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ORIGINAL ARTICLE

First molecular characterization of *Blastocystis* subtypes from domestic animals (sheep and cattle) and their animal-keepers in Ilam, western Iran: A zoonotic concern

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Abstract

A total of 360 fecal samples were randomly collected from 150 cattle, 150 sheep, and 60 humans (30 people with close animal contact and 30 individuals without close animal contact) at 10 farms in Ilam, western Iran from June 2022 to August 2023. All samples were directly examined for *Blastocystis* by zinc sulfate flotation, followed by microscopic observation. Positive samples were further subtyped using conventional PCR and sequencing methods. A mean prevalence of 5.3% (16/300) was estimated for *Blastocystis* infection among examined animals, with 6% and 4.7% for cattle and sheep, respectively. Among the people who had close and non-close animal contact, 16.7% (5/30) and 3.3% (1/30) were infected with *Blastocystis*, respectively ($p<0.05$). All 22 positive samples were successfully sequenced at the SSU rRNA locus. Accordingly, *Blastocystis* isolates infecting domestic animals in Ilam belonged to the four STs (ST1-ST3, and ST10). Of the 16 animal isolates, nine sequences (four ST10, three ST3, and two ST1) were related to cattle, and seven sequences (three ST10, two ST3, and two ST2) were isolated from sheep. Among the six human isolates, ST3 was the most predominant ST, followed by STs 1, 2, 6, and 7 (one case each). Of note, ST1-ST3 were isolated in various farms both from animals and their breeders, which indicates the possible circulation of these STs between animal and human populations.

KEYWORDS
Blastocystis sp., cattle, human, Iran, sheep, subtypes

INTRODUCTION

BLASTOCYSTIS sp. is a single-celled anaerobic parasite belonging to the Stramenopiles group (Mahdavi et al., 2022; Stensvold & Clark, 2020). It inhabits the intestinal tracts of a large number of birds and mammals, including humans, around the world (Jiménez et al., 2019). Transmission of *Blastocystis* sp. occurs via the fecal-oral route (Baek et al., 2022). Some studies suggest that water contaminated with feces may be a source of infection (Higuera et al., 2021). The number of individuals infected with this parasite is more than one billion worldwide (Asghari et al., 2020; Asghari, Sadeghipour, et al., 2021). Although the disease is more common in developing countries, surveys in developed nations typically show a prevalence above 5% in the general population (Asghari, Hassanipour, & Hatam, 2021; Gabrielli et al., 2020). Six different morphological forms of the parasite have been reported: vacuolar, granular, amoeboid, avacuolar, multivacuolar, and cystic (Asghari, Sadrebazzaz, et al., 2021; Mülayim et al., 2021). Parasites are genetically distinct despite morphological similarities. To date, a total of 32

Blastocystis subtypes (STs) have been described, based on polymorphisms in the small subunit ribosomal RNA (SSU rRNA) gene. ST1–ST32 have been described in many mammalian and avian species. However, based on current criteria for being considered a single ST, a total of 28 STs (ST1 to ST17, ST21, and ST23 to ST32) are generally widely recognized as valid STs (Oliveira-Arbex et al., 2020; Shams, Shamsi, et al., 2022). Over the past two decades, many epidemiological studies of *Blastocystis* have demonstrated that most STs have low host specificity. The infection is associated with at least 14 STs (ST1-ST10, ST12, ST14, ST16, and ST24) in humans and animals, suggesting a high likelihood of zoonotic transmission. Additionally, more than 95% of *Blastocystis* cases in humans are thought to be caused by ST1–ST4 (Güreser et al., 2022; Maloney et al., 2022). Some STs are highly host-specific, for example, ST5 commonly infects pigs while ST6 and ST7 predominate in birds. These hosts represent potential reservoirs for zoonotic infection transmission (Shams, Asghari, et al., 2022; Sheikh et al., 2020). Earlier investigations have revealed that *Blastocystis* is common in people who handle animals, including zookeepers and slaughterhouse workers, signifying those animals may pose a considerable risk as a source of transmitting *Blastocystis* to humans (Greige et al., 2018; Köster et al., 2022). The present study, the first reported to be carried out in the Ilam city, examines the first molecular characterization of *Blastocystis* sp. isolated from livestock (sheep and cattle) and their keepers/herders in Ilam, western Iran.

