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A preliminary molecular analysis of zoonotic *Blastocystis* subtype ST5 in wild boars (*Sus scrofa*) from Hatay, Türkiye

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ABSTRACT

Wild boars (*Sus scrofa*), an important component of wildlife and increasingly present in rural and urban areas, play a complex role in transmission of pathogens between wildlife, livestock, and humans, with uncertainty about the directionality of transmission. This preliminary study aimed to demonstrate the occurrence of zoonotic *Blastocystis* in wild boars for the first time in Türkiye. The real-time PCR (RT-PCR)-based positivity rate of *Blastocystis* was determined as 29.2% (7/24). Two wild boar isolates that could be successfully sequenced, as a result of barcoding PCR, showed 100% similarity to *Blastocystis* zoonotic subtype 5 (ST5) and allele 115 was detected. The findings of this first study highlight the importance of monitoring *Blastocystis* for zoonotic transmission dynamics in wildlife and the need for future studies including larger numbers of wild boar samples from different geographical regions of Türkiye..

Key words: *Blastocystis*; Türkiye; wild boar; zoonotic potential; molecular analysis

Introduction

Blastocystis is a protist that inhabits the gastrointestinal tract of various animals (mammals, reptiles, fish, and birds) as well as humans. The life cycle of this eukaryotic microorganism includes vacuolar, granular, amoeboid, and cyst forms. Transmission occurs as a result of fecal-oral ingestion of cyst forms excreted in the feces of the infected host (HUBLIN et al., 2021). Nonetheless, water sources could represent a significant reservoir that contributes to the environmental contamination cycle and thereby the transmission of *Blastocystis* from one host to another, such as a human or various animals (MASUDA et al., 2022).

Blastocystis has been associated with non-specific gastrointestinal symptoms and, less commonly, dermatological symptoms, and asymptomatic carriage is common. Recent research suggests a

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positive correlation between *Blastocystis* and microbial gut diversity, highlighting its potential symbiotic function (TAN, 2008; AUDEBERT et al., 2016; KODIO et al., 2019; AYKUR et al., 2024).

Genetic polymorphism of small-subunit rRNA (SSU rRNA) has led to the identification of at least 44 different *Blastocystis* subtypes (ST) to date, including ST1-ST17, ST21, and ST23-ST44. Among these subtypes, ST1-ST10, ST12, ST14, ST16, and ST26 have been reported in both humans and animals, demonstrating the zoonotic potential of *Blastocystis.* Due to the low host specificity of multiple *Blastocystis* STs, research on various animal species and different geographic origins is needed to better understand its biology, transmission routes, and zoonotic potential (OSORİO-PULGARIN et al., 2021; HERNÁNDEZ-CASTRO et al., 2023; SANTIN et al., 2024; ŠEJNOHOVÁ et al., 2024).

Wild boars (*Sus scrofa*), in particular, represent an important wildlife species that should be considered within the One Health framework due to their increasing interactions with livestock, humans, and natural water resources (CUNNINGHAM et al., 2017).

The wild boar population in Türkiye has grown rapidly in recent years, and their presence in urban centers required an investigation of their effects on zoonotic transmission dynamics. There are limited studies reporting pathogens in wild boars in Türkiye (ALBAYRAK et al., 2013; KESIK et al., 2021; SALTIK et al., 2022; CELIK et al., 2024; GUNYAKTI KILINC et al., 2024), but so far there have been no studies reporting the presence of *Blastocystis* in wild boars. In this study, we have aimed to examine the presence and zoonotic potential of *Blastocystis* in free-ranging wild boars in Hatay Province, as a preliminary study for the first time in Türkiye.

Materials and methods

Study area and sample collection. Hatay province (35°52′–37°04′ N, 35°40′–36°35′ E), situated in the southernmost region of Türkiye, on the Syrian border, is surrounded by the Asi River, the Amik Valley, the Amanos Mountains, the Samandağ Coast, and the Gulf of Iskenderun. Due to the combination of land and sea habitats, Hatay is one of Türkiye's most unique places in terms of ecology and biodiversity (ZEREK et al., 2023). The Antakya, İskenderun, and Kırıkhan areas of Hatay province are the areas where the wild boar materials used in this investigation were sampled (Fig. 1).

