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## SERINE PROTEASE OF *TRICHINELLA SPIRALIS* AND ITS POSSIBLE APPLICATION IN THE EARLY DIAGNOSIS OF TRICHINELLOSIS IN ANIMALS

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### Abstract

Serine proteases of parasites play a significant role in the infection of a host organism. This is especially reflected in the process of immunosuppression, in particular, during IgG hydrolysis. The importance of the performed function ensures their presence at all stages of development, which makes them key targets for early diagnosis of trichinellosis. In this study, we demonstrated that serine protease transcripts are detected in 83% of cases when mice are infected with BALB/c on days 7 and 14 after infection and are identified by molecular methods. Two groups of mice were infected with different doses of larvae (100 and 250 larvae), it was shown that the dosage did not affect the effectiveness of serine protease detection in mice, which would provide a better diagnostic effect. The transcripts presence confirms the possibility of using serine protease as a protein for the diagnosis of trichinellosis in animals and humans.

**Key words:** recombinant protein; serine protease; parasites; *Trichinella*; trichinellosis.

### Introduction

The genus *Trichinella* includes ten species and three different genotypes, which are capable of infecting more than 150 domestic and wild mammals [1, 2]. People become infected with trichinellosis when ingesting raw or undercooked meats infected with *Trichinella* larvae.

The main diagnostic methods today are serological tests, such as Western blotting and ELISA for the detection of antibodies to trichinella. These methods are valuable approaches to the diagnosis of human and animal trichinellosis [3].

The most commonly used antigens for the diagnosis of trichinellosis are excretory-secretory (ES) antigens of muscle larvae, however, cross-reactions with other parasites often occur. In addition, it should be taken into account that a variety of different antigens are expressed at different stages of development, which may explain why muscle larvae are not recognized by trichinella induced ES antibodies at the intestinal stage, and false negative results observed in the early stages of infection [4].

This is due to the fact that the biological function of the protein largely depends on the spatial structure of the protein. Different antigens can also be expressed at different stages of *T.spiralis* development. In total, 4691 proteins were identified at the adult larva, newborn larva and muscle stage, 1067 differentially expressed, including serine protease, DNase II, trypsin enzyme II of the protein family and paramiosin. These proteins are being actively studied by scientists as candidate molecules for early

diagnosis, as well as for the creation of a vaccine against trichinellosis [5].

It is possible that the most relevant and promising protein will be one that can be produced at all stages of the parasite's life cycle.

In Zhai C. and et.al. research the diagnostic effect of ES antigens at different stages of development was studied using ELISA. When mice were infected with 100 larvae after 10 dpi, antitrichinella IgG antibodies were detected using intestinal ES infectious antigens of larvae, but after 12 dpi, infection was already diagnosed with the help of antigens of encapsulated larvae [6].

The main proteins expressed at different stages of trichinella development are serine proteases [7, 8], which are a family of proteolytic enzymes that play many biological roles during parasite infestation: they are involved in worm invasion, migration, and proteolysis of various host tissues [9]. They can be important antigenic targets for the creation of serological and molecular tests for early diagnosis of the disease.

Recent studies have shown that several types of serine proteases are involved in invasion by *T.spiralis* larvae, but this factor is not fully understood. It is necessary to conduct additional studies on the stage choice of larval development, the excretion of *T.spiralis* serine proteases and to investigate their immunogenicity based on the primary, secondary and tertiary protein structures.

Thus, the objective of our research was to study the excretion of serine protease at different development stages and to predict the structure and spatial configuration of

serine protease for the development of

### Materials and Methods

All activities involving animals were carried out in compliance with high standards of biosafety and animal welfare. All protocols are implemented in accordance with the *International Guiding Principles for Biomedical Research Involving Animals* [10].

The care and use of laboratory animals were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine and Animal Husbandry Technology of the NCJSC «S. Seifullin Kazakh Agrotechnical Research University» (KATRU), Astana, Kazakhstan (Protocol No. 2 of July 20, 2020).

The experiments were carried out on the basis of the Research Platform of Agricultural Biotechnology and the Joint Kazakh-Chinese Laboratory for Biological Safety NCJSC «Saken Seifullin

a test system based on a recombinant protein.

