Original article

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STUDY OF THE PROTEIN COMPOSITION OF ANTIGEN PREPARATIONS FROM THE PATHOGEN *OPISTHORCHIS FELINEUS*, OF VARIOUS CHEMICAL NATURES.

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ABSTRACT

Opisthorchiasis is currently widespread not only in the Republic of Kazakhstan but also in many other countries, making it a significant concern in modern medicine and veterinary science. Accurate diagnosis of this disease is crucial for effective treatment. The specificity of diagnostic tests relies on antigen preparations that contain various antigens. Our research focused on studying antigens of different chemical natures derived from the pathogen *Opisthorchis felineus*. As a result, we obtained three types of antigens: excretory-secretory (ESA), egg (EA), and somatic (SA). We analyzed their protein composition and identified the number of major proteins as follows: ESA - 21, SA - 20, and EA - 33. Protein fractions with molecular weights of 105 and 250 kDa exhibited immunogenic properties. The immunogenic and antigenic properties of these antigens were confirmed. Mass spectrometry analysis of ESA, which includes 214 secretory proteins from the parasite, allowed us to identify three highly immunogenic proteins: cathepsin F, glutathione transferase, and paramyosin, through immunoprecipitation with specific sera.

Key words: *Opisthorchis felineus,* antigenic preparations, protein composition, immunogenicity, specificity

1. INTRODUCTION

Many common zoonotic pathogens can be transmitted through the consumption of raw or partially cooked meat or fish [1, 2]. Numerous fish species harbor zoonotic liver flukes, which currently infect over 45 million people worldwide. Among these, *Clonorchis sinensis*, *Opisthorchis felineus*, and *Opisthorchis viverrini* (family *Opisthorchiidae*, subfamily *Opisthorchiinae*) are the most significant. Recent studies have highlighted a strong association between chronic opisthorchiasis and the development of cholangiocarcinoma. To mitigate the risk of asymptomatic chronic infections, a rapid, reliable, and cost-effective diagnostic test is recommended for use in local hospitals [3, 4]. Although cholangiocarcinoma is rare globally, it is highly prevalent in areas where *Opisthorchis viverrini* is endemic, such as northeastern Thailand, which has the highest incidence of this cancer worldwide [5, 6].

Several diagnostic tests based on serological reactions have been developed. For instance, a native protein from *O. felineus* was used to detect specific antibodies in blood serum, but it proved unsuitable for routine diagnostics due to its high cost and low specificity. Other methods include the indirect hemagglutination test, intradermal test, and ELISA, which utilized a crude somatic extract of adult worms [7]. Among these, ELISA emerged as the most effective method, correlating well with infection intensity, though its specificity was not thoroughly assessed.

Over time, researchers have developed diagnostic tests using protein antigens from *O. felineus*, sourced from adult worms, excreta, and eggs. These proteins, with molecular weights of 105, 74, 70, and 64 kDa, respectively, have shown potential for immunodiagnostics [8-10]. Recombinant antigens are preferable for antibody detection, as they can be produced on a larger scale, facilitating the development of convenient and affordable serological tests. While detecting trematode eggs in feces or bile remains a reliable method, the absence of eggs does not rule out infection. Consequently, serological methods detecting antibodies in blood serum have been refined. Parasite antigens used in these methods can be classified into excretory (excretory-secretory) and somatic antigens [11-14]. Current advancements in opisthorchiasis diagnostics underscore the potential of studying the immunogenicity of specific proteins in the excretory-secretory and somatic preparations of *O. felineus*, and developing highly specific immunological test systems based on recombinant antigens. This study aims to analyze the protein composition of antigenic preparations from *O. felineus* of various chemical natures, and to identify immunogenic proteins through electrophoretic analysis, immunoblotting, and mass spectrometry.