Fecal samples were taken from 24 mature (>12 months) wild boars (coded as No:1-24) that were hunted within the permitted hunting season in Hatay province's Antakya (10), İskenderun (4), and Kırıkhan (10) districts. Approximately 10-20

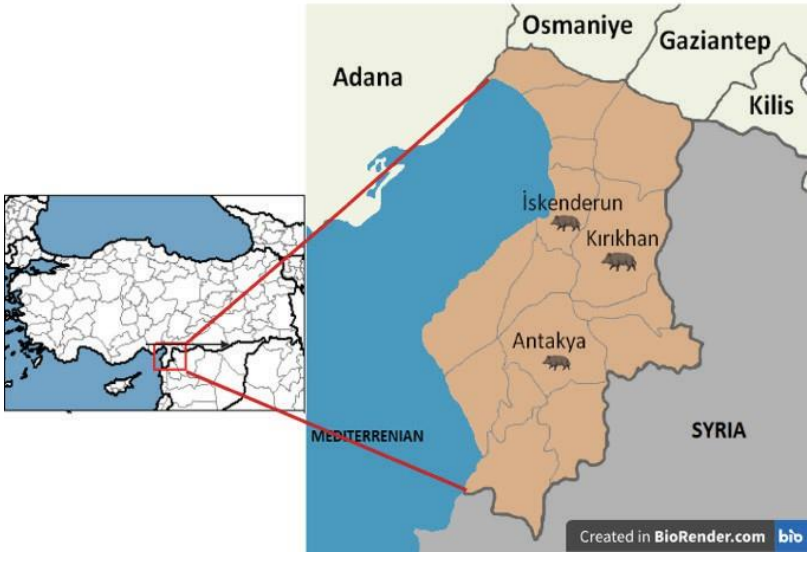

Fig. 1. Map of the Hatay/Türkiye showing the sampled areas

grams of newly collected fecal samples were removed from the rectum within 24 hours following the hunt and delivered in a cold chain to the laboratory in leak-proof containers.

DNA extraction. GeneMATRIX® stool DNA Purification Kit (EURx Sp. z o.o., Gdańsk, Poland) was used for DNA isolation from the stool samples following the kit procedure. The DNA samples obtained were stored at -20°C until molecular analysis.

Real-time PCR (RT-PCR) for the detection of Blastocystis. In this study, Blasto FWD F5, Blasto R F2 primers, and a FAM (fluorescein) probe were used for RT-PCR (STENSVOLD et al., 2012) for the small subunit ribosomal RNA (SSU rRNA) gene of *Blastocystis*. The sequence of the primers and probe is presented in Table 1. Amplification reactions (25 µl) contained 12.5 µl PCR Master Mix (Promega, USA), 0.5 µM Blasto_FWD_F5/ Blasto_R_F2 primer pairs, 0.3 µM probe, and 2 µl template DNA. Each reaction included positive (previously detected as ST3_*Blastocystis* by DNA sequencing) and distilled water as negative controls. RT-PCR protocol: 95˚C for 3 minutes, denaturation; 95˚C for 15 seconds, primer annealing, and extension step 57˚C for 1 minute (40 cycles).

DNA sequence analysis, subtypes determination. In samples determined as *Blastocystis* positive by real-time PCR and with a Ct value below 30, barcoding regions were amplified by conventional PCR and run in gel electrophoresis.

For *Blastocystis* SSU rRNA barcoding PCR (~600 bp), RD5 and BhRDr primers were used (STENSVOLD, 2013). The sequence of the primers is presented in Table 1. The thermal cycler protocol for DNA amplification was applied as follows: pre-denaturation 95˚C for 5 minutes; denaturation 94˚C for 60 seconds; primer annealing 55˚C for 45 seconds, extension step 72˚C for 60 seconds (35 cycles), and final extension step 72˚C for 5 minutes.

As a result of DNA sequence analysis, the sequences belonging to the "barcoding" region were compared with reference gene sequences in the National Center for Biotechnology Information (NCBI) using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The *Blastocystis* database (http://pubmlst.org/*Blastocystis*/ database) was also used to determine *Blastocystis* subtypes and alleles. A small subunit ribosomal RNA gene-based phylogenetic tree of *Blastocystis* was constructed using the MEGA program (version 11.0.13) with additional sequences retrieved from GenBank. The BioNJ algorithm was used to construct the phylogenetic tree.

