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MOLECULAR IDENTIFICATION OF GASTROINTESTINAL NEMATODE SPECIES IN SHEEP FROM TIMIȘ COUNTY

IDENTIFICAREA MOLECULARĂ A SPECIILOR DE NEMATODE GASTROINTESTINALE DE LA OVINELE DIN JUDEȚUL TIMIȘ

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ABSTRACT

Gastrointestinal nematodoses represent one of the main problems affecting the profitability of sheep farming, particularly parasitism with *Haemonchus contortus*, which is the most pathogenic nematode for sheep. All ruminants raised in open systems on pastures are directly exposed to the risk of infestation with parasitic elements. Depending on laboratory facilities, parasitological diagnosis can be carried out in different ways. In the present study, the aim was to molecularly identify adult nematodes found in the digestive tract of sheep from 5 flocks in Timiș County. To achieve this objective, nematodes were collected after necropsy from the digestive tract of sheep from different farms in Timiș County, located in the areas of Cadar (Tormac commune), Sânnicolau Mare, Sânandrei, Gătaia, and Cenei. These nematodes were preserved in 70% ethanol and subsequently subjected to molecular analyses to identify parasitic DNA. Through PCR analysis and sequencing, nematodes from the species *Haemonchus contortus, Teladorsagia (Ostertagia) circumcincta, Oesophagostomum venulosum,* and *Chabertia ovina* were identified in sheep from Timiș County.

Keywords: sheep, gastrointestinal nematodes, molecular identification

REZUMAT

Nematodozele gastrointestinale reprezintă una din problemele principale care afectează rentabilitatea creșterii ovinelor, în special parazitismul cu *Haemonchus contortus*, care este cel mai patogen nematod pentru ovine. Toate rumegătoarele crescute în sistem deschis, pe pășuni, sunt expuse direct riscului de infestare cu elemente parazitare. Dependent de dotările laboratoarelor, diagnosticul parazitologic se poate efectua diferit. În studiul prezent, scopul a fost de a identifica molecular nematodele adulte regăsite la nivelul tubului digestiv la ovinele din 5 turme din județul Timiș. Pentru realizarea obiectivului s-au recoltat nematode de la nivelul tubului digestiv de la ovine moarte, provenite din ferme diferite, din județul Timiș, localizate pe raza localităților Cadar (comuna Tormac), Sânnicolau Mare, Sânandrei, Gătaia și Cenei. Aceste nematode au fost păstrate în sol. etanol 70% și ulterior, au fost supuse analizelor moleculare în vederea identificării ADN-ului parazitar. Prin analiza PCR și prin secvențiere, la ovinele din județul Timiș, s-au identificat nematode din speciile *Haemonchus contortus, Teladorsagia (Ostertagia) circumcincta, Oesophagostomum venulosum* și *Chabertia ovina*.

Cuvinte cheie: ovine, nematode gastrointestinale, identificare moleculară

Sheep farming is one of the most important branches of agriculture in Romania. It represents an economic, family-orientated, and predominantly traditional activity with a strong social impact (40). Globally, sheep farming significantly contributes to improving rural economies by creating jobs near villages, especially for unskilled workers (19). In Romania and other Eastern European countries, sheep are mainly raised for meat production (18), in contrast to Mediterranean countries, where they are primarily raised for milk production (38). Romania ranks highly in terms of sheep populations and the number of sheep exported both within Europe and to other continents (8). In December 2021, Romania ranked third in the European Union for the number of sheep and goats, following Spain and Greece, with a total stock of 11,536,000, of which 9,052,200 were adult animals. Within the country, Timiș County was a leader with a stock of approximately 608,000 heads (44). The predominant breed in our country is *Tsurcana* (about 70% of the total stock), followed by breeds such as *Tsigai, Merinos of Palas, Merinos of Transilvania, Racka, Teleorman Black Head,* etc. (14, 28).

