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Original Article

Characterization of prevalence and genetic subtypes of *Blastocystis* sp. in wild and domestic Suidae of central Italy aided by amplicon NGS

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ABSTRACT

Blastocystis spp. is a common single-celled intestinal symbiont, comprising several genetic subtypes (ST) and transmissible by animal-to-animal, human-to-human, animal-to-human and, possibly, human-to-animal routes. This work was designed to explore the presence of *Blastocystis* in sympatric domestic and wild suids and their ability to carry zoonotic STs, in a condition of widespread opportunity to come in contact with the microorganism through their shared water and food resources, and other carriers. We sampled 42 and 37 stool samples from wild boars and domestic pigs, respectively. STs were first identified by PCR followed by Sanger sequencing. Sequences represented in double-band PCR products or in Sanger chromatograms displaying multiple peaks, were resolved by next generation sequencing (NGS). Twenty-six (61.9%) wild boar and 26 (70.2%) pig samples were PCR-positive, respectively. ST3, ST5 and ST15 were found in 3.8%, 38.4% and 80.8% of the positive wild boars and 11.5%, 88.5%, 11.5% of the positive pigs, respectively. ST1 was found only in pigs (3.8%). STs 5 and 15 were common in both groups of animals, but in reversed proportions, suggesting preferential colonization. We found significantly different ST distributions among wild boars and domestic pigs. This might indicate that lifestyle differences between the two populations influence their risk for contracting certain subtypes, or that ST5 and ST15 can colonize preferentially wild or domestic animals. Based on the STs described here, wild boars and domestic pigs can act as reservoirs with zoonotic potential. The ability of suids to carry zoonotic STs appears to be higher when using NGS than Sanger sequencing, and resolution of complex sequencing profiles is imperative before excluding the presence of STs of human concern.

1. Introduction

Blastocystis spp. is an intestinal anaerobic eukaryote belonging to the protozoan group Stramenopiles (Adl et al. 2019). This protozoan can be commonly found worldwide, in a diverse range of host species including humans, non-human primates, other mammals, birds and reptiles (Yoshikawa et al. 2016). Considerable controversy exists over a possible causative role of *Blastocystis* in human disorders, mainly intestinal. In fact, there are inherent difficulties in developing studies aimed at a rigorous assessment of this relationship, as thoroughly discussed in Andersen and Stensvold (2016). However, evidence for a positive correlation between the presence of *Blastocystis* and gut microbial diversity was recently put forward, suggesting that a shift from the parasite to symbiont and from the infection to colonization concepts is appropriate for describing the main feature and the acquisition of this organism, respectively (Andersen and Stensvold 2016; Lukeš et al. 2015; Stensvold and Clark 2016). Consequently, human population screenings are not considered urgent for public health purposes, but surveys of humans and synantropic animals retain their validity for a better understanding of the biology of *Blastocystis*, its dispersal routes and the evolution of its presence in human populations (Stensvold and Clark 2016).

Fecal-oral transmission of *Blastocystis* is believed to occur by animal-to-animal, human-to-human, animal-to-human and, possibly, human-to-animal routes. However, examination of professionally exposed subjects has shown that, at least for one of the genetic subtypes, the preferential transmission route is represented by human-to-human contacts (Stensvold et al. 2012). The finding of *Blastocystis* in faeces of animal hosts has led to proposals of zoonotic potential, and that these hosts may be the source of many of the isolates found in humans (Alfellani et al. 2013b). Numerous data demonstrate that the water- and environmentally-resistant infective

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cyst represents a transmissible stage of the parasite (Stensvold and Clark 2016; Yoshikawa et al. 2004), indicating that environmental reservoirs might be important in mediating host-to-host transmission and contributing to the overall parasite prevalence.

As to animal-to-animal transmission, extensive screenings of a wide range of animals kept in captive conditions (zoos) have been performed, with *Blastocystis*-positive specimens reported from taxa as different as insects, reptiles, birds and mammals (Alfellani et al. 2013b; Cian et al. 2017). Here, the connectivity between hosts may be enhanced by food sharing, waste treatment and spatial confinement. These findings indicate permissivity of the host but may not reflect normal infection rates in more natural conditions.

Extensive genetic variation among numerous *Blastocystis* sp. isolates from both humans and animals has been reported upon PCR and sequencing a 600 bp segment of the small subunit (SSU) rRNA gene (Scicluna et al. 2006), providing strong phylogenetic support for the definition of genetic subtypes (STs). A consensus on terminology has been proposed (Stensvold et al. 2007), which accounts for "alleles" within STs, i.e. molecules belonging to the same ST, differentiated by the presence of additional variants. The list of STs and alleles is maintained at https://pubmlst.org/blastocystis/. Recently, a warning has been issued for the excessive proliferation of STs, when experimental artefacts and inter-ST divergence are not taken in due account (Stensvold and Clark, 2020). Of the 17 STs currently recognized (designated ST1 to ST17), nine (ST1 to ST9) colonize humans, other mammals and birds, while 8 (ST10 to ST17) have been found only in non-human hosts (Alfellani et al. 2013a; Tan 2008). While a more limited array of ST have been recorded in each of the animal species scored so far, a precise list of STs with zoonotic potential harboured by each species is yet to be compiled, even for the species that come in closest contact with humans. Incomplete knowledge is due not only to the propagation of ST definitions and nomenclatures that do not comply with the consensus (Andersen and Stensvold 2016; Stensvold and Clark 2016), but also to detection methods with insufficient sensitivity, which lead to underestimation of isolates represented as minor components, e.g. in multiple infections. Thus, in a framework in which specificity is regarded more as a quantitative than a qualitative property, it is possible that animal species (and even breeds) which host preferentially a ST of limited concern for humans, are indeed able to convey also other STs to which humans are highly susceptible. For example, Wang et al. (2014) have reported a similar presence/absence pattern for STs among pigs and in-contact workers (Table 2 therein) in Queensland, but with reversed proportions among the two groups.