2. MATERIALS AND METHODS

Obtaining Excretory-Secretory Product (ESP). Viable *O. felineus* adults are thoroughly washed to remove traces of bile and blood using sterile saline solution. They are then placed in RPMI-1640 medium containing antibiotics and cultured at 37°C with 5% carbon dioxide for 14-16 hours. After this initial period, the medium is replaced, and cultivation continues for an additional 5-6 days. Following cultivation, the culture medium is purified by centrifugation and used as the excretory-secretory product (ESP) of the helminth.

Obtaining Somatic Antigen (SA). Adult sexually mature *O. felineus* are washed with physiological solution. The washed parasites are then placed in a 0.01 M sodium phos-

phate buffer (pH 7.2) containing 0.45% NaCl, with approximately 1000 individuals per 5 ml of solution. This mixture is kept at -20°C for 18-24 hours. After thawing for 1 hour at 37°C, the parasites are mechanically homogenized. The soluble protein fraction is separated from the tegment proteins by centrifugation at 3000 g for 30 minutes. The supernatant is carefully poured off, and the proteins are precipitated with 30% ammonium sulfate at 6-8°C for 12 hours. The precipitate is collected by centrifugation at 3000 g for 30 minutes, then dissolved in 2 ml of 0.05 M Tris-HCl (pH 8.2) containing 0.5 M NaCl. This preparation is used as the somatic antigen.

Obtaining Egg Antigen. The culture fluid containing eggs is first centrifuged at 1000 rpm for 5 minutes. The supernatant is collected as the excretory-secretory product (ESP), while the egg sediment is retained. The sediment is washed twice with sterile saline and then processed to obtain the antigen. To the egg sediment, 500 µl of 1% DOX RIPA buffer, containing protease and phosphatase inhibitors, is added. The mixture is exposed to ultrasound at 4°C with an amplitude of 40, for 30 seconds, twice. Following this, centrifugation is performed at 21,000 rpm for 10 minutes at 4°C. The supernatant obtained is analyzed for total protein composition and antigenic agents.

Protein concentration in the preparation is determined using the Bradford assay [15].

Mass Spectrometry. The excretory-secretory antigen was separated by 10% SDS-PAGE for 25 minutes and visualized using Coomassie staining before mass spectrometry analysis. The gel bands containing the separated antigen were excised and washed three times with a solution of 50% acetonitrile and 200 mM ammonium bicarbonate. Gel digestion was performed following standard protocols. Reduction was carried out with 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate at 60°C for 30 minutes, followed by alkylation with 20 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 30 minutes in the dark. Digestion with trypsin (1 µg, Promega, Madison, WI) was performed at 30°C overnight. Peptides were extracted from the gel strips three times using 60% acetonitrile in 0.1% trifluoroacetic acid at 30°C for 30 minutes each time. The solvent was then reduced to 10 µl by vacuum centrifugation. Nano-LC/MS/MS was conducted using an LTQ Nano-LC/MS/MS linear ion trap mass spectrometer (Thermo Electron Corporation) coupled with an 1100 series nano-LC system (Agilent Technologies). A 75 µm × 360 µm PicoFrit nano-LC column (New Objective, Woburn, MA), packed with 3 µm Magic C18 resin (Michrom Bioresources, Auburn, CA), was employed. Mass spectra were acquired over a 1.5-hour gradient (solvent A: 0.1% formic acid; solvent B: 90% acetonitrile in 0.1% formic acid) using data-dependent acquisition. The top eight most intense ions in each mass spectrum (mass range of 300-2000 m/z) were selected for collision-induced dissociation MS/MS. Dynamic exclusion was set to two replicates with a duration of 0.5 minutes and an exclusion period of 1.5 minutes. MS/MS spectra were analyzed using a combination of Spectrum Mill (Agilent Technologies) and manual interpretation.

