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Srovnání molekulárně diagnostických přístupů pro detekci a diferenciaci střevního prvoka *Blastocystis* sp. u lidí

Rigorózní práce

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Annotation:

Blastocystis is the most commonly found intestinal protist in the world. Accurate detection and differentiation of Blastocystis including its subtypes (arguably species) is essential for understanding its epidemiology and role in human health. We compared the sensitivity of conventional PCR (cPCR) and qPCR in a set of 288 DNA samples obtained from stool samples of gut-healthy individuals and subtype diversity as detected by next-generation sequencing (NGS) versus Sanger sequencing.

Prohlášení:

Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracovala pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

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Comparison of molecular diagnostic approaches for the detection and differentiation of the intestinal protist *Blastocystis* sp. in humans

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Abstract:

Blastocystis is the most commonly found intestinal protist in the world. Accurate detection and differentiation of *Blastocystis* including its subtypes (arguably species) is essential for understanding its epidemiology and role in human health. We compared the (i) sensitivity of conventional PCR (cPCR) and qPCR in a set of 288 DNA samples obtained from stool samples of gut-healthy individuals and (ii) subtype diversity as detected by next-generation sequencing (NGS) versus Sanger sequencing. Real-time PCR resulted in more positive samples than cPCR, revealing high fecal load of *Blastocystis* based on the quantification curve in most samples. In subtype detection, NGS was largely in agreement with Sanger sequencing but showed higher sensitivity for mixed subtype colonization within one host. This fact together with using of the combination of qPCR and NGS and obtaining information on the fecal protist load will be beneficial for epidemiological and surveillance studies.

Key words:

Blastocystis; conventional-PCR; qPCR; sensitivity; quantification; NGS;

INTRODUCTION

Blastocystis sp. is a unicellular eukaryote colonizing the gastrointestinal tract of humans and various other species. Although discovered more than a century ago, its role in human health and disease has not been fully understood. Knowledge gaps remain in its epidemiology and interaction with the host, as well as factors affecting host colonization [1-3]. *Blastocystis* may be the most common intestinal human protist in the world, colonizing more than 1 billion people [4]. In some cohorts, the prevalence of *Blastocystis* sp. may reach 100% [5]. Based on small ribosomal subunit (SSU *rRNA*) gene analysis, at least 22 subtypes (ST) exist across mammalian and avian hosts [6]. Among these subtypes, ST1–ST9 and ST12 have been found in humans, with ST1–ST4 being commonly detected [3].

Despite the numerous surveys on *Blastocystis* sp., no consensus has been reached on the choice of method(s) for detection and differentiation of the protist (reviewed in Skotarczak [7]). Moreover, in spite of the development of molecular approaches, traditional microscopic examination of ova and parasites (O&P) and xenic culturing is still commonly used in laboratories to detect *Blastocystis* [8]. However, these methods require specialized technicians [8], they are less sensitive, and do not provide subtype information [9-11]. Nevertheless, accurate detection and distinction of *Blastocystis* subtypes is essential for understanding the transmission and the role of this protist in human health. Due to their high sensitivity and specificity, molecular methods such as conventional PCR (cPCR) or real-time PCR (qPCR) are often used [7,12,13]. In addition, next-generation sequencing (NGS) is gaining prominence in detection *Blastocystis* and its subtypes [14-16].

The aim of this study was to compare (i) the sensitivity of cPCR and qPCR on a set of DNA samples obtained from stool samples of individuals with no gastrointestinal symptoms and (ii) subtype diversity detected by cPCR and Sanger sequencing versus NGS.

