Distribution of Blastocystis subtypes isolated from humans from an urban community in Rio de Janeiro, Brazil

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Abstract

Background: Blastocystis is a cosmopolitan protist parasite found in the human gastrointestinal tract and is highly prevalent in developing countries. Recent molecular studies have revealed extensive genetic diversity, which has been classified into different subtypes (STs) based on sequence analysis of small subunit ribosomal RNA gene. Blastocystis is one of the most common fecal parasites in Brazil, but the diversity of subtypes remains unknown in the country. This study aimed to determine the distribution of Blastocystis STs in an urban community in Duque de Caxias, Rio de Janeiro, Brazil.

Methods: A total of 64 stool samples positive for Blastocystis in Pavlova’s medium were subtyped by PCR and sequenced using primers targeting the small subunit rRNA gene, in addition to phylogenetic analysis and subtype-specific PCR using sequence-tagged-site (STS) primers.

Results: Endolimax nana (14%), Entamoeba complex (10.5%), Taenia sp. (0.6%), Trichuris trichiura (1.3%) and Enterobius vermicularis (1.3%) were detected in Blastocystis-positive samples. Of the 64 samples tested by PCR/DNA sequencing, 55 were identified as ST1 (42%), ST3 (49%), ST2 (7%) and ST4 (2%), and the presence of mixed ST (ST1 + ST3) infection was detected in nine samples (14%).

Conclusions: DNA sequencing and phylogenetic analysis of Brazilian Blastocystis isolates identified four different subtypes. To our knowledge, this study provided the first genetic characterization of Blastocystis subtypes in an urban area of Rio de Janeiro, Brazil. We also identified ST4 for the first time in Brazil. Further studies are necessary to determine the distribution of STs across human populations in Rio de Janeiro.

Keywords: Blastocystis, PCR, Subtypes, Epidemiology

Background

Blastocystis spp. are strictly anaerobic unicellular organisms that inhabit the gastrointestinal tract of humans as well as other mammals, birds, amphibians, reptiles, fish, arthropods and annelids [1–9]. Blastocystis is a cosmopolitan enteric protist estimated to infect over one billion people worldwide [10]. However, its prevalence rates vary widely between countries and between regions within the same country [11, 12], possibly reaching 30% in industrialized countries and 30–76% in developing nations [13–18]. In the latter, high prevalence rates may be directly related to educational deficits, poor socioeconomic status, and sanitary conditions [19–22]. Nevertheless, high rates of infection are also observed in developed countries, especially among individuals who work with animals [6, 7, 23]. Previous studies have reported that the transmission of Blastocystis is fecal-oral, and as a result the protist was included in the Water Sanitation and Health program of the World Health Organization [24].

Although Blastocystis is an emerging intestinal parasite [25], its pathogenic potential remains unclear [24]. Diagnostic limitations, extensive genetic diversity, poor understanding of the host-parasite relationship, and the
lack of an experimental model represent major bottlenecks in elucidating the clinical significance and pathogenesis of *Blastocystis*. After infection (blastocystosis), not all persons develop the clinical disease, for which common symptoms include chronic or acute diarrhea, abdominal pain, flatulence, anorexia, nausea and vomiting [26–28]. One case of enteroinvasive blastocystosis diagnosed by histopathological analysis of biopsies showed *Blastocystis* in ulcers in the cecum, transverse colon and rectum of an immunocompetent individual, indicating that the parasite has invasive potential [29]. In addition, a case of appendicitis associated with appendix lumen obstruction caused by *Blastocystis* has also been reported [30]. *Blastocystis* subtype 3 (ST3) was detected in the appendix, recto-uterine pouch, and peritoneal fluid of a nine-year-old child [31] and in splenic cysts of an immunocompetent woman [32]. Other studies have reported extraintestinal manifestations including arthritis (with full resolution of clinical symptoms after metronidazole treatment) and recurrent episodes of arthritis in immunocompetent individuals [33]. Additionally, several studies suggest an association of *Blastocystis* with urticaria and skin lesions in immunocompetent individuals [34, 35]. These findings provide strong evidence of the ability of *Blastocystis* to migrate to extraintestinal sites and of its pathogenic potential. Moreover, some epidemiological surveys have suggested a strong association between irritable bowel syndrome (IBS) and *Blastocystis*, but this association has been questioned [36, 37].