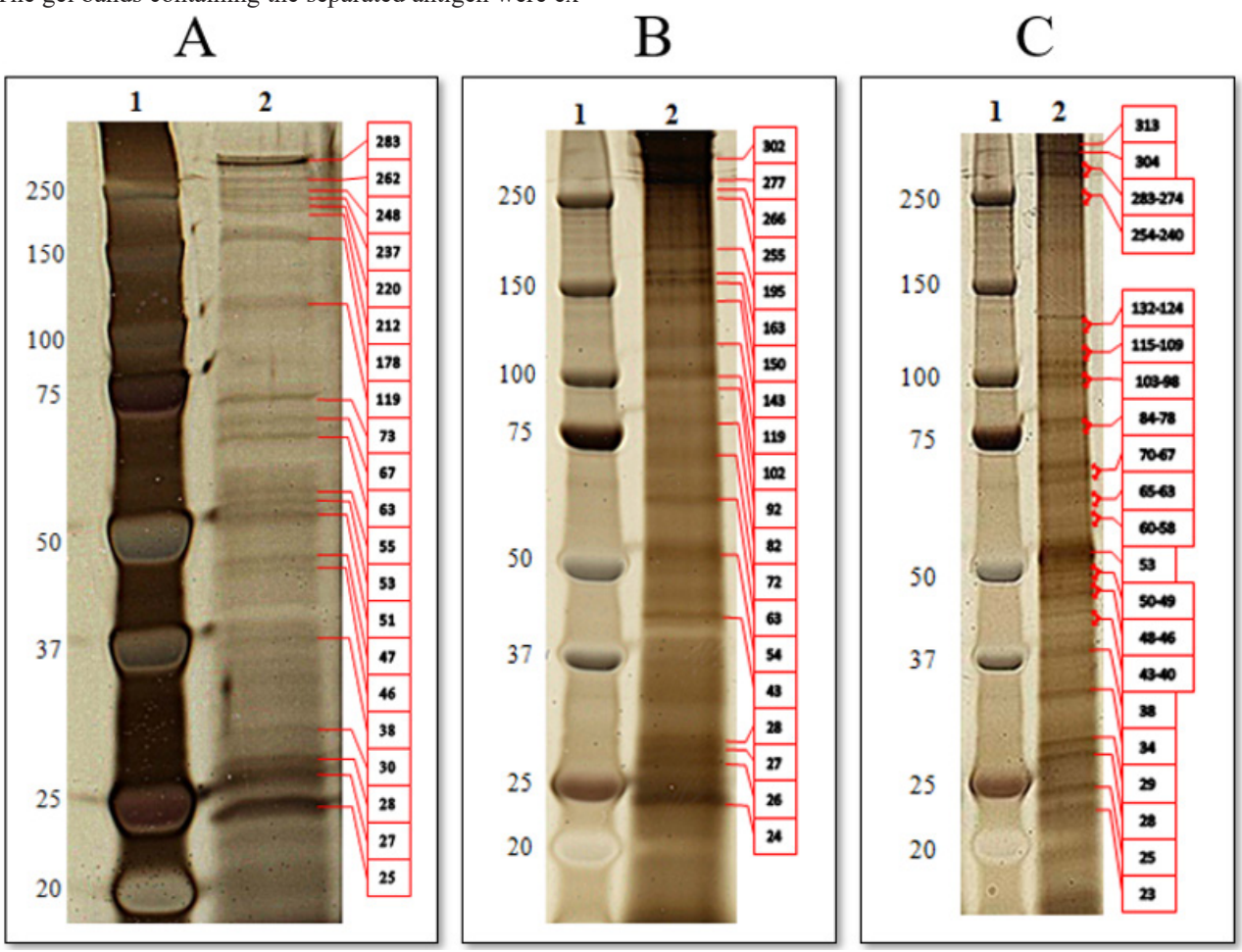

Figure 1 – Electrophoretic Separation of Antigens from *Opisthorchis felineus* Waste Products: (A) Excretory-secretory antigen, (B) Somatic antigen, (C) Egg antigen.

