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Hirudo verbana is a source of fungal isolates potentially pathogenic to humans

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As microbial contamination of leeches poses a risk of transmission of pathogens to humans, contact with non-sterile leeches causes a potential hazard to human health. The purpose of this study was to evaluate the mycological purity of the body surface, jaws/pharynx and intestines of the medical leech, *Hirudo verbana*, and the purity of aquarium water within which the leeches were incubated. Leeches were kept without feeding under optimum laboratory conditions recommended for medicinal uses. The strains of fungi were isolated according to our own methods and standard mycological procedures. Of the 150 cultures taken from 50 leeches and 50 samples of water, 152 strains of yeast-like fungi and yeasts belonging to 14 species and 3 genera were identified. The greatest number of fungal species (11) was isolated from the leech jaws, next (10) from the body surface, while the fewest species (8) were found from the samples of water in which the animals were maintained. Fungal isolates belonging to biosafety level two (BSL-2), classified as potential pathogens for humans identified were *Candida albicans*, *Candida ciferrii*, *Candida krusei*, *Candida tropicalis*, *Trichosporon asahii* and *Trichosporon asteroides*. Some other isolates with a decreased pathogenicity potential (BSL-1) also identified were *Candida guilliermondii*, *Candida famata*, *Candida lambica*, *Candida parapsilosis* and *Rhodotorula rubra.* The isolation of a high number of yeast-like fungal strains from *H. verbana* suggests that this medical leech is a vector of potentially pathogenic human fungal species.

Key words: Yeast-like fungi, *Candida* spp., leeches, *Hirudo verbana*, vector.

INTRODUCTION

All leeches are either predatory or parasitic carnivores. The majority of blood-sucking leeches live in fresh water environments. The typical habitat is a eutrophic pond with a muddy substratum with littoral vegetation (Elliott and Kutschera, 2011). Leeches feeding on the blood of numerous aquatic animals such as fishes, amphibians, water birds and mammals (including humans) are a source of pathogens for successive hosts. Additionally various microorganisms living in the natural water reservoir can colonize the leeches (Eroglu et al., 2001; Schulz and Faisal, 2010). More than 650 species of leech have been identified, but only 15 of them are used medically and are classified as medicinal leeches. In Europe, two species of leech, *Hirudo medicinalis* and *H. verbana* are mainly applied in hirudotherapy (Elliott and Kutschera, 2011). During feeding, leeches secrete different biologically and pharmacologically active substances into the wound. Leech saliva contains more than 100 bioactive compounds including coagulation inhibitors, platelet aggregation inhibitors, vasodilators, anti-inflammatory substances and a variety of enzymes, such as collagenase and hyaluronidase (Singh, 2010). Additionally, the application of leeches reduces venous congestion through active absorption of the patient's blood, and passive bleeding after detachment (Porshinsky et al., 2011).

Medical leeches are currently used in various medical specialties, especially in plastic and reconstructive

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surgery, microvascular replantation and traumatology (Porshinsky et al., 2011; Whitaker et al., 2012). The use of H. medicinalis was approved as a medical device by the U.S. Food and Drug Administration (FDA) in 2004. However, the use of leeches can be complicated by infections, especially with the bacterial genus Aeromonas Bacterial infections may vary from minor application in modern medicine greatly increased when wound complications, local abscess and cellulitis, to serious illness such as myocarditis, peritonitis, meningitis, bacteremia and sepsis (Bauters et al., 2007; Yantis et al., 2009; Bourdais et al., 2010). The extensive studies carried out on the gut bacterial flora of medical leeches show that *Aeromonas hydrophila* and *Aeromonas veronii* biovar sobria are the dominant symbiotic species living in the leech digestive tract (Worthen et al., 2006). However, these bacteria are important pathogens to humans. A high incidence of *Aeromonas* infection (2.4 to 36%) has been noted after application of medicinal leeches, despite their external decontamination before their medical use (Bauters et al., 2007). Moreover, infections with other pathogens such as *Serratia marcescens*, *Pseudomonas* spp., *Vibrio fluvialis* associated with medicinal leech therapy have also been reported (Porshinsky et al., 2011). Also, feeding leeches with fresh animal blood during the maintenance and reproductive phases poses the risk of transmission of pathogens to the patients. It has been experimentally demonstrated that many pathogens such as viruses, bacteria, and protozoan parasites from previous blood sources can survive within a leech for many months, and may be transmitted to mammalian hosts (Nehili et al., 1994; Al-Khleif et al., 2011).