MATERIALS AND METHODS

Ethics approval

The present study was approved by the Ethics Committee of Ilam University of Medical Sciences, Ilam Province, Iran (approval number: A-10-2429-20).

Study area

This cross-sectional descriptive study was conducted to examine the molecular characteristics, subtype distribution, and zoonotic potential of *Blastocystis* sp. in livestock/ruminants (cattle and sheep) and their keepers/herders in Ilam, western Iran (Figure S1). Ilam province has an area of 20,133 square kilometers, making it the 22nd largest province in Iran. The capital of this province is the city of Ilam. The geographical coordinates of this province are 33.638531° N and 46.422649° E. Ilam is located in a mountainous area, half hot and half humid. This province is one of the forest regions of Iran. The north of the province has a mountainous climate and cold winters, but in the south, the province has vast plains with hot summers. Livestock and livestock farming in Ilam is considered one of the leading economies in the region, and many residents in the province work in this sector.

Animal samples collection

From June 2022 to August 2023, fecal samples were gathered from 300 domestic animals (150 sheep and 150 goats), aged between 2 months to 4 years old, raised in 10 farms in Ilam city (Table 1). No clinical symptoms such as diarrhea were observed in the tested animals, and stool consistency was normal. Of note, the sampling date, species, age, and identification number were recorded for each animal. The specimens were transported to the laboratory of the department of parasitology and mycology at Shiraz University of Medical Sciences, Shiraz, Fars Province, Iran, in a cool box, then stored at 4°C and processed within 2 days of collection. Each fecal specimen was directly examined by saline/iodine wet mount examination. Wet smears were examined for the presence of *Blastocystis* sp. by light field microscopy at 400 and 1000X magnification. All samples were stored in 2.5% potassium dichromate solution at 4°C before DNA extraction.

Human samples collection

From the areas where animal samples were collected, 60 human stool samples (38 men and 22 women) were also gathered at the same time. Accordingly, 30 individuals as a case group (three people per farm) had close contact (such as grazing, milking, preparing, and spreading fodder between animals, etc.) with the sampled animals. Additionally, 30 individuals without close interactions with animals were also collected as part of the control population during the same period.

Microscopic screening and DNA extraction

All samples collected in the current study were screened for gastrointestinal parasites by zinc sulfate flotation, followed by microscopic observation (Boutellis et al., 2021). A sample was considered positive when at least one of the morphological forms of the parasite was detected in the stool. With respect to DNA extraction, at the starting step, the potassium dichromate was evacuated from all the fecal samples utilizing numerous washing steps with distilled water. Genomic DNA was straightforwardly extricated from around 200 mg of the fecal pellet employing a QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany), agreeing to the manufacturer's instruction. DNA was eluted in 50 ml of distilled water and prepared DNA was located at −20°C prior to utilize for polymerase chain reaction (PCR) examination.

TABLE 1 Prevalence and STs distribution of *Blastocystis* sp. based on age groups and sampled farms in the present study.

