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Evaluation of organisms present in the gut of *Hirudinaria granulosa* or Indian cattle leech used for the purpose of Hirudotherapy (*Jalukavacharana*) in Ayurveda - A Metagenomic study

Dr. Rajneesh V. Giri,[1] Dr. Gururaja Tantry,[2] Dr. Shyamprasad,[3] Dr. Premalatha S,[4] Dr. Vishwanatha[5]

[1]Professor & Head, Dept. of PG Studies in Shalya Tantra, SDM College of Ayurveda, Udupi, [2]Professor, [3]Asso. Professor, Dept. of PG Studies in Shalya Tantra, Muniyal Institute of Ayurveda Medical Sciences, Manipal, [4]Scientist, [5]Senior Research Officer, Dept. of Biotechnology & Microbiology, SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi, Karnataka, INDIA.

ABSTRACT

In Ayurveda, *Shalyatantra* envisages surgical (*Shastra Karma*) and minimally invasive surgical methods (*Anushastra Karma*) for the management of various diseases. Among minimally invasive surgery, blood letting (*Raktamokshana*) plays an important role. Hirudotherapy or therapeutic use of leeches (*Jalukavacharana*) has been lauded for its role in the management of abscess, inflammation, cellulitis, non-healing ulcers, skin diseases etc. Leeches are applied to the venous-congested sites to withdraw obstructive blood and also the presence of anticoagulating and vasodilating agents further reduce circulatory obstruction and facilitate blood flow through the area. However, the advantages of leech therapy are confounded by more recent and widely reported occurrences of leech-borne infections at the bite wound, which may cause septicaemia in the patient when left untreated. It has been observed that the infection of the surgical site with bacterial species, necessitates prophylactic antibiotic cover. The study was undertaken to check for the presence and dominance of the various classes of bacteria in the gut of Indian Cattle leech which is used for the purpose of *Jalukavacharana* by Ayurvedic surgeons. Microbes belonging to the Phylum Proteobacteria were the most dominant followed by Bacteroidetes and Firmicutes. In the phylum Proteobacteria, the abundance of bacteria belonging to the family Enterobacteriacea was observed. Presence of genera classified under Alcaligenaceae, Aeromonadaceae and Rhodospirillaceae were recorded to a significant extent. This study on the presence of microbes can probably shed light on the scientific value of *Poorvakarma* told by *Sushrutha*, where he advises to keep leeches in *Haridra* before being used for therapy to avoid transmission of infections.

Key words: *Hirudotherapy, Hirudinaria granulosa, Leech gut flora, Metagenomics, Proteobacteria, Sushrutha*

INTRODUCTION

In Ayurveda, *Shalyatantra*, the branch of surgery, holds an esteemed place of its own. *Shalyatantra* envisages surgical and minimally invasive surgical methods (*Anushastra Karma*) for the management of diseases. Among minimally invasive surgery, blood letting (*Raktamokshana*) plays an important role. Hirudotherapy or therapeutic use of leeches (*Jalukavacharana*) has been lauded for its role in the management of various diseases especially in conditions where the blood is vitiated with pitta and in patients who are very sensitive to trauma/pain inflicted during therapy like children, elderly, pregnant women etc.[1]

Address for correspondence:

Dr. Rajneesh V. Giri
Professor & Head, Dept. of PG Studies in Shalya Tantra, SDM College of Ayurveda, Udupi, Karnataka, India.
E-mail: rajneeshgiri@gmail.com

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The Western Science has been attracted to this form of treatment since decades. Leech therapy provides great therapeutic benefits during post-operative remediation, with studies demonstrating an associated decrease in the rate of graft failures and risk of amputation. Leeches are applied to the venous-congested[2] sites in order to withdraw obstructive blood while simultaneously secreting an anticoagulating agent and vasodilators to further reduce circulatory obstruction and facilitate blood flow through the area. One survey analyzed 277 case reports to quantify the efficacy of leech therapy and found that 78% of the cases resulted in success, where transferred tissue was salvaged and no complications occurred. However, the advantages of leech therapy are confounded by more recent and widely reported occurrences of leech-borne infections at the bite wound, which may cause septicaemia in the patient when left untreated. *A. hydrophila* infection subsequent to leech therapy has been well documented, accounting for 88% of leech therapy infectious complications.[3]