A recent study revealed the presence of potentially pathogenic fungal species such as *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, on the jaws and body surface of *H. medicinalis* (Biedunkiewicz and Bielecki, 2010). Moreover, some cases of chromoblastomycosis caused by *Fonsecaea* species after wild leech bites have been reported (Ungpakorn and Reangchainam, 2006; Slesak et al., 2011). As a number of fungal species have been reported as etiological agents of human disease, a classification of fungi into biosafety categories was created, and the criteria for attribution to biosafety levels (BSL). In 1996, three BSL categories were formed by the European Confederation of Medical Mycology (de Hoog, 1996). Saprotrophic fungi or plant pathogens able to induce superficial and non-invasive or mild infections belong to BSL-1, whereas BSL-2 contains species that may cause deep, opportunistic mycoses in immunocompromised patients. Pathogens causing superficial infections also classified in BSL-2. Fungi from BSL-3 are pathogens potentially able to cause severe, deep mycoses in otherwise healthy patients. The medical leech used in hirudotherapy is a potential source of many pathogenic microbes for humans. The transmission of pathogens by leeches to patient can occur in several ways. The most common way is inoculation of microorganisms with their saliva into the feeding site. Moreover, blood-sucking leeches require full contact with the wound area, resulting in the contamination of the patient with the microbiota colonizing the body surface and the jaws of the leech. Additionally, the ingested blood in the leech alimentary tract could be re-injected into the host, along with the various microorganisms, by regurgitation during the manipulation of leech removal (Yantis et al., 2009).

The aim of this study was to assess the mycological flora of the body cover, jaws, pharynx and intestine of the widely used leech, *Hirudo verbana*, in hirudotherapy.

MATERIALS AND METHODS

Hirudo verbana

Fifty (50) *H. verbana* leeches with a mean weight of 3.40 g from Natural Medicine Center - Hirudinea (Lodz, Poland) were used. The animals were starved for three months prior to delivery to our laboratory and they were appropriate for use in hirudotherapy. The leeches were placed in five capped glass containers with sterile (boiled) water at a temperature of around 7°C and pH about 8.0. Ten leeches were kept in each container with 5 L of water for three weeks. The water was changed weekly. Water samples were collected after seven days since last change of water.

Isolation of fungal strains from leeches

The fungi were isolated from the water in which the leeches had been kept for 7 days as detailed earlier. Ten 30 ml water samples, without the leeches, were collected from each of the five containers. The samples were concentrated by centrifugation at 5,000 × g for 20 min at 20°C in sterile test tubes and 1 ml volumes of the resulting suspensions were used to inoculate Petri dishes of solid Sabouraud's dextrose medium (SDA) with chloramphenicol. Two Petri dishes were used for each suspension. The plates were incubated at 37°C for 48 to 72 h and then at 24°C for five days. The fungi from the surface of the leech bodies were isolated by washing each leech in 5 ml of Sabouraud dextrose broth (SDB) and incubated at 37°C for 48 to 72 h. This was followed by transfer of 1 ml sample from each of the liquid cultures to the SDA plates and incubated at 37°C for 2 days. The leech jaws, pharynx and intestine were separately prepared under a stereomicroscope, with 20x magnification. All animals were held with sterile gloves and prepared in sterile conditions. Before dissection animals were disinfected externally with alcoholic solution of povidone-iodine accordingly as described by Hokelek et al. (2002). They were then attached to a sterile polystyrene foam plates by sterile pins. The jaws located on the anterior sucker were carefully prepared using a sterile scalpel and tweezers. The preparation of pharynx and intestine was performed according to techniques described by O'Gara et al. (1999) and Worthen et al. (2006), respectively. Three dissected jaws and one pharynx from each leech were collected into one test-tube. The jaws/pharynx and intestine were separately incubated sequentially in 3 ml of SDB and SDA plates at 37°C for 48 to 72 h each. Two SDA palates were used for each transferred 1 ml of SDB.

The mycological examinations were conducted based on procedures introduced in the Department of Diagnostics and Treatment of Parasitic Diseases and Mycoses, Medical University of Lodz (Kurnatowska and Kurnatowski, 2008). The incubated plates were subjected to macroscopic observation. Positive fungal

Table 1. Isolated yeast-like and yeast fungi species from *Hirudo verbana* and water samples.