		Age group[a]								
		Positive no./examined no. (%)					No. *Blastocystis* STs			
Characteristic	Animal species	<3	4–7	8–11	>12	Total	ST1	ST2	ST3	ST10
Farm 1	Sheep	1/7 (14.3)	−/8 (0)	1/10 (10)	−/5 (0)	2/30 (6.7)	–	1	–	1
Farm 2	Sheep	−/3 (0)	−/6 (0)	−/9 (0)	−/12 (0)	−/30 (0)	–	–	–	–
Farm 3	Sheep	1/8 (12.5)	−/10 (0)	−/8 (0)	−/4 (0)	1/30 (3.3)	–	–	1	–
Farm 4	Sheep	−/6 (0)	1/9 (11.1)	−/7 (0)	−/8 (0)	1/30 (3.3)	–	1	–	–
Farm 5	Sheep	1/9 (11.1)	1/7 (14.3)	−/7 (0)	1/7 (14.3)	3/30 (10)	–	–	1	2
Sub-total		3/33 (9.1)	2/40 (5)	1/41 (2.4)	1/36 (2.8)	7/150 (4.7)	–	2	2	3
Farm 6	Cattle	−/6 (0)	−/7 (0)	−/8 (0)	−/9 (0)	−/30 (0)	–	–	–	–
Farm 7	Cattle	1/7 (14.3)	1/6 (16.7)	−/9 (0)	1/8 (12.5)	3/30 (10)	2	–	–	1
Farm 8	Cattle	−/2 (0)	−/5 (0)	1/7 (14.3)	−/16 (0)	1/30 (3.3)	–	–	–	1
Farm 9	Cattle	−/4 (0)	1/5 (20)	1/6 (16.7)	−/15 (0)	2/30 (6.7)	–	–	1	1
Farm 10	Cattle	2/7 (28.6)	−/6 (0)	−/5 (0)	1/12 (8.3)	3/30 (10)	–	–	2	1
Sub-total		3/26 (11.5)	2/29 (6.9)	2/35 (5.7)	2/60 (3.3)	9/150 (6)	2	–	3	4
Total		6/59 (10.2)	4/69 (5.8)	3/76 (3.9)	3/96 (3.1)	16/300 (5.3)	2	2	5	7

[a]Age groups are based on months.

Molecular identification and PCR

The existence of *Blastocystis* sp. was determined by PCR targeting a 479 bp fragment of the SSU rRNA gene in DNA samples using the primers described earlier (Santín et al., 2011). PCR reactions were conducted in 25 µl reaction mixtures containing 2.5 µl of 10× buffer, 2.5 mM of MgCl2, 200 µM of each deoxyribonucleoside triphosphate (dNTPs) mixture, 0.2 µM of each primer (Eurogentec, Belgium), 0.025 U/µl of Taq DNA polymerase Hot Start (Qiagen, Courtaboeuf, France), and 5 µl of genomic DNA using the following thermocycling conditions: 95°C for 4 min, followed by 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s for 35 cycles, and a final extension 72°C for 5 min. Negative and positive controls were used to confirm our results. PCR amplicons were then verified by electrophoretic migration of PCR product on a 1.5% agarose gel using ethidium bromide. The reported nucleotide sequence data are available in the GenBank databases under the accession numbers OR611147, OR611148, OR611153, OR611709, OR611710, OR611713, OR611715, OR611717–OR611719, OR611721, OR611722, OR611724, OR611725, OR611916, OR611924, OR611925, OR611933, OR611934, OR611936, OR612929, and OR612930.

DNA sequencing and phylogenetic analysis

The PCR products were initially purified using the PureLink Genomic DNA Mini Kit (Life Technologies Corporation, New York, USA), according to the manufacturer's protocol. A bidirectional DNA sequencing was performed by forward and reverse primers using an Applied Biosystems 3130/3130xl Genetic Analyzer. The obtained DNA sequences were analyzed by Finch TV v 1.4.0 (Geospiza Inc., Seattle, WA, USA) and a Basic Local Alignment Search Tool (BLAST)-based comparison and multiple alignments was exerted between the SSU rDNA genes of the novel isolates and those previously deposited in the GenBank (National Center for Biotechnology Information) using BioEdit v 7.1.3.0 software (Ibis Biosciences, Carlsbad, CA, USA). Neighbor-joining analysis and construction of a phylogenetic tree were done using MEGA X software (The Biodesign Institute, Tempe, AZ, USA). The tree was rooted with the sequence of *Proteromonas lacertae* (U37108) as an out-group.

Statistical analysis

The collected data were analyzed using SPSS software (version 20, IBM Inc., USA). Chi-square tests were used to determine the correlation between *Blastocystis* infection and the examined variables. $p < 0.05$ was regarded as statistically significant.

RESULTS

Prevalence of *Blastocystis* sp. in animals

From the 300 animal stool samples screened by microscopy and PCR, 16 samples (5.3%) were positive for *Blastocystis*. Accordingly, the occurrence of *Blastocystis* infection in the examined cattle and sheep population was 6% (9/150) and 4.7% (7/150), respectively. The

investigated age groups associated with each host and prevalence of *Blastocystis* are shown in Table 1.

