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Antibiotic sensitivity and RAPD-PCR studies on cultivable gut bacteria from Indian Medicinal Leech—*Hirudinaria granulosa*

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Abstract

Background: *Hirudo granulosa*—an Indian cattle leech, is frequently used for the treatment of psoriasis and eczema. During treatment which followed by leech biting, it transfers some amount of gut microbes along with saliva. This may result in bacterial infection at the treatment site. Antibiotics used as post-surgical hirudotherapy is the reason for drug resistance. Drug resistance is the result of the change in the genetic makeup of bacteria. Therefore, it is necessary to study antibiotic sensitivity of gut bacteria and characterized them genetically.

Results: Fourteen bacterial isolates were obtained from unused leech in which five were Gram-negative and the other nine were Gram-positive. Similarly from the used leech, thirteen were isolated in which five were Gram-negative and the other eight were Gram-positive. Biochemical analysis reveals that isolates from unused leech saliva belong to *Pseudomonas*, *Micrococcus*, *Streptococcus*, and *Vibrio* species, while the used leech salivary bacteria were the member of genus *Pseudomonas*, *Comamonas*, *Escherichia*, *Citrobacter*, *Aeromonas*, *Providencia*, *Enterobacter*, and *Yersinia*. Antibiotic sensitivity tests for isolates indicated that chloramphenicol (30 µg) and norfloxacin (10 µg) were effective for unused leech isolates while sparfloxacin (5 µg) and Cefaclor (30 µg) were effective against used leech salivary isolates. Random primer (OPL-14)-based random amplified polymorphic DNA (RAPD) fingerprint showed twenty amplified regions among all tested bacteria. Most of the bacteria contain the tested sequence except U2, T2, T9A, and T10 which showed no amplification indicated the absence of primers sequence.

Conclusion: Chloramphenicol, norfloxacin, sparfloxacin, and Cefaclor antibiotics alone or in combination were possibly used to treat post-therapy infections. Bacteria from treated and untreated leeches were clustered at nearby branch in neighbor end-joining phylogenetic tree, which indicates the similar (but not exact) genetic makeup. Therefore, it can be concluded that these antibiotics were possibly used against most of them.

Keywords: Antibiotics, Bacterial Infection, Indian Medicinal Leech, Multidrug resistance Phylogenetic tree, Random amplified polymorphic DNA

Background

The use of living organisms for skin treatment is one of the many techniques since a long time. It includes the skin wound healing practice using leech (Pereira & Bártolo, 2016). Food and Drug Administration (FDA) approved the use of leech for clinical purposes such as compromised vasculature, since 2004 (Whitaker, Maltz, Siddall, & Graf, 2014). Pain reduction in osteoarthritis and other joint ailments are more common in leech

therapy. Other medicinal treatment includes cardiovascular diseases, hemorrhoids, and reconstructive surgeries (Das, 2014). Leech is the segmented annelids and known for its sanguivorous nature (Das, 2014). It belongs to phylum *Annelids*; class *Clitellata*, subclass *Hirudinea* (Lamarck, 1818). In general, treatment includes leech biting followed by blood-sucking activity of leech which is painless due to salivary bioactive compound hirustasin (Das, 2014; Sig, Guney, Uskudar Guclu, & Ozmen, 2017). The saliva of medicinal leech contains many bioactive compounds include anticoagulant, regulating factors for thrombin and platelet inhibition, which prevent

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the blood from coagulation during feeding (Sig et al., 2017).

Digestion of blood is completed through the action of many digestive enzymes and complex microbial community present in the gut of animal which influences the metabolism of an animal. These organisms absorb nutrients from the lumen and survive in the digestive tract condition. Moreover, they create a barrier for other microbes and prevent their proliferation on the surface (Graf, 2016). Leech biting transfers saliva along with some amount of gut microbes (Giltner, Bobenchik, Uslan, et al., 2013). Complications like bacterial infections in leech therapy were reported in 2% to 36% cases (Marden, McClure, Beka, & Graf, 2016). Such infectious bacteria considered to have come from the gut of the leech. Gut microbes reside in the mucosal membrane of the digestive tract of the leech and they transmit through saliva (Whitaker et al., 2014). Gut microbial community explains immunity of animal (here leech) and also provide information against the pathogen colonization. Moreover, gut microbes play an important role in symbiosis with host animal and also help in the digestion of nutrients (Worthen, Gode, & Graf, 2006).

Antimicrobial resistance is a serious problem nowadays for the humans, which occurred due to the extensive use of antimicrobial agents for human welfare (Beka, Fullmer, Colston, et al., 2018). Medicinal leech is treated with a prophylactic antibiotic before application for medicinal purposes. These antibiotics reduced the chances of several bacterial infections (Marden et al., 2016). It triggers the chance of multidrug resistance (MDR) and also the transmission of such MDR bacteria among the hosts (Giltner et al., 2013). Therefore, antibiotics assay and genetic characterization of gut microbes become important to conserve such multitasking therapeutic invertebrates. In the present study, an attempt was made to isolate cultivable bacteria from leech saliva and their antibiotic assay in order to propose the broad-spectrum antibiotic for promising prophylaxis. Molecular characterization by RAPD-PCR was also carried out to evaluate random sequence-based evolutionary relationship analysis.

Materials and methods

Organism of the study

Indian medicinal leech *Hirudinaria granulosa* were collected from Ahmadabad (Surekh Education, M85/1009, Panchvati apartment, Sola Road, Naranpura, opp. Rameshwar app., Near Indian oil petrol pump, between Bhuyandev to Parasnagar, Ahmadabad). Leech was divided into two categories. One, before any skin treatment named as 'unused' (Coded as UTL or U) while the second was after skin disease

treatment named as 'used' (coded as TL or T) for the present study.