Molecular analyses of the small-subunit ribosomal RNA have revealed extensive genetic diversity in the species. To date, 17 subtypes have been described (ST1-ST17), nine of which (ST1-ST9) are found both in humans and animals, while the others are exclusively found in animals [8, 38]. These subtypes have not been characterized as species, have low host specificity, and were isolated from humans and various animal hosts [39]. In humans, the most frequently detected subtype is ST3, followed by ST1, ST2, and ST4, whereas ST5-ST9 are rarely found [9, 40]. Genetic diversity varies dramatically among *Blastocystis* subtypes, which are not only separated by significant genetic distance [7, 39] but also differ in levels of intra-subtype variation [41]. Thathaisong et al. [42] detected polymorphisms in *Blastocystis* ST1 and created ST1/variant, while Melloni et al. [43] observed substantial intra-ST diversity in ST3 isolates and genetic homogeneity in ST4 isolates.

So far, studies have revealed the existence of genetic variability [44–47]. However, studies on *Blastocystis* diversity remain scarce in Brazil, where only three studies have been conducted to date [14, 48, 49]. To address this shortcoming, systematic studies focused on characterizing different isolates are necessary. In this study we report the molecular identification of four subtypes of *Blastocystis* in a Brazilian population. This study is the first to thoroughly investigate the diversity of *Blastocystis* subtypes in an urban community in Rio de Janeiro, Brazil. Our findings may be relevant to identifying sources of infection and potential routes of transmission of the parasite.

**Methods**

A coproparasitological study was conducted at the Center for Psychosocial Care (CAPS), a day-care hospital for the mentally ill in Duque de Caxias, Rio de Janeiro, Brazil.

Participation was voluntary and people could withdraw from the study at any time. To guarantee anonymity, each study participant was given a unique identification number. At the end of the study, all participants identified with enteric parasitic pathogens received proper antiparasitic drugs for treatment. Treatment was not offered to individuals infected with *Blastocystis*, because its pathogenic potential is still a topic of debate.

After written consent was obtained, fecal material from each participant was collected in a flask and transported to the laboratory. Unpreserved stool samples were requested and collected. A total of 180 unpreserved stool samples were collected at home from non-selected patients (n = 80) and their family members (n = 100), and kept at 4 °C and transported in ice packs to the Parasitology Laboratory at Oswaldo Cruz Foundation. Fresh unpreserved stool samples were fractionated into two aliquots upon receipt.

For parasitological analysis, one aliquot was processed using a spontaneous sedimentation technique (the Lutz technique or the Hoffman, Pons and Janer technique). Stool samples diluted in water were filtered through a gauze strip into a conical cup and subsequently submitted to sedimentation in tap water for 2 h [50]. The concentrated sample was analyzed by wet mounts and stained through an iodine staining procedure. In this study, we did not search for viruses or bacteria.