MATERIAL AND METHODS

In this study, we used 288 DNA samples obtained from fresh stool samples from a cohort created during a previous survey on the prevalence and diversity of *Blastocystis* in a gut-healthy human population in the Czech Republic (for more details on the collection and DNA extraction see Lhotská et al. [11]). We also used data on the positivity rate of *Blastocystis* sp. resulted from cPCR [11] for comparison with qPCR results obtained in the present study. Here, we applied the diagnostic qPCR protocol published in the study by Stensvold et al. [12]. The primers target the SSU rDNA fragment of 118 bp, which is detected by a Taqman probe. Samples were processed using a LightCycler LC 480 I (Roche, Basel, Switzerland) with a 96-well block. The cycling conditions consisted of primary denaturation (95 °C/10 min) and 37× (95 °C/15 s, 60 °C/30 s, 72 °C/30 s). The results of qPCR on *Blastocystis* were then compared with the results of conventional PCR (from Lhotská et al. [11]) using McNemar's test with Yates's correction (0.5). Statistical analysis was performed using the software SciStatCalc 2013 (https://scistatcalc.blogspot.com/2013/11/mcnemars-test-calculator.html).

36 Positive samples from qPCR were subjected to amplicon NGS to determine *Blastocystis*
37 subtypes: an informative fragment of SSU rDNA (~450 bp) was amplified, indexed and sequenced on
38 a MiSeq instrument with the Reagent Kit v2, 2×250 bp (Illumina); this was performed according to the
39 method by Maloney et al. (2019) [17] with minor modifications in Cinek et al. [15] (for more detail
40 see Supplementary data 1). These results were compared with the results on subtype diversity
41 described in Lhotská et al. [11] based on Sanger sequencing. Fecal protist load was estimated based on
42 a quantification curve generated from a dilution series of cultured *Blastocystis* ST3, which was set in
43 the range of 10^0 to 10^5 cells per one qPCR reaction: 10^0–10^1 - mild fecal protist load; 10^2–10^3 -
44 moderate fecal protist load; 10^4–10^5 - high fecal protist load (Supplementary data 2). *Blastocystis* cells
45 counts from culture were calculated using a Bürker`s chamber and then serially diluted to obtain
46 aliquots containing 10^0, 10^1, 10^2, 10^3, 10^4, and 10^5 cells, which were subsequently subjected to DNA
47 extraction according to Lhotská et al. [11]. All negative samples were checked for PCR inhibition
48 using addition of foreign DNA (obtained from tissue of experimental rats) and a specific qPCR
49 protocol (commercial primers and Taqman probe for detection of the rat gene for beta-2
50 microglobulin; Thermofisher Scientific, Waltham, MA, USA).

51 **RESULTS**

52 In this study, the prevalence of *Blastocystis* was determined by qPCR and subsequently compared with
53 the results from cPCR obtained in our previous study Lhotská et al. [11]. In the set of 288 stool
54 samples from the gut-healthy volunteers, the qPCR revealed a prevalence of 29% (83/288; Table 1)
55 compared to cPCR with the prevalence 24% (71/288). Real-time PCR revealed 12 more positive
56 samples (Table 1), our results indicate that qPCR is a more sensitive method for detecting *Blastocystis*
57 in stool samples than cPCR ($p < 0.05$; $\chi^2 = 8.26$; Table 2). There was a discrepancy between these
58 methods for two samples that qPCR evaluated as negative and cPCR as positive (Table 1). No internal
59 inhibition was detected in any of the samples.

60 We established a quantification curve (10^0-10^5 of cells / 1 qPCR reaction) to evaluate the
61 *Blastocystis* fecal load in positive samples and to extrapolate different colonization intensities from ct
62 values (ct values are displayed for each sample in Table 1). In more than half of the samples positive
63 in qPCR (52/83), colonization intensities reached 10^5 or more, with the range of ct values ranging
64 from 15 to 20 (Table 3). Fecal protist load 10^3-10^4 (range of ct values between 21 and 27) was found
65 in 13 samples, and 10^1-10^2 (range of ct values between 28 and 32) in 18 samples (Table 3). In the
66 samples positive only in qPCR (n=12), a very low fecal protist load was found, i.e., 10^1-10^2 (Table 3).