Electrophoresis and Immunoblotting. Electrophoresis was performed using a gradient polyacrylamide gel with sodium dodecyl sulfate (SDS) according to the method of J. Laemmli on a vertical electrophoresis apparatus (BioRad, USA). Commercial 4-12% gradient gels (BioRad, USA) were used for sample separation. The sample dilution buffer was prepared by adding 50 µl of mercaptoethanol to 950 µl of 4× buffer (BioRad, USA). Samples were diluted in a 1:1 ratio, boiled for 5 minutes at 100°C, and then cooled. Electrophoresis was conducted in two stages: initially at 125 V for 10 minutes, followed by 200 V for 30 minutes. After electrophoresis, the gel was removed from the plates and either stained or transferred to membranes. The immunoblotting procedure included the following steps: Electrophoresis: Conducted as described above. Protein Transfer: Proteins were transferred from the gel to a PVDF membrane using an Owl VEP-2 immunoblotting device (Thermo Scientific, USA) or a 49 BR device (BioRad, USA), according to the method of H. Towbin. Transfer was performed using Chaps buffer. Immunochemical Detection: The membrane was first incubated in a 5% BSA solution for 1 hour at room temperature. It was then washed three times for 5 minutes each in 1× PBST. Following this, the membrane was incubated for 2 hours at room temperature on a stirrer with a solution of specific antibodies, with dilutions depending on the manufacturer's instructions. The membrane was washed three more times and then incubated with a working dilution of anti-species antibodies for 1 hour. After washing, the chemiluminescent reaction was developed. The substrate solution was prepared immediately before use by mixing HRP Substrate Peroxide Solution and HRP Substrate Luminol Reagent in a 1:1 ratio.

3. RESULTS

For the studies, we selected only identified *O. felineus* adults, which were used to obtain specific antigens. To prepare the excretory-secretory antigen (ESP), viable *O. felineus* adults were thoroughly washed to remove traces of bile and blood with sterile saline. One hundred parasites were then placed in a culture medium. This resulted in 150 ml of ESP with a concentration of 1 mg/ml, 2.5 ml of egg antigen with a protein concentration of 1 mg/ml, and 2 ml of somatic antigen with a concentration of 1.5 mg/ml.

The three types of antigen preparations from *O. felineus* adults and their metabolic products were extensively studied for their protein composition, activity, and specificity. The protein composition of the antigens was analyzed by separating the samples using electrophoresis on 4-15% gradient polyacrylamide gels (PAAG). Staining was performed according to the instructions provided with the SilverQuest Staining Kit (Invitrogen). The quantitative analysis of the protein composition was conducted using Photo-Capt Version 12.4 software (VILBER LOURMAT). The detailed results of the quantitative protein composition of the antigens are presented in Figure 1.

Figure 1 shows the electrophoretic profiles of antigens obtained from various Opisthorchis felineus waste products: Excretory-Secretory Antigen: This preparation contained 21 major proteins with molecular weights of 283, 262, 248, 237, 220, 212, 178, 119, 73, 67, 63, 55, 53, 51, 47, 46, 38, 30, 28, 27, and 25 kDa. Somatic Antigen: The somatic antigen fractions revealed 20 major proteins with molecular weights of 302, 277, 266, 255, 195, 163, 150, 143, 119, 102, 92, 82, 72, 63, 54, 43, 28, 27, 26, and 24 kDa. Egg Antigen: The egg antigen was the most complex, containing approximately 33 major proteins with molecular weights of 313, 304, 283, 274, 254, 240, 132, 124, 115, 109, 103, 98, 84, 78, 70, 67, 65, 63, 60, 58, 53, 50, 49, 48, 46, 43, 40, 38, 34, 29, 28, 25, and 23 kDa. It is important to note that the electrophoresis method used primarily detects major proteins. For a more comprehensive analysis, including detection of less abundant proteins, more sensitive techniques such as mass spectrometry should be employed.

