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Molecular screening for enteric parasites and subtyping of *Blastocystis* sp. in haemodialysis patients in Slovakia

Elena Hatalová[1,A-F], Ingrid Babinská[1,A-C,E]✉, Andrea Gočálová[2,A-B], Ingrid Urbančíková[1,E-F]

[1] *Department of Epidemiology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Slovakia*
[2] *Logman Ltd. Nephrodialysis Centre in Košice, Slovakia*
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Abstract

Introduction and Objective. Intestinal parasitoses are important causes of morbidity and mortality, especially in immunocompromised individuals. In patients with chronic renal insufficiency (CRI), the accumulation of non-excreted metabolites leads to uraemia, which induces a state of immunodeficiency, increasing the incidence of infections. The aim of the study was molecular screening for enteric protozoa in patients with chronic renal insufficiency.

Materials and Method. A total of 53 samples were collected in January 2023 from patients undergoing dialysis at Logman Ltd. Nephrodialysis Centre in Košice, Slovakia. Samples were examined by polymerase chain reaction (PCR) for the presence of *Cryptosporidium parvum / Cryptosporidium hominis*, *Giardia intestinalis*, Microsporidia spp., and *Blastocystis* sp.

Results. From the 53 samples, the only pathogen identified by PCR was *Blastocystis* sp., in 13 patients (24.5 %). Sequence analyses confirmed that the most prevalent subtype (ST) among patients was ST 3 (n=9, 69.2%), followed by ST 1 (n=3, 23.1%) and ST 2 (n=1, 7.7%).

Conclusions. Molecular methods for the detection of microscopic enteric parasites are not used as a first-line diagnostic method in Slovakia. In immunocompromised patients, diarrhoea can be caused not only by a chronic disease or therapy but can also be a result of an ongoing underdiagnosed infection. Early diagnosis leads to targeted therapy and subsequent partial improvement of the quality of life. This study also shows the first insights into *Blastocystis* sp. subtype distribution in humans in Slovakia.

Key words

PCR, dialysis, Slovakia, *Blastocystis*, protozoa

INTRODUCTON

Intestinal parasitoses are important causes of morbidity and mortality, especially in immunocompromised individuals. In patients, in which immunosuppression is the result of an ongoing illness or immunosuppressive therapy, an increased risk exists of acquiring parasitic infections with a high degree of severity. Such infections are mostly caused by opportunistic pathogens, of which *Cryptosporidium* (*C.*) is one of the most commonly encountered parasites [1]. Microsporidia spp. and *Blastocystis* sp. have also emerged in recent years, mostly due to the introduction of molecular diagnostic methods [2, 3]. Infections caused by *Giardia intestinalis* (*G. intestinalis*) are present in both immunosuppressed and immunocompetent patients [4].

In patients with chronic renal insufficiency (CRI), the accumulation of non-excreted metabolites leads to uraemia, which induces a state of immunodeficiency, increasing the incidence of infections which are responsible for 48% of deaths in these individuals [5].

Blastocystis sp. is an intestinal parasite with a wide variety of hosts, including humans [6]. It is estimated that this parasite colonizes the intestine of more than one billion people worldwide, with a prevalence up to 55% and with significant differences between developed and developing countries [7, 8]. In some risk cohorts the prevalence may reach 100% [9]. The pathogenic potential of *Blastocystis* sp. is still not clear, but their presence has been reported in immunocompromised individuals with gastrointestinal symptoms, such as cancer patients, HIV/AIDS patients, and haemodialysis patients with CRI [5, 10, 11].

The most common methods for the diagnosis of enteric protozoa are based on microscopy. These methods are limited mostly by the low concentration of excreted pathogens, misinterpretation of the findings (especially in the case of *Blastocystis* sp., which occurs in four different morphological forms), and substitution for other pathogens, particularly in cases of co-infection. Another issue is the morphological identity of isolates obtained from both human and animal samples, neglecting the zoonotic potential of the pathogen [12–14].

With the use of molecular methods, such as PCR amplification using specific primers, it is possible to distinguish individual species, as well as genotypes, subtypes and/or assemblages of protozoan and fungal pathogens. It is necessary to identify individual subtypes to determine their zoonotic potential, importance for public health, pathogenicity, and sources of infection.