Kazakh Agrotechnical Research University».

*Infection of animals.* *T. spiralis* was maintained by serial passaging of BALB/c mice in the Immunochemistry Laboratory of the Agricultural Biotechnology Research Platform. The larvae were collected by artificial digestion using a standard protocol. 12 mice aged three-four months were selected for the experiment. According to the principle of analogues, two groups of experimental animals were formed. The causative agents of *T. spiralis* trichinellosis were invaded to animals of first group of six mice at a dose of 100 larvae per head and second group of six mice at a dose of 250 larvae. Animals were infected by the introduction of a per os «digest» containing trichinella larvae using a disposable pipette (Figure 1).



Figure 1 – Infection procedure of BALB/c mice by oral administration of saline solution with larvae

*Scheme of the experiment.* On the 7th and 14th days, three mice

from each group infected with *T. spiralis* were euthanized for

pathoanatomic autopsy by sequential intramuscular injection of xylazine at a dose of 1.5 mg/kg and intravenous injection of anestofol at a dose of 7.5 mg/kg. After the muscle's dissection of animal carcasses, they were examined for the parasite's presence in accordance with WHO/OIE recommendations [11]. The small intestine was longitudinally dissected

and washed three times with normal saline solution with ice, then cut with sharp scissors into 2 cm long fragments and cultured in normal saline solution at 37°C for 2.5 hours. Then the larvae released from the small intestine into a normal saline solution were collected by the Berman method [12].



Small intestine pieces



Mounting with a funnel

Figure 2 – Collection of larvae by the Baerman method from the small intestine with settling in a funnel

*Diagnosis and isolation* of the larvae of the causative agent of trichinellosis from animal muscle tissue samples was carried out by compressor trichinoscopy and digestion in artificial gastric juice (IHS), in accordance with methods of MUC 4.2.2747-10 «Methods of sanitary and parasitological examination of meat and meat products». The detected and isolated helminthological material was preserved in 70% ethanol solution.

*Isolation of RNA.* The total RNA was extracted using TRIzol reagent (Invitrogen, USA) [13] in accordance with the manufacturer's instructions. The RNA concentration

was measured using NanoDrop 2000 (Thermo Scientific, USA). Total RNA was transcribed back into the first cDNA chain using ProtoScript II First Strand cDNA Synthesis Kit (New England BioLabs, England).

*PCR conditions.* The reaction was carried out on a VerityPro amplifier (Applied Biosystems, USA) using the following primers: Trich SP F: 5'-CAGTATTGTGGAAATCCTTATTT T-3'; R: 5'-TCAGTAAAAAGAGTCAAAA TT-3'. The composition of the reaction mixture included: Taq 5X MasterMix (New England BioLabs) – 5 µl. Primer (10 mM) F – 2 µl, primer R –

2 µl, mQ water – 15 µl, cDNA - 3 µl (100 ng), the total volume of the mixture is 25 µl. PCR mode: primary denaturation - 95°C - 5 min. (1 cycle); denaturation 95°C - 30 sec.,

*Sequencing.* The nucleotide sequence was determined using the BigDye Terminator v3.1 sequencing Biosystems, USA). DNA sequences were collected and analyzed using a software package (Sequence Investigator, etc.), Finch TV v1.3.1. and using international nucleotide sequence databases (Blast, ENSEMBL, GeneBank, etc.).

*Bioinformatic analysis.* Bioinformatic methods were used to

## Results

*Larvae sampling.* At the first stage of the study in mice of groups 1 and 2, 7 and 14 days after infection with freshly isolated larvae, the carcasses of mice were dissected and the intestines and muscles of mice were examined for the presence of trichinella. Using the Berman method, live newborn larvae were sampled

annealing of primers 58°C - 30 sec., elongation 72°C - 60 sec. (30 cycles); final elongation - 72°C - 5 min. (1 cycle).

kit (ThermoFisher, USA) and the SeqStudio genetic analyzer (ThermoFisher, Applied) to predict the structures and functions of the serine protease protein.