67 Subtype diversity for all 83 qPCR-positive samples was evaluated by NGS, which detected
68 subtypes in 69 samples (69/83; Table 1 and 4). In case of the presence of one subtype in a sample the
69 NGS results were consistent with our previous results based on Sanger sequencing [11]. Indeed, the
70 great benefit of the NGS appears to be in an ability detecting mixed colonizations of different subtypes
71 in one sample. Mixed colonizations were found in five more cases compared to Sanger sequencing,

72 specifically the subtype colonization mix: ST1+ST7, ST1+ST3, ST2+ST3 (2×), ST3+ST7 (Table 4).
73 In the case of 12 samples positive only in qPCR with low fecal protist load, NGS detected subtypes in
74 only five samples, namely ST2, ST5, ST3 (2×) and ST4 (Table 4).

75 **DISCUSSION**

76 To compare the sensitivity between the two PCR-based approaches for detection of *Blastocystis*, we
77 used a dataset of 288 human stool samples obtained in the study by Lhotská et al. [11]. Revealing 12
78 more positive samples, qPCR was the most sensitive method for detection of *Blastocystis*. The overall
79 prevalence of *Blastocystis* by qPCR and cPCR was 29% and 24% (Lhotská et al. [11]), respectively.
80 Surprisingly, it appears that this is the very first study comparing the sensitivity between commonly
81 used cPCR protocol [18] and qPCR [12] for the detection of *Blastocystis* sp. Previously some studies
82 showed higher sensitivity of qPCR in comparison with classical methods such as direct-light
83 microscopy or xenic *in vitro* culture [12,13,19]. The study by Nourrison et al. [13] compared four
84 qPCR protocols for detection of *Blastocystis* sp. and found that they differed in specificity and
85 sensitivity. Furthermore, the authors recommend the qPCR protocol Stensvold et al. [12] for
86 diagnostic purposes and to add another method for subtype identification.

87 Despite higher sensitivity, qPCR scored two samples as negative, while conventional PCR
88 scored them positive; these two samples were positive for ST3 and ST8. The two false-negative results
89 by qPCR might be due to the degradation of DNA in the samples due to long-term storage and
90 repeated freeze-thawing cycles of their aliquots. These DNA samples were tested again by cPCR, one
91 sample appears to be negative and one (ST8) showed much less intensive amplicon in the
92 electrophoresis. Alternatively, the qPCR protocol might have limited sensitivity for example for ST8,
93 which was not used in the validation panel by Stensvold et al. [12], who developed the method.
94 However, the applicability of the primers and probe was validated *in silico* using the alignment in the
95 article's Fig 1 with a 100% match to ST8, so this means that, at least in theory, the assay should be
96 able to pick up this subtype. In addition, no inhibition was revealed in any sample during inhibition
97 control using the foreign DNA.

98 The advantage of qPCR-based diagnostic approach is the ability to estimate the fecal load of
99 *Blastocystis* in colonized humans based on an established quantitative curve. Our results in individuals
100 with healthy intestine (i.e., without inflammatory diseases) showed a high fecal *Blastocystis* load in
101 more than half of the samples. This fecal load ranged in values of order from 10^5 to 10^6 cells per one
102 qPCR reaction. In the 12 samples scored as positive only by qPCR, low fecal protist load was detected
103 (10^1–10^2 cells per sample). A very recent study by Cinek et al. [15] quantified *Blastocystis* in feces of
104 asymptomatic children and adolescents as one of the few. However, more studies on both healthy
105 humans and patients with inflammatory of functional bowel diseases are warranted [20]. A
106 comparison of fecal *Blastocystis* loads between healthy and sick individuals could fundamentally
107 contribute to understanding the role of *Blastocystis* sp. in the human gut ecosystem and could be

108 important for experimental studies testing the effect of *Blastocystis* sp. on gut inflammation [21]. It is
109 important to note that the quantification curve for assessing fecal *Blastocystis* load might be biased by
110 different copy number of the SSU rRNA gene in individual subtypes and life stages of *Blastocystis*.
111 This could slightly reduce the accuracy of quantification data. However, such data for *Blastocystis* and
112 its subtypes are not yet available. Nevertheless, an approximate determination of *Blastocystis* fecal
113 load can reveal trends between different human cohorts.