Species/genus	Place of isolation	BSL* biosafety levels
Candida albicans	IW, JP, BS	2
Candida ciferrii	JP, BS	2
Candida famata	JP, BS	1
Candida guilliermondii	JP, BS	1
Candida krusei	IW	2
Candida lambica	BS	1
Candida parapsilosis	JP, BS	1
Candida tropicalis	IW, BS	2
Lipomyces starkeyi	IW, JP	ND
Rhodosporandium sp.	JP, BS	ND
Rhodotorula rubra	IW, BS	1
Schizosacharomyces sp.	JP, BS	ND
Trichosporon asahii	IW	2
Trichosporon asteroides	IW	2
Trichosporonoides oedocephalis	JP	ND
Trichosporonoides sp.	JP	ND
Yarrowia lipolytica	IW, JP	ND

IW - incubation water, BS - body surface, JP - jaws/pharynx, ND - BSL not defined, *according to de Hoog (1996).

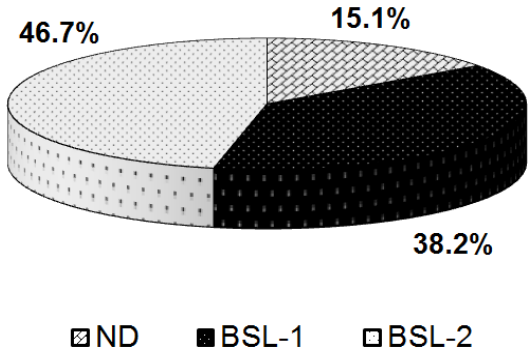

Figure 1. The percentages of fungal strains belonging to different biosafety levels (BSL) isolated from all cultures.

macrocultures were subcultured on SDA for the isolation of a pure, single colony for species identification. In order to evaluate the morphological and biochemical characteristics of the isolated fungi, the following methods were applied: direct microscopic slides, microculture technique, selective media such as Nickerson's medium and biochemical tests for example, auxanograms. Initially, the isolated fungi were determined to species/genus level on the basis of keys by De Hoog et al. (2000) and Kurtzman and Fell (2000). The axenic strains were definitively identified on the basis of the biochemical characteristics of their carbohydrate assimilation using the API 20C AUX test, according to the principle of numerical identification (Analytical Profile Index, BioMeriéux, Lyon 1990).

Statistical analysis

The differences between groups were compared by the Chi-square test or Fisher's exact test. Values of $p < 0.05$ were taken as significant. All calculations were performed using STATISTICA v. 10.0 software.

RESULTS

Fungi were found in all samples from the body surface, the leech jaws/pharynx and water samples. No fungi were recorded in the leech intestines. The presence of yeasts and yeast-like fungal species isolated from examined materials is shown in Table 1. Mycological examinations of 150 cultures from 50 leeches and 50 samples of water identified 152 fungal strains of yeast-like fungi, and yeasts belonging to 14 species and 3 genera (Table 1). A similar number of fungal species was found in the three materials. Eleven (11) species were isolated from the leech jaws/pharynx, 10 from the body surface, and 8 species were found from the samples of water in which the animals were maintained.

As seen in Table 1, fungi classified as potential human pathogens (BSL-2), species with a lowered potential of pathogenicity (BSL-1) and saprotrophic fungi (ND) were detected. Saprotrophic fungi not pathogenic to humans and animals are not classified to any BSL categories. Five species belonging to BSL-2 were isolated from the water samples. Three species with BSL level 2 were found in macrocultures from the body surface of the leeches and two from their jaws/pharynx. No statistically significant differences were found in the number of species assigned to the BSL-2 category isolated from the aforementioned materials ($P>0.05$). Nearly half of the 152 detected isolates (46.7%) belonged to BSL-2 (Figure 1). Isolates from BSL-2 and BSL-1 were found to be, respectively, 3 and 2.5 times more common than

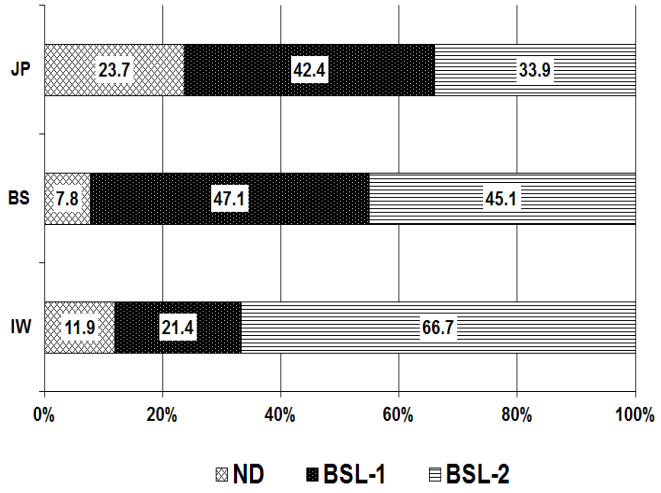

Figure 2. The percentages of fungal strains from different BSL categories isolated from water (IW), jaws/pharynx (JP) and body surface (BS) of *Hirudo verbana.*