The activity of the antigens was assessed using various ELISA variants. Sera from infected dogs were used as positive controls, while serum from a healthy animal served as a negative control. Excretory-Secretory Antigen (ESA): This antigen demonstrated good activity and specificity. For positive sera, the average titer was 1:2400 ± 0.78. Additionally, ESA showed sufficient immunogenicity to induce specific antibody formation. In ESA-immunized Syrian hamsters, the titers were 3440 ± 0.76, and in laboratory mice, the titers were 1080 ± 0.84. Somatic Antigen: The highest dilution at which a positive reaction was detected was 1:256. Egg Antigen: The highest dilution showing a positive reaction was 1:64. This result indicates that the egg antigen also has good potential for use in serological reactions.

Based on the results from previous experiments, we conducted additional work to identify immunogenic proteins within the obtained antigens that can elicit an immune response in the host organism during an opisthorchiasis infection.

Excretory-Secretory Antigen (ESA): Figure 2 shows that ESA contains a single immunogenic protein fraction with a molecular weight of approximately 105 kDa. Somatic Antigen (SA): In reactions with positive sera, SA revealed two immunogenic protein fractions, with molecular weights of approximately 105 kDa and 250 kDa. Egg Antigen: Similar results were observed with the egg antigen. Notably, the protein fraction with a molecular weight of 250 kDa exhibited the highest activity.

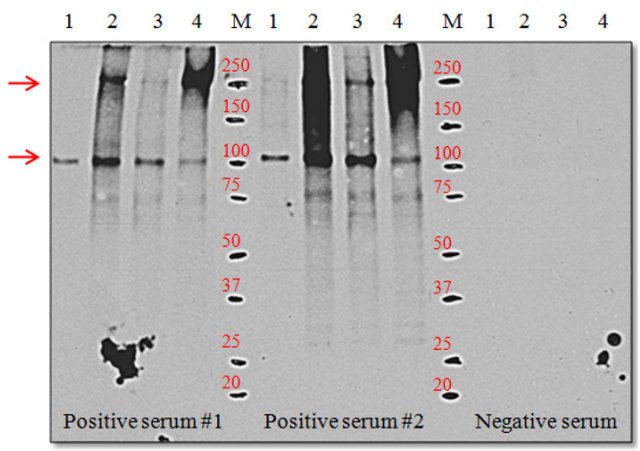

Figure 2 – Results of Immunoblotting: M – Marker, 1 – Excretory-Secretory Antigen (ESA), 2, 3 – Somatic Antigen (SA), 4 – Egg Antigen (EA).

Specificity of Antigens. Excretory-Secretory Antigen (ESA): This antigen exhibits pronounced immunogenicity

specifically towards a protein with a molecular weight of 105 kDa. Egg Antigen (EA): This antigen shows significant immunogenicity towards a protein with a molecular weight of 250 kDa. Somatic Antigen (SA): This antigen contains both protein fractions (105 kDa and 250 kDa) because it is derived from Opisthorchis adults, which produce both excretory and egg products.

Mass Spectrometric Analysis of Excretory-Secretory Product. The general composition of the excretory-secretory product of *O. felineus* was analyzed using mass spectrometry at the Whitehead Institute for Biomedical Research, Cambridge, MA, 02142. The mass spectrometry data were processed using Scaffold 4 software. In this analysis, a total of 214 proteins were identified in the excretory-secretory antigen. These proteins include not only those from the excretory-secretory secretions of the parasite but also structural proteins from the parasite's marita and internal organs. This broader protein composition likely results from the partial death and structural damage of the marita during its cultivation in nutrient media.

Identification of Immunogenic Proteins in ESA. The immunogenic proteins of the excretory-secretory antigen (ESA) were identified through immunoprecipitation. For this purpose, sera from dogs artificially infected with the Opisthorchis pathogen in previous experiments were used. The results are shown in Figure 3.