*Statistical analysis.* Statistical analysis was performed using GraphPad Prism 7.0. Statistical analysis was performed using Microsoft Excel 2010. P <0.05 was considered statistically significant.

from the intestine. Repeatedly washed areas of the intestine and saline solution after flushing were examined for the presence of larvae. Consequently, egg laying and newborn larvae were found at 7 dpi in tissue and solution samples (Figure 3).



Figure 3 – Larva of *Trichinella spiralis* during life birth (A) and newborn larvae (B) at the stage of intestinal phase of infection

I  
n mice of group 2 with a dosage of infection of 250 larvae, a larger

number of newborn larvae were observed on the intestinal walls,



whereas in group 1 with a lower dosage, larvae were found in flushes from the intestine at the egg-laying stage. This may indicate that with a higher dosage at the time of infection, the invasion process occurs faster and the larvae begin to enter the intestinal walls. It may also be due to the immune response of mice to infection, which is able to restrain larval

infestations at a lower dose. It is important to note that both experimental groups did not have the presence of encapsulated larvae in the muscles at 7 dpi. However, quantitative differences in the two groups were revealed due to the difference in the dose of larvae (Table 1).

Table 1 – Intensity of invasion of mice in experimental groups at 7 dpi

Stage of infection	Group 1	Group 2
Intestinal 7dpi		
6.7±0.8 gr	5±1.5	13±2.1
Intensity of invasion	33.5±1.2	87.1±1.7

The intensity of the invasion was significantly different depending on a dose application, which confirms the dose-dependence during infection exposure, as we described in early studies [14].

Sampling at 14 dpi significantly differed in the larvae development stage and their localization in mice group 1 and 2. So after 14 days, no larvae were found in the intestines of mice from both groups, all larvae were localized in the muscles (Figure 4).

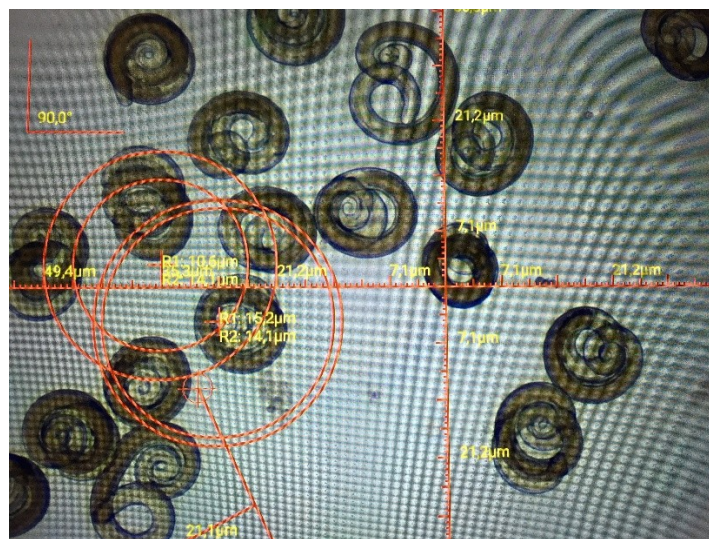


Figure 4 – Larvae of *Trichinella spiralis* from mouse muscle digestion at 14 dpi

In both group 1 and 2, the larvae reached the muscular stage, which did not show a dose-dependent effect. However, when microscoping muscles of mice from group 2, encapsulation of 4.2±1.3 larvae was already completed, when no encapsulation was detected in group 1. The intensity of invasion after 14 days averaged 20±5.7 larvae per 1 gram of muscle (Table 2).

Table 2 – invasion intensity of mice in experimental groups at 14 dpi

Stage of infection	Group 1	Group 2
Muscular 14 dpi	100 larvae	250 larvae
87.3±3.1 gr	17.2±4.1	23±7.3
Intensity of invasion	1501.6±12.7	2007.9±22.6

The invasion intensity differed slightly in the groups. However, studies have shown that the stage of larval development was different in both groups. The rate of larvae encapsulation in mice had been relatively fast in group 2, which explains the dose-dependency effect.

*Molecular confirmation of serine protease gene transcripts presence.* After studying the exact stage of larval development at

different time periods after infection, the selected larvae were subjected to total RNA cleavage and isolation from all 12 larval samples.

