

Molecular detection of *Blastocystis* from animals in Italy: subtypes distribution and implications for the zoonotic transmission

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Abstract

Background: *Blastocystis* is a common intestinal protist distributed worldwide infecting humans and a wide range of domestic and wild animals. It exhibits an extensive genetic diversity and so far, 26 subtypes (STs) have been identified in animal hosts, ten of them (ST1-ST9 and ST12) reported in humans with varying prevalence. Since several STs are common to humans and animals it has been proposed that a proportion of human infections may have a zoonotic origin. Aims of the present study were to: 1) genetically detect *Blastocystis* in faecal samples of farmed animals and wild carnivores; 2) investigate the distribution of *Blastocystis* STs in different animal hosts; 3) provide a first study on the *Blastocystis* STs circulating between animals and humans in Italy.

Methods: Fresh faecal samples (N=269) were collected from carnivores and farmed animals in different Italian provinces and submitted to genomic DNA extraction and PCR amplification followed by both sequence and phylogenetic analysis (Neighbour Joining and Maximum Parsimony)

Results: *Blastocystis* was detected in 50% of the farmed animals (42 out of 84), and 19 of them were successfully subtyped. Conversely, all the faecal samples (N=185) from domestic and wild carnivores (dogs, cats, foxes) tested in the present study, resulted negative. Phylogenetic analysis showed the finding of ST5, ST7, ST9 and ST10 in the samples from animals. The comparison with sequences of *Blastocystis* STs previously detected from humans in Italy showed the ST7, as a potential source of zoonotic transmission.

Conclusions: The present study represents the widest epidemiological survey so far performed in animals in Italy. Further epidemiological studies using molecular approaches are required to determine the occurrence and distribution of *Blastocystis* STs in other potential animal reservoirs and to define the pathways of zoonotic transmission.

1. Background

Blastocystis is a common intestinal protozoon distributed worldwide, infecting humans and a wide range of domestic and wild animals. Molecular studies based on sequence analysis of the small subunit (SSU) ribosomal RNA evidenced an extensive genetic diversity allowing the identification in mammalian and avian hosts of at least 26 divergent lineages, termed subtypes (STs), which could be considered as separate species [1, 2, 3]. Ten of the 26 subtypes, ST1 to ST9, and ST12 have been identified in human samples and have also been reported in animals so far [4, 5, 6]. Therefore, it has been proposed that a portion of human infections may result from the zoonotic transmission of the protist. Interestingly, a recent molecular epidemiological survey carried out on commercially important fish species and marine mammals from North-East Atlantic waters, detected several STs occurring in both fish and marine mammals [7]. These findings provided new insights into host range of *Blastocystis* STs including possible new STs [7]. However, the contribution of animal sources to human infection remains to be confirmed, since the direction of transmission routes to humans is uncertain.

In the recent years, the presence of *Blastocystis* has attracted attention also in Italy, where few epidemiological surveys have been published so far, demonstrating the occurrence in humans of seven STs, including those considered as zoonotic subtypes (*i.e.*, ST1, ST2, ST3, ST4, ST6, ST7, ST8) [8, 9, 10, 11]. Phylogenetic and genetic diversity analyses evidenced at the intra-ST level a high genetic homogeneity in ST4, while high values of nucleotide diversity and different haplotypes were observed in isolates identified as ST1 and ST2 [9]. This genetic variability seems to support the low host specificity of these two STs [12]. Indeed, the sequences identified as ST1 and ST2 showed 100% identity with isolates from a wide range of animals, including monkeys, cattle, pigs, dogs and non-human primates, highlighting that these subtypes of animal origin are zoonotic STs and able to infect humans at different frequencies [9, 10].

Despite a prevalence rate of about 7% has been reported in Italian population [11, 13] along with the identification of zoonotic STs in humans [8, 9, 10], studies aimed to identify the potential animal reservoirs of human *Blastocystis* infection in Italy are scant. So far, the detection of different STs from animals was carried out in few animal categories such as zoo mammals [14] and dogs [15].