Gastrointestinal nematodoses represent one of the primary issues affecting the profitability of sheep farming (10, 13, 39), especially parasitism with *H. contortus*, which is the most pathogenic nematode for sheep (6, 41). All ruminants raised in open systems on pastures are directly exposed to the risk of infestation with parasitic elements (22), thus potentially affecting their productivity (42).The most affected by helminths are the young and old animals in flocks. The decline in animal productivity can be influenced by reduced fertility, decreased milk production, loss of appetite, reduced growth rate of young animals, occurrence of diarrhoea (7), anaemia, and even death (20). In addi-

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tion to these ailments, infestations with gastrointestinal nematodes can promote secondary infections with bacteria from the digestive tract by denuding the intestinal mucosa and creating entry points for bacteria (37, 39). Among ruminants, sheep are the most affected by gastrointestinal nematode parasitism, compared to goats and cattle (30). The prevalence of parasitism depends on many factors, such as the type of farming, geographic location, rainfall patterns, temperature, type of pasture, animal species present on the same pasture, their age, and density, etc. (33).

In parasitology, conventional laboratory diagnostic methods are represented by microscopic techniques, which characterise parasites morphologically (1). Although there are many morphological differences between adult gastrointestinal strongyles, microscopic methods are inferior in providing a precise diagnosis compared to molecular methods (3, 43). Polymerase chain reaction (PCR) is an in vitro enzymatic reaction of amplification mediated by specific primers of short regions of the genome (43).

Depending on laboratory facilities, parasitological diagnosis can be performed differently. Classical identification methods using optical microscopy are the least costly compared to electron microscopy, confocal microscopy, or molecular identification methods, but they also have the lowest precision (5).

The aim of the present study was to molecularly identify adult nematode parasites found in the digestive tract of necropsied sheep from 5 sheep flocks in Timiș County.

MATERIALS AND METHODS

The study involved samples consisting of nematodes collected from the digestive tract of necropsied sheep from 5 different farms in Timiș County (Fig. 1), located in the areas of Cadar (Tormac commune), Sânnicolau Mare, Sânandrei, Gătaia, and Cenei. All necropsied sheep were over 8 years old, and the breed of the animals was predominantly *Tsurcana*, except for those from Gătaia, which were *Merinos of Transilvania*. The number of sheep in each flock ranged from approximately 300 to 700 heads.

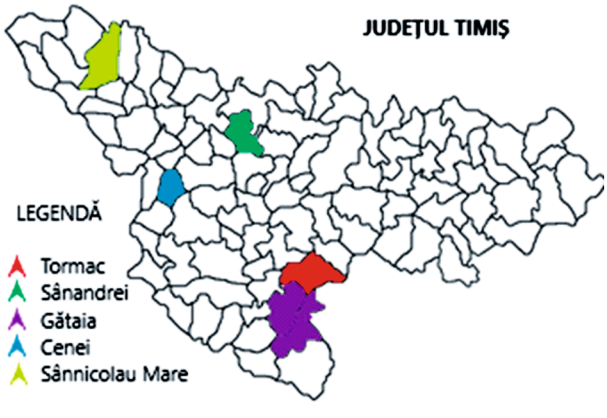


Fig. 1. Geographical location of the flocks included in the study from Timiș County

Adult gastrointestinal nematodes identified were collected and preserved in 70% ethanol (Fig. 2) and subsequently subjected to molecular analyses for parasitic DNA identification.

The DNA extraction was performed using the BIOLINE® Tissue Kit protocol (BIOLINE®). Throughout successive steps, cellular components of a different nature are eliminated, ensuring at the end of the process that DNA is precipitated to obtain purified DNA without RNA contamination.