This work was designed to explore the presence of *Blastocystis* in sympatric domestic and wild suids, in a condition of widespread opportunity to come in contact with the microorganism through their shared water and food resources. In fact, the pigs were kept in rural, family-managed farms, and fed with agricultural by-products, which could represent a proxy of the wild boars' diet in its potential for *Blastocystis* transmission. By determining the genetic subtypes in both groups of animals, we wanted to set a baseline for later comparing human subjects coming in direct contact with animals of both types, and the population at large of the same area, thus controlling for short-range heterogeneity in overall prevalence and ST occurrence (El Safadi et al. 2016). Our results add substantially to the record of the presence of *Blastocystis* in the genus *Sus*. Furthermore, by using an experimental approach with enhanced sensitivity, our results show that the examined animal populations may represent a non-negligible reservoir of strains with zoonotic potential.

2. Material studied, area description

Fecal samples used in this study were collected from domestic (pig) and wild (boar) suids (*Sus scrofa*) in the inner area of Central Apennines (provinces of Rieti, Lazio (RI) and Terni, Umbria (TR), Italy) (Fig. S1).

The geographic region under study is characterized by high variability of microclimates and habitats, and it includes several protected areas. Zootechny is widespread in the area, mainly involving

cattle, sheep and horses, and long periods of the year of wild grazing. Wood and prairie habitats are also frequented by wild ruminants such as roe deer (*Capreolus capreolus*), deer (*Cervus elaphus*) and large wild carnivores such as wolf (*Canis lupus*) and bear (*Ursus arctos*).

In this area domestic pigs are commonly reared for fattening in local family farms, sometimes featuring lodging and own farming products (farmhouse), as well as in stations which practice non intensive livestock farming. In both cases animals are fed with self-produced food or agricultural by-products, derived directly from the field. Butchery is carried out in abattoirs under sanitary control, and meat destined to familial consumption or artisanal-scale commerce of seasoned products. We analysed 37 rectal samples obtained from domestic animals directly in the farm or in abattoirs. The place and context of rearing, as well as the place of sampling are reported in Table 1.

In the same area, a considerable wild boar population is also present, which underwent a significant increase in numbers in the last decade, mainly favoured by abandon of cultivated fields. Wild boar is hunted in both game reserves and freely accessible game zones. We sampled 42 rectal samples, mainly at the meeting points of the hunting squads. The place of capture of each animal and the place of sampling are reported in Table 1.

For both domestic and wild animals, sampling was part of the sanitary control duties of the authors' Institution, to authorize meat consumption.

Samples, collected in plastic tubes, were frozen within few hours in the Institutional laboratories, for subsequent processing. Genomic DNA was extracted from approximately 250 mg of stool sample using the QIAamp DNA Stool Mini Kit® (Qiagen GmbH), according to the protocol recommended by the manufacturer, and subsequently stored at −20°. Table 1 displays each animal Id, as well as place of sampling.

3. Methods

3.1. Molecular identification

PCR reactions addressing a DNA fragment of 320 to 342 bp (depending on the subtype, ST) of the small subunit (SSU) rDNA gene (18S) used the *Blastocystis* sp. specific primers BL18SPPF1 (5'-AGTAGTCATA CGCTCGTCTCAAA-3′) and BL18SR2PP (5'-TCTTCGTTACCCGTTAC TGC-3′) (Poirier et al. 2011). Reactions were performed on 3–5 ul of DNA of each sample, in a total reaction volume of 25 ul according to standard conditions for HotStarTaq Master Mix Kit (Qiagen). These were adjusted to: initial denaturation step of 15′ at 95 °C, and 35 amplification cycles (denaturation 95 °C/1′, annealing 59 °C/1′, elongation 72 °C/1′), followed by a final elongation step of 10′ at 72 °C, approaching those reported in Cian et al. (2017) for non q-PCR with the same primers.

This DNA segment of the SSU has been shown to provide enough information for differentiating STs of *Blastocystis* sp. (El Safadi et al. 2016; Fayer et al. 2012), but only in its 3′ end it overlaps with the "barcode" stretch used at https://pubmlst.org/blastocystis/ for allele identification (see legend to Table S1). The precise length of the overlap varies between STs (e.g. 84%, 82% and 82% of the barcode for ST15, ST3 and ST1, respectively) and includes the most variable region. The excluded portion appears poorly informative.

The PCR products were considered positive when a band of about 350 bp was visible in a 1.5% agarose gel with 2 ul of GelRed® (Biotium) and exposed to UV light (UV transilluminator 2000 Bio-Rad). A human-derived sample collected at the Tor Vergata University Hospital and previously characterized as ST3 (Meloni et al. 2011) was used as positive control.

The products of PCR reactions displaying fragments of the appropriate size were purified with the QIAquick PCR Purification® kit (Qiagen) or, alternatively, with the HT ExoSAP-ITVR (Affymetrix), in accordance with the suppliers' protocols. The purified products were sequenced on both strands, using the same primers as above, with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems by

Table 1
List and features of animals considered in this study.