Aims of the present study were to: 1) genetically detect *Blastocystis* in faecal samples of farmed animals and wild carnivores collected in Italy; 2) investigate the distribution of STs in different animal hosts, and 3) provide a first comprehensive study on the STs circulating between animals and humans in Italy.

2. Methods

2.1 Source of samples

During 2018, a total of N=269 faecal samples were randomly collected from: 1) N=84 farmed animals; 2) N=74 owned and kennel cats and dogs, and 3) N= 111 foxes killed during the hunting season. Animals were sampled from different locations of three Italian provinces (Messina-ME site 1:38°15'41" N; 15°27'09" E ; ME site 2: 38°14'53" N; 15°21'86"; ME site 3: 38°00'42" N; 15°25'18" E; Catania-CT: 37°61'10" N; 15°03'10"; Rieti-RT: 42°24'29"52 N, 12°51'36"36 E)(Table 1).

Faecal samples from wild foxes were gathered during necropsy from the large intestine, whereas faeces from pets and farmed animals were directly collected from rectal ampulla to avoid environmental contamination.

Animals included in the study were in a good state of health and did not receive pharmacological treatment in at least four weeks before the sample collection.

2.2 DNA extraction and sequencing of *Blastocystis* isolates

Genomic DNA was extracted from each faecal sample, by using the Faecal DNA kit (Bioline, UK) according to the manufacturer's protocol. A fragment of about 500 bp from the SSU rDNA gene was amplified using the primers Blast 505–532 (5'-GGAGGTAGTGAC AATAAATC-3') and reverse Blast 998–1017 (5'-TGCTTTCGCACTTGTTTCATC-3') following the protocol proposed by Santín et al [16]. Positive

samples were further amplified using the primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') and BhrDr (5'-GAGCTTTTAACTGCAACAACG-3'), in order to compare sequences obtained in this study with human isolates from a previous survey [9], using the PCR-conditions described in Scicluna et al [17].

Amplicons from the barcode region of about 600 bp were sequenced using the forward amplification primer (Bio-Fab Research, Rome, Italy). The resulting chromatograms were analysed and edited in the computer software Chromas version 2.33 (Technelysium Pty Ltd, Australia). The sequences obtained were compared to the sequences of *Blastocystis* STs, previously deposited in GenBank™ and available at the website <http://pubmlst.org/blastocystis/> [18] by using the BLAST application. They were aligned with SSU rDNA sequences representing all STs using Clustal X software version 2.1 for comparative analysis and an alignment figure was carried out by using Bioedit software [19]. The subtypes (STs) were identified by determining the exact match (100%) or closest identity (99%), according to the classification of the subtypes given by Stensvold et al [20]. In order to discriminate alleles of *Blastocystis* based on 18S rRNA gene, generated sequences were subjected to online software <http://pubmlst.org/blastocystis/>.

All sequences were submitted to GenBank™ here reported with their accession numbers: MT318937, MT318938, MT318939, MT318940, MT318941, MT318942, MT318943.

2.3 Phylogenetic and genetic diversity analyses

The phylogenetic analysis of the sequence dataset obtained in the present study was carried out by using MEGA6 software [21]. The phylogenetic elaborations from the animal sequences inferred from Neighbour Joining (NJ) and (MP) Maximum Parsimony analyses were carried out considering TVM+G (G=0.207) as the best substitution model, as implemented in JModeltest2 [22]. The phylogenetic analysis considering sequences from animals (present work) and those from humans obtained from previous studies [9,10,11] was carried out by performing a NJ by MEGA6 software [21], using the same substitution model previously selected.

Blastocystis lapemi (GenBank™ accession no. AY590115) was used as outgroup to root the trees. Bootstrap values >70 were considered as a good support to the nodes of the phylogenetic trees [23].

