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Determine the Microbial Flora of Leeches in North of Iran and Designing Antimicrobial Solution to Sterile the Leeches

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Abstract
Medicinal leeches have an important and expanding role in plastic surgery and in the management of chronic venous insufficiency, but infection can complicate their use. The aim of this study were designed a new protocol to sterilize or at least to reduce the number of colonies of the bacteria on medical leeches. Bacterial flora of the surface, mouth, and gut of medical leeches was studied and bacterial identification was conducted based on morphological and biochemical tests. The susceptibilities of these isolates to 11 antimicrobial agents were investigated. Next, the leeches were immersed in susceptible antibiotic and the final results were studied. Biochemical and morphological tests indicated that *Aeromonas*spp.is the dominant culturablesymbiont in leeches collected from north of Iran. Isolates were highly susceptible to tetracycline, gentamicin, cotrimoxazole and ceftriaxone. According to results, tetracycline was superior to other antibiotic to cleansing of bacteria from leeches. *Aeromonas*spp.were eliminated completely from all leeches and statistically significant cleaning was obtained with the use of tetracycline and gentamicin solution after 4 days for 5.5 h daily.

Keywords: Microbial flora, Medical leeches, Sterilization, Antibiotic.

INTRODUCTION

Leech therapy has been known since ancient times, but it was performed needlessly for several purposes in folk medicine [1]. Its scientific use in plastic and reconstructive surgery has been well described [2, 3]. The sucking attribute of leeches is used to treat venous compromise in cases of microvascular replantation, free and conventional flaps, and trauma [4, 5]. However, their application is often complicated by infection resulting in a drop of the salvage rates below 30% [6]. The infection complication ranges from minor wound infection to flap necrosis, extensive myonecrosis, and septic shock unless empirical antibiotic prophylaxis is administrated [6, 7]. Furthermore, the necessity of prophylactic treatment with antibiotics during leech therapy was evaluated [8, 9]. In order to prevent prophylactic antibiotic administration to the patient, the immersion of leeches before application to the patient with the suitable antibiotic agents might be reasonable [8, 9].Accordion to data obtained from reported research, it seems that *A. hydrophila*, as the main component of gut flora, is the sole infective agent associated with leech therapy, the possibility of local and systemic infections caused by body surface bacterial flora of leeches cannot be dismissed [10-12]. So far, no study has been reported with regard to the body surface bacterial flora of medical leeches, while gut flora surveys have been conducted by several researchers [13-15]. On the other hand, the presence of different bacterial species in leeches collected from different areas is important in this antibiotic use, because their antibiotic susceptibility may differ according to the leech species [13]. In order to reduce the risk of infection, attempts have been made to remove or decrease the number of bacteria 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 completely the bacterial flora of the leech [1, 9, 16]. The present study attempted to isolate and identify bacteria from the body surface of medical leeches in north region of Iran and to examine their antimicrobial susceptibilities to determine which antimicrobial agents are active against the leech flora. The second goal of this study was to sterilize or at least to reduce the number of colonies of the bacteria on leeches by feeding them artificially with a blood-free antibiotic solution, with the ultimate aim of eliminating the need for chemoprophylaxis in the patients.

MATERIALS AND METHODS

Material

Antibiotics including ciprofloxacin, gentamicin, cotrimoxazole, amoxicillin, ceftriaxone, rifampin, tetracycline, clindamycin, nalidixic acid, erythromycin, nitrofurantoinwere obtained from Sigma-Aldrich, USA. Mueller-Hinton Agar and Mueller-Hinton Broth media were purchased from Merck, Germany. BALB/c mice (6-7 weeks old and 20-25 g weight) were purchased from Razi Vaccine and Serum Research Institute, Karaj, Iran.

Collection of leeches

175 wild medical leeches, which were collected from north region in Iran, were used in this study. After they were collected, the leeches were kept separately in sterile water without chlorine, which was changed daily [13]. After 10 days, the secondary pollutant of leeches was removed [13]. The animal utilization committeeapproved all procedures used in this study.

Isolation of the bacterial flora of leeches

Body surface bacteria of leeches were sampled by sterile swabs. Then the cotton swab was cut with sterile scissors and the pieces of cotton were placed into Mueller-Hinton broth (MHB) solution and incubated for 24 h at 37°C. Next, bacteriological loop was inserted into the MHB solution and one drop was collected and streaked onto Mueller-Hinton agar (MHA) [17, 18].All plates incubated for 24 h at 37°C under C02 5%.

Then, leeches were killed to obtain mouth and gut cultures [18, 19]. The leeches were anesthetized with ether in a double-floor container under sterile conditions [1, 17-19]. They were dried with sterile gauze and a full length longitudinal incision was made on the ventral surface[1, 17-19].The mouth parts and gut were identified, separated, opened, and cultured according to above mentioned [17-19].

