S
<i>Staphylococcus sciuri</i>	S	S	S	I
<i>M. resistens</i>	S	S	R	I
<i>Ochrobactrum intermedium</i>	S	I	S	S
<i>Stenotrophomonas maltophilia</i>	S	S	S	S
<i>B. fusiformis</i>	S	S	R	S
<i>Bacillus sp.</i>	S	S	S	S
<i>Bacillus cereus</i>	S	S	S	S
<i>Tsukamurella inchonensis</i>	S	R	S	S
<i>Staphylococcus saprophyticus</i>	S	S	S	S
<i>Microbacterium hydrocarbonoxydans</i>	S	S	S	I
<i>Brachybacterium sp.</i>	S	S	S	S
<i>M. morgani</i>	R	R	R	I
<i>Bacillus sp.</i>	S	S	S	S
<i>S. marcescens</i>	R	S	R	S
<i>Staphylococcus saprophyticus</i>	S	I	S	S
<i>Acinetobacter venetianus</i>	S	S	S	S

S: sensitive; I: intermediate; R: resistant.

The results showed that the bacteria such as *Moraxella sp.*, *Bacillus licheniformis*, *Stenotrophomonas maltophilia*, *Bacillus sp.*, *Bacillus cereus*, *Staphylococcus saprophyticus*, *Brachybacterium sp.*, *Acinetobacter venetianus* were susceptible to all the four antibiotics tested in the study. The results also showed that, *Serratia marcescens* resistant against doxycycline and tetracycline; *Morganella morgani* (*M. morgani*) against doxycycline, chloramphenicol, and tetracycline; *Tsukamurella inchonensis* against chloramphenicol; *Bacillus fusiformis* (*B. fusiformis*) and *Microbacterium resistens* (*M. resistens*) against tetracycline

respectively. Based on the overall susceptibility, it was observed that all the tested bacteria were susceptible to at least three of the antibiotics except *M. resistens*, *Serratia marcescens* (*S. marcescens*) and *M. morgani*. Among all the antibiotics tested, ciprofloxacin was found to be the best bactericidal agent.

### 4. Discussion

Leech therapy has been known since ancient times, but it was performed needlessly for several purposes in folk medicine[19]. Medicinal leech, *Hirudo medicinalis*, has been used to treat patients for centuries. The application of leech therapy in plastic and reconstructive surgery has been immensely reported[14,20]. The sucking attribute of leech is used to treat venous compromise in cases of microvascular replantation, free and conventional flaps, and trauma[21–23]. Medicinal leeches appear to provide one method of reducing blood coagulation, relieving venous pressure from pooling blood, especially after plastic surgery and to stimulate blood circulation[24]. Medical leeches have been shown to harbor different opportunistic pathogens. It is generally reported that the symbiotic bacteria contribute enzymes such as proteases, lipases and amylases, which play a major role in digestion and also provide vitamins of the B-complex, which are sparse in blood; and secrete antimicrobial substances, which prevent the growth of other bacteria and accordingly retard putrefaction so that the leech can store blood for long periods. According to the literatures, *Aeromonas* spp. was the most common and dominant type of bacteria isolated from medicinal leech[25–28]. The most serious complication of leech therapy is infection. The leech's digestive system contains *Aeromonas hydrophila*, a Gram-negative bacillus that enables the breakdown of ingested blood. Although most infections involving leech therapy are caused by *Aeromonas hydrophila*, infections with *Serratia marcescens*, *Aeromonas sobria*, and *Vibrio fluvialis* have been reported[29–30]. Infections can arise 2 to 11 d after therapy begins and can result in abscesses and cellulitis, which can progress in some cases to sepsis[29]. Contradictorily, in our earlier study, *Aeromonas sp.* was not observed from the two aquatic leech species. To avoid infection with indigenous leech flora, caution should be observed in applying medical leech therapy to immunocompromised patients. In the past few decades, there have been a lot of reports in the scientific literature on the inappropriate use of antimicrobial agents and the spread of bacterial resistance among microorganisms causing various infections. In the present

study, the antibiotic susceptibility to each bacterium was tested against four antibiotics. The susceptibility and resistance profile of all isolates in this study have shown that ciprofloxacin possess the higher efficacy while doxycycline, chloramphenicol, tetracycline possess lower efficacy in the study.

The immersion of leeches in ciprofloxacin before the application to the patient may be beneficial to prevent invasive infection of the patient. No evidence of antimicrobial resistance and microflora with unusual or clinically significant drug resistance patterns were identified from these leech micro flora in Malaysia. Collectively, these findings will help optimize selection of appropriate antibiotic to eliminate the bacterial flora in medically important leech species. However, further study is needed to sterilize the live leech by immersion/oral mode of administration and followed by bacterial count for the tested antibiotics.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### Comments

#### Background

Hirudotherapy is a treatment using medicinal leeches. Leeches have been used to treat patients for centuries. Presently, leeches are used to assist in the treatment of abscesses, arthritis, glaucoma, myasthenia gravis, thrombosis and some venous disorders. Medical leeches may also be used in plastic surgery and in some blood circulatory problems. During feeding, leeches secrete a complex mixture of different biologically and pharmacologically active substances into the wound. Hirudin is the prominent constituent of leech saliva. During the treatment procedure, the leeches are required to be in full contact with the wound sites, and this results in infection in the patients with the leech's bacterial flora and

causes serious diseases to those patients who undergo leech therapy. Empirical prophylactic antibiotic administration to the patient has been practiced to eradicate and suppress the growth of enteric leech micro flora and this could be a source and cause of later infection. In order to prevent prophylactic antibiotic administration to the patient, decontamination of the leech bacterial flora is imperative. In this study, authors have elucidated the antibiotic susceptibility of body surface and gut associated microflora of two local aquatic leech species (*H. manillensis* and *H. javanica*).

#### Research frontiers

This study assessed the antibiotic susceptibility of 19 body surface and gut microflora of two local aquatic leech species (*H. manillensis* and *H. javanica*) against four commercially available antibiotics (doxycycline, chloramphenicol, tetracycline and ciprofloxacin) and also suggested suitable antibiotic for sterilization of live leech used for medical.

#### Related reports

Very limited studies have been conducted on the isolation and identification of microflora from medicinal leeches and antibiotic susceptibility studies. According to the literatures, *Aeromonas* spp. was the most common and dominant type of bacteria isolated from medicinal leech. The most serious complication of leech therapy is infection.

#### Innovations and breakthroughs

Very limited attempts have been made on the eradication of gut and surface associated bacteria from leech used for medical purposes. To my knowledge, no information is available for the body surface and gut micro flora and their antibiotic susceptibility of leech. This is the first kind of study done the antibiotic susceptibility of bacteria isolated from aquatic leech species.

#### Applications

The authors have suggested based on the susceptibility and resistance profile of all isolates ciprofloxacin possess the higher efficacy while doxycycline, chloramphenicol, tetracycline possess lower efficacy in the study. The immersion of leeches in ciprofloxacin before the application to the patient may be beneficial to prevent invasive infection in patients undergoes leech therapy.

#### Peer review

This is a good study and the authors have studied the antibiotic susceptibility of body surface and gut micro flora of two aquatic leech species (*H. manillensis* and *H.*

*javanica*). The article has scientific merit.

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