The aim of the study is to assess the presence of enteric

✉ Address for correspondence: Ingrid Babinská, M.D., Ph.D., MPH, Department of Epidemiology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Slovakia, Tr. SNP 1, 040 11 Košice, Slovakia, tel.: +421 55 234 3270
email: ingrid.babinska@upjs.sk

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protozoa and fungi by molecular methods in haemodialysis patients, since molecular diagnostic of parasitoses is not a standardized method in Slovakia, and is used only as a supplementary examination.

MATERIALS AND METHODS

Population study. A total of 53 samples of faeces were collected from patients with CRI (35 males, 18 females), undergoing haemodialysis at Logman Ltd. Nephrodialysis Centre in Košice, Slovakia. Samples were obtained from adults aged 30–84 years, and stored at -20 °C until DNA extraction.

Approval for the study was obtained from the Ethics Committee of the Faculty of Medicine at the Pavol Jozef Šafárik University in Košice (Approval No. 2N/2021), and from the Ethics Committee of FMC– Dialysis Services Ltd., also in Košice. Informed consent was obtained from the patients prior to the examination. Participants were asked to complete a short questionnaire to collect the following information: demography, socio-economic status, sources of drinking water, presence of pets and farm animals, and the presence of rodents near the residence.

DNA extraction. Genomic DNA was extracted according to the protocol described by Danišová et al. (2017). Approximately 100 mg of faeces were homogenized at 6,500 rpm for 90 seconds with the addition of 0.5 mm glass beads, 1.0 mm zircon beads, and 300 µl of lysis solution in Precellys 24 homogenizer (Bertin Technologies). DNA was extracted using the DNA-Sorb-B nucleic acid extraction kit (AmpliSense, Russia) [15]. Isolated DNA was stored at –20 °C until use in PCR.

Molecular diagnostic of *C. parvum* / *C. hominis*, *G. intestinalis* and Microsporidia spp. All three diagnostic methods were carried out by nested PCR. For identification of *Cryptosporidium*, genotyping primers targeting the GP60 gene of *C. parvum* and *C. hominis* were used (GP60 F1 / GP60 R1 5′-ATG AGA TTG TCG CTC ATT ATC-3′ / 5′-TTA CAA CAC GAA TAA GGC TGC-3′ in the first reaction, and GP60 F2 / GP60 R2 5′-GCC GTT CCA CTC AGA GGA AC-3′ / 5′-CCA CAT TAC AAA TGA AGT GCC GC-3′ for the second reaction) [16]. For identification of *G. intestinalis* assemblages, primers targeting the triosephosphate-isomerase (tpi) gene were selected (AL3543 / AL3546 5′-AAA TTA TGC CTG CTC GTC G-3′ / 5′-CAA ACC TTT TCC GCA AAC C-3′ in the first reaction, and AL3544 / AL3545 5′-CCC TTCA TCG GTG GTA ACT T-3′ / 5′-GTG GCC ACC ACT CCC GTG CC-3′ in the second reaction) [17]. For identification and distinguishing Microsporidia spp. primers targeting the SSU, ITS and LSU rDNA region were selected (MSP-1 / MSP-2a 5′-TGA ATG KGT CCC TGT-3′ / 5′-TCA CTC GCC GCT ACT-3′, and MSP-3 / MSP-4a 5′-GGA ATT CAC ACC GCC CGT CRY TAT-3′ / 5′-CCA AGC TTA TGC TTA AGT YMA ARG GG-3′) [18].

Nested PCR was carried out in a Biometra Tone thermal cycler (Analytic Jena GmbH, Germany), using 5x HOT FIREPol Blend Master Mix with 7.5 mM $MgCl_2$ (Solis Biodyne, Estonia), with a total reaction volume of 20 µl and 0.1 µM concentration of individual primers. Cycling conditions were set depending on the annealing temperature of individual primers and elongation time for the expected product length. Cycling conditions for individual primers are summarized in Table 1.

Table 1. PCR protocol for *Cryptosporidium*, *Giardia* and Microsporidia identification

Operation	Temperature (primers)	Time (primers)	cycles
Initial activation	95°C	15 min	1
Denaturation	95°C	20 sec	30
Annealing	55°C / 58°C (GP60, MSP)	45 sec	
	50°C (AL)	45 sec	
Elongation	72°C	1 min 30 sec / 45 sec (GP60, MSP)	
	72°C	45 sec (AL)	
Final elongation	72°C	7 min	1

Molecular diagnostic of *Blastocystis* sp. For *Blastocystis* sp. subtyping, a semi-nested PCR was used to ensure adequate amplification of the low concentration of DNA in the isolated samples.