Culture maintenance

Leeches were stored and maintained in a transparent glass container. The container should be sufficiently big enough and filled up pure dechlorinated tap water up to 3/4th of its capacity, allowing enough space for the movement of leech. The water was changed after two successive days. The lid of the container must contain fine holes for air exchange.

Preparation of leech for experiment

The purification of leeches was carried out by putting the leeches in dechlorinated water containing a pinch of turmeric for 60 min to increase the appetite and blood-sucking power.

Saliva extraction from used and unused leech

Both used and unused leeches were starved for 3 weeks before the extraction of saliva. Starved leeches were fed with phagostimulatory solution [0.01 M Arginine in 0.15 M sodium chloride kept at 37 °C] through sterile glass funnel wrapped with ultra-violet (UV) sterilized parafilm sheets (Bemis, Neenah WI 54956). Leeches were introduced near the parafilm sheet and allowed attaching itself. After the leech becomes fully satiated, it detached itself from the parafilm. Leeches were kept in sterile ice container for 15–20 min, during the incubation time, leech became completely paralyzed and regurgitated all the solution it had already sucked. Then leeches were squeezed from the posterior end toward the anterior end in order to collect the remaining sucked solution under sterile condition. Collected saliva was centrifuged at 10,000 rpm for 15 min. Sediment was used for bacterial isolation. Leeches regained their activity on immersing them in warm dechlorinated water (37 °C) for 15–30 min.

Isolation of gut/saliva microflora

The bacterial suspension was serially diluted in sterile distilled water up to $10^{(-10)}$ dilution and 0.1 ml of inoculum was spread on the nutrient agar plate. Inoculated media plates were incubated at 37 °C for 24 h. Colonies with different morphological appearances were picked up after incubation.

Gram staining and biochemical test for bacterial isolates

All bacterial isolates collected from the saliva of unused and used leech were categorized by Gram staining (Hucker's modification). All the Gram-positive bacteria were inoculated with commercially available biochemical identification API kit (HiMedia product code: KB013).

Table 1 Colonial character of bacterial isolates from unused leech saliva

Characters	Size	Shape	Edge	Elevation	Surface	Opacity	Pigment
UTL 01	Intermediate	Round	Entire	Flat	Smooth & punctate	Oleaginous	Orangish yellow
UTL 02	Large	Irregular	Lobate	Effused	Smooth & echinate	Cretaceous	No pigment
UTL 03	Intermediate	Mycelioid	Filamentous	Flat	Smooth & squamous	Oleaginous	Creamish yellow
UTL 04	Intermediate	Elliptical	Undulated	Effused	Smooth & squamous	Oleaginous	Blue-green
UTL 05	Large	Elliptical	Articulate	Flat	Smooth & squamous	Resinous	Brown
UTL 06	Intermediate	Elliptical	Fimbriate	Effused	Smooth & papillate	Transparent	No pigment
UTL 07	Large	Round	Articulate	Umbonate	Smooth & papillate	Vitreous	No pigment
UTL 08	Large	Round	Articulate	Convex	Smooth & bullet	Oleaginous	Brick red
UTL 09	Intermediate	Round	Articulate	Effused	Smooth & squamous	Oleaginous	Orange
UTL 10	Intermediate	Elliptical	Repand	Flat	Smooth & squamous	Resinous	Brown
UTL 11	Intermediate	Round	Entire	Effused	Smooth & punctate	Cretaceous	No pigment
UTL 12	Large	Round	Entire	Flat	Smooth & punctate	Oleaginous	No pigment
UTL 13	Small	Punctiform	Entire	Umbonate	Smooth & papillate	Cretaceous	No pigment
UTL 14	Intermediate	Round	Entire	Flat	Smooth & punctate	Transparent	No pigment

Similarly, Gram-negative bacteria were inoculated on API kit (HiMedia product code: KB002) for rapid identification of the organism. Both kits were incubated at 37 °C for 48 h.

Antibiotic assays

Antibiotic assays of bacterial isolates were carried out using universal antibiotic combo disc (IC001* procured from HiMedia, Mumbai). For assays, each bacterial culture was grown in nutrient broth until OD₆₀₀ reaches up to 1.00. Next day, bacterial culture was spread on nutrient agar medium prepared in 20-cm Petri plate and the antibiotic disc was placed on the surface of the medium after inoculation. Plates were incubated at 37 °C overnight and next day, plates were checked for zone of inhibition.

[*Antibiotics on disc: norfloxacin (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), cefoperazone (75 µg), ceftazidime (30 µg), roxithromycin (30 µg), clarithromycin (15 µg), cotrimoxazole (25 µg), netillin (30 µg), cefaclor (30 µg), cefadroxil (30 µg), azithromycin (15 µg), Amphotericin/Cloxacillin (10 µg), Penicillin-G (10 units), amikacin (30 µg), sparfloxacin (5 µg), Ampicillin/Sulbactam (10/10 µg)]

DNA isolation and quantification

Bacterial genomic DNA was extracted by the modified Green and Sambrook method for bacterial genomic DNA extraction (Green & Sambrook, 2012). Briefly, bacterial (both Gram-negative and Gram-positive) cells were harvested by centrifugation at 8000 rpm for 10 min. Pellet obtained was re-suspended in 300 µl of SET buffer

