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Occurrence of larval anisakid in imported mackerel (*Scomber scombrus*) in Astana fish markets

Rabiga Uakhit[1], Nurasyl Manapov[2,3], Adilhkan Nurken[3], Ainura Smagulova[1], Aiganum Bekenova[3], Temirlan Tursynbek[3], Vladimir Kiyan[1]

[1]National Center for Biotechnology, Astana, Kazakhstan,
[2]Al-Farabi Kazakh National University, Almaty, Kazakhstan
[3]S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan

Corresponding author: Vladimir Kiyan: vskiyan@gmail.com
Co-authors: (1: RU) erken.uakhitrabiga@gmail.com; (2: NM) manapovbio@gmail.com;
(3: AN) nurkenadilkho@mail.ru; (4: AS) smagulova0114@gmail.com;
(5: AB) aiganym5555@mail.ru; (6: TT) tatursynbek@gmail.com
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Abstract

Background and Aim: This study aimed to investigate the presence of *Anisakis* larvae in mackerel fish sold in Astana markets, focusing on specimens imported from Norway and Iceland. The high prevalence of *A. simplex* (s.s.) larvae in the examined mackerel indicates the significance of this fish as a potential source of human anisakiasis.

Materials and Methods. Morphological analyses were deemed insufficient for accurate identification of Anisakis larval species, highlighting the necessity for more advanced molecular tools. Through partial sequencing of the 5.8S gene, the isolated Anisakis larvae from Norwegian Sea mackerel and Atlantic mackerel were classified as *A. simplex* (s.s.), demonstrating the efficacy of molecular methods in distinguishing closely related species.

Results. A high prevalence of *A. simplex* (s.s.) larvae was observed in 47 out of 50 examined mackerel, indicating the significance of this fish as a potential source of human anisakiasis.

Conclusion. These findings significantly contribute to understanding *Anisakis* larvae in fish within Kazakhstan and are pertinent to public health. The study underscores the importance of molecular-genomic analysis in this field and emphasizes the potential risks associated with inadequate expertise. Importantly, the study calls for further research on the genetic variability and infection rates of these parasites to monitor the status of *A. simplex* in Kazakhstan.

Keywords: Anisakis larvae; fish market; mackerel; human anisakiasis; molecular method.

Introduction

In Kazakhstan, where the annual fish consumption per capita is approximately 3.4 kg, the import of 43.000 tons of frozen fish in 2023-primarily salmon, mackerel, and herring from Norway, Iceland, and Russia-highlights the importance of understanding the risks associated with consuming raw fish. Anisakiasis, a parasitic infection caused by Anisakidae nematodes, can be contracted by humans through the consumption of raw or undercooked fish [1].

The *Anisakida*e family comprises 12 genera, seven of which are parasitic, including *Anisakis spp., Contracaecum spp., Phocascaris spp., Pseudoterranova spp., Pulchrascaris spp., Terranova spp.,* and *Sulcascaris spp.* [2, 3]. These parasites are distributed globally, predominantly in the Atlantic and

Pacific oceans and coastal waters, with cold and temperate climates, closely following the distribution of their marine hosts [4, 5].

The life cycle of *Anisakidae* nematodes involves four larval stages (L1–L4), with marine mammals such as whales, dolphins, and seals serving as the definitive hosts. Female nematodes can release up to 1.5 million eggs into the intestines of their hosts. In the L1 stage, eggs are expelled into the environment through the host's excrement. In the sea, they develop into the L2 stage and are ingested by small crustaceans and copepods. Inside these intermediate hosts, the larvae molt into the L3 stage. Copepods are consumed by marine fish, which function as paratenic hosts. The larvae embed themselves in the internal tissues of fish, particularly the liver and peritoneum, causing inflammation. When a marine mammal eats an infected fish, its life cycle is completed, and the larvae mature into adult L4 forms [2, 6].

Humans can become infected by consuming raw fish containing L3 larvae, leading to anisakiasis. Symptoms can range from gastric to intestinal, and allergic reactions are possible because of allergenic proteins produced by the nematodes [2, 5]. Mild infections can be managed with symptomatic treatment, whereas severe infections might require surgical intervention to remove the larvae. Primary treatments for anisakiasis include endoscopic and surgical removal, with some success reported for albendazole treatment [5, 7-10].

The aim of this work is to raise awareness about the risks associated with raw or undercooked fish consumption, especially in regions with significant import and consumption of frozen fish. The focus is on understanding and highlighting the parasitic infection caused by *Anisakidae* nematodes and identifying the species of the anisakid parasite. Through this work, our goal is to promote safer consumption practices and minimize the incidence of anisakiasis.

Materials and Methods

Fish sample collection and processing

To perform the research, 50 samples of imported mackerel from Norway and Iceland, destined for the fish markets of Astana, were selected. Parasitological examinations were conducted at the Laboratory of Parasitology, Faculty of Veterinary Medicine, S.Seifullin Kazakh Agrotechnical Research University. Molecular and genetic research was performed at the Laboratory of Biodiversity and Genetic Resources within the National Center for Biotechnology. Quantitative assessment of larval occurrence in fish was conducted by calculating the following metrics: prevalence = number of infected fish/numbers of examined fish, mean intensity (MI) = total number of isolated larvae per fish species/number of infected fishes of that species, and mean abundance = total number of isolated larvae per fish species/number of examined fish of that species [11]. All collected larvae were processed for microscopic examination to differentiate between type I (anisakid larvae) and type II larvae.

Morphological examination of the isolated larvae using a light microscope

The larvae isolated from each fish species were washed in saline solution for 30 min, incubated in lactic acid for 15 min, and examined using ordinary light microscopy (Fig. 1).

DNA extraction

For molecular analysis, five samples of contaminated mackerel imported from Norway and Iceland were selected. A small piece of each specimen was cut and homogenized, and the homogenate was subjected to the standard phenol–chloroform method [12], supplemented with proteinase K, to extract genomic DNA (gDNA). DNA was precipitated with ethanol, purified, dissolved in dd H_2O, and stored at −70 °C for subsequent analysis.

Polymerase chain reaction analysis

Polymerase chain reaction (PCR) was performed using the 5.8S primer pair (NC13 Forward: ATCGATGAAGAACGCAGC and NC2 Reverse: TTAGTTTCTTTTCCTCCGCT) to amplify worm gDNA [13]. PCR was conducted in a 25 µL reaction mixture containing 10× Taq buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 200 µM dNTPs (Thermo Scientific, Carlsbad, California, USA), 10 pmol of each primer, and 20 ng of extracted gDNA as the template. DNA segments were amplified by thermal cycling for 30 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 30 s). The resulting amplification products were separated by electrophoresis on a 1.5% agarose gel prepared with 1× TAE buffer and stained with 8 ng/µL ethidium bromide.

Sequencing

The PCR-amplified target gene fragment was purified using a QIAquick PCR Purification Kit (QIAGEN, Germany, Cat. No. 28106) following the manufacturer's instructions. Sequencing was conducted using a Seq Studio Genetic Analyzer (Thermo Fisher Scientific Applied Biosystems) according to the manufacturer's instructions. The resulting nucleotide sequences were visually inspected using BioEdit software version 7.0. These sequences were then compared with other sequences in the NCBI GenBank database using BLAST. The nucleotide sequences of the studied species were deposited in the NCBI GenBank database.

Phylogenetic analysis

The obtained sequences were manually edited, and sequence similarity searches were performed using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov) to compare them with GenBank reference sequences. Nucleotide sequences were aligned using the MUSCLE multiple sequence alignment program for the partial cox1 gene. A maximum-likelihood phylogenetic tree was constructed using the Tamura-Nei model with MEGA v11 software [14], with *Pseudoterranova decipiens* (AB201790) serving as the outgroup.

For detailed analyses, a pairwise distance method was used with the maximum composite likelihood model [15]. This analysis involved six nucleotide sequences with codon positions including first, second, third, and noncoding regions. For each sequence pair, all ambiguous positions were removed using the pairwise deletion option. The final dataset included 458 positions.

Results

This study analyzed 50 mackerel samples and presented findings related to anisakid larvae. Table 1 presents the prevalence, MI, and mean abundance of the larvae.

Table 1- Prevalence (P), mean intensity (MI), and mean abundance (MA) of isolated anisakid larvae in the examined fish

Fish host	No. examined	No. infected	Larvae found	P, %	MI	MA
Norway mackerel (*Scomber scombrus*)	45	42	899	93.33	21.40	19.97
Iceland mackerel (*Scomber scombrus*)	5	5	70	100	14	14

The overall prevalence of anisakid larvae was >93.33% in Norwegian mackerel and 100% in Icelandic mackerel. The MI of Norwegian mackerel was 21.40, whereas that of Icelandic mackerel was 14. The MA of Norwegian and Icelandic mackerel was 19.97 and 14, respectively.

Most anisakid larvae were encapsulated in fish muscle and visceral organs, including the peritoneum (Fig. 1A and B). Using an ordinary light microscope, all isolated larvae displayed features characteristic of Anisakis larval type 1, particularly the long ventriculus and mucron at the posterior end (Fig. 1).

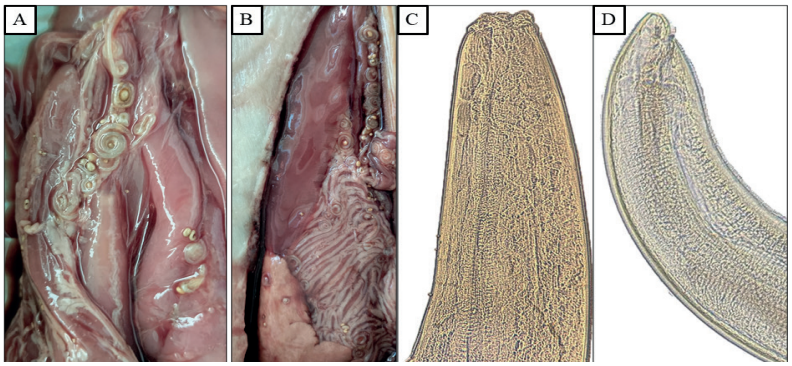

Figure 1 - Anisakis helminths observed inside mackerel. Morphology of the isolated larvae

A and B: Observed *A. simplex* helminths in the visceral organs; C: anterior end of *A. simplex*; and D: posterior end of *A. simplex*

Because the larvae within six to seven pools of each fish species exhibited nearly identical sequences of the 5.8S gene, we used three gene sequences from a single larval pool of each fish host for this analysis. Phylogenetic analysis (Fig. 2) revealed that all studied larvae belonged to anisakid larvae. Larvae isolated from Norwegian Sea mackerel (PQ047832, PQ047833, and PQ047834) and Atlantic Ocean mackerel imported from Iceland (PQ047828, PQ047829, and PQ047830) clustered closely together and were classified as A. simplex (s.s.) because they grouped near other A. simplex (s.s.) and A. pegreffii larvae (e.g., Accession numbers: AM706346.1, LC536534.2, HF680316.1, and AJ937671.1). In contrast, A. berlandi was found in separate clades [16]. Pseudoterranova decipiens (AB201790) was used as the outgroup for the rooted tree.

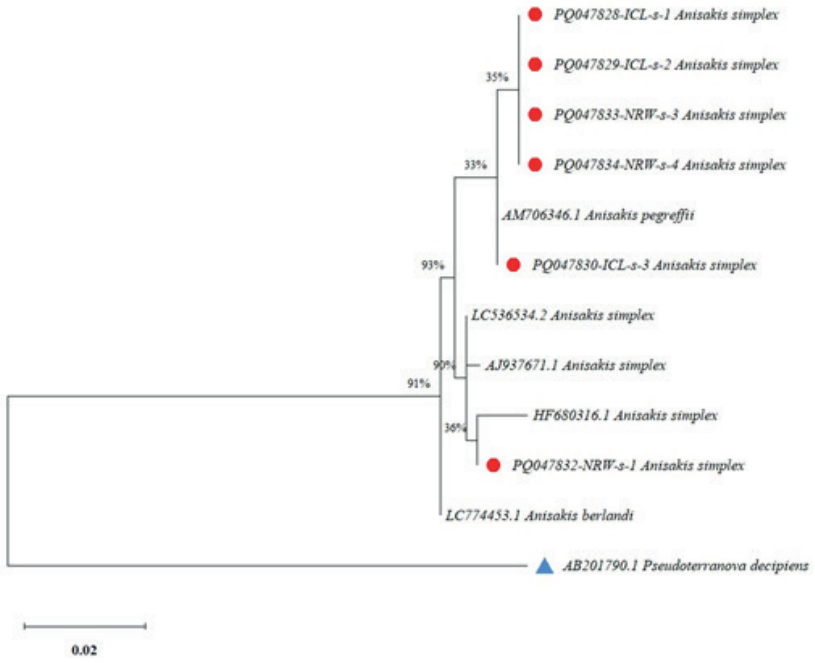

Figure 2 - Phylogenetic relatedness of the study isolates (red dot beside) to other isolates belonging to the anisakid family (divergence = 0.02)

To obtain more detailed data on nucleotide sequences, Table 2 presents the number of base substitutions per site between sequences. The data show the close relationships between the studied isolates.