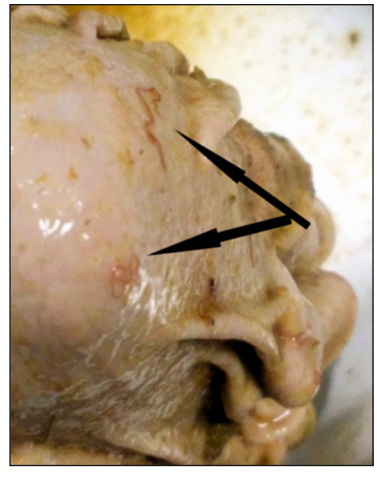


Fig. 2. Collecting gastrointestinal strongyles and their storage

DNA obtained is free from proteins, nucleases, and other contaminants or inhibitors. It is stored in a freezer at -20°C until analysis by PCR of the respective samples.

Polymerase Chain Reaction (PCR)

The PCR reaction was performed according to the technique described by Gasser et al.(1993), with some minor modifications (16). The amplification itself was carried out using classical PCR and was based on creating multiple copies of a ~300-bp segment of the ITS2 gene using the universal forward primer NC1 (ACGT CTGGTTCAGGGTTGTT) and reverse primer NC2 (TT AGTTTCTTTTCCTCCGCT).

The PCR amplification followed the protocol described in the article, modified as required for the mixture. A MyTaqTM Red Mix (BIOLINE®) Master Mix was used for the reaction. The final volume of the PCR reaction was 25 µl, consisting of 12.5 µl MyTaqTM Red Mix (BIOLINE®), 1 µl of primer NC1, 1 µl of primer NC2 (diluted to a concentration of 10 pmol/µl as per the manufacturer's protocol), extracted DNA from the sample to be analysed, and ultrapure water. The amplification program was carried out using a My Cycler thermocycler (BioRad®, Berkeley, CA, USA). The program included initial DNA denaturation at 95°C for 1 minute; 32 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final incubation at 4°C. Analysis and control of the amplicons were performed by horizontal gel electrophoresis in a 1.5%

agarose gel submerged system, using RedSafe™ (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) at 120 V and 90 mA for 60 minutes. A 100 bp DNA ladder marker was pipetted into the first well of the gel at a volume of 5 µl. To confirm the species, the PCR products were sequenced using both forward and reverse direction by the company Macrogen Europe B.V., Amsterdam, The Netherlands. A homology search was performed using the online version of the Basic Local Alignment Search Tool (BLAST) software (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on April 20, 2024) (44).

RESULTS AND DISCUSSION

During the necropsy examination, advanced emaciation of the carcasses, paleness of the mucous membranes, and presence of submandibular oedema were observed (Fig. 3).

Fig. 3. Presence of submandibular oedema

At the level of the abomasum and intestines (in all necropsied sheep), gastrointestinal strongyles were identified (Fig. 4).

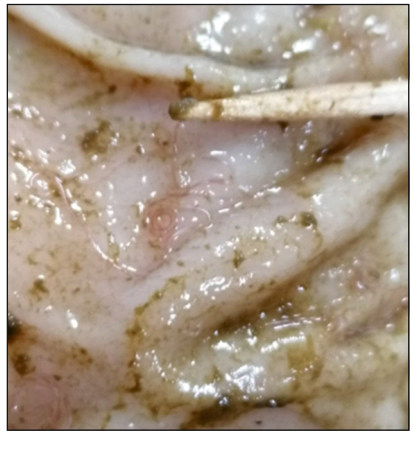


Fig. 4. Presence of gastrointestinal nematodes in the abomasum

Following molecular identification using PCR technique, gastrointestinal nematode species *Haemonchus contortus* and *Oesophagostomum venulosum* were identified in all necropsied animals from the 5 farms. *Ostertagia (Teladorsagia) circumcincta* was identified in 2 farms (Cadar and Gătaia), and *Chabertia ovina* in 3 farms (Cenei, Sânandrei, and Sânnicolau Mare). After migration of the DNA samples in the agarose gel, the gel image with the migrated DNA fragments was captured using a UV photodocumentation system (UVP®). *Haemonchus contortus* species was identified by highlighting similarities in the BLAST analysis (Fig. 5) with isolates KU558755.1 and KU 558757.1. The identified isolate of *Teladorsagia (Ostertagia) circumcincta* (Fig. 6) matched with isolates MZ323368.1 and MZ148615.1 stored in GenBank.