Table 2 Colonial morphology of bacterial isolates from used leech saliva

Characters	Size	Shape	Edge	Elevation	Surface	Opacity	Pigment
TL 01	Intermediate	Round	Erose	Effused	Smooth & punctate	Oleaginous	Blue-green
TL 02	Large	Fusiform	Auriculate	Flat	Smooth & punctate	Oleaginous	Blue-green
TL 03	Intermediate	Punctiform	Entire	Effused	Smooth & Punctate	Cretaceous	No pigment
TL 04	Intermediate	Elliptical	Lobate	Effused	Smooth & squamous	Oleaginous	Blue-green
TL 05	Intermediate	Round	Entire	Umbonate	Smooth & papillate	Cretaceous	No pigment
TL 06	Large	Elliptical	Erose	Umbilicate	Smooth & punctate	Resinous	Brown
TL 07	Large	Fusiform	Auriculate	Raised	Smooth & alveolate	Oleaginous	Blue-green
TL 08	Intermediate	Round	Repand	Raised	Smooth & squamous	Resinous	Brown
TL 09	Large	Round	Auriculate	Convex	Smooth & papillate	Cretaceous	Orange-red
TL 10	Intermediate	Irregular	Auriculate	Raised	Smooth & punctate	Cretaceous	No pigment
TL 11	Small	Punctiform	Entire	Umbonate	Smooth & papillate	Cretaceous	No pigment
TL 12	Large	Irregular	Auriculate	Umbonate	Smooth & contoured	Sebaceous	No pigment
TL 13	Intermediate	Elliptical	Fimbriate	Raised	Smooth & Echinata	Resinous	Brownish white

Table 3 Morphological characterization of isolates from unused leech under 100x magnification

Sr. no.	Code	Gram reaction	Morphological characters (100x)	Motility
1	UTL01	Gram-negative	Bacilli in clusters	Non-motile
2	UTL02	Gram-positive	Big rod in short chain	Non-motile
3	UTL03	Gram-positive	Bacilli in chains	Motile
4	UTL04	Gram-positive	Rode shape in cluster	Motile
5	UTL05	Gram-negative	Rode shape in cluster	Motile
6	UTL06	Gram-positive	Cocci in clusters	Brownian
7	UTL07	Gram-positive	Rod shape in clusters	Motile
8	UTL08	Gram-positive	Long rod in cluster	Motile
9	UTL09	Gram-negative	Rod scattered	Motile
10	UTL10	Gram-negative	Rods in clusters	Motile
11	UTL11	Gram-positive	Cocci, scattered	Non-motile
12	UTL12	Gram-positive	Big rod, single & scattered	Non-motile
13	UTL13	Gram-negative	Rods in clusters	Motile
14	UTL14	Gram-positive	Bacilli in clusters	Motile

(made up of 2 ml 1 M Tris, 1 ml 5 M NaCl, and 0.2 ml 0.5 M ethylenediaminetetraacetic acid (EDTA) in the final volume of 100 ml in double-distilled water) followed by incubation at 37 °C for an hour. In order to break open the cells and remove RNA from mixture, 10 µl of lysozyme and RNase each were added to it respectively. Mixture was incubated at 37 °C for 90 min and later, 23 µl sodium dodecyl sulfate (SDS) and 10 µl Proteinase-K was added and kept at 55 °C for 2-h incubation. Later, 85 µl of 5 M NaCl was added and after 5 min 85 µl Chloroform was added followed by mixing by inverting the tubes. Mixture was kept at room temperature for 45 min and then centrifuged at 10,000 rpm for 10 min to separate phases. Aqueous phase-upper phase was transferred to a fresh 1.5-ml Eppendorf

tube and added equal volume of 80% ethanol to precipitate DNA. Allow tubes to air dry under laminar airflow. DNA was resuspended in 40 µl tris-EDTA (TE) Buffer. DNA concentrations obtained from each bacterial sample were quantified (in ng/µl) spectrophotometrically using UV-visible spectrophotometer (Shimadzu UV VIS 1800-A114548) by checking absorption at 260 nm and 280 nm followed by calculating ratio (OD_{260}/OD_{280}).

RAPD-PCR

The purity of extracted DNA was checked by separating on 1% agarose gel. One µl of (15 ng purified) genomic DNA and 2.7 µl Master Mix (EmeraldAmp GT PCR Master mix-2X Premix, Takara, Tokyo) were taken in

Table 4 Morphological characterization of isolates from used leech under 100x magnification

Sr. no.	Code	Gram reaction	Characters	Motility
1	TL01	Gram-positive	Rod in cluster	Motile
2	TL02	Gram-negative	Bacilli in Single & Cluster	Non-motile
3	TL03	Gram-negative	Bacilli in Cluster & Duplicate	Motile
4	TL04	Gram-Positive	Rod in clusters and short chains	Motile
5	TL05	Gram-negative	Bacilli, scattered	Motile
6	TL06	Gram-positive	Rode, scattered	Motile
7	TL07	Gram-positive	Rod, single	Motile
8	TL08	Gram-negative	Bacilli in cluster	Motile
9	TL09	Gram-positive	Bacilli, scattered	Motile
10	TL10	Gram-positive	Cocci in clusters	Non-motile
11	TL11	Gram-POSITIVE	Bacilli in clusters	Motile
12	TL12	Gram-positive	Big rod in chains	Motile
13	TL13	Gram-negative	Rode, cluster	Motile