3. Results

Out of the total (N= 84) faecal samples from farmed animals (calf, chicken, cow, deer, donkey, duck, goat, horse, ostrich, peacock, pheasant, pig, sheep, turkey), 42 (50%) resulted positive at *Blastocystis* by molecular amplification. The number of positive samples varied among the animal host species, as detailed in Table 1. Conversely, all the faecal samples (N=185) from domestic (dogs, cats) and wild (foxes) carnivores tested, turned out to be negative.

A total of 19 samples from farmed animals showed a single infection with a specific ST.

The sequence analysis showed a high identity (99-100%) to homologous sequences of *Blastocystis* previously reported in GenBank™, allowing the preliminary BLAST identification of four distinct STs (i.e.

ST5, ST7, ST9, ST10). In the remaining 23 PCR products, the chromatograms revealed the presence of double signals, suggesting that mixed infections by different STs in the same host occurred. Therefore, subtypes from such samples were not assessed since the ST identification protocol followed in this study, i.e. PCR amplification coupled with sequencing, preferentially amplified and allowed to identify the predominant subtypes. Nevertheless, as previously demonstrated, the cloning of the PCR product or next generation amplicon sequencing is required to discriminate different subtypes in the same host [3, 24, 25, 26].

The sequences alignment of the four STs found in the faecal samples from animals here analysed is reported in Fig. 1. The phylogenetic analysis showed the *Blastocystis* isolates of the present study clustering in four distinct clades each of them well supported at the bootstrap analysis by both MP and NJ phylogenetic analyses (Fig. 2), also including representative sequences retrieved from GenBank™ for those STs. Thus, the tree topologies of MP and NJ were congruent in showing the *Blastocystis* isolates as belonging to the subtypes ST5 (N= 14), ST7 (N= 1), ST9 (N= 1), ST10 (N= 3) with strong robustness (from 99% to 100%). In detail, the isolates from pigs (N= 9), goats (N= 4) and deer (N= 1) clustered with ST5 reference sequences (MG000956, MF541105). The sequence from chicken (N=1) collected in the site 1/ME clustered with ST7 (KP233733) sequence deposited in GenBank™, while that from pheasant (N=1), collected in the site 2/ME, showed high identity with ST9 sequences from GenBank™ (MK861942, MK861944). Finally sequences from sheep (N=3) formed a distinct clade with ST10 deposited sequence (MF974614) (Fig.2).

Alleles were named using the *Blastocystis* 18S database [18] allowing the identification of 2 distinct alleles within the ST5 here identified from different hosts i.e. allele 115 found in samples from pigs (pig 45, 46, 47, 158, 158 and 160) and goats (goat 51, 52, 53, 54); allele 119 from pigs (pig 157, 161 and 162) and deer. In addition, the alleles 100, 129 and 152 were identified, respectively within the ST7, ST9 and ST10 isolated from chicken, pheasant and sheep (sheep 149, 151, 154), respectively (Table 1).

Table 1: Animal samples collected from various species in three Italian provinces (ME=Messina: sites 1,2,3; CT=Catania; RT=Rieti) and results of molecular identification and sequence analysis.

Host	Scientific name	Location/Province	Sample Numbers	Positive samples (%)	STs identified	Alles
<i>Farmed animals</i>						
Calf	<i>Bos taurus</i>	1/ME	3	1 (33)	untypable	
Cow	<i>Bos taurus</i>	2/ME	10	0		
Fallow deer	<i>Dama dama</i>	1/ME	1	1 (100)	5	119
Dorkey	<i>Equus asinus</i>	1/ME	2	0		
Duck	<i>Anas platyrhynchos domesticus</i>	1/ME	2	0		
Goat	<i>Capra aegagrus hircus</i>	1/ME	4	4 (100)	5	115
Chicken	<i>Gallus gallus domesticus</i>	1-2/ME	16	8 (50)	7	100
Horse	<i>Equus caballus</i>	1/ME	3	0		
Ostrich	<i>Struthio camelus</i>	1/ME	2	0		
Peacock	<i>Pavo sp.</i>	1-2/ME	7	3 (42)	untypable	
Pheasant	<i>Phasianus colchicus</i>	1-2/ME	10	6 (60)	9	129
Pig	<i>Sus scrofa domestica</i>	3/ME	13	10 (77)	5	115, 119
Sheep	<i>Ovis aries</i>	3/ME	10	9 (90)	10	152
Turkey	<i>Meleagris gallopavo</i>	1/ME	1	0		
Total			84	42 (50%)		
<i>Carnivores</i>						
Dog	<i>Canis lupus familiaris</i>	Owned-Kennel/CT	71	0		
Cat	<i>Felis silvestris canis</i>	Owned/ME	3	0		
Foxes	<i>Vulpes vulpes</i>	RT	111	0		
Total			185	0		