Prevalence of *Blastocystis* sp. in humans

From a total of 60 human stool samples evaluated by microscopy and PCR, *Blastocystis* sp. was observed in six (10%) stool samples. Among the people who had close and non-close contact (control group) with examined animals, 16.7% (5/30) and 3.3% (1/30) were infected with *Blastocystis*, respectively (Table 2).

Potential host factors associated with *Blastocystis* infection in examined animals and humans

No statistically substantial difference ($p > 0.05$) was reported between the age groups of the examined animals and the prevalence of *Blastocystis* sp. (Table 1), while regarding the prevalence and STs distribution of *Blastocystis* sp., a statistically significant difference ($p < 0.05$) between the case (people with close animal contact) and the control groups (people without close animal contact) was observed (Table 2).

Phylogenetic analysis and STs distribution of *Blastocystis* sp.

All 22 positive samples were successfully sequenced at the 18S locus. 98%–100% sequence identity was found between all sequences containing six STs (ST1–ST3, ST6, ST7, and ST10) obtained in the present study and the *Blastocystis* sequences acquired in GenBank. The phylogenetic tree of *Blastocystis* reconstructed using the neighbor-joining method and Kimura 2-parameter model demonstrated that *Blastocystis* isolates infecting domestic animals/livestock in Ilam belonged to the four STs (ST1–ST3, and ST10). Accordingly, of the 16 sequenced animal isolates, nine sequences (four ST10, three ST3, and two ST1) were related to cattle, and seven sequences (three ST10, two ST3, and two ST2) were isolated from sheep. Among the six human isolates, ST3 was the most predominant subtype (two cases), followed by STs 1, 2, 6, and 7 (one case each) (Figure 1).

DISCUSSION

The present study provided information about prevalence, STs distribution, and zoonotic significance of *Blastocystis* isolates in cattle, sheep, and humans for the first time in Ilam city, the capital of Ilam Province, western Iran. Accordingly, infection rates of *Blastocystis* were 6% (9/150) for cattle, 4.7% for sheep (7/150), and 10% (6/60) for both case and control groups of humans, which were lower than those reported previously worldwide, respectively, with 1.8%–80% in cattle (Shams et al., 2021), 5.5%–81.8% in sheep (Shams, Asghari, et al., 2022) and 0.7%–100% in humans (Bart et al., 2013; Popruk et al., 2021; Zhu et al., 2020). These reported differences in the frequency of *Blastocystis* in evaluated hosts may be related to diverse geographical areas, sample sizes, health status, husbandry procedures, host immune status, and sensitivity of the diagnostic methods.

Presently, four regions of the SSU rRNA gene are commonly employed to recognize *Blastocystis* sp. by

TABLE 2 Prevalence and STs distribution of *Blastocystis* sp. based on case and control groups and sampled farms in the present study.

	Total no.		Infected no. (%)		No. *Blastocystis* STs				
Characteristics	Case[a]	Control[b]	Case	Control	ST1	ST2	ST3	ST6	ST7
Farm 1	3	3	1 (33.3)	–	–	1	–	–	–
Farm 2	3	3	–	1 (33.3)	–	–	–	1	–
Farm 3	3	3	1 (33.3)	–	–	–	1	–	–
Farm 4	3	3	–	–	–	–	–	–	–
Farm 5	3	3	–	–	–	–	–	–	–
Sub-total	15	15	2 (13.3)	1 (6.7)	–	1	1	1	–
Farm 6	3	3	–	–	–	–	–	–	–
Farm 7	3	3	2 (66.7)	–	1	–	–	–	1
Farm 8	3	3	–	–	–	–	–	–	–
Farm 9	3	3	–	–	–	–	–	–	–
Farm 10	3	3	1 (33.3)	–	–	–	1	–	–
Sub-total	15	15	3 (20)	0 (0)	1	–	1	–	1
Total	30	30	5 (16.7)	1 (3.3)	1	1	2	1	1

[a]People with close animal contact.

[b]People with non-close animal contact.