Characterization of isolated bacteria by biochemical and physiological tests

All the isolated bacteria were identified with biochemical and physiological test include:B.A, VP, MR, SIM, TSI,Citrate utilization, N.A, TCBS%6and1%, EMB, OF [8, 9, 15].

Antibiotic susceptibility of the flora of leeches

A standard disk diffusion test was performed to determine antibiotic sensitivity. Ciprofloxacin, gentamicin, cotrimoxazole, amoxicillin, ceftriaxone, rifampin, tetracycline, clindamycin, nalidixic acid, erythromycin, nitrofurantoin was tested in this study[1, 8, 9, 13, 15, 16].

Sterilize of leeches by immersion in antibiotic solution and design new protocol to complete sterilize of leeches

The antibiotics were selected according to sensitivity results. These antibiotics include tetracycline, gentamicin, cotrimoxazole and ceftriaxone. The agents were diluted in sterile spring water into various concentrations according to their minimum inhibitory concentration (MIC) values[1, 13, 15, 16]. In the first step, immersion of leeches was performed for different concentrations of agents. A sterile glassy tube was used for the separate immersion of every leech. The tubes were filled with the solutions (Fig. 1). After an incubation period of 3 days, the leeches were anesthetized with ether in a double-floor container under sterile conditions. They were dried with sterile gauze.Samples obtained from areas of the surface body, mouth and gut were cultured in broth and then in Mueller-Hinton agar according to above mentioned surveying processes[1, 13, 15, 16]. Four leeches were separated for blood-sucking tests on mice in each group.

Leeches divided into twenty groups and each group were immersed in a solution of antibiotic daily at different time intervals (90, 150, 210, 270 and 330min) for 4 days. For example, 5 leeches immersed in gentamicin, 5 leeches immersed in tetracycline and similarly 10 leeches immersed in ceftriaxone and cotrimoxazolesolutionand after 90 min leeches werewashed with distilled water and dried with gauze. Samples obtained from areas of the surface body, mouth and gut was cultured in broth and then in Mueller-Hinton agar according to above mentioned surveying processes[1, 13, 15, 16]. Then, leaches immersed in sterile distilled water andwere allowed to rest until the next day. On the fifth and sixth day, antibiotic treatment wasn't performed on leaches.Again, samples obtained from areas of the surface body, mouth and gut was cultured to determine if leeches remain sterile after one and two days after last treatment[1, 13, 15, 16].Fisher's exact test was used to compare treated with control leeches. Twenty saved leeches were used for blood-sucking tests on mice to test the leeches' efficacy after incubation (Fig. 2). Three sedated mice anesthetized with ketamine were fixed on a tray, their abdominal hair was shaved and leeches were then applied on the blood oozing from small scratches made by a yellow syringe needle.

Figure 1. Leeches in tubes filled with antibiotic solutions

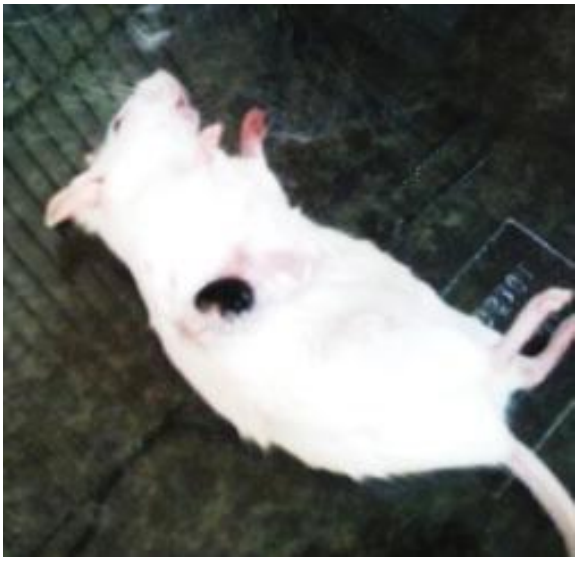

Figure 2. Leeches were used for blood-sucking tests on mice to test the leeches efficacy after incubation.

RESULTS

Characterization of isolated bacteria

According to the results of biochemical and physiological tests (Table 1),*Aeromonas*spp.,*bacillus* spp.,*Staphylococcus* spp. *and Pseudomonas* spp. and other glucosenon- fermentative gram-negative rods (NF-GNR) were isolated from the body surfaces of all leeches tested. The most common types of cultured bacteria were *Aeromonas spp*.