Sequences from animals analysed in this survey were then compared with isolates from humans identified as ST1, ST2, ST3, ST4, ST6, ST7 and ST8 in previous studies [9, 10, 11]. Phylogenetic analysis inferred from NJ analysis evidenced a highest similarity (100%) among the ST7 detected in chicken in the present study and ST7 isolates from humans previously found [9] and the ST7 reference sequence (KP233733) (99%), supported by high bootstrap value of this clade (Fig. 3). In this work, no other isolate from animal samples was found as zoonotic, while the clades representing ST5, ST9 and ST10 included also sequences from humans (Fig. 3).

4. Discussion

The present study represents the widest epidemiological survey performed so far in animals from Italy. It also increases the current knowledge on *Blastocystis* STs distribution and their circulation in 17 animal species in Italy evidencing the protist in the 50% of the analysed livestock.

Mono-subtype infections were detected in 45.23% (19/42) of the animals while in 54.76% (23/42) of the positive samples mixed infection (more than one subtype present in a single sample) has been hypothesized, as evidenced in previous surveys on animals and humans [25, 26, 27].

All the samples from dogs, cats and foxes resulted negative in this study for *Blastocystis* STs. Similar results were previously obtained in other studies, which showed dogs not infected by *Blastocystis* [28, 29, 30, 31, 32] or infected with a low or moderate prevalence value [33, 34, 35, 36, 37]. Otherwise, the prevalence reported for stray dogs from India living mostly in areas of poor sanitation and hygiene was significantly higher (24%) [38]. Recently, *Blastocystis* ST3 was identified in 21.2% faecal samples from dogs housed in 6 different shelters located in Northern Italy, thus evidencing that sheltered dogs seem to be more at risk of harbouring *Blastocystis* than owned ones [15]. Similarly, in a survey carried out in the USA, *Blastocystis* was detected in 9.7% of examined shelter dogs while no positivity was found in owned dogs [34]. Negative results observed in this study could easily be explained by a sampling bias as more than half of the dogs (42 out of 71) were from the same kennel where probably *Blastocystis* did not

circulate among dogs or in the environment, or by the daily cleaning of cages and the small number of animals *per* cage. The remaining 29 dogs were owned animals, frequently submitted to antiparasitic treatments. Anyway, this low or moderate prevalence of *Blastocystis* observed in numerous canine cohorts and confirmed also in this study, together with the absence of a dog-specific/predominant ST, strongly suggests that dogs are unlikely to be natural reservoir of *Blastocystis*. Similarly, cats seem to play a minor role in the epidemiology of *Blastocystis*, although limited studied has been conducted so far on domestic and stray cats [25, 32, 37, 40]. In the present study, only 3 feline faecal samples were examined corroborating these observations, but more specimens should be examined in order to assess the role of cat in the epidemiology of *Blastocystis*. Furthermore, *Blastocystis* was apparently absent in the investigated red fox wild population, although it was recently found in 2.2% of red foxes in Spain and about 2% of arctic foxes in China [25, 37].

Conversely, the results gathered from isolates of livestock evidenced a high percentage (50%) of farmed animals colonized by *Blastocystis*. This finding emphasizes the potential risk of the zoonotic transmission to humans. To corroborate this evidence, we compared the sequences from animals obtained in the present survey with human isolates identified as ST1, ST2, ST3, ST4, ST6, ST7 and ST8 from our previous studies [9, 10, 11].