Table 5 Biochemical characterizations of unused leech Gram-positive isolates

Sr.no.	Isolates → Name of test ↓	UTL02	UTL03	UTL04	UTL06	UTL07	UTL08	UTL11	UTL12	UTL14
1	Malanate production	-Ve	+ve	+ve	-Ve	-Ve	-Ve	-Ve	-Ve	+ve
2	V.P. test	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+ve	-Ve
3	Citrate utilization	-Ve	+ve	+ve	+ve	+ve	-Ve	-Ve	+ve	+ve
4	ONPG test	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve
5	Nitrate reduction	-Ve	-Ve	+Ve	-Ve	-Ve	-Ve	-Ve	+ve	+ve
6	Catalase test	-Ve	+ve	+ve	-Ve	+ve	-Ve	-Ve	+ve	+ve
7	Arginine test	-Ve	+ve	+ve	+ve	+ve	-Ve	-Ve	-Ve	+ve
8	Sucrose utilization	-Ve	-Ve	-Ve	+ve	-Ve	-Ve	-Ve	+ve	-Ve
9	Mannitol utilization	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve
10	Glucose utilization	+ve	+ve	-Ve	+ve	-Ve	-Ve	-Ve	+ve	+ve
11	Arabenose utilization	-Ve	+ve	+ve	-Ve	-Ve	+ve	-Ve	-Ve	+ve
12	Trehalose utilization	+ve	-Ve	-Ve	+ve	-Ve	-Ve	-Ve	+ve	-Ve
13	MR test	-Ve	+ve	-Ve	-Ve	+ve	+ve	-Ve	-Ve	+ve
14	Oxidase production	+ve	+ve	+ve	+ve	-Ve	-Ve	+ve	+ve	+ve
15	H ₂ S test	-Ve	-Ve	-Ve	-Ve	-Ve	+ve	-Ve	-Ve	-Ve
16	Gelatin hydrolysis	-Ve	+ve	-Ve	-Ve	+ve	+ve	+ve	+ve	+ve
17	Starch hydrolysis	+ve	+ve	+Ve	+ve	+ve	+ve	+ve	-Ve	+ve
18	Casein hydrolysis	-Ve	-Ve	+ve	-Ve	+ve	+ve	-Ve	-Ve	-Ve
Probable bacteria		<i>Micrococcus lylae</i>	<i>Micrococcus luteus</i>	<i>Bacillus cereus</i>	<i>Streptococcus group F</i>	<i>Bacillus megaterium</i>	<i>Bacillus coagulans</i>	<i>Micrococcus lylae</i>	<i>Bacillus cereus</i>	<i>Bacillus megaterium</i>

fresh PCR tube along with 0.6 µl of OPL-14 (random) primer [5'-TCG CTC CGT T-3'] and 5.7 µl sterile distilled water was added to get the final volume of reaction mixture to 10 µl. PCR amplification was done by keeping 94 °C for 1 min as initial denaturation step followed by 37 °C annealing for 1 min, 40 cycles of extension at 72 °C with 2 min duration and final extension for 10 min again at 72 °C (Padmalatha & Prasad, 2006).

Agarose gel electrophoresis

Agarose gel (1%) prepared in TAE buffer (1X) was used to check the purity and RAPD pattern analysis. Two µl of PCR amplicon was used for electrophoresis with 1 kb ladder agarose gel containing EtBr (5 µl/ml of gel) at 60 V. DNA banding pattern was visualized in UV trans-illuminator and was documented using BioRed Gel documentation system,

Molecular Imager®, ChemiDoc™ XRS⁺ equipped with Image Lab™ Software version 6.0.0 Bio-Red Laboratories Inc.

Phylogenetic tree construction by neighbor end-joining method

Phylogenetic relationships between isolates were evaluated by constructing the phylogenetic tree using PyElph GUI software version 1.4, according to the neighbor end-joining method.

Results

Isolation and morphological characterization of isolates

Fourteen bacterial isolates with different morphological characters were obtained from the saliva of unused leech which was coded as UTL-1 to UTL-14. Colonial characters observed visually are described in Table 1. From used leech, thirteen bacterial isolates obtained were

Table 6 Biochemical characterization of Gram-negative isolates from unused leech

Sr. no	Isolates → Name of test ↓	UTL01	UTL05	UTL09	UTL10	UTL14
1	Citrate utilization	-ve	+ve	-ve	+ve	-ve
2	Lysine test	-ve	-ve	-ve	-ve	-ve
3	Ornithin test	-ve	-ve	-ve	-ve	-ve
4	Urease production	+ve	-ve	+ve	+ve	+ve
5	Phenylalanine	+ve	-ve	-ve	-ve	+ve
6	Nitrate reduction	+ve	-ve	+ve	-ve	+ve
7	H ₂ S production	-ve	-ve	+ve	-ve	-ve
8	Glucose utilization	+ve	-ve	-ve	+ve	+ve
9	Adonitol utilization	-ve	-ve	-ve	-ve	-ve
10	Lactose utilization	-ve	-ve	-ve	-ve	-ve
11	Arabinose utilization	-ve	-ve	+ve	+ve	-ve
12	Sorbitol utilization	-ve	-ve	-ve	-ve	-ve
13	MR test	+ve	-ve	-ve	+ve	-ve
14	VP test	-ve	-ve	-ve	+ve	-ve
15	Oxidase test	+ve	+ve	+ve	+ve	+ve
16	Gelatin hydrolysis	-ve	-ve	+ve	+ve	+ve
17	Starch hydrolysis	-ve	-ve	-ve	+ve	-ve
18	Casein hydrolysis	-ve	-ve	-ve	+ve	+ve
Probable bacteria		<i>P. stutzeri</i> or <i>P. mesophilica</i>	<i>Alcaligenes faecalis</i>	<i>P. putrefaciens</i>	<i>Aeromonas hydrophila</i>	<i>Vibrio. Marinus</i>