Table 2 - Estimation of evolutionary divergence between sequences

	PQ047828	PQ047829	PQ047830	PQ047832	PQ047833	PQ047834
PQ047828-ICL-s-1 *A. simplex*						
PQ047829-ICL-s-2 *A. simplex*	0.0000					
PQ047830-ICL-s-3 *A. simplex*	0.0000	0.0000				
PQ047832-NRW-s-1 *A. simplex*	0.0000	0.0000	0.0000			
PQ047833-NRW-s-3 *A. simplex*	0.0000	0.0000	0.0000	0.0000		
PQ047834-NRW-s-4 *A. simplex*	0.0000	0.0000	0.0000	0.0000	0.0000	

Discussion and Conclusion

The aim of this study was to determine the occurrence of *Anisakis* larvae in mackerel fish widely sold in Astana markets. *A. simplex* (s.s.) larvae were found to be highly prevalent in the examined mackerel imported from Norway and Iceland. Morphological analyses alone may not be suitable for identifying Anisakis larval species [17, 18]. Although *A. simplex* (s.s.) is a well-known fish parasite associated with human disease, no prior studies have investigated the prevalence of this nematode in fish sold in Kazakhstan. Our study revealed a prevalence of *A. simplex* (s.s.) in marketed mackerel of 94%, with an MI of 20.61 larvae per infected fish (Table 1), underscoring the importance of this fish as a potential source of human anisakiasis. The high MI of *A. simplex* (s.s.) in the examined mackerel indicates significant risk of infection in humans.

Fish of the genus *Scomber* (mackerel) are widely distributed and exhibit considerable diversity. However, Atlantic and Norwegian mackerel (*Scomber scombrus*) are the most consumed *Scomber* species in the Astana market. This study provides the first insights into the occurrence of A. simplex in fish imported to and sold in Kazakhstan.

Our research demonstrated that morphological analysis can provide preliminary information about larval species, whereas advanced molecular tools offer more definitive species identification. Through partial sequencing of the 5.8S gene, we classified the isolated *Anisakis* larvae. We found that larvae from Norwegian Sea and Atlantic Ocean mackerel grouped with *A. simplex* (s.s.) and were distinct from other *Anisakis* species. This underscores the effectiveness of molecular methods in distinguishing closely related species and enhances our understanding of larval species identification.

It is evident that molecular methods such as sequencing are most effective for defining larval species. These findings make a significant contribution to our knowledge of *Anisakis* larvae in fish in Kazakhstan and have important public health implications.

Although *Anisakis* has been known since the 19th century, research on anisakiasis has notably increased recently [19]. Publications from 1970 to 1990 were limited, and research during this period was relatively stagnant. The literature output began to rise steadily between 1990 and 2000, reflecting growing interest in A. simplex. Despite this, molecular and genomic analysis of this subject remains underexplored.

Cipriani et al. [20] provided valuable insights into the distribution of Anisakis parasites in the Atlantic Ocean and Norwegian Sea, sources of mackerel imported to Kazakhstan. This report highlights the potential risks associated with a lack of awareness in this field, emphasizing the challenges posed by insufficient expertise.

Anisakiasis is a disease closely associated with local dietary habits [21]. Most reported cases (>90%) occur in Japan, with additional cases occurring primarily in European countries where raw fish dishes are common [22, 23]. There are significant gaps in research on imported fish in Kazakhstan.

Future research should focus on estimating the genetic variability and infection rates of these parasites as indicators for monitoring the status of *A. simplex* in Kazakhstan.

In conclusion, our study sheds light on the prevalence of *A. simplex* (s.s.) larvae in mackerel fish sold in Astana markets, highlighting the potential risk of human anisakiasis associated with the consumption of infected fish. Our study demonstrates the importance of using advanced molecular tools to definitively identify species in *Anisakis* larvae. Furthermore, the findings underscore the need for increased awareness and expertise in this field, particularly regarding imported fish in Kazakhstan. Future research efforts should prioritize estimating the genetic variability and infection rates of these parasites to effectively monitor the status of *A. simplex* in Kazakhstan.

Authors' Contributions

Conceptualization, VK.; methodology VK. and RU.; validation, RU., AS., AN. and AB.; formal analysis, VK., RU. and NM.; investigation, VK.; resources, VK.; data curation, AB. and AS.; writing–original draft preparation, RU., NM., VK.; writing–review and editing, VK and RU.; visualization, AB., TT. and AN.; project administration, VK.; funding acquisition, VK. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

Authors have no conflict of interest to declare.

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Keratinolytic testing for diagnosing dermatophytosis in small domestic animals

Ainura M. Smagulova , Tatyana I. Glotova

Siberian Federal Scientific Centre of Agro-BioTechnologies
of the Russian Academy of Sciences Krasnoobsk, Russian Federation

Corresponding author: Ainura M. Smagulova: smagulova0114@gmail.com
Co-authors: (1:TG) t-glotova@mail.ru
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Abstract

Background and Aim. Keratin is used as a structural element by numerous animal groups. Because of the strength and stability of keratin, few organisms can break it down and use it as a food source. Only a few insects, bacteria, actinomycetes, and fungi can use keratin as their sole carbon and nitrogen source. The enzymatic ability of fungi to degrade keratin has long been considered a key innovation in the evolution of animal dermatology.

The aim of the work is to develop keratinolytic tests for use and to identify their pathogenicity in the diagnosis of dermatomycetes of small domestic animals.

Materials and Methods. There were investigated the strains of the genera *Trichophyton, Microsporum,* and animal hair. The diagnosis for the detection of keratinolytic properties was carried out on three tests: hair perforation, nutrient media with grind animal hair and keratin hydrolysate.

Results. The authors of this article present studies for diagnosing dermatophytosis in small domestic animals for pathogenicity using three keratinolytic tests: hair perforation, the addition of ground animal hair, and keratin hydrolysate to the culture medium. The three tests demonstrated high keratinolytic activity, which manifested as the formation of pegs on hair and the appearance of a clearing zone in the culture media under the influence of keratinase. It was also established that in media containing hair and keratin hydrolysate, the growth of cultures was faster, and the cultures did not exhibit pleomorphism, which is a positive aspect in terms of the accuracy and speed of diagnosis.

Conclusion. Keratinolytic test allows to determine the pathogenicity of small domestic animal dermaticosis pathogens. The test showed that cultures do not form pleomorphisms, which contributes to the accurate identification of pathogens.

Key words: dermatophytosis; hair perforation; keratin; keratin hydrolysate; *Microsporum canis; Trichophyton benhamiae.*

Introduction

Dermatophytes are keratinophilic fungi belonging to the family Arthrodermataceae (Onygenales, Ascomycota), which includes dozens of related species that are primarily distinguished by their anamorph or asexual forms and are grouped into three classical genera: *Trichophyton, Microsporum,* and *Epidermophyton* [1-3]. Diseases caused by dermatophytes, such as dermatophytosis, are widespread across the globe, and the number of infections is increasing annually, not only in animals but also in humans. Of particular importance is the spread of dermatophytosis among small domestic animals, such as cats and dogs, which are human companions [4].

Until recently, the diagnosis of dermatophytosis was based on the analysis of clinical signs of the disease, which is unreliable because of the variable nature of dermatological lesions and their similarity to other skin conditions that mimic the symptoms of dermatophytosis [5]. Keratinolytic fungi are considered natural colonizers of keratinized substrates and play key roles in the natural hydrolysis of keratinized tissues [6]. Therefore, it was initially believed that dermatophytes obtain the nutrients necessary for their vital functions from easily hydrolyzed proteins, which make up only about 5% of the total composition of affected tissues [7]. It has been shown that keratinases perform not only nutritional functions, but are also the most important factors in the virulence of dermatophytes at the first stage of infection [8, 9]. *Aspergillus, Paecilomyces, Doratomyces, Trichoderma, Fusarium, Acremonium, Onygena, Cladosporium, Microsporum, Lichtheimia, Chrysosporium, Aphanoascus, Trichophyton,* and *Scopulariopsis* are among the recorded keratinolytic fungi [10-17]. Among fungal strains, keratinolytic activity has been widely described in dermatophytes. The keratinolytic properties of this group may reflect pathogenic tendencies and contribute to skin mycoses in humans and animals [18].

The complexity of keratin biodegradation by dermatophytes begins with sulfitolysis, which involves the intracellular generation of sulfite from cysteine catabolism via the enzyme cysteine dioxygenase (Cdo1). The keratinolytic activity and pathogenicity of this medically important group of fungi are interrelated, as during infection, dermatophytes secrete a variety of proteases that break down keratinized structures into oligopeptides as well as free amino acids, which are then used by the fungi as nutrient sources [19]. Keratin waste of agro-industrial origin presents an ecological problem, with feathers and hair from the poultry and leather industries at the forefront as they strive to meet the demands of a growing population. The authors of this article propose using bacteria and fungi that produce keratinase instead of chemical hydrolysis [20, 21].

Melentyev A.I. et al. proposed a method for diagnosing dermatomycosis by culturing samples of biological material on solid selective Sabouraud agar with the addition of an antibiotic, such as chloramphenicol and keratin hydrolysate, derived from chicken feathers. The cultivation conditions were 22 °C–24 °C for 4–8 days, followed by identification of fungal colonies at the species level. The method was effective against not only *Microsporum canis* but also *Trichophyton verrucosum* and *Trichophyton mentagrophytes*. The culture method allows isolation of pathogens and determination of its species affiliations [22].

Materials and Methods

The materials used in this study were

- strains of dermatophytes from the Trichophyton genus (No. 19 and No. 20) and Microsporum genus (No. 35 and No. 68) (isolated from cats and dogs). The strains were selected with an erased form of manifestation of the pathogen when making a diagnosis; four samples were selected from 198 samples.;
- Sabouraud glucose agar (40 g glucose, 10 g peptone, 20 g microbiological agar, and 1 L distilled water) for hydrolysis with 10% NaOH;
- pet hair (cat hair) obtained from veterinary clinics;
- equipment: Esco Class II Biological Safety Cabinet, 2010; BD-53 Thermostat, Germany, 2017; and Retsch MM 200 Vibratory Mill.

Research Methods:

Hair perforation test. To perform this test, several sterilized hairs (not chemically treated) are placed in a Petri dish containing sterile water and a few drops of 10% yeast extract are added. Then an inoculum in the form of several fragments of the isolate being tested is added and the dish is incubated for two weeks at 25 °C. A positive result is considered to be the appearance of cone-shaped perforations in the hair or erosion on the hair surface.

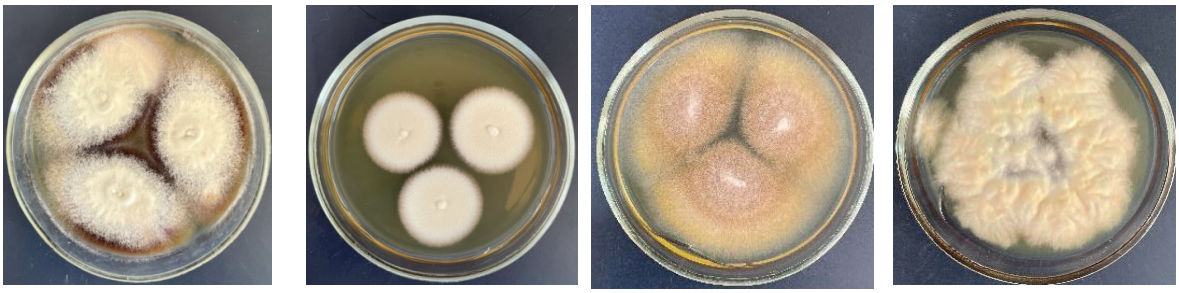
Animal hair provided by veterinary clinics was finely ground using a vibratory mill. The Sabouraud medium was supplemented with 2 g of ground hair per 100 mL of medium and autoclaved at 0.5 atm for 15 min. The prepared hair-containing medium was then poured into Petri dishes.