Id	Specimen type	Origin	Fig. S1	Provider	Collection site	ST	Method[a]
18100884/1	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST15	SS
18100884/2	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST15	SS
18100884/3	Wild boar	Bagnolo (RI)	H7	Hunting squads	Squad meeting point	ST15	SS
18100884/4	Wild boar	Posta (RI)	G5	Hunting squads	Squad meeting point	Negative on PCR	
18100884/5	Wild boar	Cittaducale (RI)	F4	Private hunter	Squad meeting point	ST15,5	NGS
19005588/2	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST15,5	NGS
19005588/3	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST15	SS
19005588/4	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST5,15	NGS
19005588/5	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST15	SS
19005588/6	Wild boar	Poggio Catino (RI)	B2	Hunting squads	Squad meeting point	ST15	NGS
19005588/7	Wild boar	Poggio Catino (RI)	B2	Hunting squads	Squad meeting point	ST5,15	NGS
19005588/8	Wild boar	Varco Sabino (RI)	F2	Private hunter	Afield	Negative on PCR	
19005588/9	Wild boar	Varco Sabino (RI)	F2	Private hunter	Afield	ST15	SS,NGS
19005588/10	Wild boar	Varco Sabino (RI)	F2	Private hunter	Afield	ST15	NGS
19005588/11	Wild boar	Morro-Labro-Leonessa-Montenero (RI)	F6	Hunting squads	Squad meeting point	ST15	NGS
19005588/12	Wild boar	Morro-Labro-Leonessa-Montenero (RI)	F6	Hunting squads	Squad meeting point	Negative on PCR	
19005588/13	Wild boar	Morro-Labro-Leonessa-Montenero (RI)	F6	Hunting squads	Squad meeting point	ST15	NGS
19005588/14	Wild boar	Morro-Labro-Leonessa-Montenero (RI)	F6	Hunting squads	Squad meeting point	Negative on PCR	
19005588/15	Wild boar	Morro-Labro-Leonessa-Montenero (RI)	F6	Hunting squads	Squad meeting point	ST15	NGS
19005946/1	Wild boar	Contigliano (RI)	D4	Hunting squads	Abattoir	ST15	SS
19005946/2	Wild boar	Contigliano (RI)	D4		Abattoir	ST15	SS
19005946/3	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST15	SS
19005946/4	Wild boar	Magliano Sabina (RI)	A3	Hunting squads	Squad meeting point	ST5,15	NGS
19005946/5	Wild boar	Varco Sabino (RI)	F2	Private hunter	Afield	ST15	SS
19005946/6	Wild boar	Varco Sabino (RI)	F2	Private hunter	Afield	ST15	SS
19005946/7	Wild boar	Monte San Giovanni (RI)	D2	Hunting squads	Squad meeting point	Negative on PCR	
19005946/8	Wild boar	Monte San Giovanni (RI)	D2	Hunting squads	Squad meeting point	ST5	SS
19005946/9	Wild boar	Poggio Catino (RI)	C2	Hunting squads	Squad meeting point	Negative on PCR	
19005946/10	Wild boar	Poggio Catino (RI)	C2	Hunting squads	Squad meeting point	Negative on PCR	
19005946/11	Wild boar	Poggio Catino (RI)	C2	Hunting squads	Squad meeting point	ST5	SS
19008011/1	Wild boar	Riserva dei laghi Longo e Ripasottile (RI)	D5	Found dead	Disposal site	ST5,3	NGS
19011401/1	Wild boar	Montopoli in Sabina (RI)	B1	Hunting squads	Squad meeting point	Negative on PCR	
19011401/2	Wild boar	Montopoli di Sabina (RI)	B1	Hunting squads	Squad meeting point	Negative on PCR	
19011401/3	Wild boar	Montisola (RI)	D4	Hunting squads	Squad meeting point	Negative on PCR	
19011401/6	Wild boar	Poggio Catino (RI)	C2	Hunting squads	Squad meeting point	Negative on PCR	
19011401/7	Wild boar	Castel Sant'angelo (RI)	F4	Hunting squads	Squad meeting point	Negative on PCR	
19086237/1	Wild boar	Pescorocchiano (RI)	H1	Private hunter	Afield	Negative on PCR	
19086237/2	Wild boar	Pescorocchiano (RI)	H1	Private hunter	Afield	Negative on PCR	
19086232	Wild boar	Posta (RI)	G5	Hunting squads	Squad meeting point	Negative on PCR	
19086235	Wild boar	Castel Sant'Angelo (RI)	F4	Hunting squads	Squad meeting point	Negative on PCR	
19086238/1	Wild boar	Amatrice (RI)	I7		Abattoir	ST5	SS
19086238/2	Wild boar	Amatrice (RI)	I7		Abattoir	ST5	SS
19005172/1	Domestic pig	Configni (RI)	B4	Private farmer	Abattoir	ST5	SS,NGS
19005172/2	Domestic pig	Torri in Sabina (RI)	B3	Private farmer	Abattoir	ST5,15	NGS
19005172/3	Domestic pig	Torri in Sabina (RI)	B3	Private farmer	Abattoir	ST1	SS
19005172/4	Domestic pig	Cottanello (RI)	C4	Private farmer	Abattoir	ST5	SS
19005172/5	Domestic pig	Casperia (RI)	C4	Private farmer	Abattoir	ST3,5	NGS
19011401/4	Domestic pig	Calvi dell'Umbria (TR)	A4	Private farmer	Abattoir	Negative on PCR	
19011401/5	Domestic pig	Calvi dell'Umbria (TR)	A4	Private farmer	Abattoir	Negative on PCR	
19100812/1	Domestic pig	Rieti (RI)	E4	Municipal abbattoir	Abattoir	Negative on PCR	
19100812/2	Domestic pig	Fara in Sabina (RI)	C1	Non intensive farm	Farm	Negative on PCR	
19100812/3	Domestic pig	Rieti (RI)	E4	Municipal abbattoir	Abattoir	Negative on PCR	
19100812/4	Domestic pig	Rieti (RI)	E4	Municipal abbattoir	Abattoir	Negative on PCR	
19100812/5	Domestic pig	Rieti (RI)	E4	Municipal abbattoir	Abattoir	Negative on PCR	
19100812/6	Domestic pig	Rieti (RI)	E4	Municipal abbattoir	Abattoir	Negative on PCR	
19100812/7	Domestic pig	Tarano (RI)	B3	Private farmer	Abattoir	Negative on PCR	
19100812/8	Domestic pig	Tarano (RI)	B3	Private farmer	Abattoir	Negative on PCR	
19100812/9	Domestic pig	Tarano (RI)	B3	Private farmer	Abattoir	ST3	NGS
19100812/10	Domestic pig	Tarano (RI)	B3	Private farmer	Abattoir	Negative on PCR	
19100812/11	Domestic pig	Configni (RI)	B4	Private farmer	Abattoir	ST5	SS
19100812/13	Domestic pig	Torri in Sabina (RI)	B3	Private farmer	Abattoir	ST5	SS
19100812/14	Domestic pig	Cottanello (RI)	C4	Private farmer	Abattoir	ST5	SS
19100812/15	Domestic pig	Magliano Sabina (RI)	A3	Non intensive farm	Abattoir	ST5,15	NGS
19100812/16	Domestic pig	Magliano Sabina (RI)	A3	Non intensive farm	Abattoir	ST15,5	NGS
19100812/17	Domestic pig	Vacone (RI)	B3	Private farmer	Abattoir	ST5	SS
19100812/18	Domestic pig	Collevecchio (RI)	A3	Private farmer	Abattoir	ST5	SS
19086221	Domestic pig	Roccantica (RI)	C3	Non intensive farm	Farm	ST5	SS
19087884	Domestic pig	Borbona (RI)	G5	Non intensive farm	Abattoir	ST5	SS
19090444	Domestic pig	Accumoli (RI)	H8	Non intensive farm	Abattoir	ST5	SS
19090448	Domestic pig	Amatrice (RI)	H8	Non intensive farm	Abattoir	ST5	SS
19090449	Domestic pig	Cittareale (RI)	G6	Farmhouse	Abattoir	ST5	SS
19096678	Domestic pig	Cottanello (RI)	C4	Non intensive farm	Abattoir	ST5	SS
19096682	Domestic pig	Cottanello (RI)	C4	Non intensive farm	Abattoir	ST5	SS
19096684	Domestic pig	Montebuono (RI)	B3	Non intensive farm	Abattoir	ST5	SS