Phylogenetic analysis evidenced a high similarity (100%) among the ST7 isolates from human patient (Hum190) with the chicken (isolate no.35) supported by high bootstrap value of this clade including also the ST7 reference sequences (Fig. 3). Accordingly, allele analysis showed the same allele (100) observed in both ST7 isolated from human and in chicken. Furthermore, the human ST6 isolates (N=7) previously detected in Mattiucci et al [9] clustered with reference sequences from chicken and humans available in GenBank™ database (Fig.3).

The identification of such zoonotic STs in edible animals as poultry emphasizes the potential risk of *Blastocystis* transmission directly from animals to humans. We cannot depict the precise mode of transmission, however, we could hypothesize that it may happen through the human handling of those farmed animals, as well as through the consumption of products of animal origin (*e.g.*, eggs or cheese) [41]. Furthermore, the identification of the allele 100 from ST7 isolates in human and chicken represents a remarkable evidence of the direct transmission of this subtype from animals to humans.

In the recent years, several studies willing to address the issue of *Blastocystis* pathogenicity in humans, suggested that it could be related to genetic differences in the subtype- or strain-level of distinct STs [42, 43], correlating the ST1, ST4, and ST7 with pathological alterations in humans, while ST2 and ST3 have been identified as non-pathogenic [43, 44, 45]. A recent multi-locus sequence typing analysis of *Blastocystis* ST3 and ST4 has provided valuable insight into genetic variation within and between the two subtypes, evidencing high or low level of genetic diversity for ST3 and ST4, respectively [46]. Similar results were obtained in our previous survey [9] where 3 haplotypes (H1, H3, H7) have been identified in ST3 isolates, while a single haplotype (H2) was observed in ST4 symptomatic patients. Thus, we suggested that intra-subtype diversity showed by ST3 and ST4 could be linked to the evolutionary history

of *Blastocystis* subtypes and that ST3 may have co-evolved with human hosts over a longer period than ST4; this latter, instead, may have extended its range to humans more recently, and, therefore, shows a low genetic variability.

In this study, we may support this hypothesis as we evidenced the same allele within the ST7, which has been reported to be strongly associated with gastrointestinal symptoms in humans and showed pathogenic properties, not observed in other STs [43, 47]. Indeed, features of gastro-intestinal symptoms were also found in human patients, here showing the same allele 100, at the ST7 (Mattiucci and Gabrielli, personal communication). Therefore, we could hypothesize that ST7, exhibiting such genetic low variability, was recently transmitted to humans and, accordingly, it shows also a higher pathogenetic role to humans.

5. Conclusion

The outcome of this study may be considered a starting point to define the distribution of *Blastocystis* subtypes in different animal hosts in Italy and to study pathways of the zoonotic transmission of the protist. However, further parasitological and molecular investigations need to be carried out with the aim to evaluate other potential animal reservoirs of *Blastocystis* in Italy. For instance, despite in this study several bird's species (chickens, pheasants, turkeys, ostriches, ducks and peacocks) have been analysed, we did not find the *Blastocystis* ST6 in any of the tested birds. Conversely, in previous surveys, ST6 was detected in human subjects (3.2% of the examined), although it is considered an "avian subtype" rarely occurring in humans [1, 6]. Also, as the sample size of some farmed animal species analysed in the current study was not large enough, further surveys should be planned including other avian species and more samples from the above tested animals as well as from other geographical areas of Italy.

Thus, it becomes evident that a greater knowledge of the evolutionary history of *Blastocystis* subtypes could, in future, explain any pathogenic aspects related to distinct subtypes. Studies on the genetic variation within and among identified subtypes may elucidate possible co-evolutionary aspects, and provide data for understanding *Blastocystis* biology, host–parasite interactions and pathogenicity to humans.