Keratin hydrolysate was obtained by mixing animal hair with 10% NaOH until the hair was fully dissolved. The resulting mixture was added to the Sabouraud medium and autoclaved at 0.5 atm for 15 min. The prepared keratin hydrolysate-containing medium was then poured into Petri dishes.

Inoculation was performed under sterile conditions in a biological safety cabinet. The cultures were incubated in a thermostat at 27 °C until colonies were formed.

Results

The studies were conducted with isolates that had previously been isolated from small domestic animals, with an erased etiology, to confirm their pathogenicity, a keratinolytic test was performed with chopped wool, keratin hydrolysate and hair perforation. In this study, strains of *Trichophyton benhamiae* and *M. canis* were used. (Figure 1).



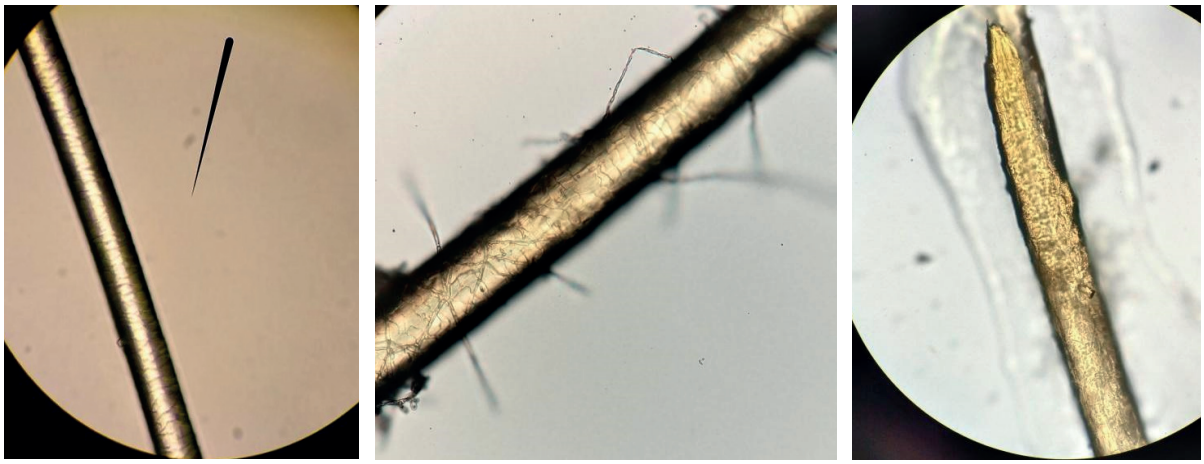
a b c d

Figure 1 – Pure cultures of *Trichophyton benhamiae* (a) No. 19 and (b) No. 20 and *Microsporum canis* (c) No. 35 and (d) No. 68

Figure 1 shows the colonies of pure cultures of *T. benhamiae* No. 19 and No. 20 and *M. canis* No. 35 and No. 68 in classical Sabouraud medium. Colony formation on this medium occurs in 18–30 days for *T. benhamiae* and *M. canis*.

To evaluate the pathogenicity of the strains, we conducted three tests for keratinolytic activity: a hair perforation test, addition of ground hair to the medium, and medium supplemented with keratin hydrolysate. The experimental results are summarized as follows.

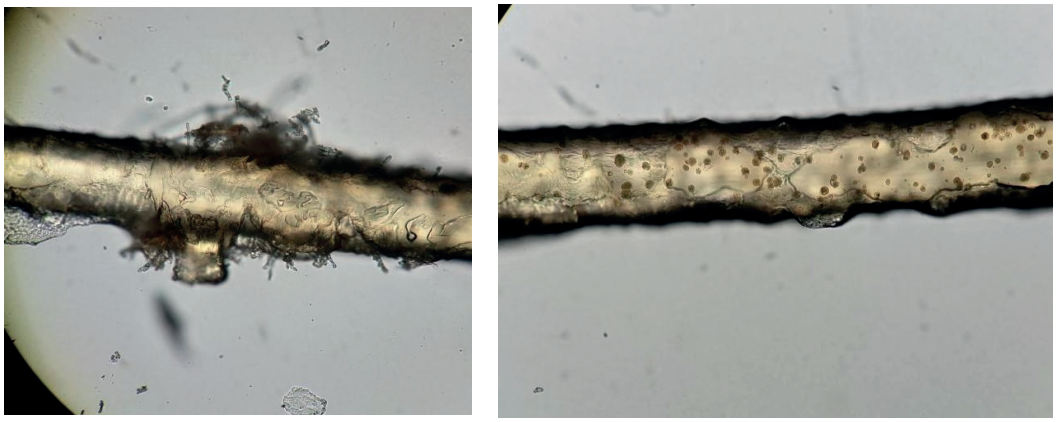
Both strains of *T. benhamiae* exhibited pronounced keratinolytic properties during the classic hair perforation test, as evidenced by abundant growth on the surface of the hair and the appearance of noticeable “pegs” or erosion on the surface of the hair (Figure 2).



Control Strain No.19 Strain No. 20

Figure 2 – Hair destruction under the action of *T. benhamiae* enzymes

Comparable results were obtained for hair infected with *M. canis.* The strains of *M. canis* exhibited pronounced keratinolytic properties during the hair perforation test, with more evident damage to the hair compared with that in the experiment with *T. benhamiae* (Figure 3).

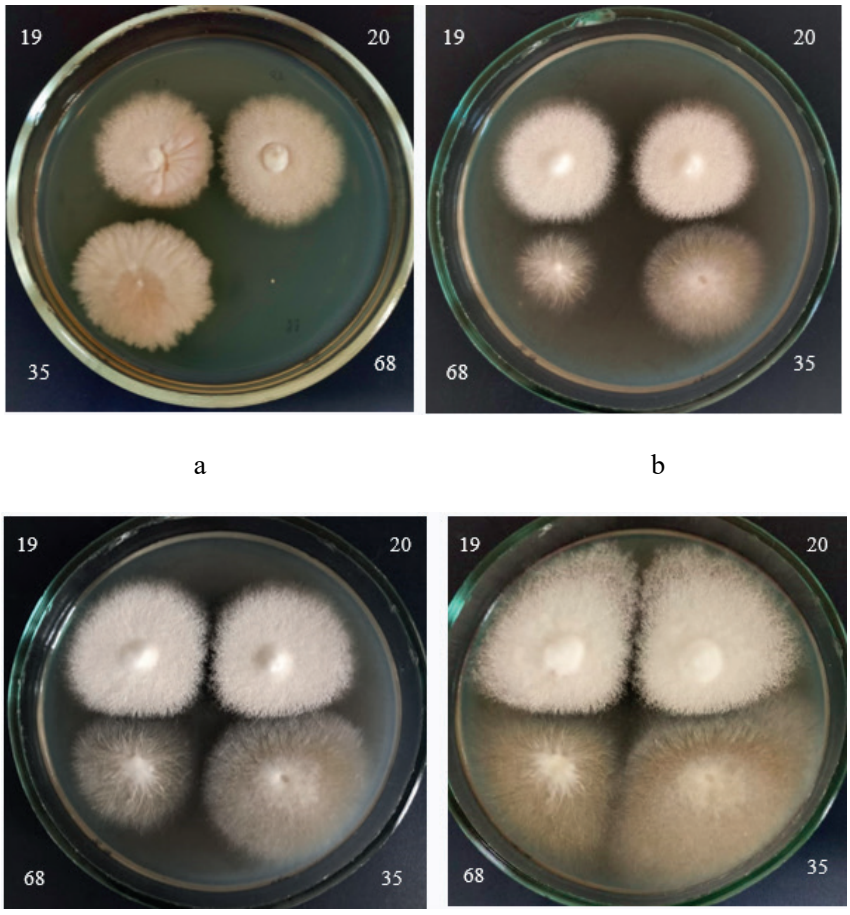


Strain No. 35 Control

Figure 3 – Hair damage under the action of keratinolytic enzymes of *M. canis*

To further identify the keratinolytic properties of dermatophyte cultures, we prepared Sabouraud media with the addition of keratin hydrolysate from cat hair and ground cat hair.

We observed more pronounced species differences in the manifestation of cultural–morphological characteristics in fungal strains grown on media with keratin. The surface of the colonies on control medium was more delicately structured and velvety, whereas that on medium supplemented with keratin was more granular and dense (Figure 4).



a b

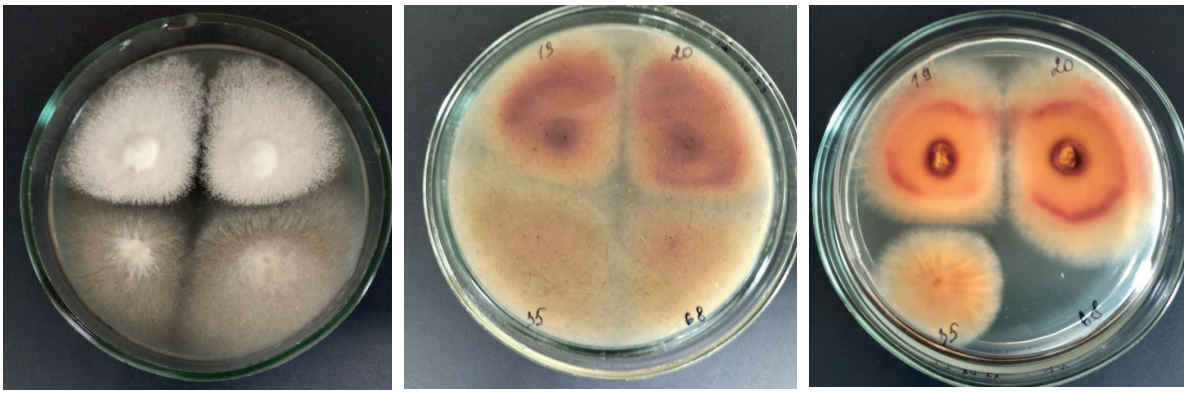
c d

a – control, 8th day; b – colonies, 8th day; c – colonies, 10th day; d – colonies, 14th day.

Figure 4 – Growth of dermatophyte colonies on Sabouraud medium with added keratin hydrolysate

As seen in Figure 4, on media with cat keratin hydrolysate (b–d), the fungal strains accumulated biomass more quickly, formed aerial and substrate mycelium and spores more actively, and exhibited different colony colors and structures compared with those on the control medium without keratin hydrolysate (a), with this effect being particularly pronounced in *T. benhamiae.*

The growth of dermatophyte cultures on Sabouraud media using the method with ground cat hair also indicated that the addition of hair affected the manifestation of cultural properties in fungal colonies (Figure 5).



a b c

a) front; b) back; c) control

Figure 5 – Characteristics of growth of *T. benhamiae* and *M. canis* strains on modified media with added ground hair

As seen in Figure 5, on the underside of colonies grown on media with chopped hair (b), there is more pronounced pigmentation on the reverse side, with zonal pigment accumulation and higher pigment intensity, especially in *T. benhamiae* strains.

Additionally, upon growth and colony formation, a more distinct zone of transparency around each strain was observed, which manifested as a more intense background color in the medium. This phenomenon is believed to be related to the thinning and destruction of hair keratin within the substrate by the keratinolytic enzymes of the fungi, which alters the direction of light dispersion. The presence of hair particles reduces the transparency of the nutrient medium, resulting in light refraction. The absence or thinning of hair particles allows light to partially pass through the boundary of the medium, thereby changing the direction of light dispersion. If the medium is transparent, the image is visually reflected in a more intense shade of the background.

Thus, we identified the keratinolytic activity of the *T. benhamiae* and *M. canis* fungal strains in relation to domestic animal hair. The pronounced keratinolytic properties of these strains provide evidence of their etiological roles in the development of skin pathology and its derivatives in domestic cats and dogs.

