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Identification of Bacteria and Select Viruses Harbored by Medicinal Leeches (*Hirudo verbana*)

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ABSTRACT

Leeches have been used to complement medical therapies for many years, however there is little data on the microorganisms they may harbor as part of their flora. The study aims were to (1) identify the presence of bacteria and (2) the presence of Hepatitis B and C viruses (HBV and HCV) in medicinal leeches using traditional bacteriological assays, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) and Quantitative real-time PCR (RT-qPCR). Samples obtained from the body surface, intestine and jaws from 10 *Hirudo verbana* leeches were aseptically cultured using traditional microbiological assays. Bacterial isolates were identified using the MALDI-TOF technique and the presence of HBV and HCV was analyzed using RT-qPCR. The primary bacterium isolated from the sampled leeches were *Aeromonas veronii* (*A. veronii*) which was isolated from the jaws, gut and body surface of all leeches. Other bacteria isolated at a lower frequency from various parts were *Chryseobacterium gleum*, *Ochrobactrum anthropi*, *Moraxella osloensis*, *Microbacterium oxydans*, *Kytococcus sedentarius*, *Rhizobium radiobacter*, *Staphylococcus hominis*, *Citrobacter* and *Bacillus*. No anaerobic bacteria or hepatitis viruses were detected. Interestingly, some of the bacterial species identified in this study have been implicated in hospital acquired infections and are of particular risk to immunocompromised patients. The recovery of potential human pathogens from within medicinal leeches is a public health concern and consequently their use should be restricted and avoided in susceptible individuals or a prophylactic treatment should be applied.

1. Introduction

Historically, leeches have been incorporated in many traditional medicinal treatments in many countries including Egypt, China and India (Maetz et al., 2012). Despite the historical usage of leeches in medicine, its acceptance in current medicine is relatively recent, when in

2004 it was approved for the treatment of venous congestion by the US Food and Drug Administration (Nelson and Graf, 2012; Rados, 2004).

The European medicinal leech is the most widely utilized leech in modern medical

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approaches (Nelson and Graf, 2012). Phylogenetic analysis has indicated that the medical leech, primarily marketed as *Hirudo medicinalis* is actually comprised of three main species: *H. medicinalis*, *Hirudo verbana* and *Hirudo orientalis* (Apakupakul et al., 1999; Siddall and Bureson, 1998; Siddall et al., 2001; Trontelj and Utevsky, 2005). Recently, a new medicinal leech species, *Hirudo sulukii* has been identified and was found in the wetlands of southeast Anatolia in Turkey (Saglam et al., 2016). Although more than 700 species of leeches have been identified, the two most widely used leeches in medicine are *H. verbana* and *H. medicinalis* (Siddall et al., 2007).

The use of leeches (*H. medicinalis*) in modern day therapeutics and medicine is increasing, particularly in post-operative procedures, microsurgery healing and plastic surgery (Whitaker et al., 2003). *H. verbana* is also being used in cosmetic surgical procedures. The leech's digestive tract has been reported to harbor several bacterial species including *Aeromonas veronii* biovar *sobria* and *Aeromonas hydrophila* (*A. hydrophila*). The organisms aide with the digestion of blood ingested by the leech (Maetz et al., 2012). Additionally, *A. hydrophila*, which is part of the normal gut flora of medicinal leeches, produces the proteolytic enzymes required to digest blood. Leeches lack the ability to do this independently (Nonomura et al., 1996). These bacteria can be pathogenic to humans (Maetz et al., 2012), where wounds infected by *Aeromonas* spp. can result in major complications, such as cellulitis, local abscess, and septicaemia (Fenollar et al., 1999; Whitaker et al., 2003).

Infection by *Aeromonas* spp. occurs as a result of colonization of open wounds and this could potentially occur during the attachment of the leech (Bauters et al., 2007). *Aeromonas* infection rates vary in patients (n=200) treated with leeches from 2.4% to 4.1% (Sartor et al., 2002). Another study found that *Aeromonas* infection rate was up to 20% (Eroglu et al., 2001). The children, old and immunocompromised persons are at the greatest risk of contracting these infections and the subsequent development of systemic infections including arthritis, peritonitis, myocarditis, meningitis, bacteremia, cellulitis and septicaemia (Fenollar et al., 1999; Whitaker et al., 2003). This risk was also highlighted by the

fact that meningitis has been reported as a secondary sequelae to *Aeromonas* infection associated with medical leech therapy (Ouder Kirk et al., 2004). Wound infections with *A. hydrophila* and *A. sobria* have also been reported from the use of leeches in medicine (Fenollar et al., 1999). Despite their increased use in modern medicine, there is little published data describing the microbiome of the medicinal leeches. Furthermore, there are few reports on this subject in Turkey (Aydin et al., 2004; Eroglu et al., 2001; Ozcan and Temiz, 2019). Therefore, the aims of this study were to fill these knowledge gaps by (1) identifying the main bacteria harbored by leeches and (2) determining the prevalence of Hepatitis B and C viruses (HBV and HCV) in leeches.