Bioinformatic Analysis of Mass Spectrometry Results. Bioinformatic analysis of the mass spectrometry data was conducted using Scaffold-4 software and the international PubMed database. This analysis revealed that, using positive serum, the highest number of immune complexes formed with three proteins: cathepsin F, glutathione transferase, and paramyosin.

Thus, the comprehensive composition of the excretory-secretory antigen was confirmed to include 214 proteins, with cathepsin F, glutathione transferase, and paramyosin identified as the most immunogenic.

4. Discussion

Opisthorchiasis is currently widespread not only in the Republic of Kazakhstan but also in many other countries, making it a significant issue in modern medicine and veterinary science. The immunology of trematode infections remains one of the least studied areas, with limited observations and minimal practical data available in the literature.

One major challenge in this field is the restricted access to excretory and protein components of parasites. The infectious forms of the pathogen, essential for studying immunological mechanisms, are often insufficient. Additionally, growing the parasite in vitro poses significant difficulties, further hindering progress in this research area. The immunology of *Opisthorchis* infections is closely linked with serology and immunodiagnostics, focusing on specific antigens and immunoglobulins as key components [16].

In medical and veterinary laboratories, opisthorchiasis is diagnosed using physical, coprological, hematological, and

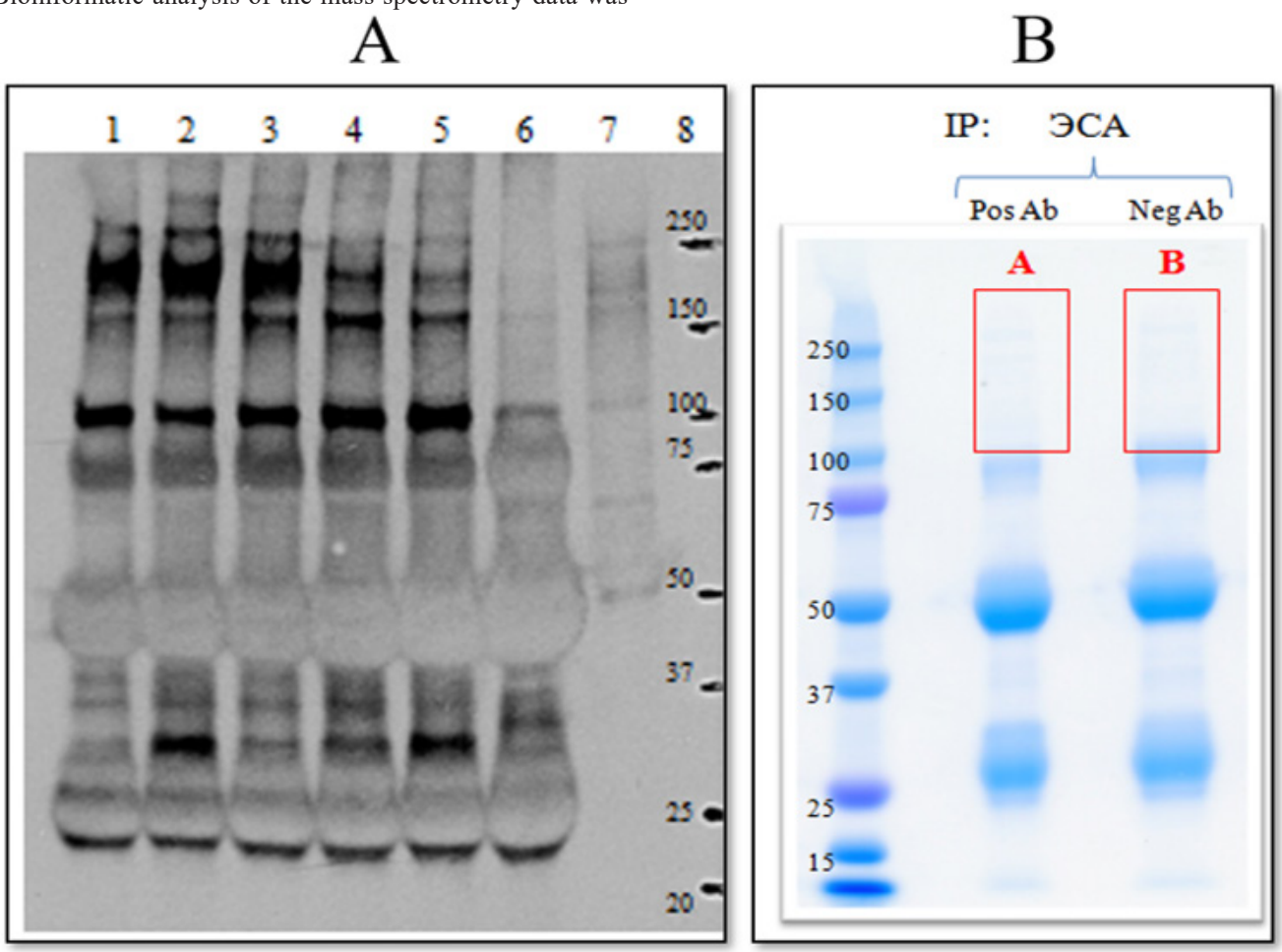

Figure 3 – Immunoprecipitation results: A – immunoblotting: 1, 2, 3, 4, 5 – positive dog sera, 6 – negative dog serum, 7 – ESA, 8 – marker; B – protein immunoprecipitation for mass spectrometry.

serological data. However, the limitations of these diagnostic methods, such as the cyclic release of parasite eggs, varied manifestations of liver disease, and low antibody titers in some serum samples, complicate accurate diagnosis. Consequently, highly sensitive enzyme-linked immunosorbent assay (ELISA) methods are increasingly employed for diagnosis. ELISA diagnostics rely on purified specific antigens.