Results

Out of 24 wild boar fecal samples examined, 7 (29.2%) were RT-PCR positive (samples No. 2, 4, 9, 12, 13, 18, and 23). Three positive samples were from wild boars in Kırıkhan, two from İskenderun, and two from the Antakya district. Barcoding PCR,

Table 1. Sequence of primers and probes used in RT-PCR and barcoding PCR techniques

Technique	Primer/ Probe name	Sequence (5'→3')	Reference
RT-PCR	Blasto FWD F5	GGTCCGGTGAACACTTTGGATTT	STENSVOLD et al., 2012
RT-PCR	Blasto R F2	CCTACGGAAACCTTGTTACGACTTCA	STENSVOLD et al., 2012
RT-PCR	FAM Probe	FAM-TCGTGTAAATCTTACCATTTAGAGGA-MGBNFQ	STENSVOLD et al., 2012
Barcoding PCR	RD5	ATCTGGTTGATCCTGCCAGT	STENSVOLD, 2013
Barcoding PCR	BhRDr	GAGCTTTTTAACTGCAACAACG	STENSVOLD, 2013

targeting the *Blastocystis* SSU rDNA region, was performed on all RT-PCR-positive samples.

Six samples showed the expected ~600 bp band on an agarose gel (Fig. 2) and were subjected to sequence analysis. Among the RT-PCR-positive samples, samples with a high threshold cycle (Ct: 28.4) value showed no band due to the low parasite burden.

Two isolates (No. 12 and 13) exhibited 100% sequence similarity with *Blastocystis* ST5 from *Sus scrofa domesticus* (Accession Nos. MK801375.1 and MK801369.1) in Germany, with allele 115 detected in both. Phylogenetic analysis confirmed the identification of ST5 (Fig. 3). The *Blastocystis* sequences from these two samples were deposited in GenBank under accession numbers PQ666459 and PQ666460.

Four isolates displayed mixed subtypes, indicated by double peaks in the sequencing chromatogram.

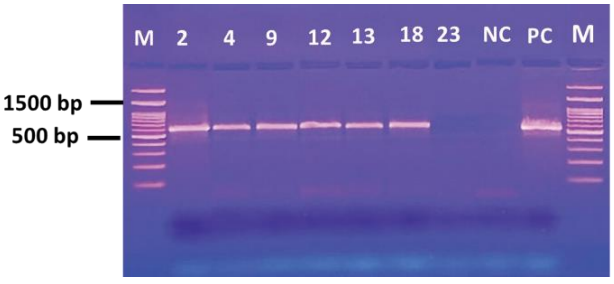

Fig. 2. RT-PCR positive samples showing the expected ~600 bp band on the agarose gel. M: Marker, PC: Positive control, NC: Negative control

Discussion and conclusions

The wild boar is one of the most widely distributed mammals in the world, with high adaptability to various habitats (SALTIK et al., 2022). As in many parts of the world, such as Eurasia, North Africa, and the Americas, wild boar populations in Türkiye have been increasing rapidly in the last decade (CELIK et al., 2024). Determined as a significant component of wildlife, wild boars also serve as reservoirs for various etiological agents, including bacteria, viruses, and parasites such as