2. Materials and Methods

2.1. Samples

Ten medicinal leech (*H. verbana*) samples were purchased from a collector company. The vendors stated that the leeches were collected from their marshland habitat. Each leech sample was examined morphologically and was identified as *H. verbana* (Govedich et al., 2019; Sağlam, 2004). Each leech was anaesthetized for 20 min at 4 °C in 10% ethanol and the surface, jaws and intestinal regions were aseptically carefully dissected using a stereo microscope (Tasiemski et al., 2015).

2.2. Bacteriological culture

The dissected samples were then cultured onto blood agar added with 5% sheep blood and Eosin Methylene Blue Agar (EMB) (Oxoid, UK). The agar plates were incubated for 24 to 48 h at both 20°C and 37°C. For the isolation of anaerobic bacteria BD Brucella Blood Agar (BD Difco, USA) with Hemin and Vitamin K1 was used and the plates were incubated at 37 °C for 24 to 72 hours in an anaerobic jar using a gas pack (Becton, Dickinson, USA) (Nonomura et al., 1996). Single colonies were selected from original sample plates and subcultured on new agar. After that colonies were chosen for Matrix-Assisted Laser Desorption/Ionization -Time of Flight Mass Spectrometry (MALDI-TOF) using the VITEK MS database (v2.0) (bioMérieux, France) following the manufacturer's instructions.

2.3. MALDI-TOF MS Analysis

The bacterial isolates were prepared by sub-culturing the colonies from original plates onto Blood agar (Oxoid, UK) and incubated at 37 °C for 24 hours. The next day, an individual colony was selected using a sterile toothpick and transferred onto two separate spots of the MALDI-TOF disposable plate. The spots were then coated with 1 µL of matrix solution (α -Cyano-4-hydroxycinnamic acid, CHCA) and allowed to air dry for 1-2 minutes at room temperature (Vlek et al., 2012). An extract of *Escherichia coli* ATCC 8739 was utilized as a reference strain for the calibration process. Samples were analyzed on the VITEK MS MALDI-TOF mass spectrometry (bioMerieux, France) to obtain the mass spectra with a charge ratio between 2,000 and 20,000 Daltons. Each spot was irradiated with a total of 500 laser shots (at 50 Hz) in 5 shot stages, each from a different position around the target spot (automatic mode). For each sample, the spectrum was gathered by the mass spectrometer together with the Acquisition Station software (Vitek MS version 2.0). The results were then transferred to the VITEK MS Analysis Server (Myla version 4.4) where the identification results were listed (Dubois et al., 2012; Westblade et al., 2013).

The VITEK MS identification system was performed in the selected colonies by comparing the characteristics of the obtained spectrum to the VITEK MS v2.0 reference database (Westblade et al., 2013).

2.4. DNA extraction and quantitative Real Time PCR (RT-qPCR)

Nucleic acids from the leeches were extracted by the QIASymphony DSP Virus/Pathogen Midi Kit (Qiagen, Germany) following the manufacturer's guidelines. The presence of HBV and HCV in the extracted samples were detected and quantified using Artus HBV RG and Artus HCV RG (Qiagen, Hilden, Germany) RT-qPCR kits, according to the manufacturer's protocols. Amplification was conducted by a Rotor Gene 6000 platform (Qiagen, Germany).

3. Results

Figures 1a and 1b shows the proportion of the identified bacteria by MALDI-TOF MS at

37°C and 20°C respectively for each sample part (jaw, intestine and surface). No anaerobic bacteria were isolated from any sample tested. Results revealed that all of score values of isolates were 99.9% and considered as a strong identification. The most common species isolated was *A. veronii* regardless of sample part type (i.e. jaw, intestine and surface) and incubation temperature. This was followed by *Ochrobactrum anthropi* (*O. anthropi*) with a total of 7 isolates of which 6 were isolated at 20°C two from the jaw, intestine and surface. The 3rd most common bacteria isolated *Chryseobacterium gleum* (*C. gleum*) (n=5) with a single isolation from each sample part at both 20°C and 37°C, except for jaw at 20°C. The only other organism that was isolated more than once was *Staphylococcus hominis* (*S. hominis*) from the jaw at 37°C. The most common species, *A. veronii* was isolated at similar rates (n=12 and n=13) at both temperatures (37°C and 20°C, respectively). All leeches were found to be negative for both HBV and HCV using RT-qPCR test.