BLASTN 2.8.0+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.

Database: Nucleotide collection (nt)
47,193,206 sequences; 175,291,025,312 total letters

Query= 211224-026_C10_399_P3_NC2 1 2286

Length=2286

Sequences producing significant alignments:		Score (Bits)	E Value
KU558755.1	Haemonchus contortus isolate CVASU_MAMHc 1 internal t...	477	7e-130
KU558757.1	Haemonchus contortus isolate CVASU_MAMHc 3 internal t...	470	1e-127
KU558759.1	Haemonchus contortus isolate CVASU_MAMHc 5 internal t...	468	4e-127
AB908961.1	Haemonchus contortus genes for 18S rRNA, ITS1, 5.8S r...	466	1e-126
MG669349.1	Haemonchus contortus voucher Haemonchus contortus fro...	464	5e-126
KC998714.1	Haemonchus contortus isolate field variant 2 5.8S rib...	462	2e-125
HQ844231.1	Haemonchus contortus 18S ribosomal RNA gene, partial ...	462	2e-125
MF784941.1	Uncinaria sp. sample_4_LZ-2017 isolate African white ...	459	2e-124
KU558758.1	Haemonchus contortus isolate CVASU_MAMHc 4 internal t...	459	2e-124
MF784940.1	Uncinaria sp. sample_3_LZ-2017 isolate African white ...	457	9e-124
KM586651.1	Haemonchus contortus isolate GD140916.3 5.8S ribosoma...	457	9e-124
AB682686.1	Haemonchus contortus genes for SSU rRNA, ITS1, 5.8S r...	457	9e-124

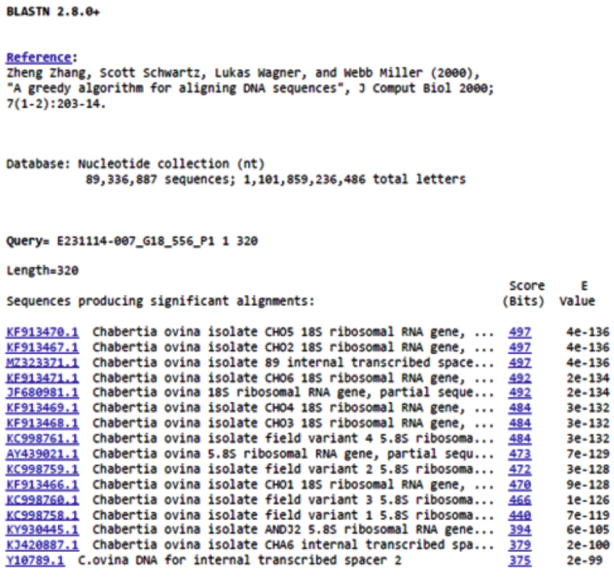
Fig. 5. BLAST analysis of the sequences obtained following amplification of extracted DNA for *Haemonchus contortus*

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Teladorsagia circumcincta isolate 34 internal transcribed spacer 2 and large subunit ribosomal RNA gene, partial sequence	Teladorsagia circumcincta	254	254	94%	7e-63	91.05%	308
Teladorsagia circumcincta isolate TCSeran 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	Teladorsagia circumcincta	254	254	94%	7e-63	91.05%	343
Teladorsagia circumcincta internal transcribed spacer 2 and 28S ribosomal RNA gene, partial sequence	Teladorsagia circumcincta	254	254	94%	7e-63	91.05%	308
Ostertagia trifurcata isolate R2 internal transcribed spacer 2 and large subunit ribosomal RNA gene, partial sequence	Ostertagia trifurcata	248	248	94%	3e-61	90.53%	290
Ostertagia lyrata isolate 233 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Ostertagia lyrata	265	265	93%	3e-66	92.15%	854
Teladorsagia circumcincta 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Teladorsagia circumcincta	257	257	93%	6e-64	91.53%	2001
Teladorsagia circumcincta isolate Fars 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	Teladorsagia circumcincta	252	252	93%	3e-62	91.01%	356
Teladorsagia circumcincta 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Teladorsagia circumcincta	252	252	93%	3e-62	91.01%	946