114 In epidemiological studies on *Blastocystis* sp. in humans, the identification of its subtypes
115 plays an important role [11,22-24]. Because different *Blastocystis* subtypes colonize different hosts
116 and apparently differ in geographical distribution, surveys aimed at subtype determination might help
117 reveal transmission pathways and potential sources of specific subtypes in a particular area. To date,
118 most studies used Sanger sequencing for subtype identification [11,25,26] which may have limitations
119 in detecting mixed subtype colonizations. Here, we subjected all 83 qPCR-positive samples to NGS
120 analysis to determine subtypes. We found that subtype diversity was largely consistent with the results
121 of Sanger sequencing by Lhotská et al. [11], in which Sanger sequencing was used. In 12 samples
122 identified as positive only by qPCR, the NGS revealed subtypes only in five samples (ST2, ST5, 2×
123 ST3, and ST4; Table 4) which was probably caused by low fecal load of *Blastocystis* (i.e., 10^1-10^2).
124 Remaining seven samples were confirmed by Sanger sequencing from qPCR amplicons (118 bp),
125 however, without information about subtypes.

126 Although epidemiological studies usually describe colonization of an individual with only one
127 subtype of *Blastocystis* sp. [11,23,27], mixed subtype colonization appears to be more common
128 [7,14,28]. This situation is in part caused by limitations of some of the current molecular tools, which
129 preferentially amplify the predominant subtypes present in a sample [17]. Here, the NGS-based
130 approach showed higher sensitivity in determining mixed subtype colonization than a combination of
131 methods, such as conventional PCR and Sanger sequencing (for more details see Lhotská et al. [11]).
132 While Lhotská et al. [11] revealed a single case of mixed infection, NGS detected five more cases of
133 mixed colonisation, specifically ST1+ST7, ST1+ST3, ST2+ST3 (2×) and ST3+ST7.

134 From a diagnostic point-of-view, our results support the fact that qPCR is the most suitable
135 method for detecting the presence of *Blastocystis*. NGS alone cannot achieve the qPCR sensitivity,
136 mainly due to the known signal crosstalk between individual samples in a sequencing run [e.g., 29].
137 Although this issue can be alleviated by using unique dual indexing, it cannot be eliminated, so very
138 low read counts do not necessarily indicate presence of the organism. Thus, the role of NGS in the
139 *Blastocystis* diagnostics is primarily in the determination of its subtypes and disentangling mixed
140 colonizations. Of the 83 total qPCR-positive samples, the NGS revealed subtypes in 69 samples.

141 **Conclusion:** To understand the epidemiology of *Blastocystis* sp. it is necessary to establish a gold
142 standard method for detection and subtype differentiation. A review of the *Blastocystis* literature so far
143 suggests that detection and differentiation has not yet been harmonized [7]. The findings of the present

study showed that qPCR is a suitable tool for the highly sensitive detection of *Blastocystis* sp., and the NGS approach enables accurate assessment of the subtype diversity, in particular, mixed subtype colonization. We believe that the combination of these two approaches will be beneficial for future epidemiological surveys and surveillance studies on *Blastocystis*.

CONFLICT OF INTEREST: Authors declare no conflict of interest.

ETHICS STATEMENT: The studies involving human participants were reviewed and approved by Ethics Committee of the Biology Center of the Czech Academy of Sciences (reference number: 1/2017). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. All data were anonymized and processed according to valid laws of the Czech Republic (e.g., Act no. 101/2000 Coll and subsequent regulations). In case of the rat tissue used for testing of the internal inhibition, we used samples from the experiment approved by the Committee on the Ethics of Animal Experiments of the Biology Centre of the Czech Academy of Sciences (České Budějovice, permit no. 33/2018) and by the Resort Committee of the Czech Academy of Sciences (Prague, Czech Republic) according to strict accordance with Czech legislation (Act No. 166/1999 Coll. on veterinary care and on changes of some related laws, and Act No. 246/1992 Coll. on the protection of animals against cruelty), as well as the legislation of the European Union.