4. Discussion

Previous studies have showed that *Aeromonas* is the predominant bacteria in *H. medicinalis* and was exclusive to the crop of the digestive system (Büsing et al., 1953; Jennings and van der Lande, 1967). Here, *A. veronii* was also prominent and represented 53.2% (25/47) of all bacterial spp. isolated from leeches. A study carried out by Nehili et al. (1994) demonstrated the presence of 11 species of bacteria of 9 genera in the digestive system of *H. medicinalis* (Nehili et al., 1994). Similarly, this study identified a total of 8 single different bacterial species and 3 sets that were assigned to one of two possible species. It has been reported that the blood of *H. medicinalis* leeches purchased in German pharmacies comprised up to eleven various species of bacteria (Nehili et al., 1994).

Medical leeches have been reported to harbor opportunistic pathogens (Nonomura et al., 1996). Whitlock and colleagues in 1983 first demonstrated the presence of *A. hydrophila* on the leech's body and that there may be link to a risk of infection by leech attachment (Whitlock et al., 1983).

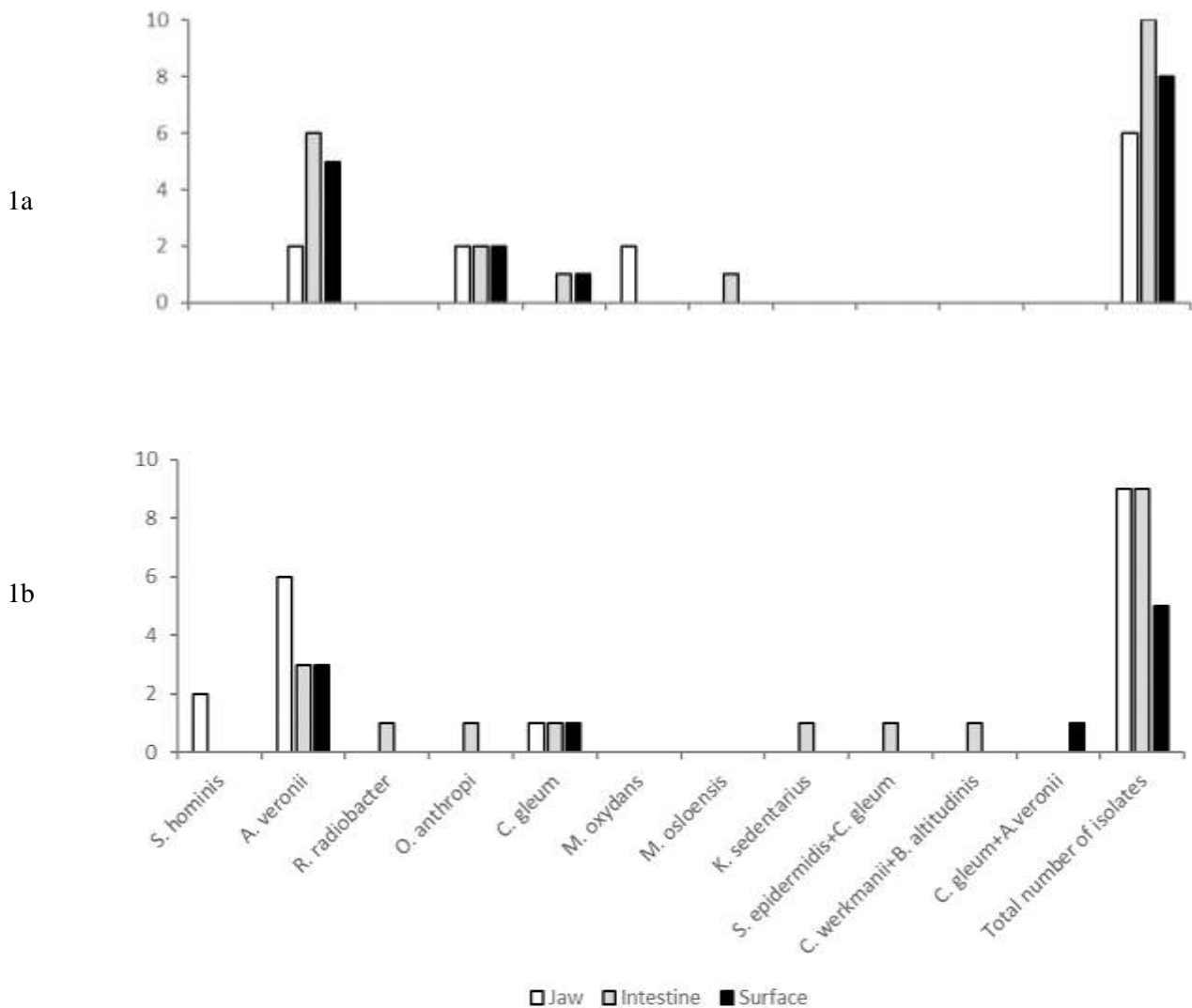


Figure 1. The number of aerobic bacterial species isolated from different parts (jaw, intestine and surface) of ten dissected leeches (*Hirudo verbana*) incubated at temperatures of 20°C (1a) and 37°C (1b) and speciated using MALDI-TOF.