Table 7 Biochemical characterization of Gram-negative isolates from used leech

Sr. no.	Isolates → Name of test ↓	TL02	TL03	TL05	TL08	TL13
1	Citrate utilization	+ve	-ve	+ve	+ve	+ve
2	Lysine utilization	-ve	-ve	-ve	-ve	-ve
3	Ornithin test	-ve	-ve	-ve	-ve	+ve
4	Urease production	-ve	-ve	+ve	+ve	+ve
5	Phenylalanine utilization	-ve	-ve	-ve	+ve	+ve
6	Nitrate reduction	-ve	-ve	-ve	-ve	-ve
7	H ₂ S test	-ve	-ve	-ve	-ve	+ve
8	Glucose utilization	-ve	-ve	+ve	+ve	+ve
9	Adonitol utilization	-ve	-ve	-ve	-ve	-ve
10	Lactose utilization	-ve	-ve	+ve	-ve	-ve
11	Arabinose utilization	-ve	-ve	+ve	+ve	+ve
12	Sorbitol utilization	-ve	-ve	-ve	-ve	-ve
13	MR test	-ve	+ve	-ve	-ve	-ve
14	VP test	-ve	-ve	-ve	-ve	-ve
15	Oxidase production	+ve	+ve	-ve	+ve	+ve
16	Gelatin hydrolysis	-ve	+ve	+ve	-ve	-ve
17	Starch hydrolysis	+ve	+ve	-ve	-ve	+ve
18	Casein hydrolysis	-ve	-ve	-ve	+ve	+ve
Probable bacteria		<i>P. putida</i>	<i>V. salmonicida</i> or <i>E. coli</i> (inactive)	<i>Providencia rettgeri</i> or <i>Ent. agglomerans</i>	<i>Burkholderia gladioli</i>	<i>Citrobacter diversus</i>

Table 8 Biochemical characterization of used leech Gram-positive isolates

Sr. no.	Isolates → Name of Test ↓	TL01	TL04	TL06	TL07	TL09	TL10	TL11	TL12
1	Malonate utilization	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
2	V.P. test	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
3	Citrate utilization	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
4	ONPG test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5	Nitrate reduction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
6	Catalase production	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
7	Arginine test	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve
8	Sucrose utilization	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
9	Mannitol utilization	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
10	Glucose utilization	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
11	Arabenose utilization	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
12	Thehalose utilization	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
13	MR test	-ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve
14	Oxidase test	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
15	H ₂ S production	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
16	Gelatin hydrolysis	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve
17	Starch hydrolysis	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
18	Casein hydrolysis	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve
Probable bacteria		<i>Bacillus megaterium</i>	<i>Bacillus megaterium</i>	<i>Corynebacterium</i> spps.	<i>Flavobacterium</i> spps.	<i>Bacillus</i> spps.	<i>S. cohnii</i>	<i>Bacillus pumilus</i>	<i>Bacillus coagulans</i>

coded as TL-1 to TL-13 and colonial characteristic of each isolated were presented in Table 2. Each of the isolates was categorized according to their Gram's reaction.

Out of fourteen isolated from unused leech, five were Gram-negative while the other nine were Gram-positive. Gram-negative used leech salivary isolates were five while rests of eight were Gram-positive (Tables 3 and 4).

Biochemical characterization of isolates

Bacterial cultures isolated from leech were categorized as Gram-positive and Gram-negative. Biochemical characterization of each isolates were described in Tables 5, 6, 7 and 8. Positive biochemical tests of each isolates were compared with the manual provided with identification kit for preliminary identification. The list of probably identified bacteria was also included in Tables 5, 6, 7 and 8.

Bacteria isolated from unused leech were quite different from those found in used leech. Interestingly, it was observed that certain species of bacteria which were not present initially become part of the digestive tract of

leech after treatment. In the present experiment, leech was starved for 15 days to remove possible bacteria which were carrying temporarily. *Bacillus* species was found in both types of leech saliva more commonly. *Pseudomonas* species were present higher in number before the treatment. *Micrococcus* species which was found in unused leech which completely disappears in used leech. *Vibrio* species were also found in both the leech.

Antibiotic assay of isolates

The antibiotic assay was carried out for the bacteria isolated from the leech saliva (Figs. 1 and 2). Clear zone around the antibiotic disc indicated that bacteria were sensitive at a given concentration. It was observed that all isolates were sensitive to most of the antibiotics. In general, chloramphenicol (30 µg) and norfloxacin (10 µg) showed highest sensitivity for bacteria from unused leech. While bacteria isolated from used leech showed the highest sensitivity towards sparfloxacin (5 µg) followed by cefaclor (30 µg).