Primers targeting the SSU rDNA were used (Blast 505–532 / Blast 998–1017 5′-GGA GGT AGT GAC AAT AAA TC-3′ / 5′-TGC TTT CGC ACT TGT TCA TC-3′) [19]. Master mix for PCR was performed as previously described. Cycling conditions were 95 °C for 15 minutes, followed by 30 cycles of 95 °C for 20 seconds, 54 °C for 45 seconds, and 72 °C for 45 seconds. Final elongation was included at 72 °C for seven minutes. The PCR product from this reaction was used as a template for the second reaction with the same primers and conditions.

Gel electrophoresis and DNA sequencing. Final products after PCR (450 bp with primers GP60 F2/R2, 530 bp with primers AL3544/AL3545, 508 bp for *Enterocytozoon* spp., and 305 bp for *Encephalitozoon intestinalis* with primers MSP-3 / MSP-4a, 479 bp with primers Blast 505–532 / Blast 998–1017), were evaluated by gel electrophoresis in 1.5% agarose gel dyed with GoodView in TBE buffer. Positive samples were sent for DNA sequencing.

Phylogenetic analysis. Final sequences were compared with homologous sequences stored in the GenBank (NCBI) using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Consensus sequences were edited and assembled using the BioEdit programme. After assembly, sequences were aligned using Alignment ClustalW2 with all reference sequences from GenBank. Phylogenetic trees were constructed using Maximum likelihood method in the MEGA X programme. Bootstrap values were calculated from 1,000 replicates. *B. lapemi* was used as an outgroup.

RESULTS

The basic characteristics of the haemodialysis patients enrolled in this study and the characteristics of *Blastocystis*-positive patients are presented in Table 2.

After evaluation by gel electrophoresis, none of the 53 samples showed positivity for *C. parvum* / *C. hominis*, *G. intestinalis,* or Microsporidia spp.. *Blastocystis* sp. was confirmed in 13 patients (24.5 %). From the *Blastocystis*-positive patients, 76.9% reported secondary education, 69.2%

Table 2. Basic characteristics of patients enrolled in the study, and characteristics of *Blastocystis*-positive patients

Characteristics	Sample (N=53) n (%)	Positive (N=13) n (%)
Age, mean (ST)	65 (12.2)	61.5 (14.6)
Sex		
Male	35 (66)	7 (53.8)
Female	18 (34)	6 (46.2)
Education		
Elementary	10 (18.9)	2 (15.4)
Secondary	38 (71.7)	10 (76.9)
University	5 (9.4)	1 (7.7)
Residence		
City	26 (49.1)	4 (30.8)
Country	27 (50.9)	9 (69.2)
Source of water		
Community water supply	46 (86.8)	10 (76.9)
Private well	10 (18.9)	3 (23.1)
Natural spring	2 (3.8)	0 (0)
Bottled water	46 (86.8)	12 (92.3)
Water fountains	24 (45.3)	8 (61.5)
Domestic animal breeding	21 (39.6)	5 (38.5)
Animal husbandry nearby	6 (11.3)	2 (15.4)
Rodents near residence	21 (39.6)	8 (61.5)

reported living in the countryside, and 61.5% reported the presence of rodents near their residence. Sequence analyses confirmed the most prevalent subtype among patients was ST 3 (n=9, 69.2%), followed by ST 1 (n=3, 23.1%) and ST 2 (n=1, 7.7%). Phylogenetic analysis of samples positive for *Blastocystis* sp. is represented in Figure 1.

Sequences obtained in this study were submitted to GenBank under accession numbers OQ913675, OQ913677, OQ913680, OQ913681, OQ913685, OQ913686, OQ913700, OQ913704, OQ913706OQ913708, OQ913709, OQ913715 and OQ947043.