Siddall and colleagues demonstrated that the gut of various species of medicinal leech harbors various *Aeromonas* spp. (Siddall et al., 2007). Another study suggested that *H. verbana* is most frequently related to *A. veronii* while *H. medicinalis* and *H. orientalis* were indicated to transport *A. hydrophila* and *A. veronii* and/or *A. jandaei*, respectively (Tasiemski et al., 2015). Our findings indicated that *A. veronii* was the most commonly isolated organism from medicinal leeches tested, regardless of anatomical part examined. Other bacteria (*C. gleum*, *Kytococcus sedentarius* (*K. sedentarius*),

Microbacterium oxydans (*M. oxydans*), *Moraxella osloensis* (*M. osloensis*), *O. anthropi*, *Rhizobium radiobacter* (*R. radiobacter*), *S. hominis*) were also present but at a much lower frequency. It has been suggested that the gut flora of medical leeches was only inhabited by *A. hydrophila* but this species was not isolated in the present study. A few studies have isolated gram negative rods such as *Pseudomonas* spp. and *Flavobacterium* spp. but at low frequency (Bickel et al., 1994; Richerson et al., 1990). A study performed in the digestive system (anterior and posterior crop, and intestine) of *H.*

verbana obtained from the Danube river by culture-independent and dependent methods reported that culture-independent attempt found that the prevalent bacterial taxon was *Mucinivorans* spp., followed by *Aeromonas* spp. (Neupane et al., 2019). New for *H. verbana*, several low abundance taxa, such as *Aquabacterium*, *Burkholderia*, *Fluviicola*, *Hydrogenophaga*, *Phreatobacter*, *Taibaiella*, *Wolinella* and unidentified *Chitinophagia*, were determined (Neupane et al., 2019). In the same study, the aerobic culturing method showed *A. veronii* (Proteobacteria), as the most prevalent taxon tracked by several *Pseudomonas* and *Acidovorax* spp. No important diversities in the bacterial communities were showed among various parts of the digestive system of individual leeches (Neupane et al., 2019). A case report published in Turkey have suggested infection with *Acinetobacter* spp. present in the superficial flora of the leech after hirudotherapy in a female patient with osteoarthritis of the knee. In addition, same authors also suggested that these bacteria, which are generally resistant to many antibiotics, should be considered when selecting the prophylactic agent (Gonen et al., 2013).

Human infections have been related with *Aeromonas* spp. particularly *A. hydrophila* from leeches (Whitlock et al., 1983). However, the species, other than *Aeromonas*, identified in this study still pose the risk of human infection, caused by leech usage and include *K. sedentarius*, *O. anthropi*, *R. radiobacter*, *M. oxydans*, *C. gleum*, *S. hominis* and *M. osloensis*. Further research on the epidemiology of leech-associated human infections is required.

Our results are concordance with a previous study that also reported that *A. veronii* was the most predominant bacterial species obtained from the intestines of *H. verbana* (Litwinowicz and Blaszkowska, 2014). Other studies using molecular methods have suggested that two species, *A. veronii* biovar *sobria* and *Rikenella*-like bacterium, predominate in the digestive system of medicinal leeches (Litwinowicz and Blaszkowska, 2014; Nelson and Graf, 2012; Silver et al., 2007; Worthen et al., 2006). Since the anaerobic, non-pathogenic *Rikenella* is difficult to detect using traditional culture the characterization of its presence in the leech environment was

performed using the analysis of specific rRNA (Litwinowicz and Blaszkowska, 2014). No anaerobes were isolated from leeches in the present study and this is consistent with previously published research that suggests that anaerobic organisms are only a minor part of leeches microflora (Nonomura et al., 1996). A negative result in the determination of HBV and HCV using RT-qPCR test kits could also be expected. A study found, using light and electron microscopy, that viruses survive in the ingested blood of leeches; where the blood in the guts of African leeches were collected in Cameroon with positive for HIV and hepatitis B (Nehili et al., 1994).

Conclusion

This is a preliminary study to identify the microorganisms present in the medicinal leech, *H. verbana* by the use MALDI-TOF MS. It also investigates if leeches in Turkey carry HBV and HCV by using RT-qPCR. Several different potentially pathogenic aerobic bacterial species were isolated from leeches in this study with *A. veronii* being the most predominant. We employed a small numbers of leeches because of limited breeding and farming leeches in Turkey. However, our results indicate that when considering the use of leeches in medicine, further larger scale studies are necessary to assess the possible risks to human health by the presence of leeches microbiome and from different geographical regions. The inclusion of wild caught leeches in any medical treatments should be prohibited along with the use of medicinal leech therapy in immunocompromised persons.

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Refereces

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