The average RNA concentration was 873 ng/ml. After the reverse transcription reaction and cDNA synthesis, PCR was performed using a specific primer. The results of PCR were visualized by electrophoresis (Figure 5).

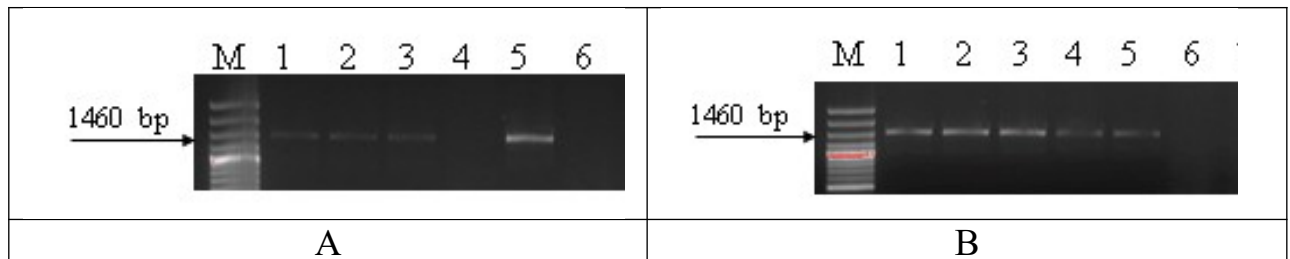


Figure 5 – Electrophoretic analysis of serine protease gene transcripts presence in larvae at 7 dpi (A) and 14 dpi (B)

According to molecular studies, the presence of serine protease transcripts was determined in all two groups of mice, regardless of the dose and the period of infection. Larvae selected at different stages of development and encapsulation in 83% showed the presence of transcripts of the serine protease gene. The data obtained are consistent with the data of recent studies of Song Y.Y. et.al. (2022), which showed that serine proteases play a key role in the invasion, growth and survival of *T. spiralis* in the host body and that they

can be the main target molecules-candidates for the creation of vaccines and early diagnosis of trichinellosis [15].

After sequencing of the serine protease gene, the nucleotide sequence (1450 bp) was determined  
ATTAGCATACGCATTAAGCATG  
AAACGCTGGCACCCCTTTGGCA  
TACCTTTTCACAATGCATTTCTG  
TTGTTTTGTATTATTATTAAGGA  
AACATTTTCACAGTATTGTGGA  
AATCCTTATTTTGAACCATATTT  
GACAAATCCACACTAATTCGAA  
CCAAATTGTTGGTGAATGGGTT

GCAAGGCCATATTCATTTCCAT  
 GGACTGTTTCATGTATTAGCTCAT  
 ATTTCTGGATTCTGGTATGAAAT  
 TCTTGTGGAGGCAGTCTGATTTT  
 TTTTGACTATACAAACGCCAGT  
 GATACTGTCCTCACTTCATCCCA  
 TTGTGTTAGAGTAAACAATCGT  
 CTTGTGGATGCAAATGCTATAA  
 CTGTGACAGCAGGTGCATTTAA  
 TATAAGGGAATTAACGAACCC  
 CACAGAGTCACTTCAAAGTCC  
 TGGCATACATGTCAGATAATTT  
 TGGTGACGTCGGTAAACCAAAT  
 GACGTCGCTATGTTGCGTTTAA  
 AAGTAAAGATTCCGCATTCTCA  
 CTACATCAGTTCAGTCTGTTTGC  
 CATATCCATTCCAAGAGATACC  
 ATATGGAGAAACGTGTTTTCTTT  
 GTTGGGGTTTCACTAGAGGAAG  
 ACCACTGTCTGAATTGCGTCAG  
 GTTGGAAATCCCAATTTTACGAA  
 GCAGCAACTGCCGATTTACTGA  
 TCGGTATGATATTTTTTGCAG  
 GTGATATGGGTGAAGGAAATTA  
 TTCTTTCAAATTGATTCGGGAG  
 GACCTCTAGTTTGTAATTTAA  
 TGATTCCTATGTTCAAATTGGCA  
 TAGTTAGTTTTGGTTACAACCAT  
 GCTGGAAAGCACCACCCTGGTA  
 TTTAATCAAAGTTCCTTATTAT  
 TTGAATTGGATATACAATCAAC  
 TGTCATCCGCTTCCTGATTCATT  
 TAACTCTTCAGATATCGGAGGC