In Ayurveda, before *Jalukavacharana*, *Acharya Sushrutha* has described a very important pre-operative preparation of leeches, so as to prevent the onset of various complications in humans. He has described a method of keeping the leeches in *Haridra* (turmeric) to reduce the untoward effects of leech bite.[4]

Hence this study has been taken up to check the scientific background of pre-operative preparation of leeches, so as to verify the necessity of prophylactic antibiotic cover based on the presence of microorganisms in the gut of leech.

NEED FOR THE STUDY

In Ayurveda, though leeches are used extensively, no work has been done to date to check for the presence of organisms in leeches in India so that the possibility of their transmission to humans via hirudotherapy can be evaluated.

AIM

To study the leech gut flora and prove the need ofprecautionary steps for *Jalukavacharana* as mentioned in Ayurveda

OBJECTIVES

To check for various organisms (esp. the presence of Aeromonadaceae and Enteobacteriaceae family members) present inthe gut of cattle leech commonly used in *Jalukavacharana* in Ayurveda.

MATERIALS AND METHODS

Leeches were collected from Karnataka and Goa states of India. They were collected and stored in fresh water in transparent glass bottles. The water was changed every day. The bottle was cleaned once a week.

The selected leeches were kept in conical flasks and about 5ml of ether was poured on a cotton ball and the mouth of the flask was covered. It was kept in situ till the leeches became motionless.

The dissection of the leeches was carried out in laminar hood. The leeches were weighed and those with same weight were selected, wiped with sterile cotton dipped in saline and fixed on sterile wax tray. It was dissected with proper aseptic measures and the samples from the gut were collected in sterile microfuge tubes. Guts were pooled (2-4/set) and sent for metagenomics analysis.

16S Metagenome analysis

Four sets namely GB5, GB9, LB5 comprising of 2 guts/each set and LB14 (guts from four leeches pooled) collected in the microfuge tube, were stored at -20ºC. The samples were packed and transported under cold conditions (enveloped in ice-pack) to Genotypic Technology (P) Ltd., Bangalore for metagenomic analysis.

DNA Extraction

Isolation of DNA from leaf tissues was performed using QiagenDneasy Blood and tissue Kit (Qiagen,

Catalog #69504) as per manufacturer recommended protocol.

DNA Quality Control

The purity and concentration of the DNA was assessed using ND1000 nanodrop spectrophotometer

(Thermo Scientific, MA, USA.)

Workflow for V3-V4 Library Preparation:

Library Preparation and Sequencing

16S V3-V4 metagenome libraries were prepared using 16S Bacteria V3-V4 region-specific targeting proprietary primers at Genotypic Technology Pvt. Ltd., Bangalore, India. Briefly, 20 ng of genomic DNA (Quantification method: Nanodrop spectrophotometer) was amplified for 26 cycles using KAPA HiFiHotStart PCR Kit (KAPA Biosystems Inc., Boston, MA USA). The forward and reverse primer concentration is kept at 0.2 uM each. The amplicons were analyzed on 1.2% agarose gel. 1ul of round 1 PCR amplicons were used for Indexing PCR (round 2). Here, the round 1 PCR amplicons were amplified for 10 cycles to add Illumina sequencing barcoded adaptors (Nextera XT v2 Index Kit, Illumina, U.S.A.). Round 2 PCR amplicons were analyzed on 1.2% agarose gel (Figure 1: Round 2 PCR).