The establishment of industrial production for ELISA-based diagnostic tests requires a substantial number of reagents, including antigens and antibodies. Currently, antigens are obtained by culturing *Opisthorchis* maritas in vitro from artificially infected laboratory animals. However, challenges such as the difficulty in infecting and maintaining biological models, along with ethical concerns regarding animal use in biomedical research, highlight the need for alternative approaches. Therefore, molecular genetic methods for producing antigen preparations are increasingly preferred.

To date, there is limited data on the immune response of the host organism to individual protein antigens of *O. felineus* [17]. However, significant research has been conducted on major antigens of *O. viverrini* and other trematodes in the *Opisthorchiidae* family, which are known to elicit a strong humoral immune response in humans. Notable among these antigens are vitellin and cathepsin F [18-26].

Nosov A.A. et al. (2012) investigated the antigenic and functional properties of the major proteins vitellin and cathepsin F in *O. felineus*. Their study demonstrated the antigenicity of recombinant proteins with respect to sera from individuals with opisthorchiasis [27]. The antigens produced by these methods exhibit high specificity, homogeneity, and activity, making them valuable components for designing diagnostic tests, including ELISA.

Thus, this area of research holds significant relevance and promise, not only in the Republic of Kazakhstan but also in other regions where opisthorchiasis is prevalent.

CONCLUSION

As a result of our work, three types of antigens were obtained: excretory-secretory (ESA), egg (EA) and somatic (SA). Their protein composition was studied and the number of major proteins was determined: ESA - 21, SA - 20 and EA - 33. Protein fractions with a molecular weight of 105 and 250 kDa have immunogenic properties. Immunogenic and antigenic properties of the obtained antigens were proven. The composition of ESA, which includes 214 protein substances of the secretory nature of the parasite, was studied by mass spectrometry. Immunoprecipitation using specific sera allowed us to identify the three most immunogenic proteins - cathepsin F, glutathione transferase and paramyosin.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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ИЗУЧЕНИЕ БЕЛКОВОГО СОСТАВА *АНТИГЕННЫХ ПРЕПАРАТОВ ВОЗБУДИТЕЛЯ OPISTHORCHIS FELINEUS РАЗЛИЧНОЙ ХИМИЧЕСКОЙ ПРИРОДЫ.*