Fig. 6. BLAST analysis of the sequences obtained following amplification of extracted DNA for *Teladorsagia (Ostertagia) circumcincta*

Molecular analysis of *Chabertia ovina* isolates was highlighted in the results shown in Figure 7, while for *Oesophagostomum venulosum* the result is presented in Figure 8.

Specifically, all necropsied animals showed clusters of gastrointestinal nematodes (100% prevalence of parasitism) in the abomasum, with the abomasal mucosa exhibiting an exudative-haemorrhagic appearance. The animals had pale mucous membranes due to anaemia primarily caused by intense parasitism with *H. contortus*, a hematophagous parasite (34).



BLASTN 2.8.0+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.

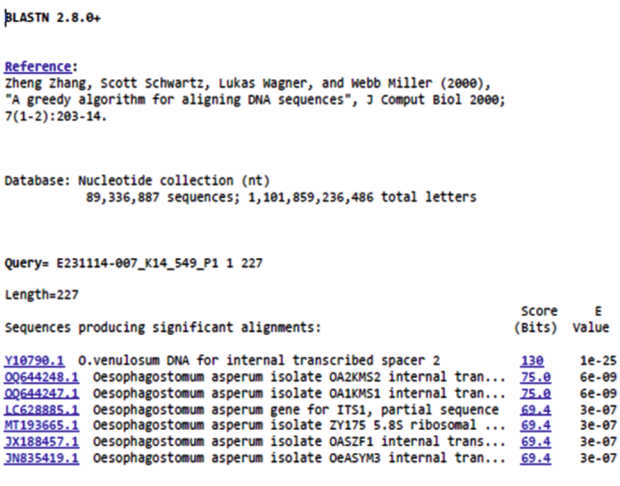
Database: Nucleotide collection (nt)
89,336,887 sequences; 1,101,859,236,486 total letters

Query= E231114-007_G18_556_P1 1 320

Length=320

Sequences producing significant alignments:		Score (Bits)	E Value
KF913470.1	Chabertia ovina isolate CHO5 18S ribosomal RNA gene, ...	497	4e-136
KF913467.1	Chabertia ovina isolate CHO2 18S ribosomal RNA gene, ...	497	4e-136
MZ323371.1	Chabertia ovina isolate 89 internal transcribed space...	497	4e-136
KF913471.1	Chabertia ovina isolate CHO6 18S ribosomal RNA gene, ...	492	2e-134
JF680981.1	Chabertia ovina 18S ribosomal RNA gene, partial seque...	492	2e-134
KF913469.1	Chabertia ovina isolate CHO4 18S ribosomal RNA gene, ...	484	3e-132
KF913468.1	Chabertia ovina isolate CHO3 18S ribosomal RNA gene, ...	484	3e-132
KC998761.1	Chabertia ovina isolate field variant 4 5.8S ribosoma...	484	3e-132
AY439021.1	Chabertia ovina 5.8S ribosomal RNA gene, partial sequ...	473	7e-129
KC998759.1	Chabertia ovina isolate field variant 2 5.8S ribosoma...	472	3e-128
KF913466.1	Chabertia ovina isolate CHO1 18S ribosomal RNA gene, ...	470	9e-128
KC998760.1	Chabertia ovina isolate field variant 3 5.8S ribosoma...	466	1e-126
KC998758.1	Chabertia ovina isolate field variant 1 5.8S ribosoma...	440	7e-119
KY930445.1	Chabertia ovina isolate AND32 5.8S ribosomal RNA gene...	394	6e-105
KJ420887.1	Chabertia ovina isolate CHA6 internal transcribed spa...	379	2e-100
Y10789.1	C.ovina DNA for internal transcribed spacer 2	375	2e-99

Fig. 7. BLAST analysis of the sequences obtained following amplification of extracted DNA for *Chabertia ovina*



BLASTN 2.8.0+

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.