(*continued on next page*)

Table 1 (*continued*)

Id	Specimen type	Origin	Fig. S1	Provider	Collection site	ST	Method[a]
19096685	Domestic pig	Montasola (RI)	C3	Non intensive farm	Farm	ST5	SS
19096691/1	Domestic pig	Tarano (RI)	B3	Non intensive farm	Abattoir	ST3	SS
19096691/2	Domestic pig	Tarano (RI)	B3	Non intensive farm	Abattoir	ST5	SS
19096698	Domestic pig	Casperia (RI)	C3	Non intensive farm	Abattoir	ST5	SS
19096706	Domestic pig	Magliano Sabina (RI)	A3	Non intensive farm	Abattoir	ST5	SS

a. SS = Sanger sequencing; NGS = Amplicon next generation sequencing.

Life Technologies, Foster City, CA), following the manufacturer's protocols, and run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems by Life Technologies).

Sequences represented in double-band PCR products or in Sanger chromatograms displaying multiple peaks, were resolved by next generation sequencing (NGS) with a protocol similar in principle but developed independently from that in (Maloney et al. 2019) and addressing a different sequence target.

Briefly, 1–2 ul of a 1:100 dilution of the first PCR product were subjected to a second amplification with HotStarTaq Master Mix kit (Qiagen), using the primer pair BL18SPPF1Ftail (5'-TCGTCGGCAGCG TCAGATGTGTATAAGAGACAGAGTAGTCATACGCTCGTCTCAAA-3′) and BL18SR2PPRtail (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGTCTTCGTTACCCGTTACTGC-3′). These primers are identical to those used in the first reaction (see above) but 5′-extended with tails suitable for Illumina sequencing. The nested design was adopted to reach a final quantitative yield suitable for the sequencing pipeline. PCR conditions were as follow: initial denaturation of 15′ at 94 °C, 30 amplification cycles (denaturation 94 °C/30″, annealing 60 °C/35″, elongation 68 °C/50″), followed by a final elongation step of 2′ at 68 °C. Product purification, indexing, quantity normalization and paired-end sequencing on Illumina Miseq platform were performed in outsourcing at BMR Genomics (Padua, Italy; https://www.bmr-genomics.it/).

Raw data were supplied in fastq format. Quality was assayed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and the raw reads (forward and reverse) of the samples that passed the quality control were filtered with Trimmomatic (Bolger et al. 2014), using the following parameters: Leading and Trailing with phred-score > 27, minimum length of reads 100 bp, and average quality of reads > 27. The analyses described above were performed on the Galaxy Project (Afgan et al. 2018) platform server. The resulting reads for each sample were subsequently processed with Qiime2 (Bolyen et al. 2019) according to the following pipeline: sequences were refined with the Cutadapt plug-in (Martin 2011) to delete the primer regions; the vsearch plug-in (Rognes et al. 2016) created the contigs of the reads (join-pairs tool, default parameters). Then the unique sequences (100% identity) were identified in each sample and their absolute frequency calculated with the dereplicate-sequences tool (vsearch).