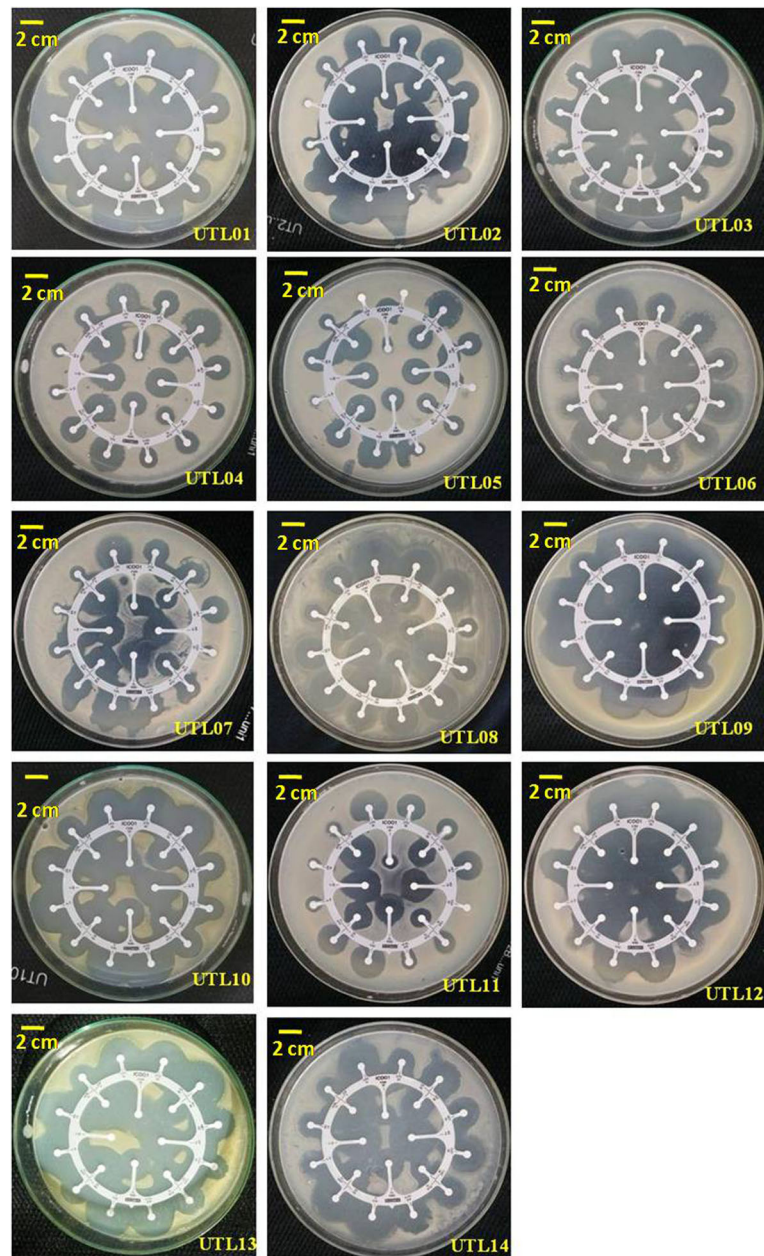


Fig. 1 Antimicrobial assay of bacteria from untreated leech

RAPD-PCR and phylogenetic tree

In the present study, RAPD-PCR was performed using random primer OPL-14 to screen intra-species variation among the isolates from both used and unused leech. Variation was screened with respect to random primer OPL-14 (Chacon, Martinez, Wachsman, et al., 2013; Fernanda, Da, Biscola, et al., 2016). There were 20 amplified band patterns were observed using OPAL14 primer (Fig 3). The variation in band patterns for each successfully characterized isolate as indicated by RAPD-PCR possibly suggests the existence of sequence within the species

(Adeleke, Olaitan, Abiona, et al., 2014). Figure 3 showed the RAPD band pattern with a ladder (1 kb) at the first lane.

Phylogenetic tree construction (Fig. 4) by neighbor end-joining method reveals that isolate U7, U4, and U3 were phylogenetically different than U11, U8, U10, U9, U12, and U6. Similarly, T9B, T8, T12, and T10 contain different genetic makeup than T4 and T1. Branches of these isolates indicated that they were evolved to survive under similar conditions. U2, T2, T9A, and T10 showed no amplification, which indicated absence of primers sequence.

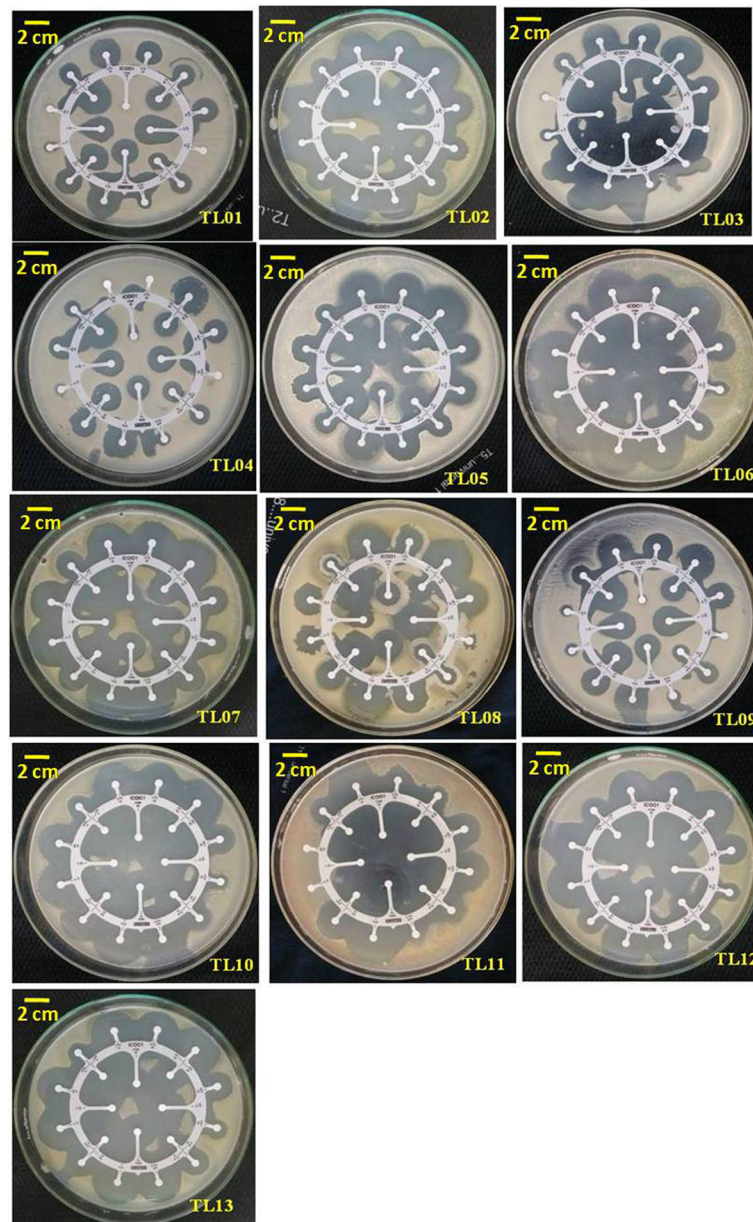


Fig. 2 Antibiotic assay of bacteria isolated from treated leech

Discussion

Herudotherapy or leech therapy for skin diseases is an ancient process (Das, 2014). These blood-sucking animals serve the human kind for since long. The saliva of medicinal leech contains many bioactive compounds and it was reviewed in detail in the past years (Siddall, Trontelj, Utevsky, Nkamany, & Macdonald-III, 2007; Das, 2014; Sig et al., 2017). These bioactive compounds are of many medicinal properties for which leeches were exploited (Siddall et al., 2007). But saliva does not only contain bioactive compounds but it is also the habitat of some gut microbes. Such gut microbes are normal flora