DISCUSSION

The elongation of human life and expanded life expectancy also lead to the increased occurrence of life-long conditions, such as chronic renal failure [5, 20]. The loss of renal functions leads to uraemia, changing natural and acquired immunity. This has a negative impact on neutrophil chemotaxis, phagocytosis, and T-cell functions, creating a state of immunosuppression, which leads to increased susceptibility to infections [21]. In recent decades, there has

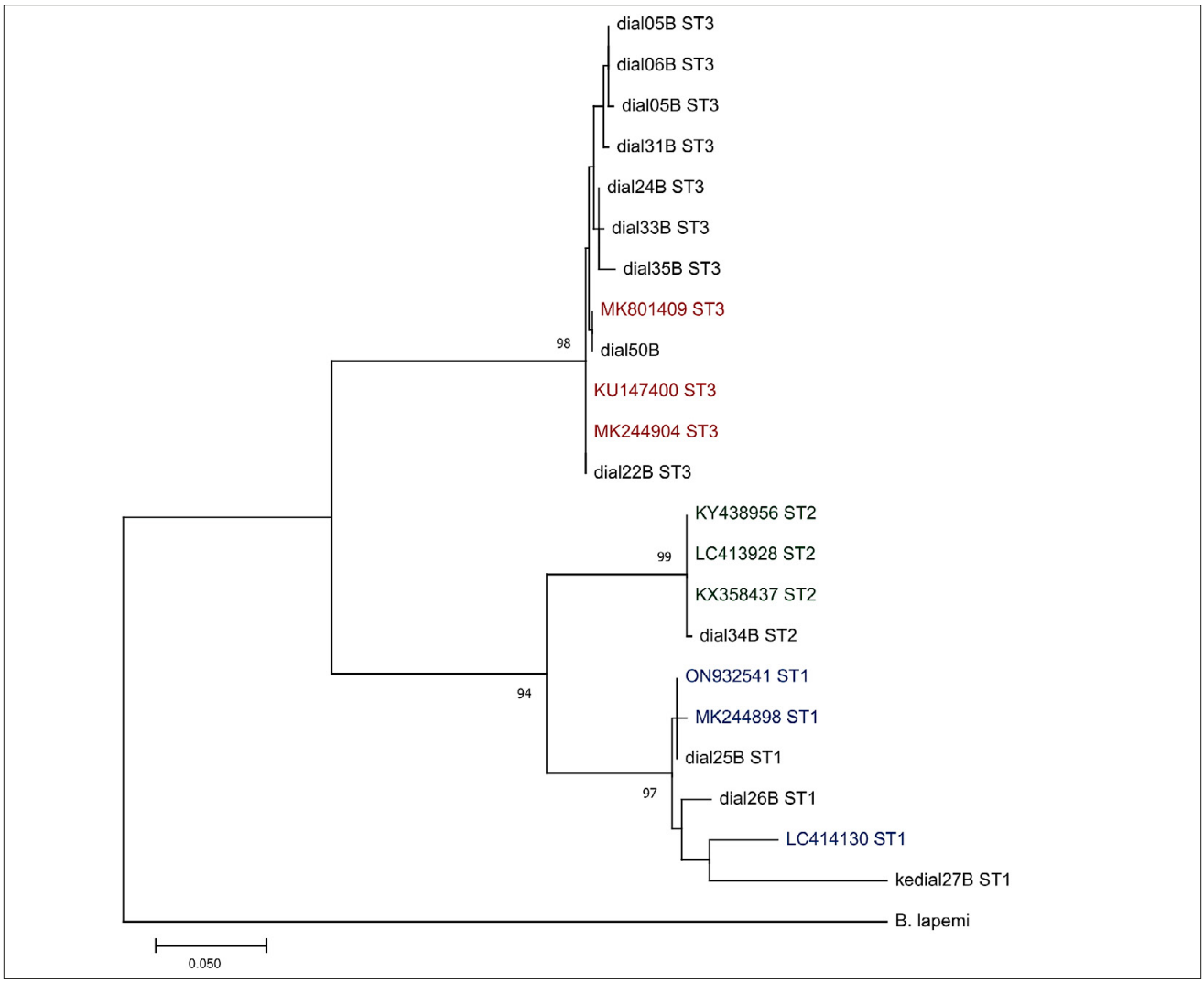

Figure 1. Phylogenetic tree based on a maximum likelihood method analysis of the SSU rDNA region of *Blastocystis* sp.. Isolates obtained from haemodialysis patients (represented as "dial") forming clades with reference sequences obtained from GenBank, represented by accession numbers and respective subtype, and marked by color (red for ST3, dark green for ST2 and dark blue for ST1). *B. lapemi* was selected as an outgroup

been an increase in the number of studies regarding parasitic infections in patients with CRI undergoing dialysis. These studies show significant infection rates in these patients, mainly caused by enteric protozoa [5, 22].