Lastly, as *Blastocystis* has been reported as a neglected waterborne protist and recently detected in tap water [45] and drinking water treatment plant [48], further investigations are needed to assess this source of transmission to human and animal hosts also in Italy.

In conclusion, these findings represent only the tip of the iceberg about the epidemiology of *Blastocystis* in Italy and suggest the need to apply molecular and phylogenetic analyses to demonstrate the transmission dynamics among humans, animals and environment of this so far debated plastic protist.

Abbreviations

Neighbour Joining (NJ), Maximum Parsimony (MP), Subtype (ST), Small subunit (SSU).

Declarations

Author's contribution

Simona Gabrielli: Conceptualization, Data curation, Writing- Original draft preparation, Funding acquisition; **Federica Furzi:** Investigation, Software, Writing- Reviewing and Editing; **Emanuele Brianti, Gabriella Gaglio, Ettore Napoli, Laura Rinaldi, Renato Aco Alburquerque:** Resources; Writing- Reviewing and Editing; **Michela Paoletti:** software, Writing- Reviewing; **Simonetta Mattiucci:** Conceptualization, Data curation, Writing- Reviewing and Editing, Project administration, Funding acquisition.

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Availability of data and materials

All data generated or analysed during this study are included in this published article. The raw datasets are available from the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