Direct cultivation of *Blastocystis* from the aliquot of stool samples was performed using Pavlova’s medium (1.29 g/l of sodium phosphate dibasic, 0.42 g/l of potassium phosphate monobasic, 7.27 g/l of sodium chloride and 1.46 g/l of yeast extract) supplemented with 10% adult bovine serum (ABS) and penicillin-streptomycin (1000 IU/ml and 500 μg/ml, respectively) and incubated at 37 °C [51]. Screening of *Blastocystis* cultures was performed using a standard light microscopy in the first seven days of incubation. When typical forms of the parasite were observed, *Blastocystis* culture aliquots were frozen at -20 °C until used for DNA extraction.
Molecular characterization
Total DNA of each isolate was extracted from each culture of Blastocystis suspension using the Qiap DNA stool Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer’s recommendations. DNA was stored at -20 °C until use. To amplify segments from an approximately 500 bp fragment of genus-specific small subunit ribosomal DNA (SSU rDNA), we used the following primers: forward Blast 505-532 (5’-GGA GGT AGT GAC AAT AAA TC-3’) [52] and reverse Blast 998-1017 (5’-TGC TTT CGC ACT TGT TCA TC-3’), following the protocol described by Santin et al. [8]. The PCR reaction was performed in a final volume of 50 µl and each reaction contained 100 mM of Tris-HCl (pH 9.0), 500 mM of KCl, 1.5 mM of MgCl₂, 200 µM of each dATP, dGTP, dCTP, and dTTP; 0.2 µM of primer; 1.5 U of Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.05% of bovine serum albumin (BSA); and 5 µl of the DNA sample. Cycle conditions were an initial activation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, with a final extension step at 72 °C for 7 min. PCR amplification reactions were performed in a Veriti™ 96-well thermal cycler (AB Applied Biosystems, Foster City, CA, USA). PCR products were electrophoresed in 1.5% agarose gels in a tris-borate EDTA buffer, stained with Gelred (Biotium Inc., Hayward, CA, USA), and photographed under UV transillumination. Amplicons were purified using the Wizard® SV gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), and sequenced in both directions using the PCR Clean-Up System kit (Promega, Madison, WI, USA) and sequenced in both directions using the ABI 3730 Sequencing Platform. Bi-directional sequences formed using the BigDye® Terminator v.3.1 Cycle sequencing reactions were performed using the Wizard® SV gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and sequenced in both directions using the ABI 3730 Sequencing Platform. Bi-directional sequences were assembled and edited using SeqMan (DNASTAR software package, DNASTAR Inc., Madison, WI, USA). DNA sequences of Blastocystis subtypes were downloaded from GenBank and a multiple sequence alignment was performed using the ClustalW algorithm of MEGA software version 6.0 [53]. Bayesian Inference (BI), Maximum Likelihood (ML), and Neighbor-joining analyses of SSU rDNA data were conducted to explore the relationships between taxa using MrBayes [54], PHYML [55] and MEGA software [53], respectively. Nucleotide substitution models were chosen based on the Akaike Information Criterion in jModelTest [56] and MrModeltest [57]. ML analysis was conducted using the Hasegawa-Kishino-Yano Model with gamma distribution (HKY + G) [58] with four parameters and unequal base frequencies including rate variation among sites (-InL = 24,599.056), which was predicted as the best model of nucleotide substitutions by the Akaike Information Criterion (AIC). For Bayesian analysis, the HKY + G model was determined by the Bayesian Information Criterion (BIC) as the best-fit substitution model. Proteromonas lacertae (U37108) was used as an out-group [59, 60].

Subtype sequences with mixed traces indicative of mixed infections were analyzed by PCR using subtype-specific sequence-tagged-site (STS) primers (Table 1), according to Yoshikawa et al. [61].

Results
A total of 180 participants aged 1–85 years (80 patients and 100 patients’ family members) provided adequate stool samples for the study; 105 (58.3%) were male (mean age: 52 years) and 75 (41.7%) were female (mean age: 48 years). Overall, based on light microscopy examination, 82 (45%) participants were found to be infected with one or more species of intestinal parasites. Of the 180 samples tested by the culture method for the presence of Blastocystis, 64 (35.5%) were positive, whereas microscopy examination was less effective in detecting Blastocystis, with only 49 (27%) samples showing positive results. The frequency of Blastocystis infections was 32.5% (26/80) among patients and 38% (38/100) in family members. Among the 64 Blastocystis-positive participants, 18.3% (33/180) of individuals (14 patients and 19 family members) presented poly-parasitism. Endolimax nana (14%), Entamoeba coli complex (10.5%), T. suis sp. (0.6%), Trichuris trichiura (1.3%) and Enterobius vermicularis (1.3%) were detected in the Blastocystis-positive samples. Of the 31 participants singly infected with Blastocystis, only five family members reported symptoms: two individuals reported intermittent episodes of diarrhea, while the other three respectively presented flatulence, bloating and abdominal discomfort.