Discussion and Conclusion

The method of diagnosing dermatophytes by inoculating clinical material on the Sabouraud medium with keratin hydrolysate and finely chopped hair allows for a reduction in the time required to isolate and identify dermatophyte fungal cultures. Moreover, pleomorphic changes in cultures were not observed when using the proposed media.

In studying the keratinolytic properties of dermatophytes, characteristic hair damage was identified, confirmed by the research of Čmoková, A. (2020), who demonstrated that *T. benhamiae* exhibits pronounced enzymatic activity in the breakdown of hair keratin, indicating that it belongs to the *T. mentagrophytes* complex. The *M.canis* strains exhibited strong keratinolytic properties, with hair damage being more pronounced in the perforation test compared with the experiment with *T. benhamiae.* These results complement the findings of Čmoková A. (2020) and Ajello L. (1967), who described hair perforation and peg formation under the action of keratinase in *T. mentagrophytes* and *M. canis* [23, 24].

According to Li Q. (2021), complete keratin biodegradation requires an appropriate microorganism strain, properly optimized cultivation conditions, and a keratinase degradation system that includes a mixture of enzymes. Considering the influence of easily accessible carbon and energy sources, such as glucose and xylose, on the overall keratinolytic process, including proteolysis, sulfitolysis, and keratinolytic attack, the addition of glucose primarily stimulated protease and keratinase activities in the cultures of the three strains of *Trichophyton ajelloi* [25].

In addition to classic dermatophyte fungi, saprophytic fungi are used in studies aimed at identifying keratinase enzymes. For example, *S. Timorshina* et al. (2022) evaluated 32 strains of micromycetes belonging to the genera *Aspergillus, Chaetomium, Cladosporium, Paecilomyces, Penicillium,* and *Ulocladium* using three agarized media with various protein substrates: keratin, casein, and gelatin. On the basis of the study results, the following fungi of the genus Aspergillus demonstrated effective keratin hydrolysis: *Aspergillus amstelodami, Aspergillus chevalieri, Aspergillus clavatus, Aspergillus fischeri, Aspergillus ochraceus, Aspergillus sydowii,* and *Cladosporium sphaerospermum* [26].

Among various fungi exhibiting keratinolytic abilities, there also mentioned dermatophytes and related fungi from the Chrysosporium group [27]. In addition to these fungi, some authors [28] report that keratinolytic abilities are also manifested by ubiquitous mold fungi such as *Fusarium sp.* Among dermatophytes, anthropophilic species such as *Trichophyton rubrum,* zoophilic species such as *Trichophyton verrucosum,* and geophilic, including species such as *Trichophyton terrestre, T. georgie, T. ajelloi, Microsporum gypseum* and *M. fulvum* are distinguished [29].

Authors Yu R.J. et al. (1968) conducted an experiment on the isolation and purification of extracellular keratinase of *T. mentagrophytes*. In their research, they used horse hair and guinea pig hair. Cultivation of *T. mentagrophytes* was carried out in a keratin medium consisting of horsehair and a separate medium of guinea pig hair. According to the results of the experiment, keratinase enzyme were obtained using chromatography with a molecular weight of approximately 48,000 from horse hair. An experiment with guinea pig hair gave a negative result [30]

Giudice M.C. et al. (2012) conducted studies to evaluate the activity of extracellular proteolytic enzymes keratinase and elastase in the geophilic fungus *Microsporum gypseum* isolated from various soils in Brazil. According to the results of the experiment, the geophilic fungus *M. gypseum* showed low enzymatic activity, compared with isolates obtained from human and veterinary sources [31].

In addition to fungi, in industry uses microbial hydrolysis. Thus, in the publication R. Bhari et al. (2021), microorganisms of microbial origin were used for keratin hydrolysis. The bioconversion of these dreaded wastes into value-added protein hydrolysate using keratinolytic microbes is an effective way to recycle and manage hard-to-process keratin waste. There is a complete catalog of keratinolytic microbes (*Bacillus* sp. *Amycolatopsis* sp. и *Streptomyces* sp.), known to dissolve feather protein in a short time under mild conditions, and their number is constantly increasing [32].

Currently, pet owners turn to veterinary clinics, who treat the first signs of dermatomycosis pathogens, which subsequently leads to a latent form of the disease. Our research is aimed at allowing veterinarians and mycological laboratory workers to use keratin media to test pathogenicity, which will help in making a diagnosis and prescribing timely treatment.

Thus, we have identified keratinolytic activity in the fungal strains *T. benhamiae*: No. 19 and No. 20; and *M. canis:* No. 35 and No. 68 in relation to the hair of small domestic animals, and also conducted a hair perforation test, where hair destruction was visible, which indicates the pathogenicity of the fungal strains. The use of media containing ground animal hair and keratin hydrolysate allows for determining the pathogenicity of dermatophyte pathogens, and it has been proven that the fungi do not exhibit pleomorphism. This factor enables accurate identification of the pathogen and contributes to the correct diagnosis. The presence of pronounced keratinolytic properties in these fungal strains is evidence of their etiological role in the development of skin pathology and its derivatives, as identified in domestic cats and dogs.

Authors' Contributions

AS and TI: Concept development, design and planning of the study, data collection and analysis, critical review of the article and final approval, research, statistical analysis. TI: Conducted the final revision and proof reading of the manuscript. AS and TI: Conducted a comprehensive literature search

and conducting research. All the authors have read, reviewed and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Development of conventional designations for veterinary epidemic significant objects

Yersyn Y. Mukhanbetkaliyev[1], Aizada A. Mukhanbetkaliyeva[1], Asem Zh. Abenova[1], Maxat A. Berdikulov[2], Sarsenbay K. Abdrakhmanov[1]

[1]Faculty of Veterinary and Animal Husbandry Technology,
S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan,
[2]RSE to the REM «National Reference Center for Veterinary Medicine» Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan,
Astana, Kazakhstan

Corresponding author: Yersyn Y. Mukhanbetkaliyev: ersyn_1974@mail.ru
Co-authors: (1: AM) aizada.1970@mail.ru; (2: AA) asem.abenova.1993@mail.ru;
(3: MB) berdikulov.ma@mail.ru; (4: SA) s_abdrakhmanov@mail.ru
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Abstract

Background and Aim. Epidemiologically significant objects are objects related to veterinary activities, requiring strict control and supervision, since violation of sanitary and hygienic requirements imposed on them, deterioration of the epidemiological situation at these objects can contribute to the emergence and spread of diseases, which in turn will lead to large negative economic, environmental and social consequences. This research aims to develop conventional designations of objects that have epidemiological significance in the country for their subsequent use in cartographic analysis, forecasting, and risk assessment of the spread of infectious diseases.

Materials and Methods. Conventional forms were developed for almost all objects that may have any epidemiological significance. These facilities were relatively categorized into 4 areas: veterinary, agricultural, municipal, and other epidemically significant facilities. Conventional forms of designation of epidemically significant objects were developed using the CorelDraw program.

Results. In total, symbols have been developed for 48 epidemiologically significant objects, including 14 veterinary, 17 agricultural, 7 municipal, and 10 other objects. The shape of all symbols was defined as a quadrangle with equal sides, while each group of symbols has its color for the border of the figure. The design and drawing of each symbol were developed in such a way that both specialists and ordinary users could visually understand what this sign meant.

Conclusion. The developed symbols will allow more effective use of information and communication technologies for forecasting and assessing the risk of spreading the most relevant contagious diseases in our country. This will subsequently allow more effective preventive and anti-epizootic measures.

Key words: epidemiologically significant objects; epizootic situation; mapping; prevention; symbols.

Introduction

It is a well-known fact that in many socially significant zoonoses, one of the main factors that significantly influence the epizootic process of a particular nosological unit is epidemiologically significant veterinary objects. In this regard, the role of the areas of epizootiology and epidemiology that study potentially dangerous (epidemic significant) veterinary objects, as well as the characteristics and patterns of the spread and manifestation of infectious diseases in various natural and socioeconomic conditions, has increased to assess the epizootic risk and rationalize the systems of anti-epizootic

measures to reduce the risk of animals and the population becoming infected with socially significant infections [1, 2, 3]. Now, Kazakhstan is experiencing a complex epizootological and epidemiological situation for certain infectious diseases [4, 5, 6]. In recent years (2020-2024), the following diseases have been registered in the country among the most dangerous diseases of animals and humans: foot-and-mouth disease, anthrax, rabies, brucellosis, pasteurellosis, etc. In addition, isolated cases of soil and natural focal infections dangerous to humans and animals are registered annually in the Republic of Kazakhstan, such as tularemia, rabies, Aujeszky's disease, tuberculosis, listeriosis, sheep pox, leptospirosis, enterotoxaemia, etc. [7-10].

In this aspect, it is obvious that one of the factors that significantly influence the epizootic situation for socially significant zoonoses in a particular region of the country are epidemiologically significant veterinary facilities and their safety. Epidemiologically significant objects are veterinary objects (cattle burial grounds, Beccari pits, animal carcass disposal points and other biological waste, slaughterhouses, etc.) that require strict control and supervision, because failure to comply with sanitary and hygienic requirements imposed on them, deterioration of the epidemiological situation at these objects can contribute to the emergence and spread of diseases, which in turn will lead to major negative economic, environmental and social consequences [11]. In the established practice, the identification of epidemiologically significant objects is carried out taking into account communities and the main mechanisms of transmission of pathogens. Russian scientists propose classifying epidemiologically significant objects: agricultural, veterinary, municipal, and others [12]. Information on epidemiologically significant veterinary objects is one of the important parameters necessary for assessing and interpreting the manifestation of the epizootic process and planning anti-epizootic measures. Therefore, the definition and identification of epidemiologically significant veterinary objects will make it possible to compile a single register of data on such objects, with their characteristics and degree of potential danger. Based on the above, the purpose of this research is to develop conventional designations of objects that have epidemiological significance in the country for their subsequent use in cartographic analysis, forecasting, and risk assessment of the spread of infectious diseases.

Materials and Methods

The studies were conducted at the Faculty of Veterinary and Animal Husbandry Technology, S. Seifullin Kazakh Agrotechnical Research University, using the software of the laboratory of «Risk Analysis and Forecasting in Veterinary». Primary data on existing epidemically significant objects in the country were collected through expeditionary visits to the administrative districts of 17 regions. Conventional forms were developed for almost all objects that may have any epidemiological significance. Such objects are conventionally classified into 4 areas: veterinary epidemically significant objects (veterinary stations, clinics, pharmacies, laboratories, cattle burial grounds; including anthrax, Bekkari pits, biological enterprises, etc.); agricultural (industrial) epidemically significant objects (livestock enterprises, enterprises for the production of agricultural products, processing plants, places of animal slaughter: slaughterhouses, sites, etc.); municipal epidemically significant objects (landfills, transport hubs (railway and bus stations, airports), recycling plants, exhibitions, etc.) and other objects (zoos, nature reserves, game reserves, hunting farms, nurseries, etc.).

Conventional forms of designation of epidemically significant objects were developed using the CorelDraw program [13].

For this purpose, a platform for the developed conventional designation is created in the CorelDRAW program, the parameters of the drawing are specified (shape, size, resolution, color differences of the background, border, and sign symbolizing a certain object). In some cases, when creating the symbol, we used the default settings of the CorelDRAW program itself or data from the Internet, social networks, and other platforms. You can browse and search for default settings. The symbol for each epidemiologically significant object was developed so that both specialists and ordinary users in most cases could visually understand what this symbol denotes. When developing the signs, we took into account the nature of the activity of a particular object, previous experience in developing similar signs in various countries [14], special symbols, signs, drawings, and letters that visually inform and convey the meaning of this symbol.

Results

As a result of the conducted research, conditional values of 48 epidemiologically significant objects have been developed, including 14 veterinary, 17 agricultural, 7 municipal, and 10 other objects. To differentiate and quickly determine which group of objects a particular sign belongs to, each group of symbols has its color of the edging of the figure. The shape of all symbols was defined in the form of a quadrangle, with equal sides. The drawings inside the square reflect the characteristic features of each epidemiologically significant object.

The symbols of veterinary facilities are presented in the form of a quadrangle with a blue border (Figure 1). In total, 14 symbols have been developed for objects related to veterinary activities, including veterinary offices, clinics, pharmacies, laboratories, animal burial grounds, biothermal pits, etc.