GAAGAATCTGATTGTCCAGATG  
 ATTGTTACCACCCTTGGCGATCC  
 GTCTTCAAACATTTTAAACATCG  
 CAAGGCGTCATTCCGAAATCGT  
 CCACCGTATTCACATTCCTCA  
 GACTTACAATGAATGAGAATCG  
 TCCACCACCACCTCCAGATTCTC  
 AAAATTTTGATATGGAATCTTTG  
 GAAAGTACTGAAGGTGATCCTA  
 GTGATTGGTCTCCATATTCAACT  
 AACCAGCATTACCAATCAAATT  
 ATGATGGATCTCAGACAGGCAA  
 AGGAAATCGTCCACCGTATTCA  
 CATTACACAGACCTACAATGA  
 ATGAGAATCGTCCACCACCACC  
 TCCAGATTCTCAAATTTTGACT  
 CAAATTACTGAAGCCATGCAGC  
 TTTGCGGCATTGTCATTCTTTAG  
 TTTTGACGGAGCGCAAGCATCA  
 CTCCAATACAATTATATATAAG  
 GAATGGACTTTTAAAAGAATTA  
 GCAATTAATATAATTCTGTAAG  
 TTTTAAAATGCATTGTATGTTA  
 ATAAAATGAATTGCATCAC

Next, a bioinformatic analysis of the serine protease sequence was performed, including an alignment of the cDNA sequence of serine proteases in GeneBank with the determination of the amino acid sequence in the primary structure obtained in the Mega 11.0 program.

Species/Abbrv	*	*	*	*	*	**
1. EU302801.1	TACGCATTAAG	-CATGAAACGCTGGCACCCCTTTGGCA	TACCT	.....	TTTCACAATGCATTTCTGTTGTTTTGTATT	
2. EU302800.1	TACGCATTAAG	-CATGAAACGCTGGCACCCCTTCGGCA	TACCT	.....	TTTCATAATGCATTTCTGTTGTTTTGTATT	
3. Our sample	TACGCATTAAG	-CATGAAACGCTGGCACCCCTTTGGCA	TACCT	.....	TTTCACAATGCATTTCTGTTGTTTTGTATT	
4. JN166799.1	TATACCTCAATGCACGAGGCTTGGATTCCTTTTTCT	TATCTGCAGCTTCTCCAT	-	CACTAGGGGCGTTT	.....	
5. EU245046.1	TACACGTTAAT	-CATGAAACGCTTTCCACCCTTCGGCA	TATTT	.....	TTTCATTATGCACCTTCTGATGTTTTGCATT	
6. L29506.1	TACG	-ATTAAG	.....	TATCT	-AAGAATCCCAATTAATAACCATTTTACAAA	
7. D89871.1	TCCGGGCTACA	...GAGCTCCTGGCGCTCATCGCCTCT	GGCT	CCAGCCTTGTCTTC	CGGGGCTGACCCCTTTGGGTC	



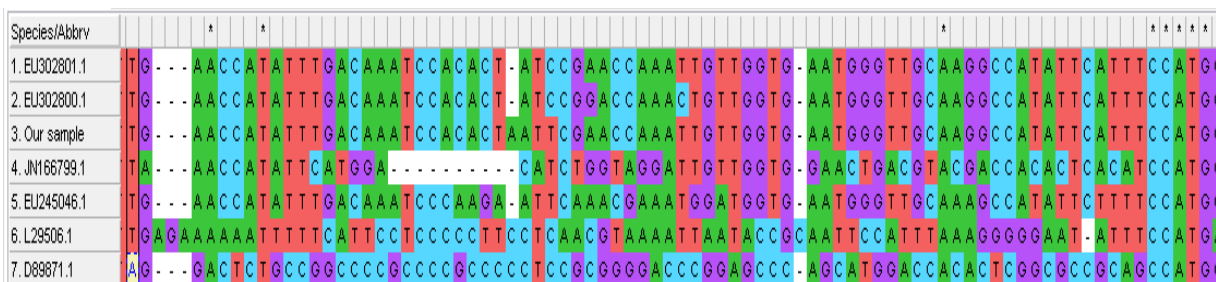


Figure 6 – Alignment of the nucleotide sequence of serine protease in the Mega 11.0 program