Table 1 Sequence-tagged site (STS) primer sets used in this study targeting SSU rRNA gene of Blastocystis

| Primer pair | Target subtype | Primer type | Sequence (5’-3’)
|-------------|----------------|-------------|-----------------
| SB83 | ST1 | F | GAAGGACTCTCTGACGAGTA
| | | R | GTCGAATGAAAGGAGC
| SB340 | ST2 | F | TGGCTCTGTCTTCTCAGCT
| | | R | TTCCTCACACTCCGGTCT
| SB227 | ST3 | F | TAGATTTGTGTTGAGAGA
| | | R | TTAGAGTGAAGGAGGAGAAGAG
| SB337 | ST4 | F | GTCTTTCCCTGCTTTATTTCTG
| | | R | AATTGGGTCTGCTTTCTTG
| SB336 | ST5 | F | GTGGGTAGAGGAAGGAAAACA
| | | R | AGAAACTGCTGAGAAGGAGAT
| SB332 | ST6 | F | GCTACGACACTTACTAC
| | | R | CCATTTCAGACACCACAT
| SB155 | ST7 | F | ATCCGCTTACATTTCTGCC
| | | R | ATCCGACACTTCCAGAT


Typing of Blastocystis

APCR amplicon of approximately 500 bp of the SSU rDNA was successfully generated in all 64 samples. DNA sequencing revealed single-subtype infection in 55 isolates. Those 55 sequences showed a high identity (97–100%) to the reference sequences previously published by GenBank, and were identified as four distinct subtypes: ST1, ST2, ST3 and ST4. Blastocystis ST3 was the most prevalent subtype, followed by ST1, ST2 and ST4. In addition, mixed infections were detected in 14% of the samples, similar to the prevalence reported in other countries, which ranged between 2.6–14.3% [6, 12, 19, 46, 49, 62–64]. According to Alfellani et al. [9], single infections are typically reported, whereas mixed infections have been reported in less than half of the studies, at an overall prevalence of only 6%, suggesting a possible under estimation compromising the validity of results and their epidemiological interpretation. Our phylogenetic trees revealed four well-defined clades that grouped isolates into four different subtypes, bearing similarity to those reported in previous studies. In Brazil, even though only three studies have published ST data, the geographical distribution of Blastocystis subtypes varies across regions. ST1 was the most prevalent subtype (followed by ST2 and ST3) among an indigenous population from Mato Grosso, Brazil, whereas a study conducted in two small fishing villages situated along the Tietê River in the state of São Paulo, Brazil found a higher incidence of ST3 isolates, followed by ST1 [14, 48, 49]. Globally, subtypes ST1–ST4 have been identified as the most common subtypes in humans [6, 9, 19, 36, 61, 65–70]. Even though it has been suggested that ST3 is the only subtype of human origin [11, 60, 70], it has been detected in several other hosts including pigs, cattle, and non-human primates [3, 9, 65, 71, 72]. Several studies across the world have identified ST3 as the predominant Blastocystis subtype [9, 12, 18, 19, 67, 69, 72–80]. Our study identified ST4 in one only sample. This contrasts with previous studies showing that ST4 is common in European and African populations [9, 38, 64, 81–85].

Discussion

Blastocystis is one of the most prevalent unicellular protozoan species found in human fecal specimens [10]. In our study, ST3 was the most prevalent subtype, followed by ST1, ST2, and ST4. In addition, mixed infections were detected in 14% of the samples, similar to the prevalence reported in other countries, which ranged between 2.6–14.3% [6, 12, 19, 46, 49, 62–64]. According to Alfellani et al. [9], single infections are typically reported, whereas mixed infections have been reported in less than half of the studies, at an overall prevalence of only 6%, suggesting a possible under estimation compromising the validity of results and their epidemiological interpretation. Our phylogenetic trees revealed four well-defined clades that grouped isolates into four different subtypes, bearing similarity to those reported in previous studies. In Brazil, even though only three studies have published ST data, the geographical distribution of Blastocystis subtypes varies across regions. ST1 was the most prevalent subtype (followed by ST2 and ST3) among an indigenous population from Mato Grosso, Brazil, whereas a study conducted in two small fishing villages situated along the Tietê River in the state of São Paulo, Brazil found a higher incidence of ST3 isolates, followed by ST1 [14, 48, 49].