All representatives of unique sequences were then compared via BLAST (blastn 2.7.1, Galaxy Project platform) with the 18 s-rRNA-barcode dataset published in the Blastocystis Sequence Typing website (Jolley et al. 2018) for ST assignment.

In order to set a baseline for ST recognition in NGS data, we used a previously microscopically diagnosed human sample found to carry an ST3 *Blastocystis* infection (Meloni et al. 2011), as well as a pig sample (19,005,172/1, ST5) producing the lowest background and no double peaks in the Sanger electropherogram, suggestive of the presence of a single *Blastocystis* sp. isolate. The developed protocol has the advantage of being fast and producing material to be sequenced highly enriched for the proper genomic target. However, it is prone to polymerase errors that might be magnified in the two consecutive PCR rounds. We thus imposed stringent thresholds for data cleaning, by considering the largest clusters of identical reads bearing also the proper bases at key positions for the subtype identification. NGS of these samples produced a unique sequence type with overwhelming frequency (68.8% of 111,845 valid reads and 44.9% of 118,207, respectively) with a 100% match with the respective Sanger results. When considering all groups of identical sequences represented in 1% or more of reads, the human sample produced 5 groups (amounting to 82.0%), all assigned to ST3, with an inter-group divergence ranging 0.3–1.0%. The pig sample produced 4 such groups, all assigned to ST5 (amounting to 54.3%), with an inter-group divergence ranging 0.3–0.7%. We notice here that the average inter-ST divergence, determined on the 150 sequences available at publmlst.org/blastocystis is an order of magnitude greater, ranging 10–21% in pairwise comparisons between ST1, ST3, ST5 and ST15. This gap in divergence ensures that artefacts in the amplification protocol here used do not reach a level comparable with inter-ST divergence. Furthermore, the finding of arrays of substitutions diagnostic for alternative STs rule out that variant read clusters are artefactual.

The equality of ST distributions in different sets of animals was tested by Contingency Chi square with the chisq.test function in R, which returns an exact probability value.

The set of sequences newly described in this work was deposited in GenBank and received Acc. n. MT821041-MT821076 for Sanger sequencing and MT820953-MT821040 for NGS.

3.2. Phylogenetic reconstruction

In order to give each variant the same weight and avoid the bias potentially introduced by the hierarchical ST assignment, all sequences were analysed in the frame of the reference panel reported at http://entamoeba.lshtm.ac.uk/blastorefseqs.htm.

The sequences were analysed and processed using Geneious R7 software (Biomatters Ltd) and aligned with MAFFT, G-INS-i algorithm (Katoh et al. 2002) implemented therein. Maximum likelihood analysis was performed with the software IQtree (Nguyen et al. 2014) with 1000 bootstrap replicates The nucleotide substitution model was identified with JModelTest2 (Darriba et al. 2012). Sequences of *Blastocystis apemi*, *B. cycluri* and *B. pythoni* were included, ignoring their purported status of distinct species. *Protoopalina intestinalis* (AY576544), *Opalina triangulata* (MK872804), *O. undulata* (MF434114) and *Proteromonas lacertae* (U37108.1) were used as outgroups (Noël et al. 2005). The resulting tree is shown in Figs. S2–S5.

4. Results

Of the 42 and 37 stool samples from wild boars and domestic pigs, respectively, 26 (61.9%) and 26 (70.2%) revealed the presence of PCR products compatible with *Blastocystis,* with the specific primers used. Sanger sequencing of the amplified material from both boars and pigs confirmed that it was obtained from *Blastocystis* (Table 1). Sequences obtained with this method could be assigned to 2 (ST5 and ST15) and 3 (ST1, ST3 and ST5) subtypes, respectively. In particular, ST15 was unequivocally identified because of the presence of the 27 and 5 bp insertions in positions corresponding to 130–131 and 256–257 of the reference Acc. AF408426.2. The internal sequence of these segments matched perfectly the ST15 reference in our set.

Eighteen samples were subjected to the NGS procedure (Table 1), which revealed 10 (56%) instances of multiple colonization. For wild boars, 5 out of 6 cases involved ST15 and ST5, the latter being the most represented among reads in 3 cases. The remaining case displayed the presence of ST5 and ST3 (Table 1). For domestic pigs multiple colonization always involved ST5, with either ST15 (3 cases) or ST3 (1 case).

Table 2
Overall prevalence and infection by subtype.

	n. samples	PCR-positive (%)	Mixed infections (%[a])	By subtype			
				ST1 (%[a])	ST3 (%[a])	ST5 (%[a])	ST15 (%[a])
Wild boar	42	26 (61.9)	6 (23.1)	0	1 (3.8)	10 (38.4)	21 (80.8)
Domestic pig	37	26 (70.2)	4 (15.4)	1 (3.8)	3 (11.5)	23 (88.5)	3 (11.5)

[a] Percent of infected animals.

The overall distribution of STs is reported in Table 2. The occurrence of STs in wild boars and domestic pigs was significantly different (Chi square test $p = 1.2E-04$). In particular, ST15 and ST5 were by far the most common in wild boars and domestic pigs, respectively. ST3 was found at low frequency in both and the single observation of ST1 came from a domestic pig.