Based on the nucleotide sequence of the gene, the amino acid sequence of the protein was constructed:

#Sequence 1 Amino acid chain:

ISIRIKHETLAPLWHTFSQCISVVLYYY\*GNIFTVLWKSFL\*TIFDKSTLIRTK  
 LLVNGLQGHIFHGLFMY\*LIFLDSGMKFLWRQSDFF\*LYKRQ\*YCPHFIPL  
 C\*SKQSSCGCKCYNCDSRCI\*YKGIKRTPQSHFKSPGIHVR\*FW\*RR\*TK\*RR  
 YVAFKSKDSAFSLHQFSLFAISIPRDTIWRNVFSL LGFH\*RKTTV\*IASGWNP  
 NFTKQQLPIY\*CV\*YFLRR\*YG\*RKLFFPN\*FGRTSSL\*IK\*FLCSNWHS\*FW  
 LQPCWKAPPWYLIKSSLLFELDIQSTVIRFLIHLTLQISEAKNLIVQMIVTTL  
 GDPSSNILNIARRHSEIVHRIHIHSDLQ\*MRIVHHHLQILKILWNLWKVLKV  
 ILVIGLHIQLTSITNQIMMDLRQAKEIVHRIHIHTDLQ\*MRIVHHHLQILKILT  
 QITEAMQLCGIVIL\*F\*RSASITPIQLYIRNGLLKE LAINIIL\*VFKMHCMLIK\*I  
 AS

A graphical representation of the protein subunits distribution in the structure of the larval cuticle membrane was obtained using the Phyre 2.0 Internet resource (Figure 7).

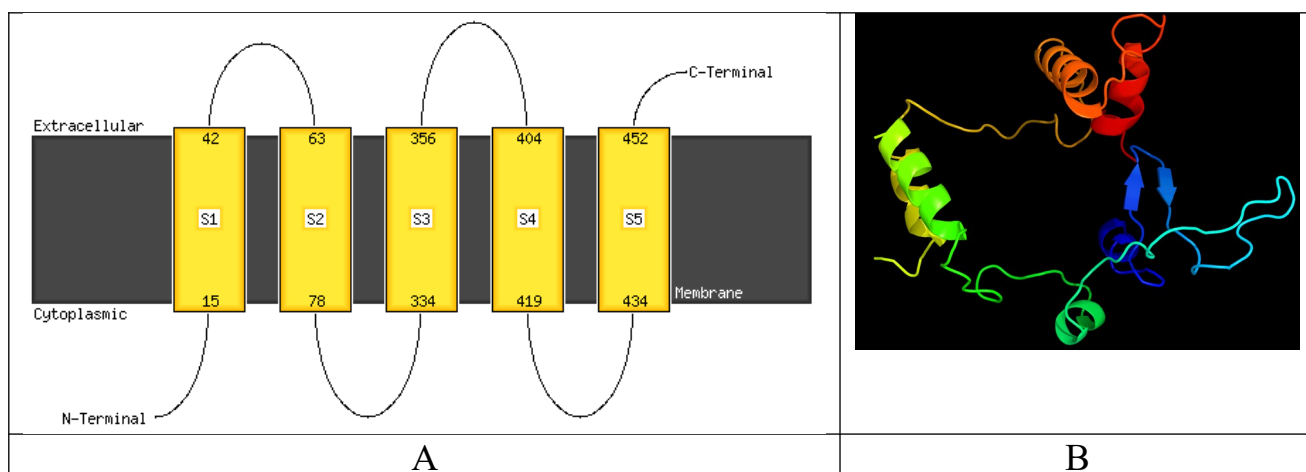


Figure 7 – Graphical representation of the distribution of protein structural units in the membrane thickness (A) and the spatial configuration of the protein (B)