Illumina Adapter Sequences: 5’ - AATGATACGGCGACCACCGAGATCTACAC [i5] TCGTCGGCAGCGTC

5’ - CAAGCAGAAGACGGCATACGAGAT [i7] GTCTCGTGGGCTCGG

[i5, i7] – Unique dual index sequence to identify sample-specific sequencing data (Table 1)

The pooled library was sequenced on Illumina Miseq for 300 bp paired end chemistry according to the manufacturer’s protocol.

Data pre-processing and quality check The Illumina paired-end reads having dual indexed barcodes specific to the sample were demultiplxed using bcl2fastq tool allowing 1 mismatch in the index sequence. The generated illumina paired end reads (275*2) were quality checked using FastQC tool.

The reads with adapter and low quality bases were removed using ABLT script. The high quality paired end reads (70% of the bases having phred score (Q>30)) with primer sequences where stitched using fastq-join tool.

Analysis Methodology

The stitched reads were analysed using QIIME v 1.9.0 software. The query sequences were mapped against the curated chimera free 16s rRNA database (Greengenes v 13.8) using UCLUST method and

OTUs (Operational Taxonomic Unit) were identified at >=97% sequence similarity. The generated biom was rarefied at a depth 60,000 sequences/sample with a step size (10 default) and alpha diversity indexes were calculated using various metrics. The absolute abundance distribution of the microbial community including the rare OTUs in each sample was calculated.

RESULTS AND DISCUSSION

The distribution and abundance of various phyla in the gut flora is presented in Table 1.

Table 1: Distribution and dominance of various phyla of bacteria in Leech gut flora

Phylum	Population, %			
	GB5	GB9	LB5	LB14
Proteobacteria	79.4488	87.6363	51.3058	95.5694
Bacteroidetes	17.0818	6.3862	15.1624	0.1257
Firmicutes	0.2082	2.6254	25.3854	0.4555
Actinobacteria	1.2172	0.9506	1.0749	0.0931
Acidobacteria	0.7322	-	-	0.0033
Chloroflexi	0.4355	0.0062	-	-
Cyanobacteria	0.2928	1.7647	1.0827	0.0041
Planctomycetes	0.258	0.5878	-	0.0057
AD3	0.1141	-	-	-
Verrucomicrobia	0.1109	-	-	0.0049
WPS-2	0.0528	-	-	-
Gemmatimonadetes	0.0248	-	-	0.0008
Elusimicrobia	0.0106	-	-	-
GAL15	0.0077	-	-	-

Fusobacteria	0.0029	-	-	-
WS3	0.0018	-	-	-
TM7	-	-	1.0935	0.0008
Synergistetes	-	0.0378	0.3863	0.7323
fbp	-	-	-	-
Tenericutes	-	-	0.133	0.0008
OD1	-	0.0031	0.0156	0.0188
TM6	-	0.0006	-	-
SR1	-	0.0006	4.3603	2.9839
Euryarchaeota	-	0.0006	-	-
[Thermi]	-	-	-	0.0008
Total	100	100	100	100

It was seen that bacteria belonging to the Phylum Proteobacteriae were the most common organism presentin the gut of the Indian cattle leech generally used for *Jalukavacharana*. Percentage of bacteria belonging to Firmicutes and Bacteroidetes was also significantly higher. The phylum Proteobacteria includes a wide genera of microbes. Proteobacteria are Gram-negative and the divisions include Alphapoteo bacteria, Betaproteo bacteria, Gammaproteo bacteria, Deltaproteo bacteria, Epsilonproteo bacteria and Zetaproteo bacteria. Many pathogenic and opportunistic organisms such as *Escherichia, Salmonella, Vibrio, Helicobactespp, Pseudomonas aerugions, Aeromonashydrophila* and *Aeromonasveronii,* biovarsobria etc. are included in this phylum. In various recent works it has been observed that *H. medicinalis* used after surgery has led to Aeromonas infections, most commonly with *A.veronii*, which can lead to local cellulitis even progressing to subcutaneous abscess and sepsis.[5] Bacteroidetes are found in the guts and on the skin of animals. Bacteroidia, Cytophagia and Flavobacteria are the classes of Gram-negative, non sporeforming anaerobic/aerobic, rod-shaped bacteria classified under the phylum Bacteroidetes. Some members of the genus Bacteroides are opportunistic pathogens. GB5 and GB9 showed presence of members belonging to this phylum in abundance. Most microbes categorized under *Firmicutes* have Gram-positive cell wall structure.