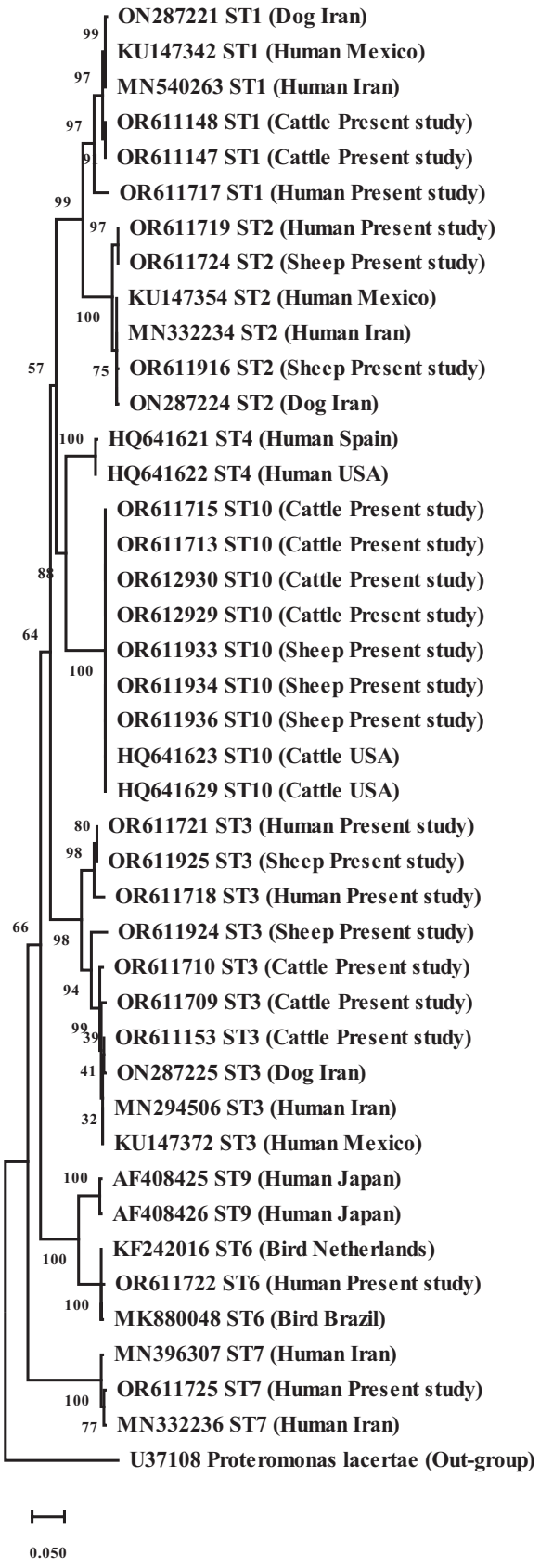

FIGURE 1 The phylogenetic tree was built using the neighbor-joining method and sequences from GenBank. It revealed that the *Blastocystis* STs infecting animals and humans in this study belong to STs 1–3, 6, 7, and 10. The percentages of replicate trees with associated taxa clustered in the bootstrap test (1000 replicates) are indicated next to the branches.

PCR (Clark et al., 2013). Primers aimed at amplifying the Santín region of *Blastocystis* were confirmed to have the advantage of greater specificity and sensitivity than primers for the Barcode and Stensvold regions, respectively (Santín et al., 2011). Meanwhile, it was reported that Barcode region amplification primers are capable of amplifying SSU-rRNA genes from other eukaryotes (mainly fungi) in the absence of *Blastocystis*, especially when screening extracted DNA directly from feces (Stensvold, 2013). Barcoding is only suitable for molecular characterization of known positive samples, not for screening (Stensvold & Clark, 2016a, 2016b). Therefore, in the present study, to avoid PCR amplification of *Blastocystis* false positives, the primers designed by Santín et al. were used to screen for *Blastocystis* in stool samples (Santín et al., 2011).