Figure 1 – Symbol of veterinary epidemiologically significant facilities

Almost all designations of veterinary facilities are made on a white background, except for the designation of the animal burial ground, which is made on a brown background. This is done to distinguish between a cattle burial ground and an anthrax burial, which have the same pattern of identification but differ in the color of the pattern and the background substrate.

The symbols of agricultural objects also have the shape of a square but with a green border (Figure 2). All signs of this category are made on a white substrate. In total, 17 symbols have been developed for objects related to agricultural activities. This category includes livestock enterprises, enterprises for the production and processing of agricultural products, and places of slaughter of animals: slaughterhouses, sites, etc.

The symbols of livestock enterprises are indicated in the form of a graphic image of the type of animal to which a particular enterprise belongs. Enterprises producing and processing agricultural products are marked with stylized and graphic drawings of the corresponding products (cheese, milk, meat, canned food, etc.). The figures denoting various animal slaughter enterprises were left in the form of the same signs that were adopted in the veterinary service on the territory of the former Soviet Union.

Figure 2 – Symbol of agricultural epidemiologically significant objects

Communal facilities were designated in the form of a quadrangle with a yellow border (Figure 3).

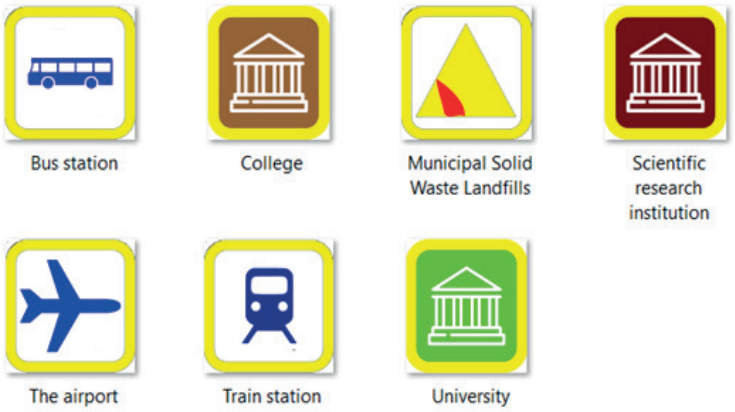

Figure 3 – Symbol of municipal epidemiologically significant facilities

At this stage, 7 conditional signs have been developed, but in the future, their number will be increased with the addition of new objects that will have epidemic significance. The symbols have been developed for the main transport hubs (railway station, bus station, airport), which are available in cities and large settlements and can have a significant impact on the epidemiological situation in the region for certain infections. A sign has also been defined to designate a solid waste landfill in the form of a stylized yellow triangle denoting waste recycling and covering the lower left part of the triangle with a stylized red flame denoting disposal. Signs of educational and scientific institutions (university, college, research organization) related to veterinary medicine, which can also affect the epidemiological situation, are designated in a single style, as a graphic representation of the facade of an ancient building, which in most cases is associated with an educational and scientific institution. At the same time, the sign of the university is full of green, the sign of the college on brown, and the sign of the research organization is on a dark burgundy background.

The symbols of other epidemiologically significant objects are also presented in the form of a quadrangle with a red border (Figure 4). At this stage, 10 symbols have been developed, but in practice, there may undoubtedly be more, so new signs for objects of this category will be added in the future. At this stage, signs have been developed for such facilities as zoos, nature reserves, hunting farms, nurseries, places for animal exhibitions, etc.

All designations of other epidemiologically significant objects are made on a white background, in the form of a graphic image or a stylized drawing denoting the activities of a particular enterprise or organization.

Figure 4 – Symbol of other epidemiologically significant objects

Discussion and Conclusion

Current areas of veterinary science include research on predicting the occurrence and spread of socially significant and hazardous infectious animal diseases. Often, epizootics of such infections pose a real threat to the health and life of the population and also lead to huge economic losses in agriculture.

The territory of Kazakhstan has historically been considered unfavorable for many diseases of infectious etiology common to humans and animals. And if some nosological forms have a natural focal character, then other diseases are anthropological, that is, the development of the epizootic process of such diseases depends directly on the activity of a person [4, 5, 15, 16]. In this regard, objects that a person uses in animal husbandry, in the processing of livestock products, may potentially turn out to be the link where the causative agent of the disease can be transmitted directly or through transmission factors to susceptible animals [2, 8, 17].

The practice of using symbols for veterinary facilities of epidemic importance was used earlier in the USSR and Russia [14]. To compile an epizootic map of a certain administrative unit (region, district), conventional signs of diseases of infectious etiology and veterinary facilities with epidemic significance were developed. But these signs are outdated and inapplicable in modern computer-analytical programs.

The development of such symbols requires their formativeness and the possibility of their application on various platforms (in particular, in ArcGIS) for their visualization, identification, and systematization.

The conducted research allowed us to develop 48 conditional definitions of epidemiologically significant facilities, including 14 veterinary, 17 agricultural, 7 municipal, and 10 other facilities. Primarily, symbols were developed for those objects that can have the greatest impact on the epizootic process of a particular disease of infectious etiology. Undoubtedly, this work will continue and in connection with the development and diversification of the livestock industry, the changing market conditions of livestock products, and new facilities will appear for which symbols will be developed.

In practice, knowledge of the location and epidemiological characteristics of such facilities will allow the veterinary service of the subject and administrative districts to control and rationalize the program of animal husbandry improvement, to optimally distribute the means and efforts of veterinary services to prevent the introduction and spread of particularly dangerous animal diseases into the territory of the region, district or locality.

Veterinary facilities of epidemiological importance require strict accounting and control over their sanitary and epidemiological condition. Since such objects constantly carry a potential threat of the emergence and spread of diseases of infectious etiology, dangerous for both animals and humans. The developed symbols are classified into four categories of objects: veterinary, agricultural, communal, and others.

These symbols will make it possible to use information and communication technologies more effectively to predict and assess the risk of the spread of the most relevant diseases of infectious etiology for our country, which in the future will allow for more effective preventive and anti-epizootic measures.

Authors' Contributions

YM and SA developed the concept and design of the study. AM and AA conducted a comprehensive literature search, analyzed the collected data, and drafted the manuscript. SA and MB: Performed final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

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Assessment of genetic diversity using microsatellite markers and milk productivity of Mugalzhar horses

Shinara N. Kassymbekova[1], Kairat Zh. Iskhan[2], Serikbay S. Rzabaev[3], Zhanat Zh. Bimenova[1], Dinara I. Kabylbekova[1], Spandiyar A. Tursunkulov[2,3]

[1]Department of obstetrics, surgery and reproductive biotechnology, Faculty of Veterinary, Kazakh National Agrarian Research University, Almaty, Kazakhstan.
[1] N.O. Bazanova Department of Physiology, Morphology and Biochemistry, Faculty of Veterinary, Kazakh National Agrarian Research University, Almaty, Kazakhstan
[2]Head of the Horse Breeding Department of Aktobe Agricultural Academy, Honorary Chairman of the Republican Chamber of Horse Breeds of Productive and Universal Directions
[2,3]Department of Veterinary medicine, S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan

Corresponding author: Kassymbekova Sh. Nikolaevna: kasymbekova-s@mail.ru
Co-authors: (1: KI) Kayrat_ishan@mail.ru; (2:SR) rzabaev@mail.ru;
(3: ZhB) zhan_225@mail.ru (4:DK) dna_03@bk.ru; (5: ST) 8104@bk.ru
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Abstract

Background and Aim. At "Chromtau Beef" LLP in Aktobe region, Mugalzhar horse breed is primarily used for meat and milk production. This largely depends on improving breeding techniques through introduction genetic achievements in farm and identifying and realizing genetic potential for productivity and breeding quality in production. The purpose this research was to study the zootechnical and genetic characteristics of modern population horses Mugalzhar breed using DNA markers.

Materials and Methods. In this study, we studied polymorphism microsatellite markers to assess genetic differentiation sample Mugalzhar horses. Data on 17 microsatellite loci 20 heads horses from Aktobe population were used. The results were compared using GenAlEx 6.503 program: FST was calculated using matrix distances between alleles; according to matrix distances between genotypes.

Results. The study zootechnical characteristics revealed that horses did not differ from breed standard in measurements. The milk production linebred mares varied considerably during 105 days lactation (average milk production Bekzat, Bau and Paluantora mares was 1701.0, 1492.05 and 1431.15 litres, respectively). Higher productivity was observed at 2-3 months lactation, after which milk yields gradually decreased and more sharply towards end lactation. Estimates genetic differentiation breed were obtained: FST = 0.028, matrix distances between genotypes = 0.015. A high correlation of FST and GD with Nei polocus differentiation scores was found (100). The inbreeding coefficient Fis for studied loci was found to have negative value (-0.014), which shows predominance heterozygous genotypes in Mugalzhar breed horse population.

Conclusion. The coefficients variability (Cv) for both size and live weight are low, indicating uniformity horses. Our research has shown that Mugalzhar mares from different lines have different levels milk production. Simulation Mugalzhar breed with GD close to zero resulted in increase in number alleles per locus by 40%. It is advisable to use results obtained in development breeding measures to preserve diversity of breed.

Key words: DNA marker; horse breeding; Mugalzhar horse breed; selection; STR.

Introduction

Horse breeding in Kazakhstan has always held a special place among other branches of animal husbandry. Productive horse breeding is essential for the population of the Aktobe region, where three local breeds are raised: Kushum, Mugalzhar, and Kazakh horse Zhabe type [1]. All three breeds are highly valued for their adaptability to the steppe, desert, and semi-desert conditions of Kazakhstan. These hardy breeds have excellent meat and dairy qualities [1].

At the LLP "Chromtau Beef" in the Aktobe region, the Mugalzhar breed is primarily used for meat and milk production. To increase productivity, it is necessary to constantly improve these horses. This largely depends on enhancing breeding techniques through the introduction of genetic achievements in the farm and identifying and realizing the genetic potential for productivity and breeding quality in production [2]. DNA analysis using microsatellite markers has been widely used in animal species to identify individuals, verify paternity, and preserve endangered animals. It has also been used to study the genetic diversity and origin of local animals and promote their conservation [2-3].

Countries around the world have used microsatellite markers since the mid-1990s to track the origin and hereditary characteristics of local animals. This information is important for improving production on farms and promoting the conservation of these animals [4, 5].

An important aspect of increasing production on farms is improving the reproduction of horses. In 2020-2023, the output of foals per 100 mares was 79-81 heads.

In the future, "Chromtau Beef" LLP plans to increase the number of Mugalzhar horses and improve their quality through targeted breeding work.

The dietary value, nutritional benefits of horse meat and koumiss compared to other types of agricultural products, the high profitability of their production, and the low cost contribute to an increase in production of these valuable food products in Kazakhstan [6].

Mares of different types and lines have different levels of productivity, both in terms of quantity and quality, under the same conditions of feeding and care. A comparative study of their economically useful characteristics has contributed to the selection of mares suitable for specific conditions, opening up additional opportunities for increasing dairy productivity [1-3].

The aim of these studies was to investigate the zootechnical and genetic characteristics of modern livestock from the domestic horse population of the Mugalzhar breed, using DNA markers. This allowed us to identify polymorphisms, as well as identify typical alleles present in representatives of this breed, in order to select a sample of horses for future genome-wide research.

Materials and Methods

Scientific and economic experiments were conducted at the seasonal kumis farm of "Chromtau Beef" LLP in the Aktobe region in 2023.

There were three groups of mares involved in the experiment, each with 5 members. Each mare was measured and weighed, and four measurements were taken: height at the withers, oblique body length, chest circumference, and metacarpal circumference [7].

Milking was done manually, five times a day with breaks of 2-2.5 hours between each milking. Mares were only milked during the day and kept together with their foals at night in the pasture. Commercial milk production was determined during lactation using the method of controlling milk yields twice a month for two days.

The milk productivity was calculated by taking into account the milk sucked by the foal at night, according to the formula proposed by I.A. Saigin [8]:

$$Yc = Yd / t \times 24, \quad (1)$$

where, Yc – estimated amount of milk in 24 hours, kg; Yd – the actual amount of milk received per day, kg; t – the time period from the beginning to the end of milking mares during the day, hours; “24” – hours per day

All experimental data were analyzed using biometric methods according to N.A. Plokhinsky [9].