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Table 2  Blastocystis subtypes identified in the present study

| Subtype | No. of isolates | Isolate code and GenBank accession number |
|---------|----------------|-----------------------------------------|
| ST1     | 23             | RTDC1 (KU892242); RTDC4 (KU892241); RTDC5 (KU892222); RTDC8 (KU892243); RTDC17 (KU892244); RTDC27 (KU892225); RTDC29 (KU892233); RTDC32 (KU892227); RTDC35 (KU892237); RTDC36 (KU892229); RTDC37 (KU892235); RTDC38 (KU892236); RTDC39 (KU892230); RTDC40 (KU892238); RTDC41 (KU892231); RTDC42 (KU892240); RTDC49 (KU892232); RTDC50 (KU892226); RTDC52 (KU892239); RTDC56 (KU892228); RTDC60 (KU892223); RTDC66 (KU892224) |
| ST2     | 4              | RTDC44 (KU892245); RTDC45 (KU892246); RTDC67 (KU892247); RTDC69 (KU892248) |
| ST3     | 27             | RTDC2 (KU892249); RTDC3 (KU892250); RTDC6 (KU892251); RTDC10 (KU892252); RTDC12 (KU892253); RTDC20 (KU892254); RTDC22 (KU892255); RTDC23 (KU892256); RTDC24 (KU892257); RTDC25 (KU892258); RTDC26 (KU892259); RTDC28 (KU892260); RTDC30 (KU892261); RTDC31 (KU892262); RTDC33 (KU892263); RTDC34 (KU892264); RTDC43 (KU892265); RTDC51 (KU892266); RTDC54 (KU892267); RTDC57 (KU892268); RTDC58 (KU892269); RTDC59 (KU892270); RTDC61 (KU892271); RTDC62 (KU892272); RTDC63 (KU892273); RTDC64 (KU892274); RTDC65 (KU892275) |
| ST4     | 1              | RTDC9 (KU892276) |
| ST1–ST3a | 9              | RTDC13; RTDC15; RTDC18; RTDC21; RTDC47; RTDC48; RTDC53; RTDC68; RTDC70 |

*Subtype-specific sequence-tagged-site (STS) primers*
Rodents have been proposed as the reservoir host of this subtype [58, 86–88]. In Colombia, ST4 has been identified in a few humans and non-human primates [85, 86]. However, the molecular epidemiology of Blastocystis is incompletely known in other South American countries. To better understand the genetic diversity and determine the prevalence of Blastocystis subtypes in South America, further research with larger sample sizes is required.

After ST3, ST1 was the most frequent subtype detected in our study. ST1 has low specificity and is found in a wide range of animals, including dogs, chickens, cattle, pigs and non-human primates [3, 63]. Some studies have suggested that ST1 is linked to zoonotic transmission [89, 90]. Blastocystis is transmitted by the fecal-oral route [1] and several studies have suggested that contaminated water may be a source of Blastocystis infections [19, 20, 88, 91, 92]. A study conducted in China...
reported a high infection rate (32.6%) and found that inadequate water intake was a predictor of Blastocystis infection [19]. Also, Blastocystis has been detected in samples of soil and drinking water [93]. Overall, many human settlements are characterized by poor hygiene habits, extensive interpersonal contact and increased dependency on care, which provide favorable conditions for the circulation and transmission of parasites and increased susceptibility to parasitic infections. This was revealed by reports of enteric parasitic infections in mental institutions, which indicate that rates of intestinal parasitic infections are higher among institutionalized persons than in the general population, even in developed countries [94]. It should be noted that we analyzed unprocessed stool samples from non-selected adults and identified Blastocystis in 46% of family members, compared to a lower figure of 32.5% among institutionalized patients. Therefore, at least in our study population, transmission through cyst-contaminated water appears to be more common than transmission at the care center [87, 88, 90]. The presence of intestinal parasites in the population highlights the need for a more comprehensive epidemiological study in the region to support public policies aimed at educating the public about intestinal parasite prevention. Over the last few years, scientific interest has increased tremendously with respect to the clinical relevance of Blastocystis infection and molecular epidemiology.