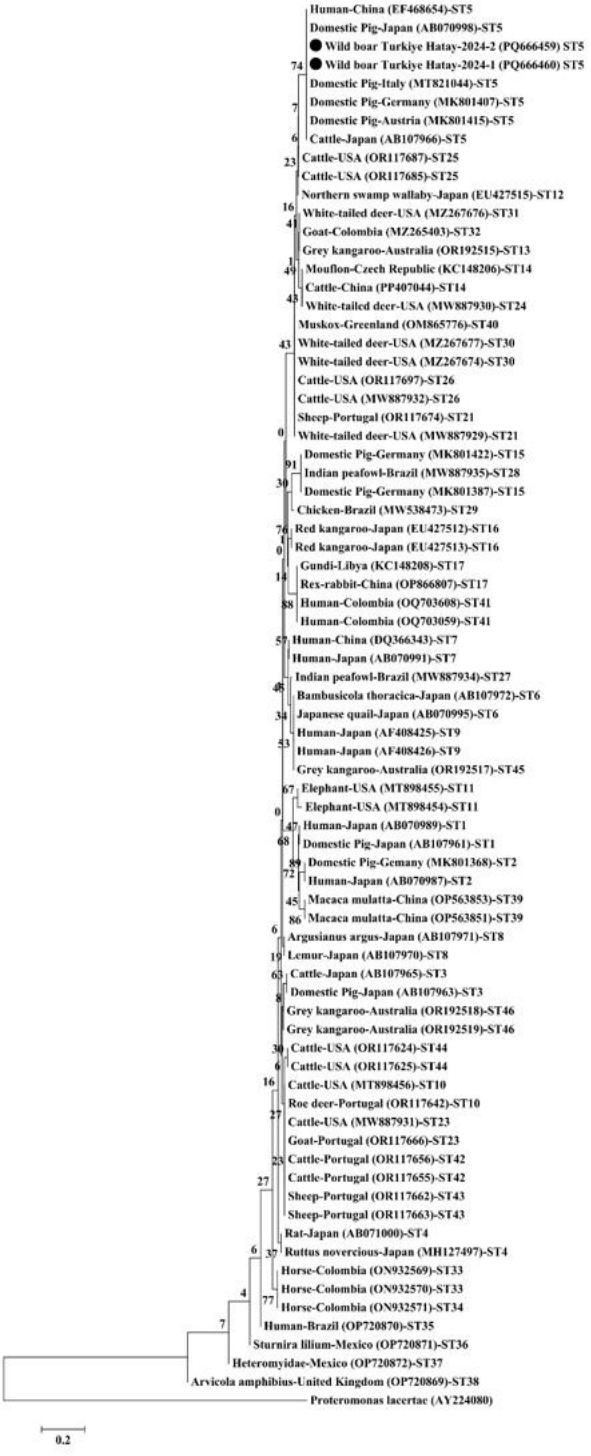

Fig. 3. Phylogenetic tree based on *Blastocystis* "small subunit ribosomal RNA gene" using MEGA program

Blastocystis (KÖSTER et al., 2024). Nowadays when wild boars have come down to suburban and urban areas across the country, there is a higher probability that wild boars may spread *Blastocystis* to livestock, water sources, and ultimately to humans.

There are limited studies on the epidemiology of *Blastocystis* in wild boar populations worldwide (KÖSTER et al., 2024). However, *Blastocystis* has been detected in several waterborne protozoan outbreaks, emphasizing the importance of including environmental transmission routes in epidemiological assessments through a One Health approach (BOURLİ et al., 2023).

Globally, prevalence rates of up to 62% of *Blastocystis* have been reported in free-ranging wild boars, with prevalence rates reaching 34.3% in European countries (KOSTER et al., 2024). ST5 was the most frequently identified subtype in wild boar *Blastocystis* studies, accounting for 79.7% of cases. The fact that ST5 is also the most frequently reported subtype in pigs (DANIŠOVÁ and VALENČÁKOVÁ, 2021; MASUDA et al., 2022) was interpreted as representing an adapted ST of the *Suidae* family (KÖSTER et al., 2024).

Additionally, *Blastocystis* ST5 has been detected in humans (reviewed in KUMARASAMY et al., 2023; MALATYALI et al., 2023) and small ruminants, cattle, companion animals such as dogs, domestic and wild birds, non-human primates, and zoo animals (reviewed in ALFELLANI et al., 2013; HUBLIN et al., 2021; SHAMS et al., 2022a; SHAMS et al., 2022b). More information is needed to understand the prevalence of *Blastocystis* in wild boars and its potential impact on animal and public health.

Our study confirmed the presence of *Blastocystis* (29.2 %) in wild boars in the southern part of Türkiye for the first time, and suggests that this may be the case in other regions as well. The ST5 subtype was found in two wild boar samples in this study using Sanger sequencing of *Blastocystis* SSU rRNA, which matches findings from other studies on domestic and wild boars (DANIŠOVÁ and VALENČÁKOVÁ, 2021; MASUDA et al., 2022; KÖSTER et al., 2024). In Türkiye, ST5 has been documented in only three studies: in humans (MALATYALI et al., 2023), cattle (ÖNCÜ ÖNER et al., 2022),and sheep (ÇAKIR et al., 2022). Although these data are not sufficient to comment on the cross-species transmission dynamics of ST5 in Türkiye, they are important in emphasizing the need for studies on this subject in different hosts.

Also in this study, four isolates showed mixed subtypes defined by double peaks in the *Blastocystis* sequencing chromatogram. The double peaks often seen in the chromatogram data of *Blastocystis* sequences from Sanger sequencing of stool samples suggest that there are mixed subtypes, which may come from different *Blastocystis* STs in a single host (MELONİ et al., 2012; HİGUERA et al., 2021). Similar to our study, there are numerous studies reporting mixed infection in both humans (reviewed by FUSARO et al., 2024) and animals (reviewed by SHAMS et al., 2021; HUBLİN et al., 2021; SHAMS et al., 2022a; SHAMS et al., 2022b). The identification of mixed infections indicates that we need to do more research on the biology and epidemiology of *Blastocystis*, which will require additional analyses, such as PCR product cloning or NGS to distinguish subtypes among the mixed infections (MASUDA et al., 2022).