Microsatellite markers and DNA genotyping data from 20 Mugalzhar horse heads were used for scientific research. The STR - genotyping process was carried out in the Genetics laboratory of the Kostanay Regional University named after Akhmet Baitursynov, using the ABI 3100 genetic analyzer from Applied Biosystems. Genotyping was performed at 17 DNA loci, including VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB23, ASB2, HTG10, HTG7, HMS3, HMS2, ASB17, LEX3, and HMS1.

PCR products were analyzed using an automatic gene analyzer (ABI 3100 Genetic Analyzer, USA), and subsequent electrophoresis was conducted on POP 7 polymers (Applied Biosystems, USA). Based on the data from a series of peaks, the sizes of alleles (in base pairs) were determined for each marker, using the results of an equine test conducted by the International Society of Animal Genetics (ISAG), and the GeneMapper software from Applied Biosystems (USA). The study of the genetic characteristics of the Mugalzhar horse breed was conducted using population-genetic analysis based on the frequency of types and alleles for 17 microsatellite loci. The level of polymorphism, effective number of alleles at each locus, and subpopulation inbreeding coefficient (Fis) were calculated. Allele frequencies and number of alleles per locus were estimated through direct calculation based on observed genotypes. Observed heterozygosity (Hobs), expected heterozygosity (He), number and frequency of allelic variants, and polymorphism information content (PIC) values for the breed were also calculated using GenAlEx 6.503 [10].

Results

In "Chromtau Beef" LLP, foaling of mares took place in mid-March and early April. Milking of the mares began in early May, which is a month after foaling.

For a complete zootechnical characterization of the development and body type of the mare, measurements and weights were taken. The data from these measurements and live weights are presented in Table 1.

Table 1- Measurements and live weight of mares from different lines

Indicators	Measurement parameters, centimetre (cm)				Live weight, kg
	Height at the withers	Oblique body length	Chest circumference	Metacarpal circumference	
Paluantora line 136-91					
M+m	144.6±0.37	151.8±0.43	184.3±0.68	19.3±0.12	493.5±2.36
lim	143-145	149-152	183-185	19-20	470-500
σ	0.84	1.12	1.58	0.27	7.85
C_v	0.58	0.74	0.86	1.40	1.59
Bau line 208-96					
M+m	145.2±0.40	152.5±0.49	183.8±0.71	19.0±0.10	490.2±2.12
lim	144-146	148-152	181-184	18.5-19.5	460-495
σ	0.79	1.09	1.52	0.22	7.66
C_v	0.54	0.71	0.83	1.16	7.56
Bekzat line 187-91					
M+m	144.1±0,46	151.2±0.51	183.2±0.74	19.1±0.08	485.3±2.41
lim	143-145	148-152	180-183	19-20	450-490
σ	0.75	1.06	1.49	0.21	7.62
C_v	0.52	0.70	0.81	1.10	1.57

From the data in Table 1, we can see that the experimental mares, which were acquired from the Mamyr-Aktobe farm when they were 2 years old, and which had already reached the age of 5 at the farm of "Chromtau Beef" LLP, did not differ from their line representatives. Mares from the Bau 208-96 and Paluantora 136-91 lines exceeded the mares from the Bekzat 187-91 line in height by 1.1 and 0.5 cm at the withers and in oblique body length by 1.3 and 0.6 cm, respectively, and in chest circumference by 0.6 and 1.1 cm. In terms of live weight, the Paluantora mares exceeded the Bau and Bekzat mares by 3.5 and 8.2 kg.

The coefficients of variability (Cv) for both size and live weight are low, indicating the uniformity of the horses. Our research has shown that the Mugalzhar mares from different lines have different levels of milk production (Table 2).

Table 2 - Milk production of mares from different lines

Live weight, kg	Actual milk yield		Milk		
	yield per day	For 105 days of lactation	Per day	For 105 days of lactation	Per 100 kg of live weight
493.5	Paluantora line 136-91				
	5.68±0.15	596.4±4.92	13.63±0.31	1431.15.15 ±20.3	290
490.2	Bau Line 208-96				
	5.92±0,17	621.6±5.92	14.21±0.35	1492.05.05±22,6	304
485.3	Bekzat Line 187-91				
		708.7±4.50	16.20±0.43	1701.0±28.3	351

From the data in Table 2, we can see that for 105 days of lactation, the average milk production of mares from the Bekzat, Bau, and Paluantora lines was 1701.0, 1492.05, and 1431.15 liters, respectively.

The commercial milk yield from mares in each line was 708.7, 621.6, and 596.4 liters for the Bekzat, Bau, and Paluvantora lines, respectively. Thus, the Bekzat mares produced 14% more milk than the Bau mares and 19% more than the Paluvantora mares.

According to the milk production index (per 100 kg of live weight), the Bekzat and Paluvantora lines showed high performance, with 351 and 290 liters per 100 kg, respectively, while the Bau line was slightly lower at 304 liters.

It is worth noting that the milk production of linear mares varied significantly during 105 days of lactation. Higher productivity was observed at 2–3 months of lactation, after which milk yield gradually decreased and more sharply towards the end of lactation (Table 3).

Table 3- Change in milk production of mares of different lines s by months of lactation, liters

Milk production indicators	Lactation month			
	May II	June III	July IV	August V
Paluantora line 136-91				
Per day	14.40±0.31	15.17±.23	14.16±0.27	13.10±0.26
Per month	432.0±5.08	470.22±7.20	438.90±5.24	170.36±3.36
Bau Line 208-96				
Per day	13.78±0.26	14.45±0.32	13.63±0.27	12.53±0.29
Per month	413.28±7.74	447.90±4.93	422.60±4.98	162.86±2.07
Bekzat Line				
187-91 Per day	16.42±0.033	14.48±0.35	16.35±0.32	14.57±0.28
Per month	492.70±4.12	542.06±5.50	506.97±5.02	189.43±3.39

From the data in Table 3, we can see that the milk production of mares from the Bekzat, Bau, and Paluantora lines at the 2nd month of lactation was 492.7, 413.28, and 432 liters, respectively. At the 5th month, these values were 189.43, 170.36, and 262.86 liters.

This suggests that with seasonal milking and the selection of dairy rather than meat mares on farms producing koumiss, it is possible to increase milk productivity significantly.

To identify the genetic diversity and relationships among individuals in the Mugalzhar horse breed, 20 horses with the desired phenotype were genotyped using 17 microsatellite markers. Microsatellites are useful in identifying the paternity of animals [11-14] and studying the structure of populations [15-17]. They have been widely used in breeding control programs for cattle, pigs, horses, and dogs. In most countries, these controls are based on microsatellite typing, standardized through regular comparative tests conducted by the International Society for Animal Genetics (ISAG).

Horses of the Mugalzhar breed stand out for their high genetic diversity in microsatellite loci. When we analyzed 17 microsatellite loci in these horses, we found 122 different alleles, with the number of alleles per locus ranging from 9 (AHT4) to 12 (ASB17) (Table 4).

Table 4 - Genetic characteristics of horse polymorphism of the Mugalzhar breed based on 17 DNA microsatellites

Locus	N	Na	Ne	I	Ho	He	uHe	Fis
AHT4	20	9.000	5.594	1.915	0.800	0.821	0.842	0.026
AHT5	20	7.000	5.442	1.777	0.750	0.816	0.837	0.081
ASB17	20	12.000	6.897	2.163	0.900	0.855	0.877	-0.053
ASB2	20	9.000	5.594	1.892	0.850	0.821	0.842	-0.035
ASB23	20	7.000	4.188	1.651	0.600	0.761	0.781	0.212
CA425	20	7.000	2.909	1.330	JUST 0.700	0.656	0.673	-0.067
HMS1	20	6.000	2.712	1.286	0.500	0.631	0.647	0.208
HMS2	20	6.000	5.031	1.684	0.650	0.801	0.822	0.189
HMS3	20	6.000	3.865	1.512	0.800	0.741	0.760	-0.079
HMS6	20	7.000	4.848	1.733	0.900	0.794	0.814	-0.134
HMS7	20	5.000	2.974	1.297	0.750	0.664	0.681	-0.130
HTG10	20	6.000	2.532	1.141	0.600	0.605	0.621	0.008
HTG4	20	6.000	3.636	1.507	0.800	0.725	0.744	-0.103
HTG6	20	5.000	3.042	1.322	0.600	0.671	0.688	0.106
HTG7	20	6.000	3.636	1.450	0.750	0.725	0.744	-0.034
LEX3	20	9.000	6.061	1.982	0.500	0.835	0.856	0.401
VHL20	20	9.000	6.015	1.924	0.900	0.834	0.855	-0.079

N - sample size, Na- number of alleles, Ne - the number of effective alleles, I-information index, Ho - observed heterozygosity, He - expected and uHe -unbiased expected heterozygosity and Fis the coefficient of inbreeding.

The genetic structure of the Mugalzhar breed is characterized by a relatively high variability in the frequency of occurrence of alleles (Fig.1)

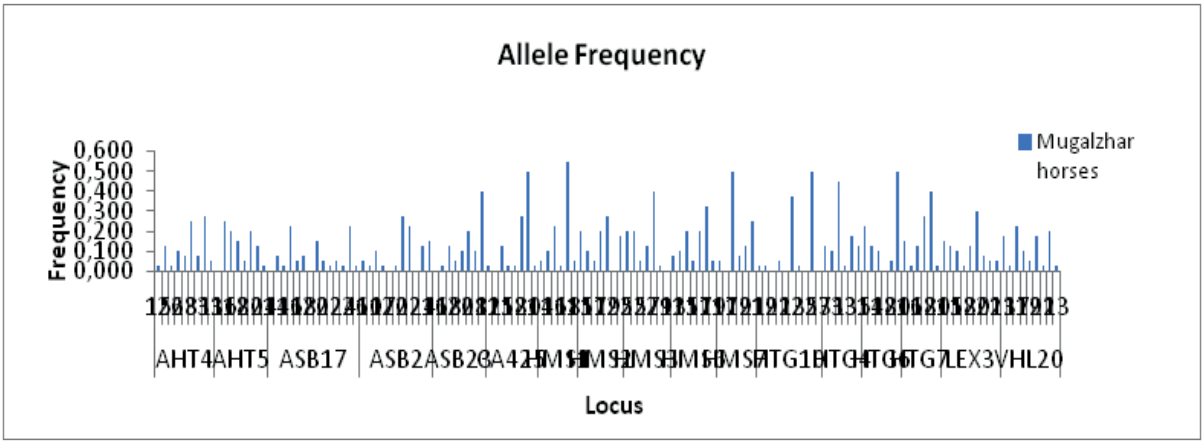

Figure 1- Allele frequencies for a population of Mugalzhar horses (n=20) with a plot by loci for codominant data

The average number of effective alleles across all studied loci, or the average level of polymorphism (Ne), is of interest in order to preserve genetic intra-breed diversity. In the Mugalzhar horse breed, the average number of effective alleles per locus was found to be 4,410. The polymorphism level of the tested loci ranged from 1,286 to 1,982, with the minimum and maximum observed heterozygosity values being 0.500 (HMS1) and 0.900 (ASB17) (Fig. 2). The average inbreeding coefficient (Fis) for the studied loci was found to have a negative value of -0.014, indicating the predominance of heterozygous genotypes within the population of Mugalzhar horses.

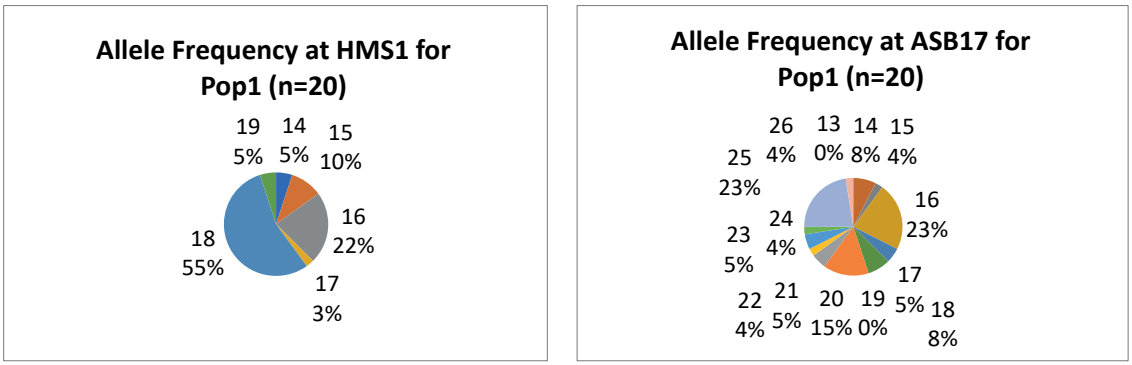

Figure 2- Frequency of alleles by population for HMS 1 and ASB 17

Discussion and Conclusion

122 alleles of STR loci were identified in the tested horses of the Mugalzhar breed, which indicates a significantly high level of polymorphism in microsatellite DNA. Within the sample, the observed heterozygosity (Ho) exceeds the expected heterozygosity (He), indicating the preservation of genetic diversity. The inbreeding coefficient (Fis) showed negative values, indicating a low level of inbreeding.