Proteobacteria being the major phylum, the distribution of various classes of bacteria belonging to this phylum present in abundance is shown in the tables below (2, 3, 4 and 5). The designation for classes is as follows,

AlphaPB- Alphaproteobacteria BetaPB- Beta proteobacteria; GammaPB- Gammaproteo bacteria; DeltaPB- Deltaproteo bacteria; EpsilonPB- Epsilonproteo bacteria.

Table 2: Distribution of the bacteria belonging to phylum Proteobacteria in GB5.

Class	**Order**	**Family**	**Genus**	**Species**	**%**
GammaPB	Enterobacteriales	Enterobacteriaceae	Morganella	Unclassified	42.1366
GammaPB	Enterobacteriales	Enterobacteriaceae	Unclassified	Unclassified	21.1301
BetaPB	Burkholderiales	Alcaligenaceae	Achromobacter	Unclassified	6.0753
BetaPB	Burkholderiales	Alcaligenaceae	Unclassified	Unclassified	3.4586
GammaPB	Enterobacteriales	Enterobacteriaceae	Morganella	morganii	2.0638
GammaPB	Aeromonadales	Aeromonadaceae	Unclassified	Unclassified	1.6253
AlphaPB	Rhizobiales	Brucellaceae	Ochrobactrum	Unclassified	0.5494
AlphaPB	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes	Unclassified	0.5007
GammaPB	Enterobacteriales	Enterobacteriaceae	Erwinia	Unclassified	0.4329
AlphaPB	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Unclassified	0.2725
Alpha	Rhodospiri	Rhodospirill	Unclassifi	Unclas	0.16

PB	llales	aceae	ed	sified	93
GammaPB	Xanthomonadales	Xanthomonadaceae	Unclassified	Unclassified	0.1368
GammaPB	Xanthomonadales	Sinobacteraceae	Unclassified	Unclassified	0.1221
GammaPB	Enterobacteriales	Enterobacteriaceae	Trabulsiella	Unclassified	0.0755
GammaPB	Enterobacteriales	Enterobacteriaceae	Enterobacter	arachidis	0.0746

Table 3: Distribution of the bacteria belonging to phylum Proteobacteria in GB9.

Class	Order	Family	Genus	Species	%
GammaPB	Enterobacteriales	Enterobacteriaceae	Unclassified	Unclassified	30.37
GammaPB	Enterobacteriales	Enterobacteriaceae	Morganella	Unclassified	25.024
BetaPB	Burkholderiales	Alcaligenaceae	Achromobacter	Unclassified	13.421
GammaPB	Aeromonadales	Aeromonadaceae	Unclassified	Unclassified	10.507
GammaPB	Enterobacteriales	Enterobacteriaceae	Morganella	morganii	1.2346
BetaPB	Burkholderiales	Alcaligenaceae	Unclassified	Unclassified	0.9946
BetaPB	Burkholderiales	Comamonadaceae	Unclassified	Unclassified	0.9332
AlphaPB	Rhodobacterales	Rhodobacteraceae	Unclassified	Unclassified	0.8619
GammaPB	Xanthomonadales	Xanthomonadaceae	Unclassified	Unclassified	0.8266
AlphaPB	Rhizobiales	Methylobacteriaceae	Unclassified	Unclassified	0.6033
AlphaPB	Rhizobiales	Brucellaceae	Ochrobactrum	Unclassified	0.5382
GammaPB	Enterobacteriales	Enterobacteriaceae	Erwinia	Unclassified	0.2642
GammaPB	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Unclassified	0.2325

Table 4: Distribution of the bacteria belonging to phylum Proteobacteria in LB5.