saprotrophic strains (ND). Figure 2 shows the percentage distribution of fungal strains according to BSL categories and their place of isolation. There was a significant difference in number fungal strains isolated from the different biological samples (Chi^2 = 15.058, df = 4, P = 0.00458).

In the water samples, the largest number of strains (28 strains; 18.4% of total isolates) belonging to BSL-2 was detected, and it was significantly different from the numbers of strains from BSL-2 isolated from the jaws/pharynx (Chi^2 = 10.564, df = 1, P = 0.00115) and body surface of leeches (Chi^2 = 4.326, df = 1, P = 0.0375). Similar numbers of BSL-1 isolates (P>0.05) were identified from cultures of the jaws/pharynx (25) and body surface (24), while only 9 strains from BSL-1 class were discovered in water samples. The most frequently detected fungi were *C. albicans* (20.4%), *C. parapsilosis* (17.1%) and *C. tropicalis* (13.2%). *C. albicans* strains were found in three examined materials; 31 strains were isolated from the body surface of the leech (11), its jaws/pharynx (13) and also the water samples (7).

Six biochemical phenotypes of *C. albicans* were assigned with different numerical assimilation profiles (Analytical Profile Index, bioMérieux, Lyon 1990), and among the isolates, two codes dominated: 2576154 (35.5%) and 2576174 (29.0%) as shown in Table 2. From five biotypes of *C. tropicalis*, phenotype 2556375 dominated, whereas code 6756175 was isolated most often from strains of *C. parapsilosis*. Some species occurred less frequently: *C. guilliermondii*, *C. famata*, *C. ciferri*, *Rhodotorula rubra*, *Lipomyces starkeyi*, *Yarrowia lipolytica* and *Trichosporonoides oedocephalis*. Other species/genera such as *C. krusei*, *C. lambica*, *Trichosporon asahii* and *Trichosporon asteroides*, *Rhodosporandium* sp., and *Schizosacharomyces* sp. were isolated occasionally.

DISCUSSION

In this study, the six fungal species isolated from water samples, body surface and jaws/pharynx of *H. verbana* were classified as BSL-2 and five isolates as BSL-1, despite the leeches being kept in sterile laboratory conditions. A study by Biedunkiewicz and Bielecki (2010) also identified potentially pathogenic *Candida* species on the body surface and jaws of *H. medicinalis*. It should be noted that the leeches used in this and the study aforementioned were cultured in two different leech farms, and so had never been in contact with the natural environment. Fungal colonization of these leeches was probably a consequence of the non-sterile conditions associated with breeding, growing or transport. This could be because *Candida* species are ubiquitous that is, may be found in fresh water, soil, fruit, animals or humans (Schauer and Hanschke, 1999) and are the most common fungal pathogens that infect humans.

The sources of microbial contamination leeches at the leech farms could be leech tanks and water, the ground where leech cocoons are incubated, blood meal given to growing animals or farm workers, especially their hands. *C. albicans* is considered an opportunistic pathogen which frequently colonises human skin (Kim and Sudbery, 2011). It was confirmed that human hands are an important route for transmission of fungi from one person to another, and from people to inanimate surfaces, and hand hygiene still remains the major preventive measure against nosocomial infections (Yildirim et al., 2007).

C. albicans strains were detected from all examined materials with the exception of *H. verbana* intestine. Because most of these strains were isolated from leeches kept in laboratory conditions, it can be assumed that the animals were first colonized by them during farming or transport and then the sterile water used has been secondarily contaminated. The absence of fungal strains in leech intestines is most likely due to the intensive colonization of their digestive tract by symbiotic bacteria, which inhibit the growth of other microorganisms (Worthen et al., 2006). In our study, numerous bacteria belonging to the genus *Aeromonas* were isolated from intestinal cultures. Among six of the *C. albicans* strains isolated from *H. verbana*, two biochemical phenotypes (2576154, 2576174 - API-20C AUX) were predominant and they were detected both from cultures of jaws/pharynx and body surface, as well as from water samples. These same *Candida* strains, with numerical assimilation profiles 2576174 and 2576154, were found most frequently by other authors in both people with fungal skin colonisation and candidosis (Williams et al., 2000; Glowacka, 2002). In particular, a strain of *C. albicans* coded 2576174 is the most common strain observed in people with symptomatic candidoses (Williams et al., 2000). The API-20C AUX test is used successfully in epidemiological studies (Kurnatowska and Kurnatowski, 2008). A comparative analysis of the assimilation phenotypes of strains of the same species

Table 2. Numerical identification codes (Analytical Profile Index, bioMérieux, Lyon, 1990) of isolated strains (n = 129) belonging to BSL class 1 or 2 from biological materials of *Hirudo verbana* and water samples.