Other researchers [18] in the genetic monitoring of the Mugalzhar horse breed of two inbred types (Kozhamberdi and Kulandi), the allele pool of horses was investigated for 16 DNA microsatellites and from 5 to 13 alleles were identified. The data obtained characterised the polymorphism of each of the markers. The highest level of polymorphism in Mugalzhar horses was observed in the ASB17 locus, which also corresponds with our results.

Evaluation of selection and genetic parameters of the main economic and biological traits of horses of the Mugalzhar breed, gives the opportunity to obtain a complete productive and breed characteristic of the horse stock, both zootechnical and genotypic analysis by DNA-technology, which in modern conditions are included in the organisation of centralised breed registration.

Selection work in productive horse breeding is a continuous process. The practical significance of this work is to confirm the origin of animals by 17 STR loci and to identify unrelated individuals in the sample of horses of the Mugalzhar breed of the Aktobe population, which includes representatives of outstanding lines of this horse breed. Further, whole genome sequencing (WGS) of the obtained DNA samples will allow conducting research and searching for breed-specific SNP polymorphisms.

The Mugalzhar breed is characterized by a high level of genetic diversity, as evidenced by the high observed heterozygosity and low inbreeding values. This indicates that the breed has the potential for further improvement in terms of productivity and can be used as a source of genetic diversity for other breeds. Breeding should continue along the most promising lines, such as the Bekzat and Bau lines, to maximize the benefits of heterosis on a molecular genetic level. The data obtained will be used for future comparisons with the allele pool of other local breeds in order to update the genetic database.

Authors' Contributions

ShK, KI and SR: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. ZhB, DK and ST: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscrip.

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Determination of the residual content of veterinary drugs in raw materials of animal origin by enzyme immuno assay method

Maxat A. Berdikulov[1], Aigul S. Omarova[1], Assem B. Axeitova[1], Botagoz Zh. Aitkozhina[2], Dinara K. Zhanabayeva[2], Gulzhan N. Yessembekova[2]

[1]National Reference Center for Veterinary Medicine of Veterinary Control and Supervision Committee of the Ministry of Agriculture of the Republic of Kazakhstan, Astana, Kazakhstan
[2]S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan

Corresponding author: Maxat A. Berdikulov: berdikulov.ma@mail.ru
Co-authors: (1: AO) omarovaac@mail.ru; (2: AA) asya.astana@mail.ru; (3: BA) botagoz_aitkozhina@mail.ru; (4: DZh) dinara.kausar.berik@mail.ru; (5: GY) gulzhan_nk@mail.ru
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Abstract

Background and Aim. The article deals with the problem of the use of residual veterinary drugs in animal husbandry and poultry farming. In the modern world, the use of antibiotics in animal husbandry is a common problem. However, their use is often uncontrolled and can lead to contamination of food products of animal origin with medicines. Within the framework of this work, the contamination of raw materials of animal origin and food products sold on the territory of Kazakhstan has been studied.

Materials and Methods. The studies were conducted by enzyme-linked immunoassay (hereinafter referred to as ELISA) to determine the permissible levels of residual content of veterinary drugs. The studies took into account the content of residual amounts of pollutants such as chloramphenicol, sulfamethazine, nitrofuran 3-amino-5morpholinomethyl-2oxazolidinone (AMOZ), bacitracin in raw materials of animal origin: horse meat, pork, poultry, beef, lamb. The use of the ELISA method for the analysis of the content of antibiotics in meat allows for accuracy and reliability in determining the residues of veterinary drugs. This is necessary to comply with regulatory requirements and ensure the safety of meat products consumption.

Results. Our results show that the residual levels of antibiotics in animal raw materials are within the permissible limits. This is an important conclusion for food safety.

Conclusion. Based on the studies conducted in this work, monitoring of antibiotic residue in animal raw materials, it can be concluded that, their use in livestock and food production should be monitored continuously.

Keywords: chloramphenicol; ELISA; meat; nitrofuran; sulfamethazine.

Introduction

Among a number of substances that can contaminate food raw materials and foodstuffs, veterinary drugs used both for the treatment of animals and as growth stimulants occupy an important place. Antibiotics remain the most potent drugs used in veterinary medicine [1].

Antibacterial drugs are widely used in the cultivation of productive animals, so it is necessary to monitor their content in food at all stages of production. For these purposes, many laboratories use the method of immuno-enzyme analysis.

The term "antibiotics" covers a wide range of chemicals that are produced naturally, semi-synthetically and synthetically and are used to inhibit bacterial growth or destroy them [2].

It should be noted that antibiotics with a similar chemical structure have a similar antimicrobial spectrum [3].

The systematic intake of antibiotics into the human body with food is extremely harmful, most often they can cause various allergic reactions, dysbacteriosis, metabolic disorders, impaired kidney function, suppress the activity of certain enzymes, and inhibit the intestinal microflora.

The possibility of toxic, teratogenic and mutagenic effects is also not excluded [4].

Conjugates of antigens and antibodies with various proteins, synthetic polymers, enzymes and their substrates and cofactors are used in ELISA [5].

The method of enzyme immunoassay continues to develop. On the one hand, the range of research objects is expanding, and on the other, analysis methods are deepening and improving. This leads to simplification of the procedure, reduction of reagent consumption and reduction of analysis time. The development of the enzyme immunoassay method is also influenced by the chemistry of high-molecular compounds, cellular and genetic engineering, which changes the technology of obtaining reagents for this method. To determine antibiotics, a solid – phase enzyme immunoassay is used, based on the competition of a free antibiotic from the sample and an immobilized antibiotic in the solid phase during reaction with specific antibodies. After separating the unbound reagents, the quantity of antibodies interacting with the immobilized antigen is determined, using secondary antibodies labeled with peroxidase. The amount of secondary antibody conjugate bound to the antibodies is measured using a substrate and chromogen mixture. The antibiotic concentration in the sample is inversely proportional to the optical density measured from the enzymatic reaction product [6, 7].

To confirm the results obtained, an instrumental method of highperformance liquid chromatography with mass selective detection (HPLC MS/MS) is used.

According to the study of Arsene M.M.J and others (2021), antibiotics are the most important compounds in the field of veterinary medicine and animal husbandry. They are substances that can destroy or inhibit the growth of bacteria. Their use is almost inevitable in the treatment of bacterial infections in both animals and humans [8].

According to Arsène M.M.J. et al (2021), maintaining a waiting period and conducting physico-chemical tests are necessary to ensure that residue levels of antibiotics or their analogues do not exceed the acceptable limits (MRL) before food is released for sale. This measure is extremely important for public health, since antibiotic residues in food and the rise of antibiotic resistance are a serious problem [9].

According to Busch G. et al (2020), with the increasing use of antibiotics in agriculture, the focus is on their safety and effectiveness. This is of great importance as it aims to protect consumers from serious infections that can be transmitted to humans through contact with infected animals, consumption of contaminated food or spread in the environment [10].

On the territory of Kazakhstan, the residual content of antibacterial drugs in raw materials of animal origin is regulated by regulatory documents, such as:

Technical Regulations of the Customs Union "On food safety" (TR CU 021/2011) [11].

Technical regulations of the Customs Union (TR CU 034/2013) "On the safety of meat and meat products" [12].

Technical Regulations of the Eurasian Economic Union (EAEU TR 051/2021) "On the safety of poultry meat and processed products" [13].

"Unified sanitary-epidemiological and hygienic requirements for products (goods subject to sanitary-epidemiological supervision (control)" Approved by the Decision of the Commission of the Customs Union dated May 28, 2010 No. 299 [14].

The purpose of this work was to determine the content of the residual amount of antibiotics in food products of animal origin sold on the territory of the Republic of Kazakhstan.

The scientific novelty of the work consists in the fact that by the authors conducted monitoring studies to study the extent of distribution of raw materials and animal products containing residual amounts of antibiotics such as chloramphenicol, sulfamethazine, bacitracin, nitrofurans AMOZ.

Meat containing residual antibiotics should be sent to the manufacture of canned meat and vegetable meat, with the exception of canned food for baby food.

Materials and Methods

The study was conducted at the testing center of the National Veterinary Reference Center for Veterinary Control and the Supervision Committee of the Ministry of Agriculture of the Republic of

Kazakhstan (NVRC). This center is accredited in the accreditation system of the Republic of Kazakhstan in accordance with the requirements of GOST ISO/IEC 17025-2019 "General requirements for the competence of testing and calibration laboratories". The object of the study was animal samples received by the NVRC to determine the content of the residual amount of antibiotics in them. The study considered the residual content of pollutants such as chloramphenicol, sulfamethazine, nitrofuran (3-amino-5-morpholinomethyl-2-oxazolidinone, AMOZ), and bacitracin in animal-derived raw materials, including horse meat, pork, poultry, beef, and lamb.

Sample preparation and result processing were conducted according to standards MG–4.1.1912-04, MG 4.1.2158-07, MP No. KZ 06.03.00127-2021, and ST RK 2.638-2019.The determination of the residual content of pollutants in raw materials of animal origin was carried out in accordance with the methodology and instructions for use by the ELISA method, using such commercial test systems: "Algimed Techno" LLC, manufactured in the Republic of Belaarus; Elabscience Biotechnology Inc, manufactured in China; I'screen, Eurofins Technologies, manufactured in Hungary.

One of the main stages of the analysis can be distinguished: sample preparation and processing of the obtained results. For example, the sample preparation of bacitracin.

The progress of the study from sample preparation to calculation is reflected in Table №1.

Table 1- Bacitracin sample preparation

Sample preparation
Bacitracin
Meat samples without adipose tissue were crushed on a homogenizer, 1gram sample of homogenized sample was taken into a 15 ml centrifuge tube with a screw cap, 2.0 ml of 75% methanol solution was added and stirred on a vortex at maximum speed for 15 minutes. The test tube was centrifuged on a Thermo centrifuge at an acceleration of 4000 rpm at room temperature (20-25 °C) for 10 minutes. Aliquots of 100 µl of the supernatant were taken with a dispenser and transferred to 2ml test tubes. In test tubes with aliquots of the supernatant, we add 400 µl buffer to dilute the samples mixed on a vortex for 1 minute. For the analysis, 100 µl were used for each well of the plate.
Preparation of a microplate photometer (according to the operating instructions)
Application of standards and test samples. In two parallel wells of the microtitration plate, we dispense 100-µl of each standards in ascending concentration order. In corresponding wells, we place parallel samples of each test sample
Incubation of the Plate. Cover the plate with a film, incubate it at 20-25 °C for 60 minutes, then remove the film and quickly invert the plate to discard the liquid.
Rinsing the plate uncover the sealer carefully remove the liquid in each well. Immediately add 250µl wash buffer to each well and wash. Repeat wash procedure for 3 times, 30s intervals/time. Invert the plate and pat it against think absorbent paper.
Adding a substrate Solution for staining. Immediately after washing, add 100 µl of TMB substrate solution to each well and mix the contents with gentle circular motions on the plate surface.
Subsequent incubation. Cover the plate with a film, incubator it at 20-25 °C for 20minutes in a dark place
Completion of the staining reaction. Immediately after the end of the incubation time, 100 µl of stop reagent is added to each well to stop the enzymatic reaction

Continuation of table 1

OD Measurement: determine the optical density (OD value) of each well at 450 nm with a microplate reader. This step should be finished in 10 min after stop reaction.