of them or might becomes from the environment in which the leeches live. These microfloras might be pathogenic to humans. For example, *Aeromonas* which is commensal in the leech digestive system is pathogenic for human (Marden et al., 2016). It reported to cause pneumonia, gastroenteritis, and septicemia (Das, 2014). Therefore, medicinal leech, before the treatment of skin diseases, treated with antibiotic solutions to eliminate possibilities of post-operational bacterial infections (Das, 2014). Microevolution process changes the genetics of these bacteria as they divide in a very short time and the problem of drug resistance arises. Nowadays, due to the

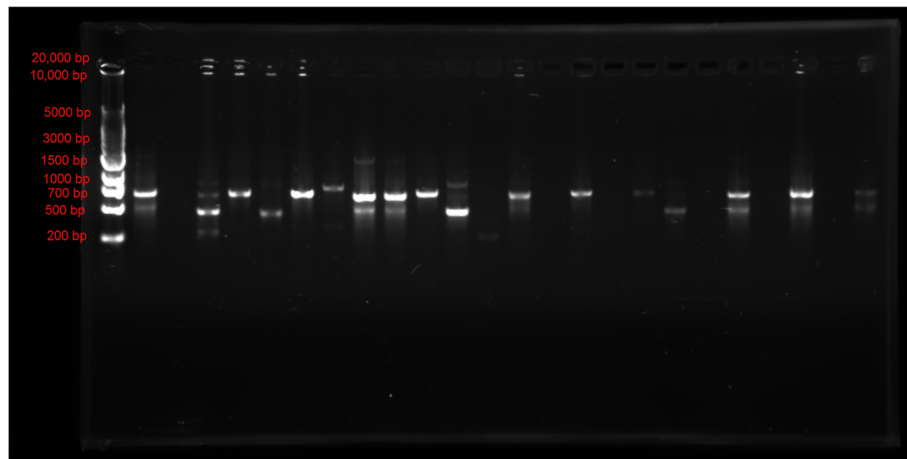


Fig. 3 RAPD fingerprint of all isolates using OPL-14 as a random primer

excessive use of many antibiotics, these drug resistance phenomena changed in multidrug resistance (MDR). The bacterium which was sensitive to a certain drug becomes resistant to those antibiotics. MDR become the global threat (Giltner et al., 2013). MDR is a genetic phenomenon. Therefore, it becomes important to evaluate the salivary microbiota for their genetic profile and antibiotic sensitivity. Conventional microbiological techniques were used to isolate the bacteria from the saliva

of the leech. For the present study, two types of leech were used. One leech was before the skin treatment and another was after the skin treatment. Bacterial isolates were also tested biochemically to find out the genus name of them. It was noted that salivary bacterial makeup was partially changed. *Aeromonas* and *Burkholderia* were isolated from used leech which was thought to be present in the digestive tract of leech as symbiotic bacteria (Graf, 2016; Ott, Dacks, Ryan, & Rio, 2016;