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254

Table 1. **Comparison of the sensitivity of conventional PCR and qPCR from the entire dataset of human samples (n=288).** In addition, we also evaluated the success of *Blastocystis* detection by Next-generation sequencing (NGS) only in a set of qPCR-positive samples (n=83).

# sample	methods				# sample	methods			
	PCR	qPCR	Ct value	NGS		PCR	qPCR	Ct value	NGS
B1	+	+	15	+	**B2**	+	+	19	+
B13	+	+	15	+	**B19**	+	+	19	+
B24	+	+	15	+	**B115**	+	+	19	+
B59	+	+	15	+	**B126**	+	+	19	+
B68	+	+	15	+	**B184**	+	+	19	+
B195	+	+	15	+	**B220**	+	+	19	+
B201	+	+	15	+	**B374**	+	+	19	+
B226	+	+	15	+	**B417**	+	+	19	+
B235	+	+	15	+	**B86**	+	+	20	+
B312	+	+	15	+	**B292**	+	+	20	+
B339	+	+	15	+	**B277**	+	+	21	-
B371	+	+	15	+	**B303**	+	+	21	+
B373	+	+	15	+	**B380**	+	+	21	+
B9	+	+	16	+	**B300**	+	+	22	+
B37	+	+	16	-	**B375**	+	+	22	+
B42	+	+	16	+	**B418**	+	+	22	+
B45	+	+	16	+	**B424**	+	+	22	+
B49	+	+	16	+	**B431**	+	+	23	+
B120	+	+	16	+	**B33**	+	+	24	+
B225	+	+	16	+	**B36**	+	+	24	+
B327	+	+	16	+	**B313**	+	+	24	+
B343	+	+	16	+	**B365**	+	+	24	+
B352	+	+	16	+	**B55**	+	+	26	+
B364	+	+	16	+	**B144**	-	+	28	+
B412	+	+	16	+	**B345**	+	+	28	+
B15	+	+	17	+	**B405**	+	+	29	+
B30	+	+	17	+	**B356**	+	+	30	-
B65	+	+	17	+	**B372**	-	+	31	+
B82	+	+	17	+	**B10**	-	+	32	-
B99	+	+	17	+	**B35**	-	+	32	-
B113	+	+	17	+	**B38**	-	+	32	-
B185	+	+	17	+	**B41**	-	+	32	+
B336	+	+	17	+	**B50**	-	+	32	-
B341	+	+	17	+	**B54**	-	+	32	-
B353	+	+	17	+	**B62**	-	+	32	-
B363	+	+	17	+	**B114**	-	+	32	+
B31	+	+	18	-	**B189**	-	+	32	-
B224	+	+	18	+	**B240**	-	+	32	+

B231	+	+	18	+	B248	-	+	32	-
B393	+	+	18	-	B398	-	+	32	-
B397	+	+	18	+	B425	+	+	32	+
B413	+	+	18	+					

TABLE 2. Comparison of results of qPCR (Stensvold et al., 2012) and conventional PCR (Scicluna et al., 2006) in detection of *Blastocystis* sp. using McNemar test ($p < 0.004$; $\chi^2 = 8.265$).

		qPCR		
		positive	negative	
cPCR	positive	69	2	71 (25 %)
	negative	14	203	217 (75 %)
		83 (29 %)	205 (71 %)	288

TABLE 3. Evaluation of fecal load of *Blastocystis* sp. in human samples based on the established quantification curve (set in the range of 10^0 to 10^5 cells per 1 qPCR reaction)**.**

Estimated fecal protist load[1]	Number of samples/Number of positive samples	Ct value range
10^1-10^2	18/83	28-32
10^3-10^4	13/83	21-27
10^5 -10^6	52/83	15-20

[1]Number of cells per 1 qPCR reaction.

TABLE 4. Comparison of *Blastocystis* subtype data in a set of 83 qPCR-positive samples obtained by Sanger sequencing (results obtained in previous study Lhotská et al., 2020) and next-generation sequencing (NGS).