4.1. Variation within STs

In order to put all additional variation represented in our dataset in the frame of the overall *Blastocystis* ST diversity, we constructed a tree which included an entire reference panel. We were particularly interested in possible artifactual diversity introduced by PCR and emerging upon NGS. We then included in the analysis all read clusters above 1% obtained in the 18 specimens sequenced by NGS. The aim was to test the null hypothesis that all sequences fall securely within one of the clades on which ST definition relies, and that the sometime numerous single nucleotide variants differentiating NGS sequence clusters as compared to reference sequences did not lead to ambiguous ST assignment. Under this hypothesis one would expect little intra-ST variation as contrasted with large inter-ST variation. The tree depicted in Figs. S2-S5 clearly shows that all our sequences fall within the clades corresponding to the four ST here recorded (Table 2). This holds true also for different STs recovered from the same sample (mixed infections). Finally, sequences derived from the same sample (by NGS) and assigned to the same ST display a distance from a reference invariably below 0.02. We then conclude that variation possibly introduced during amplification does not alter ST assignment.

5. Discussion

Emerging data call for the introduction of sensitive methods for the recognition and identification of *Blastocystis* isolates in biological samples. For example, a 16-fold increase in the detection of mixed infections was detected when comparing Next Generation to Sanger sequencing results in samples obtained from cattle (Maloney et al. 2019). Optimally, such methods would also be capable of assigning the phase of multiple variants occurring along a DNA segment, i.e. of reconstructing the particular arrangement of variant nucleotides along each molecule.

Wyzelich et al. (2019) introduced a metagenomic approach to analyse the parasitic content of pig faeces. Their approach exploited RNA rather than DNA, leveraging the abundance of rRNA material in the former. This approach has the potential of detecting a wide spectrum of organisms, and is unbiased, as far as there is no preferential amplification of any target sequence.

Conversely, our approach was based on DNA, and analysed PCR material potentially deriving from *Blastocystis* rDNA, with the aim of resolving the individual molecules generated by PCR. This strategy was adopted to avoid the laborious procedure of cloning the products of the first PCR into recombinant plasmids and sequencing them individually. Such a procedure is not only time-consuming but does not guarantee that molecules represented at few percent or less are indeed captured. We addressed a 330 bp fragment, internal to those initially analysed by Scicluna et al. (2006) and Santín et al. (2011). Within this smaller segment, variants diagnostic for the different subtypes are clustered in few stretches of adjacent positions. The length of our sequence target ensures that paired-end reads cover it to completion on both strands, enhancing the final quality. This approach is rapid and exploits the multiplexing of several samples, leading to abated costs and the possibility of extending the procedure to large screenings.

Comparisons between the results obtained with both Sanger and NGS sequencing indicate that ST assignment obtained with the latter is reliable. As to mixed ST infections, we arrived at defining the following requirements to recognize them in the NGS dataset for a given specimen: a) the predominant sequence type is positively assigned to a known subtype; b) there are other sequence types represented at least in 1% of valid reads; c) one or more of the groups in b) are positively assigned to a known subtype different form that in a) (Table S1). The finding of the same ST15 allele in samples analysed by Sanger sequencing, NGS in the presence of single ST and NGS in mixed infections, further confirms that the artefacts introduced by consecutive PCR rounds do not obscure the main sequence type. We expect that the incidence of artefacts will be further reduced by using high-fidelity polymerases. At any rate, our protocol led to the identification *of Blastocystis* STs in all of the 10 cases of unreadable, mixed Sanger profiles (13% of all specimens). The impact of this kind of missed detection in other studies based on Sanger sequencing alone is undetermined.

With this tool in hands, we reported on the occurrence of *Blastocystis* sp. and its genetic subtypes in wild and domestic suids collected in a well-defined geographic region of Central Italy, with opportunities to share contaminated soil, food and water. Previous studies from different countries have reported the overwhelming occurrence of ST5 in domestic and wild swine (Lee et al. 2020; Pintong et al., 2018; Stensvold et al. 2009a; Wang et al. 2014; Wylezich et al. 2019; Yan et al. 2007), while this ST is uncommon in humans. Contact with domestic pigs has been reported as a risk factor of colonization in humans (Stensvold et al. 2009b; Wang et al. 2014; Yan et al. 2007). We found drastically different ST distributions among domestic pigs and wild boars. As to wild boars, this is the first report on a European feral population. We found 3 STs, of which ST15 was by far the most common (80.8% prevalence). Instead, a low prevalence of infected animals was reported in South Korea, with the exclusive presence of ST5 (Lee et al. 2020). ST15 was reported in camels and primates (Alfellani et al. 2013b; Cian et al. 2017), thus displaying a wide range of hosts. As to domestic pigs, we found 4 STs, the most common of which was ST5 (88.5% prevalence). In both Stensvold et al. (2009a) and Wang et al. (2014) a high prevalence of ST5 in pigs was found, with a small percentage of ST3 carriers and an overall less diverse distribution of types. In the zoo survey by Cian et al. (2017) other arctiodactyla were not found to carry ST5, whereas ST3 was found in the oryx. The occurrence of ST1, 2, 3, 5 and 15 in pigs was reported (Wylezich et al. 2019), with a distribution not significantly different to that reported in the present study (Chi square $p = 0.08$). In particular, in both studies, ST3 and ST15 in pigs were found only in mixed infections with ST5. With the caveat that ST5 is anyway present in the majority of pigs, this prompts the question of whether this ST is associated to conditions which enable also the colonization by other STs. In cattle, too, ST3 was found more often as a minor component in mixed than in single infections (Maloney et al. 2019).

It is reasonable to assume that wild and domestic animals in this study were exposed to similar contaminants from the environment through water, food, contact with other carriers or any combination of these. Yet, ST15 predominated over ST5 in wild boars, whereas the reverse was true for pigs. At present our data do not allow to distinguish between two, non-mutually exclusive hypotheses. The first is that ST5 and ST15 can colonize preferentially wild or domestic animals. This

would represent an unusual case un sub-specific heterogeneity in predisposition, for which literature data are scanty. The second is that lifestyle differences between wild and domestic animals lead to divergent opportunities for contact and successful colonization by ST5 or ST15. Host/environment interactions are known to impact on parasite infectivity on both the broad and narrow geographic scales, as repeatedly exemplified (Fecchio et al. 2019; Han et al. 2015; Jackson and Tinsley 2005). This phenomenon for *Blastocystis* and wild and domestic host species is yet to be explored.