The zoonotic risk of *Blastocystis* has been predicted by several previous studies (Asghari et al., 2019). In early 1999, in an epidemiological study of *Blastocystis* in humans, it was found that animal handlers had a significantly higher infection rate than healthy individuals who did not work with animals (Salim et al., 1999). There are also a number of studies evaluating the potential for zoonotic transmission at the molecular level, based on the fact that the same subtypes have been detected in humans and animals in the same regions. In Australia, ST5 has been detected in pigs and piggery workers (Parkar et al., 2010; Wang et al., 2014). In Nepal, ST6 was found in animals (cattle and goats) and their breeders (Lee, Chye, et al., 2012; Lee, Tan, et al., 2012), while ST2 was detected in monkeys and in-contact children (Yoshikawa et al., 2009). However, recent results of SSU rRNA gene analysis of *Blastocystis* isolates from humans and non-human primates reveal not only the general characteristics of ST1 but also the cryptic host specificity of ST2 (some ST2 haplotypes have a preference for either humans or NHP) (Villanueva-Garcia et al., 2017). In the present study, the sequencing of 22 positive *Blastocystis* samples revealed that the cattle and sheep examined were infected with STs 1, 3, and 10 and 2, 3, and 10, respectively. In line with a previous study (Boutellis et al., 2021), the examination of human samples showed that the prevalence of *Blastocystis* infection in the case group with STs 1–3 and 7 was remarkably high compared to the control group with only ST7 [16.7% vs. 3.3% ($p < 0.05$)]. Of note, although a relatively low prevalence of *Blastocystis* was reported in the investigated hosts in the present study, subtypes 1–3 were isolated in farms 1, 3, 7, and 10 both from animals and their keepers/breeders, which indicates the possible circulation of these STs between animal and human hosts and the possible transmission of zoonotic infection.

In the present study, the only case isolated from the human control group and one of the STs isolated from the case group were ST7 [100% (1/1)] and ST6 [16.7% (1/6)], respectively, which are zoonotic STs and are more specific to birds (Maloney et al., 2020; Wang et al., 2018). Among animal and human *Blastocystis* isolates, the most predominant STs reported were ST10 [43.7% (7/16)] and ST3 [33.3% (2/6)], respectively, which is completely consistent with the results of global meta-analysis studies (Shams et al., 2021; Shams, Asghari, et al., 2022), accordingly, 14 genetically diverse STs of *Blastocystis* (ST1–ST5, ST7, ST10, ST12, ST14, ST15, ST21, ST23, ST24, ST26) have been reported in sheep, and the highest pooled prevalence was related to ST10 [57.8% (95% CI: 43.7%–70.8%)], followed by ST14 [28.4% (95% CI: 20.2%–38.4%)], and ST7 [21.1% (95% CI: 4.5%–60.3%)]. Furthermore, 16 STs were isolated from cattle, with ST10 as the highest-reported [32.3% (95% CI: 21.6–45.3)] as well as ST24 and ST25 as the lowest-reported STs [1.4% (95% CI: 0.2–9.1)].

CONCLUSION

The present study represents the frequency, STs distribution, and zoonotic potential of *Blastocystis* for the first time in common livestock (cattle, sheep) and their keepers/breeders in Ilam city, western Iran. *Blastocystis* was relatively more prevalent in cattle (6%) compared to sheep (4.7%) in Ilam. Identification of potentially zoonotic subtypes 1–3 in both animals and their keepers/breeders suggests the possibility of zoonotic transmission. Nevertheless, the low infection rates of *Blastocystis* and the small percentage of potentially zoonotic subtypes imply a minimal risk of these livestock in the transmission of *Blastocystis* to humans. Although the pathogenicity of *Blastocystis* remains unclear, health education to reduce zoonotic risk can be given to farmers, breeders, and veterinarians having close contact with these livestock. Overall, more epidemiological data is required to ultimately identify the potential animal reservoirs of human infection and add to our understanding of the circulation of *Blastocystis* STs in animal and human populations.

AUTHOR CONTRIBUTION

A.A. and M.S. planned and designed the study. A.A., A.B., A.M., S.S., and L.S. were involved in sample collection and methodology. A.A., M.S., and A.B. conducted the molecular analysis. A.A. and M.S. wrote the manuscript, with A.A. revising it. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available in the online version.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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