Class	Order	Family	Genus	Species	%
AlphaPB	Rhodospirillales	Rhodospirillaceae	Elstera	litoralis	17.133
AlphaPB	Rhizobiales	Phyllobacteriaceae	Aminobacter	Unclassified	9.7056
AlphaPB	Rhizobiales	Phyllobacteriaceae	Mesorhizobium	Unclassified	9.6851
BetaPB	Methylophilales	Methylophilaceae	Unclassified	Unclassified	8.0536
BetaPB	Burkholderiales	Comamonadaceae	Unclassified	Unclassified	1.6305
AlphaPB	Rhodospirillales	Rhodospirillaceae	Unclassified	Unclassified	1.3957
AlphaPB	Rhodobacterales	Rhodobacteraceae	Unclassified	Unclassified	0.5194
GammaPB	Xanthomonadales	Xanthomonadaceae	Unclassified	Unclassified	0.5008
AlphaPB	Rhizobiales	Methylobacteriaceae	Unclassified	Unclassified	0.4235
GammaPB	Enterobacteriales	Enterobacteriaceae	Unclassified	Unclassified	0.3159
AlphaPB	Rhizobiales	Xanthobacteraceae	Labrys	Unclassified	0.312
AlphaPB	Rhizobiales	Phyllobacteriaceae	Unclassified	Unclassified	0.1946
AlphaPB	Rhodobacterales	Hyphomonadaceae	Oceanicaulis	Unclassified	0.1878
AlphaPB	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium	Unclassified	0.1673

Table 5: Distribution of the bacteria belonging to phylum Proteobacteria in LB14.

Class	Order	Family	Genus	Species	%
GammaPB	Enterobacteriales	Enterobacteriaceae	Morganella	Unclassified	88.18
GammaPB	Enterobacteriales	Enterobacteriaceae	Morganella	Morganii	6.3597
EpsilonPB	Campylobacterales	Helicobacteraceae	Wolinella	Succinogenes	0.6294
DeltaPB	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	Unclassified	0.0996
GammaPB	Enterobacteriales	Enterobacteriaceae	Unclassified	Unclassified	0.0653
DeltaPB	Myxococcales	Unclassified	Unclassified	Unclassified	0.0596
AlphaPB	Rhizobiales	Methylobacteriaceae	Unclassified	Unclassified	0.0302
AlphaPB	Rhodospirillales	Rhodospirillaceae	Elstera	Litoralis	0.0171
BetaPB	Burkholderiales	Comamonadaceae	Delftia	Unclassified	0.0155
GammaPB	Pseudomonadales	Moraxellaceae	Acinetobacter	Schindleri	0.0057
GammaPB	Pseudomonadales	Moraxellaceae	Acinetobacter	Unclassified	0.0049
GammaPB	Oceanospirillales	Alcanivoracaceae	Alcanivorax	Unclassified	0.0049
GammaPB	Chromatiales	Ectothiorhodospiraceae	Unclassified	Unclassified	0.0049
AlphaPB	Rhodospirillales	Acetobacteraceae	Unclassified	Unclassified	0.0049
AlphaPB	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	Unclassified	0.0049

Members of the enterobacteriaceae family which belong to class Gammaproteo bacteria were found to predominate the gut flora. Bacteria belonging to the genus *Morganella* were found to predominate. Morganella species are also known to be opportunistic pathogens capable of causing nosocomial infections. GB5 and GB9 samples showed presence of species belonging to Aeromonadaceae family. Presence of *Aeromonastaiwanensis* and *Acaviae* was recorded in the guts of leeches from set GB9.

CONCLUSION

The presence of above organisms has been identified in the gut of leech. Further studies have to be taken up to see which of these organisms can be neutralized by adopting the *Poorvakarma* of keeping leeches in *Haridra* before *Jalukavacharana* to prove the safety of the procedure to the scientific community.

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