6. Conclusion

In summary, three potentially zoonotic STs (Alfellani et al. 2013a; Ramírez et al. 2014) were detected in aggregated wild boars and domestic pigs, of which ST1 and ST3 are commonly observed in humans, whereas ST5 is seldom observed. It is to be stressed that, in our series, 2 of the 4 observations of ST3, one of the most common subtype found in humans, came from mixed infections, that would have escaped detection under ordinary Sanger sequencing. The rates of mixed infections detected in this study are to be considered a lower bound, as some of the cases resolved by Sanger sequencing alone may have also harboured other STs at low frequency. This work suggests that the ability of suids in carrying zoonotic STs unveiled by NGS is higher than Sanger sequencing, and resolution of complex sequencing profiles is imperative before excluding the presence of STs of human concern.

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Authors' contributions

V.R., P.C., A.N. conceived the study;
V.R., R.F., M.P. performed the experiments;
M.M.d.F., D.D.C, F.B. provided essential reagents;
A.N., P.C. supervised the work;
V.R., A.N. wrote the paper;
M.M.d.F., D.D.C, F.B.,P.C. critically reviewed the manuscript.
All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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References

Adl, S.M., Bass, D., Lane, C.E., Lukeš, J., Schoch, C.L., Smirnov, A., Agatha, S., Berney, C., Brown, M.W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., del Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A.A., Hoppenrath, M., James, T.Y., Karnkowska, A., Karpov, S., Kim, E., Kolisko, M., Kudryavtsev, A., Lahr, D.J.G., Lara, E., Le Gall, L., Lynn, D.H., Mann, D.G., Massana, R., Mitchell, E.A.D., Morrow, C., Park, J.S., Pawlowski, J.W., Powell, M.J., Richter, D.J., Rueckert, S., Shadwick, L., Shimano, S., Spiegel, F.W., Torruella, G., Youssef, N., Zlatogursky, V., Zhang, Q., 2019. Revisions to the classification, nomenclature, and diversity of eukaryotes. J. Eukaryot. Microbiol. 66, 4–119.

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B.A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A., Blankenberg, D., 2018. The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res. 46, W537–W544.

Alfellani, M.A., Stensvold, C.R., Vidal-Lapiedra, A., Onuoha, E.S.U., Fagbenro-Beyioku, A.F., Clark, C.G., 2013a. Variable geographic distribution of *Blastocystis* subtypes and its potential implications. Acta Trop. 126, 11–18.

Alfellani, M.A., Taner-Mulla, D., Jacob, A.S., Imeede, C.A., Yoshikawa, H., Stensvold, C.R., Clark, C.G., 2013b. Genetic diversity of *Blastocystis* in livestock and zoo animals. Protist 164, 497–509.

Andersen, L.O.B., Stensvold, C.R., 2016. *Blastocystis* in health and disease: are we moving from a clinical to a public health perspective? J. Clin. Microbiol. 54, 524.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857.

Cian, A., El Safadi, D., Osman, M., Moriniere, R., Gantois, N., Benamrouz-Vanneste, S., Delgado-Viscogliosi, P., Guyot, K., Li, L.-L., Monchy, S., Noël, C., Poirier, P., Nourrisson, C., Wawrzyniak, I., Delbac, F., Bosc, S., Chabé, M., Petit, T., Certad, G., Viscogliosi, E., 2017. Molecular epidemiology of *Blastocystis sp.* in various animal groups from two French zoos and evaluation of potential zoonotic risk. PLoS One 12, e0169659.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.

El Safadi, D., Cian, A., Nourrisson, C., Pereira, B., Morelle, C., Bastien, P., Bellanger, A.-P., Botterel, F., Candolfi, E., Desoubeaux, G., Lachaud, L., Morio, F., Pomares, C., Rabodonirina, M., Wawrzyniak, I., Delbac, F., Gantois, N., Certad, G., Delhaes, L., Poirier, P., Viscogliosi, E., 2016. Prevalence, risk factors for infection and subtype distribution of the intestinal parasite *Blastocystis* sp. from a large-scale multi-center study in France. BMC Infect. Dis. 16, 451.

Fayer, R., Santin, M., Macarisin, D., 2012. Detection of concurrent infection of dairy cattle with *Blastocystis*, *Cryptosporidium*, *Giardia* and *Enterocytozoon* by molecular and microscopic methods. Parasitol. Res. 111, 1349–1355.

Fecchio, A., Wells, K., Bell, J.A., Tkach, V.V., Lutz, H.L., Weckstein, J.D., Clegg, S.M., Clark, N.J., 2019. Climate variation influences host specificity in avian malaria parasites. Ecol. Lett. 22, 547–557.

Han, B.A., Schmidt, J.P., Bowden, S.E., Drake, L.M., 2015. Rodent reservoirs of future zoonotic diseases. Proc. Natl. Acad. Sci. U. S. A. 112, 7039–7044.

Jackson, J.A., Tinsley, R.C., 2005. Geographic and within-population structure in variable resistance to parasite species and strains in a vertebrate host. Int. J. Parasitol. 35, 29–37.

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 3, 124.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.

Lee, H., Seo, M.-G., Oem, J.-K., Kim, Y.-S., Lee, S.-Y., Kim, J., Jeong, H., Jheong, W.-H., Kim, Y., Lee, W.-J., Kwon, O.-D., Kwak, D., 2020. Molecular detection and subtyping of *Blastocystis* detected in wild boars (*Sus scrofa*) in South Korea. J. Wildl. Dis. 56, 662–666.