The current study aimed to assess the presence of intestinal protozoan and fungal pathogens (*C. parvum* / *C. hominis*, *G. intestinalis*, Microsporidia spp., and *Blastocystis* sp.) by molecular methods in a group of patients with acquired immunodeficiency caused by uraemia associated with CRI.

The only pathogen identified in the panel was *Blastocystis* sp., which was present in 24.5% of samples. The occurrence was higher in females (33.3%) than in males (20%). Results of this study suggest that patients living in the countryside and patients reporting the presence of rodents near their residence are seen as having a higher risk for acquiring *Blastocystis* infection. However, considering the small number of patients in this study, the results should be generalized with caution.

There have been several studies published identifying *Blastocystis* sp. in haemodialysis patients. In Brazil, Kulik et al. (2008), Gil et al. (2013) and Gama et al. (2018) assessed the prevalence of *Blastocystis* sp. in dialysis patients to be 20.9%, 24.5%, and 41.2%, respectively. In Turkey, Karadag et al. (2015) identified *Blastocystis* sp. in 23.9% of samples from dialysis patients. Other studies from Saudi Arabia and Iran showed *Blastocystis* sp. positivity in dialysis patients ranging from 4.4–14.1%. In all studies mentioned above, conventional diagnostic methods were used, including the native-Lugol method, formalin-ether concentration technique, and trichrome staining [5, 20, 22–27]. The wide variety of *Blastocystis* sp. prevalence in the conducted studies may be related to different geographic regions, demographics, socio-economic status, or different diagnostic methods used for detection [28].

Fewer studies were focused on subtyping individual *Blastocystis* isolates in dialysis patients. In 2020, Gulhan et al. published a study focused on subtype distribution and molecular characterization of *Blastocystis* isolates in dialysis patients in Turkey. Seven samples positive after native-Lugol and trichrome staining were subjected to subtyping. From these, six samples were subtyped successfully, with the following subtypes: ST1 in one sample, ST2 in two samples, ST3 also in two samples, and a combination of ST3+ST6 in one sample [2]. Silva et al. (2020) subtyped *Blastocystis* sp. in three groups of transplant candidates, from which the highest occurrence of *Blastocystis* sp. was found in renal transplant candidates (31.4%), with a dominant prevalence of ST1 (50%), followed by ST3 (37.5%), and equally by ST2 and ST7 (6.25%) [29].

The most prevalent subtype identified in our study was ST3 (69.2%), followed by ST1 (23.1%) and ST2 (7,7%). Worldwide, *Blastocystis* infections are mostly caused by subtypes 1–4, with ST3 being the most frequently isolated subtype in epidemiological studies [30–34]. The results obtained in our study are consistent with these findings.

To date, more than 20 *Blastocystis* subtypes have been described in animal and human hosts, according to the diversity of the SSU rDNA region [35]. Twelve subtypes ST1-ST10, ST12, and ST14 have been identified in humans. Eleven of these subtypes were also identified in animal hosts (except ST9, which is considered a strictly human pathogen), suggesting zoonotic potential. All other subtypes were identified only in animal hosts [28, 36–40].

Currently, there are only three studies from Slovakia focused on molecular identification and subtyping of *Blastocystis* sp., conducted on wild, domestic and Zoo animals. In these studies, ST5, ST7, ST10 and ST12 were identified [41–43]. Even though these subtypes are also zoonotic, they differ from the subtypes identified in our study, suggesting different subtype distribution in the population of animals and humans in Slovakia.

The limitation of this study is the small group of patients, therefore, even with complete anamnesis and completed questionnaires, no statistically significant statements can be extrapolated from the data.

CONCLUSION

Molecular methods for diagnosing enteric pathogens are gradually replacing conventional diagnostic methods, especially in industrialized countries. In Slovakia, molecular diagnosis of intestinal protozoa and fungi are still not used as a first-line method.

This study was aimed at the molecular detection of intestinal protozoan and fungal pathogens in haemodialysis patients, and is also the first study subtyping *Blastocystis* sp. in humans in Slovakia. Although the study was carried out on a small population, the results should encourage facilities dealing with immunocompromised patients to utilize molecular diagnostics. In these patients, diarrhoea can be caused not only by a chronic disease or therapy but can also be a result of an ongoing underdiagnosed infection. Early diagnosis in these patients can lead to targeted therapy and subsequent partial improvement in the quality of life.

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