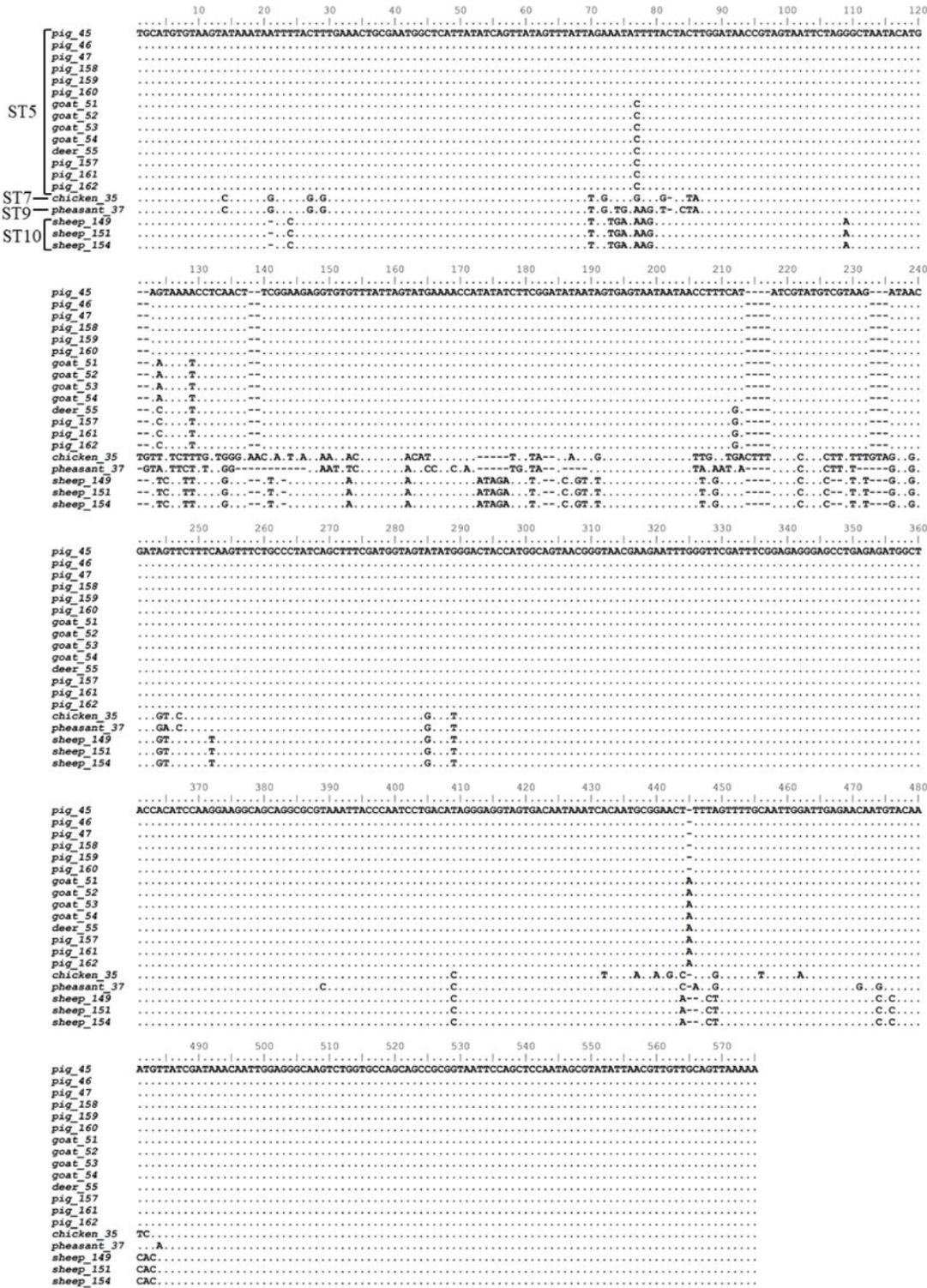


Figure 1

Multiple sequence alignment inter- and intra-subtype carried out with Bioedit software and performed by using sequences obtained from animals analysed in this survey. Dots represent identity and dashes correspond to gaps.

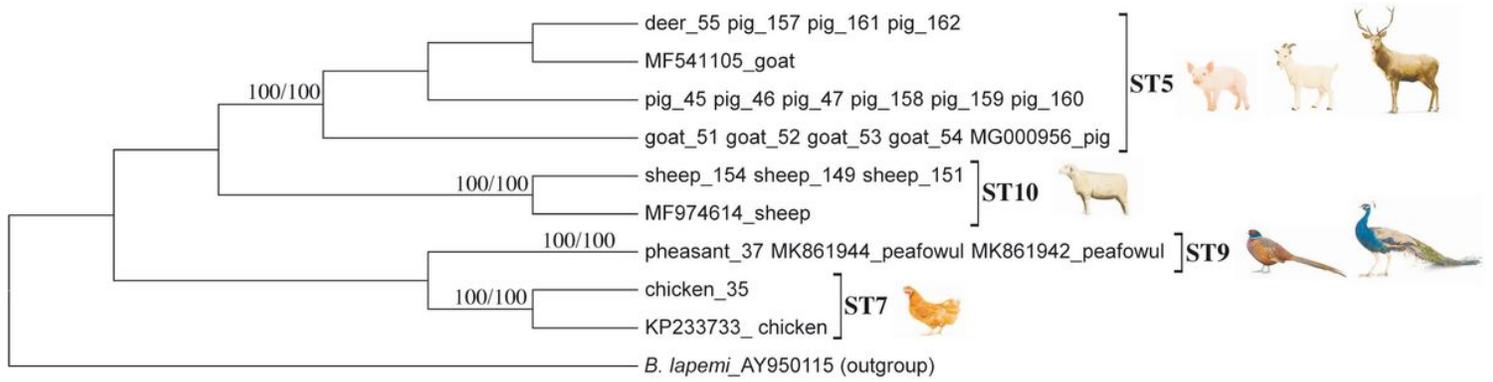


Figure 2

NJ and MP condensed phylogenetic consensus trees obtained from sequences at the SSU rDNA gene of *Blastocystis* samples from animals analysed in this study, in comparison with the available STs previously deposited in GenBank™. NJ and MP bootstrap values are reported, respectively, at the nodes. The phylogenetic trees were rooted using *Blastocystis lapemi* (GenBank™ accession no. AY590115) as outgroup.