Lukeš, J., Stensvold, C.R., Jirků-Pomajbíková, K., Wegener Parfrey, L., 2015. Are human intestinal eukaryotes beneficial or commensals? PLoS Pathog. 11, e1005039.

Maloney, J.G., Molokin, A., Santin, M., 2019. Next generation amplicon sequencing improves detection of *Blastocystis* mixed subtype infections. Infect. Genet. Evol. 73, 119–125.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17.

Meloni, D., Sanciu, G., Poirier, P., El Alaoui, H., Chabé, M., Delhaes, L., Dei-Cas, E., Delbac, F., Luigi Fiori, P., Di Cave, D., Viscogliosi, E., 2011. Molecular subtyping of *Blastocystis* sp. isolates from symptomatic patients in Italy. Parasitol. Res. 109, 613–619.

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2014. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274.

Noël, C., Dufernez, F., Gerbod, D., Edgcomb, V.P., Delgado-Viscogliosi, P., Ho, L.-C.,

Singh, M., Wintjens, R., Sogin, M.L., Capron, M., Pierce, R., Zenner, L., Viscogliosi, E., 2005. Molecular phylogenies of *Blastocystis* isolates from different hosts: implications for genetic diversity, identification of species, and zoonosis. J. Clin. Microbiol. 43, 348.

Pintong, A.-R., Sunyanusin, S., Prasertbun, R., Mahittikorn, A., Mori, H., Changbunjong, T., Komalamisra, C., Sukthana, Y., Popruk, S., 2018. *Blastocystis* subtype 5: predominant subtype on pig farms, Thailand. Parasitol. Int. 67, 824–828.

Poirier, P., Wawrzyniak, I., Albert, A., El Alaoui, H., Delbac, F., Livrelli, V., 2011. Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies. J. Clin. Microbiol. 49, 975.

Ramírez, J.D., Sánchez, L.V., Bautista, D.C., Corredor, A.F., Flórez, A.C., Stensvold, C.R., 2014. *Blastocystis* subtypes detected in humans and animals from Colombia. Infect. Genet. Evol. 22, 223–228.

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 18, e2584.

Santín, M., Gómez-Muñoz, M.T., Solano-Aguilar, G., Fayer, R., 2011. Development of a new PCR protocol to detect and subtype Blastocystis spp. from humans and animals. Parasitol. Res. 109, 205–212.

Scicluna, S.M., Tawari, B., Clark, C.G., 2006. DNA barcoding of *Blastocystis*. Protist 157, 77–85.

Stensvold, C.R., Clark, C.G., 2016. Current status of *Blastocystis*: a personal view. Parasitol. Int. 65, 763–771.

Stensvold, C.R., Clark, C.G., 2020. Pre-empting Pandora's box: *Blastocystis* subtypes revisited. Trends Parasitol. 1952.

Stensvold, C.R., Suresh, G.K., Tan, K.S.W., Thompson, R.C.A., Traub, R.J., Viscogliosi, E., Yoshikawa, H., Clark, C.G., 2007. Terminology for *Blastocystis* subtypes – a consensus. Trends Parasitol. 23, 93–96.

Stensvold, C.R., Alfellani, M.A., Nørskov-Lauritsen, S., Prip, K., Victory, E.L., Maddox, C., Nielsen, H.V., Clark, C.G., 2009a. Subtype distribution of *Blastocystis* isolates from synanthropic and zoo animals and identification of a new subtype. Int. J. Parasitol. 39, 473–479.

Stensvold, C.R., Lewis, H.C., Hammerum, A.M., Porsbo, L.J., Nielsen, S.S., Olsen, K.E.P., Arendrup, M.C., Nielsen, H.V., Mølbak, K., 2009b. *Blastocystis*: unravelling potential risk factors and clinical significance of a common but neglected parasite. Epidemiol. Infect. 137, 1655–1663.

Stensvold, C.R., Alfellani, M., Clark, C.G., 2012. Levels of genetic diversity vary dramatically between *Blastocystis* subtypes. Infect. Genet. Evol. 12, 263–273.

Tan, K.S.W., 2008. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. Clin. Microbiol. Rev. 21, 639–665.

Wang, W., Owen, H., Traub, R.J., Cuttell, L., Inpankaew, T., Bielefeldt-Ohmann, H., 2014. Molecular epidemiology of *Blastocystis* in pigs and their in-contact humans in Southeast Queensland, Australia, and Cambodia. Vet. Parasitol. 203, 264–269.

Wylezich, C., Belka, A., Hanke, D., Beer, M., Blome, S., Höper, D., 2019. Metagenomics for broad and improved parasite detection: a proof-of-concept study using swine faecal samples. Int. J. Parasitol. 49, 769–777.

Yan, Y., Su, S., Ye, J., Lai, X., Lai, R., Liao, H., Chen, G., Zhang, R., Hou, Z., Luo, X., 2007. *Blastocystis* sp. subtype 5: a possibly zoonotic genotype. Parasitol. Res. 101, 1527–1532.

Yoshikawa, H., Yoshida, K., Nakajima, A., Yamanari, K., Iwatani, S., Kimata, I., 2004. Fecal-oral transmission of the cyst form of *Blastocystis hominis* in rats. Parasitol. Res. 94, 391–396.

Yoshikawa, H., Koyama, Y., Tsuchiya, E., Takami, K., 2016. *Blastocystis* phylogeny among various isolates from humans to insects. Parasitol. Int. 65, 750–759.