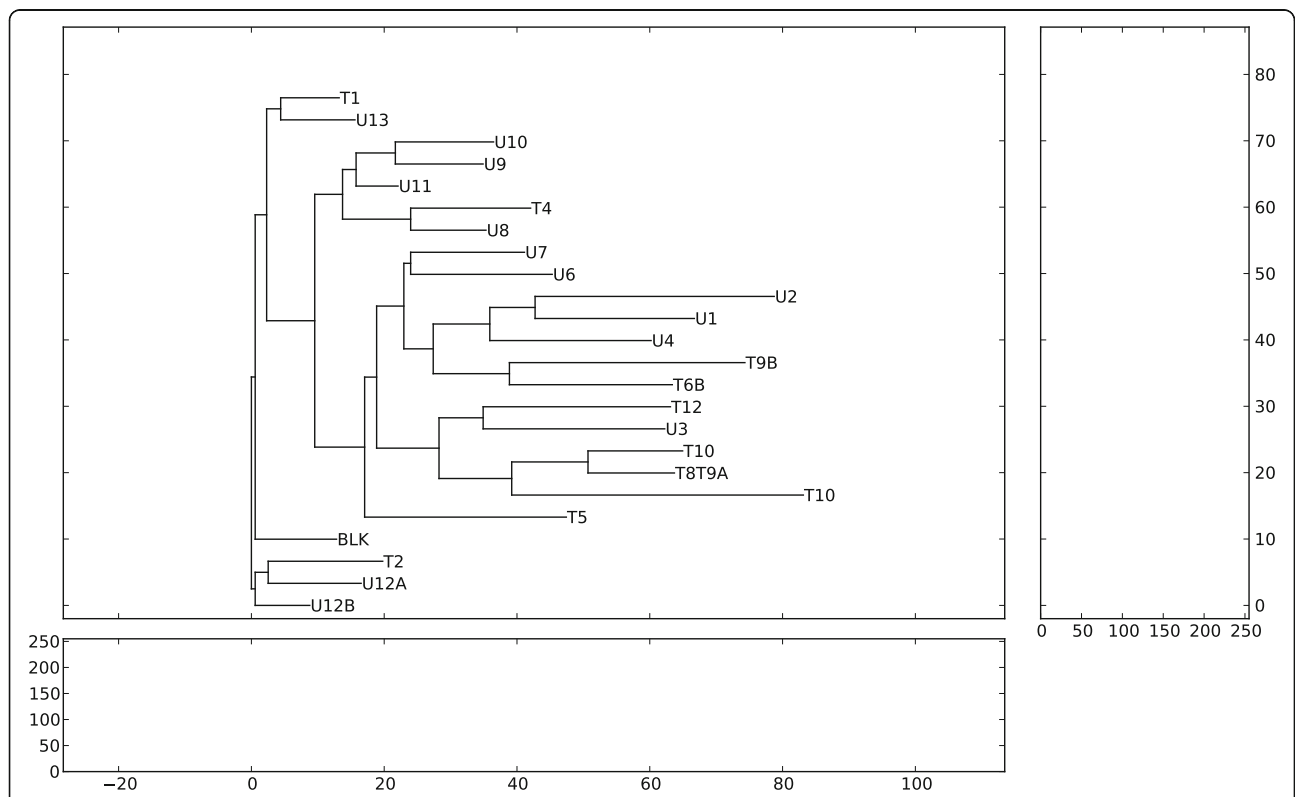


Fig. 4 Phylogenetic tree constructed by PyElph Software Version 1.4 through neighbor end-joining method

Paoletti, Mazzon, Martinez-Sañudo, et al., 2013; Worthen et al., 2006). Leech received bacteria that belong to genera *Citrobacter*, *Comamonas*, *Corynebacterium*, *Providencia*, *Escherichia*, and *Enterobacter*. *Burkholderia* present in the midgut of many crypts (Paoletti et al., 2013). Bacteria which belong to *Escherichia* and *Enterobacter* might have come from water in which leech was maintained. *Comamonas* was isolated from the mucosal secretion of *H. verbena* (Ott, Rickards, Gehrke, & Rio, 2014).

Antibiotic sensitivity of all of them was analyzed by disc diffusion technique using a commercially available antibiotic combo disc containing 20 antibiotics. The technique is equally important because it also gives the comparative results of each antibiotic tested. Each bacterium was sensitive to most of the antibiotics. Most of the bacterial infections which resulted from leech therapies were due to *Vibrio*, *Pseudomonas*, and *Aeromonas*. These infections would be treated with oral antibiotics, for example, fluoroquinolones, trimethoprim, aminoglycosides with cephalosporins—3rd generation (Das, 2014; Gunawan, Wibowo, Bunawan, & Turner, 2015). Cefepime, cotrimoxazole, vancomycin, co-trimoxazole, aztreonam, ampicillin and metronidazole were reported as treatment for *Aeromonas* infection developed due to leech therapy (Giltner et al., 2013; Marden et al., 2016). Recently, *Aeromonas* species resistant from fluoroquinolones and ciprofloxacin was isolated (Beka et al., 2018) which raised the need of a new combination of known antibiotics for the treatment.

The RAPD technique constitutes an efficient tool for the study of DNA polymorphism. It involves the amplification of random segments of genomic DNA by PCR using short-single primers of arbitrary sequence. RAPD requires very small quantities of DNA while no cloning, sequencing or hybridization is necessary. The possible number of RAPD patterns was estimated by the changes of one or more clear bands or band size. The RAPD technique, therefore, surveys (scans) numerous loci in the genome, which makes this method particularly attractive for analyzing the genetic distance and similarity between closely related species (Ling et al. 2007). Phylogenetic tree on the basis of OPL-14 confirmed that the bacterial genera isolated from each leech were able to survive in the same environmental conditions. The change in genetic makeup expressed as a new branch in phylogenetic tree.

Conclusion

Bacterial culture isolated from unused and used leech were morphologically, biochemically, and genetically characterized. Present study was conducted to isolate the cultivable bacterial culture from 'unused' and 'used' Indian medicinal leech (*Hirudinaria granulose*) and

characterized them morphologically, biochemically and genetically. It was found that bacterial makeup in both the leech was different. Biochemical characterization reveals that isolates were belong to different genera viz *Bacillus*, *Pseudomonas*, *Micrococcus*, *Aeromonas*, *Citrobacter*, *Vibrio*, *Providencia*, *Escherichia*, *Enterobacter* etc. Bacteria from unused leech were more sensitive to chloramphenicol and norfloxacin while bacteria from used leech showed the highest sensitivity towards sparfloxacin and cefaclor which suggested that these antibiotics may serve as prophylactic for post-leech therapy bacterial infections. RAPD analysis using random primer OPL-14 (5'- TCG CTC CGT T-3') illustrated that most of the genera isolated from both types of leeches contain primer sequence. Most of the bacteria from isolated from each leech showed clustering in phylogenetic tree indicated that they were of similar origin and genetic makeup with respect to tested random primer. Therefore, these antibiotics possibly used against them.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s41936-020-00143-5>.

Additional file 1. Area of Zone of inhibition of Unused leech isolates in cm.

Additional file 2. Area of Zone of inhibition of Used leech isolates in cm.

Abbreviations

µg: Microgram
EDTA: Ethylenediaminetetraacetic acid
FDA: Food and Drug Administration
MMolar: Molar
MDRM: Multidrug resistance
PCR: Polymerase chain reaction
RAPD: Random amplified polymorphic DNA
Rp: Rotation per minute
SDS: Sodium dodecyl sulfate
TE buffer: Tris-EDTA buffer
UV: Ultraviolet

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Authors' contributions

DP and JS participated in the design and concept of the study, molecular experimentations, data analysis and drafted the manuscript. KK carried out isolation and biochemical characterization of isolates. JS, PP, and SM were involved in molecular experimentation for RAPD profile and antibiotic sensitivity testing; MN was involved in conception, design, and coordination of the study and also in preparation of the final draft of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset(s) supporting the conclusions of this article is (are) included in the article. The conclusions are based on the data generated from the current study. The author can be contacted for any additional supporting data required by the journal.

Ethics approval and consent to participate

Not applicable as the study relates to the use of leech of this species only. The authors declare that no animal was sacrificed for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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