We should note that the source of DNA (feces vs culture) used in PCR may in principle imply a bias in subtyping. We used DNA from cultured Blastocystis, and it is possible that parasite culture could have led to a selection of subtypes of Blastocystis. However, this was not observed in a previous study by Stensvold et al. [38] which involved mixed infections. The authors stated that xenic in vitro culture (XIVC) has very little or no impact on subtype distribution or variation within a given specimen. Moreover, the authors recommended that short-term XIVC should be used for cost-effective screening of Blastocystis infection from fresh fecal specimens, to generate valid prevalence estimates and to identify isolates. When PCR is based on DNA extracted from feces, the consequence may be false-negative results or decreased PCR efficiency due to a series of factors, including low amount of template DNA in the test sample, inadequate removal of polymerase inhibitors and poor DNA recovery after extraction and purification steps. As a reasonable solution to the presence of inhibitors or degraded target DNA in samples, constitutive genes may be used in parallel assays to evaluate the integrity of the DNA template. PCR is such a powerful tool that even a single molecule of template can be amplified. However, it is difficult to amplify rare templates when they are mixed with similar, but more abundant, templates sharing amplification primers. Nonetheless, no study has evaluated the sensitivity range of PCR using a panel of mixed Blastocystis infection samples (multi-ST infections). Moreover, little is known about ST gene targets and how conserved they are both within, and between, subtypes. Had DNA from feces been available for each of the Blastocystis samples in our study, it would have been possible to answer some of these important questions.

It has been suggested that pathogenicity may be related to specific Blastocystis subtypes, but this association is still inconclusive and often contradictory. Many previous reports on the potential pathogenicity of Blastocystis have been limited by a lack of definition of symptoms and more comprehensive investigations able to exclude other infectious agents of diarrhea [91, 95, 96]. Unfortunately, we could not estimate correlations between subtype distributions and symptoms due to the high proportion of asymptomatic individuals, in addition to the fact that individuals had symptoms that could be attributed to other causes (co-infections with pathogenic and commensal intestinal protozoans as well as helminths). In symptomatic individuals not infected by any other protozoans or helminths, the symptoms of Blastocystis infection were intermittent episodes of diarrhea, flatulence (in the case of ST2 infection), and flatulence, bloating, and abdominal discomfort but no diarrhea (in the case of ST1, ST2 and ST3). However, other possible causes of clinical symptoms must be considered, such as viral and bacterial infections not evaluated in this study.

Conclusions
To our knowledge, we conducted the first molecular study of human Blastocystis infection in Rio de Janeiro, Brazil. Blastocystis isolates were highly genetically divergent. Of the subtypes identified, ST3 was the most common, followed by ST1, ST2 and ST4 (ST4 was identified for the first time in Brazil). However, these results cannot be generalized to the entire Rio de Janeiro population. Further studies are required to determine the distribution of STs in the general population. Nonetheless, our study is a contribution to the understanding of the molecular epidemiology, transmission patterns and genetic diversity of Blastocystis at a regional and global scale.

Abbreviations
AIC: Akaike Information Criterion; B1: Bayesian Inference; BIC: Bayesian Information Criterion; ML: Maximum Likelihood; MLST: multilocus sequence typing; NJ: Neighbor-joining; SSU rDNA: small subunit of the ribosomal RNA gene; ST: subtype

Acknowledgments
The authors would like to thank the DNA Sequencing PDTIS/FIOCRUZ platform facilities and we also gratefully acknowledge the dedicated efforts of the Center for Psychosocial Care (CAPS) staff who participated actively in the study, particularly Nilzete de Araújo Ribeiro Costa.
Funding
This study was supported by Carlos Chagas Filho Research Foundation of State of Rio de Janeiro (Faperj), the National Council for Scientific and Technological Development (CNPq) and Oswaldo Cruz Foundation (PAPES-VF/IOCruz). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials
The data sets supporting the conclusions of this article are included within the article. All sequence data that support the findings of this study have been deposited in the GenBank database under the accession numbers: KU892222–KU892276.

Authors’ contributions
SHLC and MHW conceived and designed the study. SHLC and BRJ obtained the samples and conducted the in vitro cultivation and microscopy examination. BVC was responsible for the molecular analyses and SHLC and IR data analysis and interpretation. BVC, LDCM and HLCs prepared the first draft of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Prior to data collection, the study protocol was reviewed and approved by the Human Ethics Committee at Oswaldo Cruz Foundation (protocol number. 632/11). Study participants (patients and their family members) were informed about the study’s aims, potential risks and benefits. Participants provided written informed consent followed by signatures of two external witnesses. Individuals unable to provide consent were represented by their caregivers, relatives or legal tutors.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Received: 10 January 2017 Accepted: 6 October 2017
Published online: 25 October 2017

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