# sample	subtype		# sample	subtype	
	Sanger sequencing	NGS		Sanger sequencing	NGS
B1	ST3	ST3	**B225**	ST1	ST1
B2	ST1	ST1	**B226**	ST1	ST1
B9	ST1	ST1	**B231**	ST3	ST3 + ST1
B10	-	-	**B235**	ST3	ST3
B13	ST1	ST1	**B240**	-	ST3
B15	ST3	ST3	**B248**	-	-
B19	ST3	ST3	**B277**	ST7	-
B24	ST6	ST6	**B292**	ST7	ST7
B30	ST3	ST3	**B300**	ST4	ST4
B31	ST3	-	**B303**	ST7	ST7
B33	ST3	ST3	**B312**	ST3	ST3
B35	-	-	**B313**	ST3	ST3
B36	ST1	ST1	**B327**	ST2	ST2
B37	ST2	-	**B336**	ST3	ST3
B38	-	-	**B339**	ST1	ST1
B41	-	ST3	**B341**	ST3	ST3
B42	ST1	ST1	**B343**	ST5	ST5
B45	ST1	ST1 + ST7	**B345**	ST6	ST6
B49	ST1	ST1	**B352**	ST3	ST3 + ST2
B50	-	-	**B353**	ST1 + ST3	ST1 + ST3
B54	-	-	**B356**	ST3	-
B55	ST3	ST3	**B363**	ST3	ST3
B59	ST4	ST4	**B364**	ST3	ST3 + ST2
B62	-	-	**B365**	ST7	ST7 + ST3
B65	ST4	ST4	**B371**	ST4	ST4
B68	ST3	ST3	**B372**	-	ST4
B82	ST2	ST2	**B373**	ST4	ST4
B86	ST3	ST3	**B374**	ST2	ST2
B99	ST3	ST3	**B375**	ST1	ST1
B113	ST2	ST2	**B380**	ST3	ST3
B114	-	ST2	**B393**	ST7	-
B115	ST7	ST7	**B397**	ST2	ST2
B120	ST1	ST1	**B398**	-	-
B126	ST6	ST6	**B405**	ST6	ST6
B144	-	ST5	**B412**	ST2	ST2
B184	ST3	ST3	**B413**	ST4	ST4
B185	ST6	ST6	**B417**	ST2	ST2

B189	-	-	**B418**	ST2	ST2
B195	ST3	ST3	**B424**	ST3	ST3
B201	ST3	ST3	**B425**	ST2	ST2
B220	ST3	ST3	**B431**	ST4	ST4
B224	ST1	ST1			

SUPPLEMENTARY DATA 1: Detailed description of the Next-generation sequencing protocol for *Blastocystis*.

Positive samples from qPCR were subjected to NGS to determine *Blastocystis* subtypes according to the method by Maloney et al. (2019). Briefly, amplicons of an informative region (~450 bp) of the SSU rDNA gene were generated using overhang primers, purified, and provisioned with indices and sequencing adaptors using a limited number of PCR cycles with combinatorial indices (Nextera XT Index Kit v2 Set A and D, Illumina, San Diego, CA, USA). The amplicon libraries were purified and equalized using on the SequalPrep plates (Thermo, Waltham, MA, USA), pooled, supplemented with 20% PhiX control to balance the amplicon signal, and sequenced on a MiSeq instrument with the Reagent Kit v2, 2x250 bp (Illumina). The ensuing sequences were downloaded from BaseSpace as demultiplexed fastq files, and processed using the USEARCH10 program (Edgar et al. 2010): primers were trimmed, reads were filtered for quality, and unique sequences defined as zero-radius operational taxonomic units, denoised, their frequencies were tabulated, off-target amplicons were removed and subtypes of *Blastocystis* identified by clustering with a reference set of representative sequences as described in Cinek et al. (2021).

References:

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SUPPLEMENTARY DATA 2: Quantification curve used in qPCR diagnostic protocol for evaluation of the fecal *Blastocystis* load in human DNA samples (in LightCycler LC 480 I; Roche, Basel, Switzerland). The curve was set in the range of 10^0 to 10^5 cells per 1 qPCR reaction based on the *Blastocystis* ST3 culture.