Database: Nucleotide collection (nt)
89,336,887 sequences; 1,101,859,236,486 total letters

Query= E231114-007_K14_549_P1 1 227

Length=227

Sequences producing significant alignments:		Score (Bits)	E Value
Y10790.1	O.venulosum DNA for internal transcribed spacer 2	130	1e-25
OQ644248.1	Oesophagostomum asperum isolate OA2KMS2 internal tran...	75.0	6e-09
OQ644247.1	Oesophagostomum asperum isolate OA1KMS1 internal tran...	75.0	6e-09
LC628885.1	Oesophagostomum asperum gene for ITS1, partial sequence	69.4	3e-07
MT193665.1	Oesophagostomum asperum isolate ZY175 5.8S ribosomal ...	69.4	3e-07
JX188457.1	Oesophagostomum asperum isolate OASZF1 internal trans...	69.4	3e-07
JN835419.1	Oesophagostomum asperum isolate OeASYM3 internal tran...	69.4	3e-07

Fig. 8. BLAST analysis of the sequences obtained following amplification of extracted DNA for *Oesophagostomum venulosum*

Because all animals were elderly, they had weakened immune systems, which allowed parasitism with *H. contortus* to lead to their demise.

Timiș County is situated in a temperate climate zone, known for the specific prevalence of gastrointestinal nematode parasitism (29). The combination of abundant rainfall and high temperatures facilitated the development of parasite populations.

In 2014, studies across Timiș County in sheep demonstrated varying prevalence rates of gastrointestinal nematode parasitism among different species: 0-75% for *H. contortus*, 0-100% for *Trichostrongylus colubriformis*, 0-100% for *Teladorsagia circumcincta*, 0-66.66% for *Nematodirus filicollis*, 0-75% for *Cooperia curticei*, 0-60% for *Bunostomum trigonocephalum*, and 0-100% for *Chabertia ovina* (12).

In Romania, recent studies on the prevalence of gastrointestinal strongyles in sheep have shown significant variation across different regions of the country. In southern Romania, the prevalence was found to be 92.06% (2), while in the Banat and Crișana regions, it was 77.8% and 45.9%, respectively (37). Older studies have indicated a prevalence range of 70-100% in Dobrogea (4), 30-35% in Bistrița-Năsăud (17), 92.8% in the South, 85.7% in the North-East (29), and 85% in the western part of the country (21).

In neighbouring countries such as Serbia, Bulgaria, and Moldova, the prevalence of gastrointestinal parasites in ruminants has been recorded between 64.1% and 98.5% (11, 24, 32). Other studies have reported prevalences of 100% in Spain and Turkey (27, 35), 80% in Kenya (26), 63.2% in Nigeria (15), 63% in Colombia (25), 68.7% in India (36) and 72% in Papua New Guinea (23).

These studies highlight the significant impact of climatic conditions, management practices, and other factors on the prevalence and distribution of gastrointestinal parasites in ruminant animals. They underscore the importance of monitoring and managing these parasites for the overall health and productivity of livestock, both in Romania and globally.

The practice and use of genetic methods are important for the easy identification of specific parasitism and for managing parasitic diseases effectively (31).Molecular information serves as remarkable markers in detecting genes responsible for the emergence of chemoresistance, thereby enhancing long-term parasitological management (9).

CONCLUSIONS

The prevalence of gastrointestinal nematodes in necropsied sheep from the farms included in the study was 100%. Through PCR analysis and sequencing, nematodes of the species *Haemonchus contortus*, *Teladorsagia (Ostertagia) circumcincta*, *Oesophagostomum venulosum*, and *Chabertia ovina* were identified in sheep from Timiș County.

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