Species (No. of strains)	Code	Number of strains	
		n	% ± SD
Candida albicans (n = 31)	2576154	11	35.5 ± 8.59
	2576174	9	29.0 ± 8.15
	2576074	5	16.1 ± 6.60
	2566174	3	9.7 ± 5.31
	2572174	2	6.5 ± 4.42
	2576174	1	3.2 ± 3.16
Candida ciferrii (n = 11)	6701366	6	54.5 ± 15.01
	6671366	4	36.4 ± 14.07
	6643176	1	9.1 ± 8.67
Candida famata (n = 9)	2576773	5	55.6 ± 16.56
	6756373	2	22.2 ± 13.85
	6756773	2	22.2 ± 13.85
Candida guilliermondii (n = 9)	6756377	6	66.7 ± 15.71
	6676371	3	33.3 ± 15.71
Candida krusei (n = 3)	1000005	3	100 ± 0.0
Candida lambica (n = 2)	2400004	2	100 ± 0.0
Candida parapsilosis (n = 26)	6756175	9	34.6 ± 9.32
	6756135	7	26.9 ± 8.60
	2756175	5	19.2 ± 7.72
	2656175	3	11.5 ± 6.26
	6756171	1	3.8 ± 3.75
	6756131	1	3.8 ± 3.75
Candida tropicalis (n = 20)	2556375	7	35.0 ± 10.66
	2556175	5	25.0 ± 9.68
	2576175	5	25.0 ± 9.68
	6556175	2	10.0 ± 6.71
	2552174	1	5.0 ± 4.87
Rhodotorula rubra (n = 12)	6402073	7	58.3 ± 14.23
	2610062	5	41.7 ± 14.23
Trichosporon asahii (n = 5)	2744775	3	60 ± 21.91
	2767735	2	40 ± 21.91
Trichosporon asteroids (n = 1)	3364325	1	100 ± 0.0

isolated from different parts of human body or several persons can help determine intra- and inter-human transmission of pathogenic fungi. Moreover, horizontal transmission (environment-people) of *C*. *albicans* strains was confirmed on the basis of the identity of digital codes of strains isolated from human skin lesions and the sanitary devices with which they had contact (Glowacka, 2002). In our studies, *Candida* strains (*C. albicans* and *C. tropicalis*) detected in water samples were found to have the same numerical assimilation profiles as those seen in cultures of biological materials of *H*. *verbana.* Hence, the water in which the leeches were being kept was contaminated by strains colonizing the body surface and/or jaws. Among the non- *C. albicans* leech isolates,

predominant assimilation phenotypes of *C. tropicalis* and *C. parapsilosis* were frequently detected in human biological materials collected from patients with nosocomial mycoses (Ng et al., 2001). Our results suggest that fungal assimilation biotypes colonising leech jaws/pharynx and body surfaces may be the cause of wound complications occurring during hirudotherapy. Hence, the maintenance of sterile conditions for the culture, transport and storage of medical leeches is of paramount importance.

Today, leech therapy is indicated in plastic and reconstructive surgery to relieve venous congestion and to improve the microrevascularization of flaps or replants, with a 60 to 83% increase in success rate (Bourdais et al., 2010; Whitaker et al., 2012). Moreover, the postoperative application of leeches carries the risk of microbial infection. In the presence of infection as a complication of the medicinal use of leeches, the success rate for flap salvage may decrease to over 30% (Whitaker et al., 2012). Hence, the sterility of the leeches used in hirodotherapy is a fundamental aspect of patient safety. The results of the present study underline the importance of maintaining sterile conditions not only during storage of medical leeches but also during their development and growth in leech farms.

Conclusion

The identification of fungi and yeast-like fungi on the body surfaces and jaws/pharynx of *H. verbana* kept under optimum laboratory conditions implies that this leech can act as a vector of these potential human pathogens.

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