Table 1. Biochemical and physiological characteristics of isolates

Test	Isolate No.								
	Type strain	1	2	3	4	5	6	7	8
β-galactosidase	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	+	+	-	+	-	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-
Citrate utilization	+	-	-	-	-	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-
Tryptophan deaminase	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+
Acetoin production	+	+	-	+	+	+	+	+	+
Gelatinase production	+	+	+	+	+	+	+	+	+
Utilization of glucose	+	+	+	+	+	+	+	+	+
mannitol	+	+	+	+	+	+	+	+	+
inositol	-	-	-	-	-	-	-	-	-
sorbitol	-	+	-	-	-	-	-	-	-
rhamnose	-	-	-	-	+	+	-	-	+
sucrose	-	+	+	+	+	+	+	+	+
melibiose	-	-	-	-	-	-	-	-	-
amygdalin	+	-	+	+	-	+	+	+	+
arabinose	+	+	+	+	+	+	-	+	+
oxidase	+	+	+	+	+	+	+	+	+
NO_2 production	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Growth on MacConkey	+	+	+	+	+	+	+	+	+

Antibiotic susceptibility of the flora of leeches

The susceptibilities of 5 bacterial isolates bacteria to 11 antimicrobial agents are given in Table 2. According to data obtained from MIC test, ceftriaxone, gentamicin, tetracycline and cotrimoxazole have most effect to decrease of growth bacteria isolated of leeches. The appropriate concentrations for most effective antibiotics were determined. This determination was based on maximum decrease of colony count (Table 2). They were 128 µg/ml for ceftriaxone, 1 µg /ml for gentamicin, 1 ≤ µg /ml for tetracycline and 128µg/ml for cotrimoxazole.

Antibiotics and concentrations (µg/ml)		Colony count		
		Surface	Mouth	Gut
CIP	10	Intense[a]	105	60
	20	–	25	100
	100	100	–	–
	500	–	–	–
TMP/SMX	100	–	5	–
	200	Intense	Intense	Intense
	500	–	–	25
	1000	Intense	Intense	Intense
CRO	500	125	–	4
	1000	–	–	–
	10000	–	–	–
C	50	100	10	Intense
	100	Intense	2	Intense
	500	2	–	50
	1000	20	–	–
	10000	Intense	5	3

[a] >200 colony/plate

Sterilize of leeches by immersion in antibiotic solutionand **design new protocol to complete sterilize of leeches**

After immersion of leaches into MIC concentrations of antibiotics, the results of bacterial growth showed the complete sterilization of leaches. However, all leaches were sterilized, but all leeches lost sucking ability after immersion in MIC concentrations of antibiotic agents for 3 days. Therefore, this protocol is unsuccessful. In the second step for optimum eradication of bacteria from leeches, incubation time of leaches into antibiotic solutions decreased. Leaches were divided to four groups and immersedto antibiotic solution for short period (30, 45, 60, 75 min). After given time, samples obtained from areas of the surface body, mouth and gut was cultured in broth and then in Mueller Hinton agar according to above mentioned surveying processes. Again, this protocol was failed because bacteria were growth on all plate, so leaches was not sterile.

In this experimental, it was decided to immerse the leeches in antibiotic solutions daily for short time during 4 days. According to figure 4,in the gentamicin and tetracycline incubation group, most elimination of bacteria was obtained after 90 min incubation, but cotrimoxazole has least effective antibiotic to eliminate the bacteria from leech. It is interesting that tetracycline and gentamicin have again the greatest effect on bacterial eradication of leaches after 5.5h incubation, and cotrimoxazole was least antibiotic effect. These results repeated for 24, 72 and 92 h too.On the fifth day, antibiotic treatment was discontinued. Due to decrease of antibiotic dose, the bacterial elimination from leaches decreased in ceftriaxone and cotrimoxazole groups. However,thetetracycline and gentamicin incubation group exhibited significant antibiotic effects after 24 h from the last antibiotic treatment.Tetracycline reduced the number of leech associated aeromonads to undetectable levels for extended periods (2 day after last treatment). So,according to statistical analysis, tetracycline was superior to gentamicin according to the cleaning of bacteria from leeches (P<0.005). All leeches saved for sucking tests, sucked blood successfully.

DISCUSSION

In this study, the susceptibilities of 117 bacterial isolates to 11 antibiotic agents were studied. Gentamicin, cotrimoxazole, ceftriaxone andtetracyclinewere good active against isolates from medical leeches.Our research results showed the effectiveness of gentamicin and tetracycline has been very successful, so after 3 days from last treatment, leeches were completely free of microbes.In contrast, ceftriaxone and cotrimoxazole have weak effect and they are not reliable for disinfectingof leech.The results showed that leeches still kept their disinfection 24 h after of last treatment with tetracycline and gentamicin.It donate that leeches can be used for leech therapy for patients even one days after antibiotic treatment, the.On the fifth day, the return of normal bacterial flora of leeches can be seen. However, the tetracycline incubation grouphas been maintained itself disinfection after 48 h of last treatment, so tetracyclineisstill reliable.

CONCLUSION

In conclusion, the results of the present study suggest that the strategy of feeding leeches artificially with antibiotics reduces the number of aeromonas to undetectable levels for extended periods especially for tetracycline treatment.

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