Although there are limited studies on *Blastocystis* in wildlife, ST5 has been reported in wildlife other than wild boars, including short-nosed monkeys, long-tailed gorals (CHEN et al., 2021), bison, gray wolves (KACZMAREK et al., 2021) and wild rodents (GAO et al., 2024).

SHAN et al. (2024) also investigated the presence, molecular characterization, and zoonotic potential of *Blastocystis* among free-living sympatric rodents on pig farms and detected ST5 in 40.7%. These data strengthen the possibility that ST5, known as an adapted ST of the *Suidae* family, is carried from the nature to the farms by these wild small mammals. Additionally, they identified two distinct alleles (allele 115 and allele 119) among the ST5s found in these wild small mammals. In our study, ST5 displayed the presence of allele 115 in both wild boar isolates. These studies suggest that the source of ST5 in farm animals and humans may originate from wild animals which transmit this subtype within the population and to environmental sources.

Although One Health approaches to wildlife-related diseases are still relatively new, the evidence collected suggests that a comprehensive approach including all free-living species should be developed and implemented by stakeholders (CUNNINGHAM et al., 2017). Findings highlight the importance of monitoring *Blastocystis* for zoonotic transmission in wildlife to better assess public health risks.

The most obvious limitation of this preliminary study investigating the presence of *Blastocystis* in wild animals is the small sample size. However, the findings of this preliminary study are important as they highlight the need for future studies which will include a larger number of wild boar samples from different geographical regions in Türkiye. In addition, only two isolates could be sequenced in our study; advanced analyses, such as next-generation sequencing, are needed to demonstrate the presence of potential mixed subtypes.

In conclusion, the data obtained from our study provide preliminary information that emphasizes the importance of including wildlife in the investigation of *Blastocystis* transmission routes. Therefore, future wildlife field research should be continued in other regions of Türkiye, including different hosts and ecological components. Research on wildlife diseases is advancing our knowledge of parasitic diseases in wild animals, which might provide advantages for wildlife conservation and the prevention of zoonotic infections originating from wildlife.

Ethical statement and animal welfare

The decision that this study is not subject to the approval of the Animal Experiments Local Ethics Committee was made by the Mustafa Kemal University Local Ethics Committee (Decision number: 03-04/2024).

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

Authorship contribution statement

EAÖ, AZ, İE and FDA conceived and planned the experiments. EAÖ, AZ and İE carried out the experiments. EAÖ, FDA, AZ, MŞ contributed to the interpretation of the results. EAÖ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon a reasonable request.

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ÖZTÜRK, E. A., A. ZEREK, İ. ERDEM, M. ŞEVIK, F. DOĞRUMAN-AL: Prva molekularna analiza zoonotskog organizma *Blastocystis* podtip ST5 u divljih svinja (*Sus scrofa*) s područja Hatay, Turska. Vet. arhiv 96, 165-173, 2026.

SAŽETAK

Divlje svinje (*Sus scrofa*), kao važna populacija među divljim životinjama koja se sve više približava ruralnim i urbanim područjima, imaju složenu ulogu u prijenosu patogena između divljih životinja, stoke i ljudi, pri čemu je teško odrediti smjer prijenosa među vrstama. Ovo je preliminarno istraživanje imalo za cilj po prvi put pokazati pojavu zoonotskog organizma *Blastocystis* u divljih svinja u Turskoj. Primjenom PCR-a u stvarnom vremenu otkriveno je 29,2% pozitivnih uzoraka (7/24). U dva izolata iz divljih svinja s otkrivenim alelom 115 provedeno je uspješno sekvenciranje i potvrđena 100%-na podudarnost s podtipom 5 - *Blastocystis (*ST5). Rezultati istraživanja naglašavaju važnost praćenja patogena *Blastocystis* za dinamiku prijenosa zoonoza u divljih životinja kao i potrebu za budućim istraživanjima koja bi uključila veći broj uzoraka od divljih svinja iz različitih geografskih područja Turske.

Ključne riječi: *Blastocystis*; Turska; divlja svinja; zoonotski potencijal; molekularna analiza