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АБСТРАКТ

Описторхоз в настоящее время имеет значительное распространение не только в Республике Казахстан, но и во многих других странах мира, поэтому представляет одну из острых проблем современной медицины и ветеринарии. Диагностика данного заболевания имеет решающее значение для дальнейшего лечения пациентов. Специфичность используемых диагностических тестов зависит от антигенных препаратов, к которым относятся антигены. Наши исследования были нацелены на изучение антигенов различной химической природы, полученных из возбудителя *Opisthorchis felineus*. В результате проведенной нами работы было получено три вида антигенов: экскреторно-секреторный (ЭСА), яичный (ЯА) и соматический (СА). Изучен их белковый состав и определено количество мажорных белков: ЭСА – 21, СА – 20 и ЯА – 33. Иммуногенными свойствами обладают белковые фракции с молекулярной массой 105 и 250 кДа. Доказаны иммуногенные и антигенные свойства полученных антигенов. Методом масс-спектрометрии изучен состав ЭСА, в состав которого входит 214 белковых веществ, имеющих секреторную природу паразита. Иммунопреципитация с использованием специфических сывороток позволила идентифицировать

три наиболее иммуногеных белка – катепсин F, глютатион-трансфераза и парамиозин.

Ключевые слова: *Opisthorchis felineus,* антигенные препараты, белковый состав, иммуногенность, специфичность.

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OPISTHORCHIS FELINEUS ҚОЗДЫРҒЫШЫНЫҢ ХИМИЯЛЫҚ ТАБИҒАТЫ ӘРТҮРЛІ АНТИГЕНДІК ПРЕПАРАТТАРДЫҢ АҚУЫЗДЫҚ ҚҰРАМЫН ЗЕРТТЕУ

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АБСТРАКТ

Қазіргі уақытта описторхоз тек Қазақстан Республикасында ғана емес, сонымен қатар әлемнің көптеген елдерінде кең таралған, сондықтан описторхоз саласындағы зерттеулер қазіргі заманғы медицина мен ветеринарияның өзекті мәселелерінің бірі болып табылады. Описторхозды диагностикалау пациенттерді одан әрі емдеу үшін өте маңызды. Практикада қолданылатын диагностикалық сынақтардың телімділігі антигеннің антигендік препараттарына байланысты. Біздің зерттеуіміз Opistorchis felineus қоздырғышынан алынған әртүрлі химиялық табиғаттағы антигендерді зерттеуге бағытталған. Атқарылған жұмыстың нәтижесінде антигендердің үш түрі алынды: экскреторлық-секреторлық антиген (ЭСА), жұмыртқа антигені (ЖА) және соматикалық антиген (СА). Олардың ақуыздық құрамы зерттеліп, негізгі белоктардың саны анықталды: ЭСА – 21, ЖА – 20 және СА – 33. Молекулярлық массасы 105 және 250 кДа белок фракцияларында иммуногендік қасиет болатыны анықталды. Алынған антигендердің иммуногендік және антигендік қасиеттері дәлелденді. Масс-спектрометрия көмегімен секреторлық табиғаты бар 214 ақуыздық затты қамтитын ЭСА құрамы зерттелді. Телімді сарысуларды қолдану арқылы иммунопреципитация реакциясының көмегімен иммуногенді жоғары үш ақуыз – катепсин F, глутатионтрансфераза және парамиозин анықталды.

Кілтті сөздер: *Opisthorchis felineus,* антигенді препараттар, ақуыз құрамы, иммуногендік, телімділік.