According to the data, the serine protease consists of 1,450 bp,

while the amino acid sequence includes 483 amino acids. The protein

is represented by 5 subunits, alternately located in the thickness of the cuticle, while the sections of the molecule N-terminal end – 1-14, located in the cytoplasm, S1-S2 – 43-62 – associated with extracellular space, S2-S3 – 79-333 – located in the cytoplasm, S3-S4 – 357-403 – binds the protein to the extracellular space, S4-S5 – 420-433 – is concentrated in the cytoplasm and the C-terminal site – 31 amino acids - exits into the extracellular space. The location of the serine protease determines its functions as the main proteolytic

### **Discussion**

In recent years, trichinellosis has become a new and emerging parasitic disease, and the severity of trichinellosis in humans ranges from subclinical to lethal [17]. Early diagnosis of infection is crucial for the timely and effective treatment of trichinellosis, since anthelmintic drugs are much more effective against adult helminths in the intestine than against encapsulated larvae in the muscles [18, 19]. Therefore, it is important to identify antigens recognized by the host's immune system at an early stage of infection. These immunodominant antigens can be developed as biomarkers for early diagnosis of trichinellosis or even as potential vaccines for better control of this zoonotic foodborne disease.

Serine proteinases are important members of the superfamily of proteolytic enzymes that are widely distributed in organisms. Serine proteases have two main structural folds: trypsin-like domains and subtilisin-like domains. Most trypsin-like domains play an important role in

enzyme involved in the process of larval invasion, in the processes of secretion, repair, morphogenesis and differentiation. Importantly, it is the exact protein that is able to cause an immune response to invasion in the host body. It has been proved that the immunosuppressive effect of parasites on the host organism largely depends on the activity of proteolytic enzymes capable of hydrolyzing the IgG hinge region and heavy chains of immunoglobulins of all classes, as well as cleaving interleukin 1 $\beta$  [16].

helping parasites to invade, digest, shed, and hydrolyze proteins [20].

Previous studies have shown that *Trichuris muris* serine proteinase can disrupt the integrity of the cell membranes of the intestinal epithelium, which is associated with the hydrolysis of the mucous barrier of the intestinal surface of the host [21].

In our study, it was shown that serine protease is present in 83% of cases when infected with *Trichinella* larvae. This indicator is sufficient to identify the disease at different stages of development, including intestinal. The intestinal stage did not reveal a dependence on the dose of larvae during infection. However, the number of larvae accelerated the process of invasion and encapsulation in the muscles.

Sun G.G. and et.al. research it was shown that recombinant serine protease of trichinella has an immunogenic property, which proves the presence of specific antitrichinella IgG in 100% of infected pigs [22].

An earlier study of this group of scientists on mice showed that specific antitrichinella IgG in infected mice was detected by ELISA based on recombinant serine protease protein after 7 dpi, and the level of positive antibodies reached 100% at 10 dpi, while ELISA on excretory-secretory antigens did not allow detecting 100% of infected mice up to 16 dpi [23].

Analysis of the protein sequence of serine protease showed that the protein is intracellular, has extracellular structures. The resulting complete amino acid sequence and spatial structure will help to better isolate this protein and understand its functions.

Recently, the conduct of proteomic analyses has been of great

### **Conclusion**

Studies have shown that serine protease is expressed in the larva of trichinella both at the stage of intestinal infection and at the stage of muscle larvae. Serine protease transcripts at 7 and 14 dpi were detected in 83% of infected mice. The invasion intensity showed a significant dose-dependence. The conducted bioinformatic analysis demonstrated that the location of serine protease in the membrane of the cuticle of larvae is a key factor in its use as a diagnostic component. Thus, serine protease is a promising protein for early serodiagnosis of trichinellosis.

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interest to scientists, since it allows us to determine not only the protein, but also its region as an immunogenic site using the example of the entire protein diversity represented in the ES serum. For example, scientists from China have identified 185 T proteins. and several enzymes (for example, adult-specific DNase II, serine protease and serine protease inhibitor) that can act as invasion-related proteins and early diagnostic antigens of trichinellosis [24].

Thus, parasitic serine proteases involved in reproduction, coagulation and associated with larval invasion of the intestinal mucosa may be potential targets for vaccines against trichinella, as well as antigens in the design of serological tests for early diagnosis of trichinellosis.

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