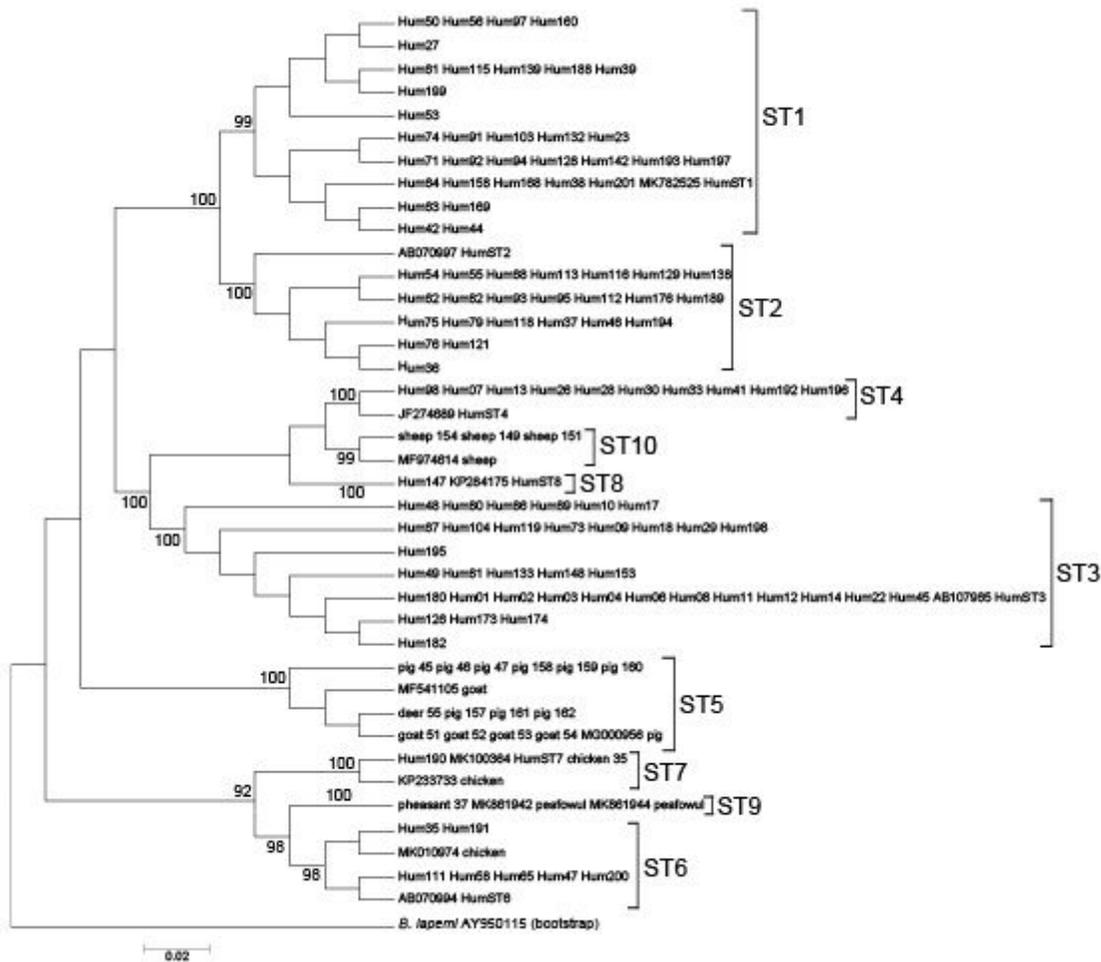


Figure 3

NJ phylogenetic tree obtained from sequences at the SSU rDNA gene of *Blastocystis* samples from animals analysed in this study, in comparison with human isolates identified as ST1, ST2, ST3, ST4, ST6, ST7 and ST8 from previous studies (Mattiucci et al., 2016; Fontanelli-Sulekova et al., 2019; Gabrielli et al., 2020) and available STs previously deposited in GenBank™. NJ bootstrap values are reported, respectively, at the nodes. The phylogenetic tree was rooted using *Blastocystis lapemi* (GenBank™ accession no. AY590115) as outgroup.