Processing measurement results

Kit specification

Detection limit: 9.00 ppb.
Dilution ratio: 15, Cross–reaction rate - bacitracin-100%, zinc bacitracin -100%

Calculation

The result of measuring the optical density is expressed as a percentage of the optical density of the well with a zero standard (% absorption) according to the formula:

Absorption (%) = B/B0*100%

B – Average absorbance of standard or sample;

B0 – Average absorbance of 0 ppb Standard. Calibration curves are plotted on semi-logarithmic paper based on the relative absorption values calculated for standard solutions and the corresponding known values of antibiotic concentrations of mcg/kg (mcg/l), which should be linear in the main data range.

The concentration of the antibiotic in the studied samples is determined by the calibration curve of the measured relative optical density, respectively, Fig. 1,2.

Results

Study results. In 2023, 4,286 samples, of which beef meat - 2,109, horse meat -372, lamb-804, poultry - 498, pork - 503, were analyzed. Of these, 837 samples were examined in Astana, 850 samples in Akmola region, 662 samples in Pavlodar region, 581 samples in Karaganda region, 671 samples in North Kazakhstan region, and 685 samples in Kostanay region. Based on the results of laboratory studies within the framework of monitoring studies "Veterinary measures and food safety".

In Kazakhstan, the residual content of veterinary drugs in animal derived raw materials is regulated by the technical regulations "On Food Safety" (TR CU 021/2011) and "On the Safety of Meat and Meat Products" (TR CU 034/2013). The permissible level and limit of detection of veterinary drugs in raw materials of animal origin by the ELISA method are shown in Table No. 2.

Table 2 - Permissible level and limit of detection of veterinary drugs in raw materials of animal origin by the ELISA method

The studied samples	Name of the study	Permissible the level of antibiotics, mg/kg according to TR CU 021/2011, 034/2013	Limit of detection of veterinary drugs
Meat	Bacitracin	not allowed (< 0,02 mg/kg)	9 ppb
	Chloramphenicol	not allowed (< 0,0003 mg/kg)	0,0125 ppb
	Nitrofuran AMOZ	not allowed < 0,1 mg/kg	0,05 ppb
	Sulfamethazine	< 0,1 mg/kg	0,5 ppb

Table 2 presents the detection limits for bacitracin, chloramphenicol, nitrofuran, and sulfamethazine in animal-derived raw materials. In accordance with the requirements of TR CU 021/2011, 034/2013, the permissible level of antibiotics in meat is strictly regulated. According to the regulations TR CU 021/2011 and 034/2013, the content of bacitracin in meat is not allowed above the permissible level. The ELISA method allows the detection of bacitracin at a detection limit of 9 ppb (parts per billion). The maximum permissible level of bacitracin in meat is less than 0.02 mg/kg.

In accordance with regulations, the content of chloramphenicol in meat is not allowed < 0,0003 mg/kg. The ELISA method has a detection limit of 0.0125 ppb for chloramphenicol. The presence of nitrofurans in meat is not allowed < 0,1 mg/kg. The ELISA method can detect AMOZ with a limit of 0.05 ppb. The allowable level of sulfamethazine in meat is below 0.1 mg/kg. The detection limit of this substance by the ELISA method is 0.5 ppb.

Discussion and Conclusion

In a comparative aspect, the sensitivity of enzyme immunoassay methods to determine the presence of antibiotics has been studied. The calibration curve for bacitracin is shown in Figure 1. The results of the optical density of graduated solutions for bacitracin are shown in Table -3.

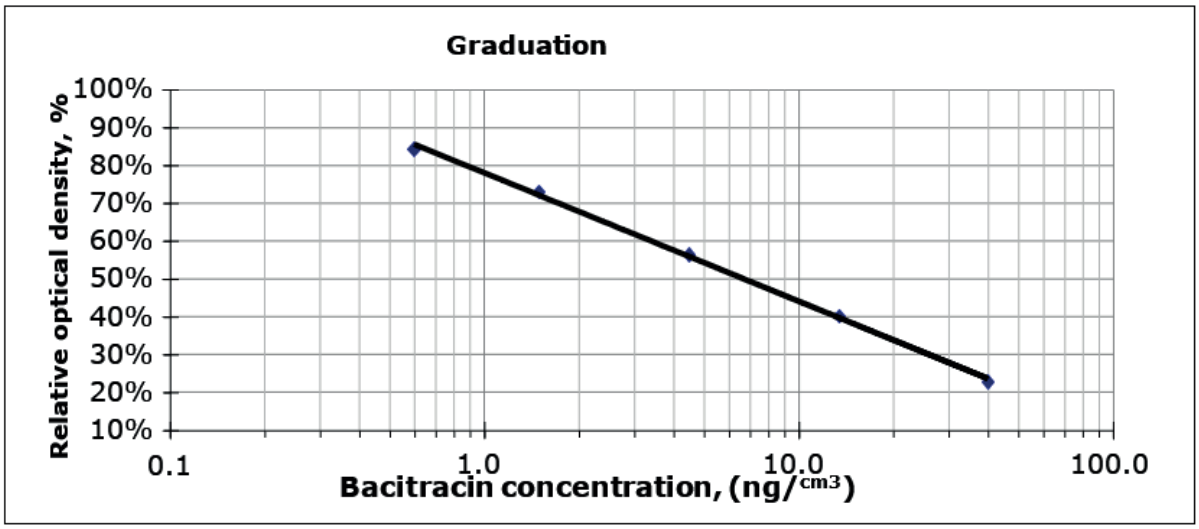

Figure 1- Calibration curve for bacitracin

Table 3 - Optical density of graduated solutions for bacitracin

Std. (ng/ml)	O.D.1	O.D.2	Bi	C.V. (%)	B/Bo (%)
0	2.797	2.913	2.855	2.86%	100.00
0.6	2.377	2.422	2.399	1.34%	84.04
1.5	2.037	2.156	2.097	4.01%	73.44
4.5	1.668	1.683	1.676	0.63%	58.69
13.5	1.075	1.073	1.074	0.13%	37.62
40.0	0.649	0.699	0.674	5.27%	23.61

As can be seen from Table 3, concentration and optical density as the concentration of bacitracin increases, there is a decrease in optical density (O.D.), which indicates an inverse relationship between the concentration and the measured optical density. The coefficients of variation for each standard are within acceptable values, which indicates good repeatability of measurements. B/Bo (%) shows how the optical density of each standard correlates with the density of the zero standard, which is key for constructing a calibration curve.

These data can be used to construct a calibration curve that will accurately determine the concentrations of bacitracin in samples with unknown concentrations. An accurate and repeatable methodology for measuring optical density using ELISA ensures reliable determination of bacitracin residues in animal raw materials.

Table 4 - Average content of antibacterial drugs in meat

The studied samples	Antibiotic	The permissible level of antibiotics, mg/kg according to TR CU 021/2011, 034/2013	The average content in all samples, mg/kg
Beef	NitrofuranAMOZ	< 0.1 mg/kg	< 0.0000115
	Chloramphenicol	< 0.0003mg/kg	< 0.0000125
	Bacitracin	< 0.02 mg/kg	0.0014
	Sulfamethazine	< 0.1 mg/kg	< 0.0005
Horse meat	Nitrofuran AMOZ	<0.1 mg/kg	< 0.00005
	Bacitracin	<0.02 mg/kg	< 0.009
	Chloramphenicol	< 0.0003 mg/kg	< 0.000075
	Sulfamethazine	<0.1 mg/kg	< 0.0005
Lamb	Nitrofuran AMOZ	< 0.1 mg/kg	< 0.00005
	Chloramphenicol	<0.0003mg/kg	< 0.000013
	Bacitracin	<0.02 mg/kg	0.0029
	Sulfamethazine	<0.1 mg/kg	0.0005
Pork	Nitrofuran AMOZ	< 0.1 mg/kg	0.00005
	Chloramphenicol	<0.0003mg/kg	< 0.000056
	Sulfamethazine	<0.1 mg/kg	0.0005
	Bacitracin	<0.02 mg/kg	< 0.009
Poultry	Chloramphenicol	<0.0003mg/kg	0.000013
	Nitrofuran AMOZ	< 0.1 mg/kg	0.00005

It can be seen from Table 4 that the studied samples of the residual content of antibacterial drugs are contained within the permissible limits. In the beef samples studied, the average content of all antibiotics except bacitracin is significantly below permissible levels. Bacitracin is present in amounts not exceeding acceptable levels.

In horse meat samples, the average content of all studied antibiotics is significantly below permissible levels. The average content of all antibiotics in lamb samples is below permissible levels, and some antibiotics are present in trace amounts. The average content of nitrofuran AMOZ in pork is below permissible levels, but they are present in quantities close to the detection limit.

In the studied poultry samples, all the studied antibiotics are present in trace amounts, significantly below permissible levels. The average content of antibiotics in meat samples of various animal species is within the permissible levels established by regulatory acts. These results indicate compliance with veterinary standards and a safe level of residual antibiotics in meat.

Authors' Contributions

AB and MM: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. ShG, EY and AH: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

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Improvement of direct microscopy method for differential diagnosis of skin mycoses

Gulshat E. Bailina[1], Aziza E. Nesipbaeva[2], Elena V. Kukhar[3]

[1]Department of Veterinary medicine, Faculty of Veterinary and Livestock Technology, S.Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan
[2]Department of Microbiology and Biotechnology, Faculty of Veterinary and Livestock Technology, S.Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan
[3]Research platform of agricultural biotechnology, S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan

Corresponding author: Gulshat E. Bailina: gulshat_010@mail.ru
Co-authors: (1: AN) nessipbayeva2510@mail.ru; (2: EK) kucharev@mail.ru
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Abstract

Background and Aim.In the practice of veterinary doctors, a number of methods for diagnosing dermatomycoses are used, methods of direct and luminescent microscopy are undeservedly ignored. Comparative analysis of staining methods for improving the method of direct microscopy, rapid and reliable detection of spores and mycelium in biomaterial, identification of pathogens and differential diagnosis of skin mycoses is of crucial importance for the effective treatment of animals

Materials and Methods. 47 clinical samples of wool and skin taken from farm animals were examined for dermatomycetes. Diagnosis was performed using direct microscopy, fluorescence microscopy with calcofluor, and culture medium culture Saburo.

Results. During the study of 47 samples of biological material, it was found that during microscopy the pathogen was detected in 10 cases (21.3%), during KOH microscopy - in 15 cases (29.9%), during KOH microscopy with other dyes in 11 cases (23.4%), during KOH microscopy with color calcofluor in 25 cases (53.2%). The efficiency of the KOH microscopy method with white calcofluor in comparison with direct microscopy was 60% higher, in comparison with KOH microscopy with other dyes - by 56%, classical KOH microscopy by 43.8%.

By the culture method, pathogens were detected in 28 cases (59.6%), of which 7 *Trichophyton spp.,* 1 *Microsporum spp.,* 14 *Aspergillius spp.,* 6 yeast. Growth was absent in 19 samples (40.4%), bacteria were detected in 3 samples (6.3%). In comparison with KOH microscopy with calcofluor white, positive results were confirmed in 89.3%.

Conclusion. The use of coloring with various dyes of morphological elements of micromycetes in pure cultures allows you to identify mycelial hyphae of various thicknesses, spores, conidia, other morphological structures and carry out a quick preliminary identification of dermatomycetes and opportunistic mold fungi in smears.

Keywords: dermatomycete; dye; macroconidia; microscopy; mycelium; spore.

Introduction

Early diagnosis of infections caused by classic dermatophytes or opportunistic micromycetes is critical for prompt, effective treatment. Diagnostic problems have been evident since these infections were first identified. Routine clinical assays often yield nothing. Developing specific serological and biochemical tests has been attempted for years, but progress has been slow. The lack of early diagnosis is associated with problems in treatment, since the inability to make an accurate diagnosis does not allow forchoosing the right strategy and tactics fortreatment measures. In addition, the clinical manifestations of skin mycoses are diverse (inflammation, allergic reaction) and depend on the animal'sbody, which makes it very difficult to diagnose diseases [1, 2, 3].

Laboratory results that allow the pathogen to be detecteddirectly in the diagnostic material are decisive in diagnosing mycoses. Early laboratory diagnostics involves using methods that include tests with a Wood lamp, direct microscopy with alkali, inoculation of clinical material on nutrient media of various compositions, and microscopic examination [4].

Fungal microscopy is a mandatory procedure for making a preliminary diagnosis, while obtaining positive microscopy results that confirmthe presence of infection regardless of the results of other diagnostic methods [5]. Usually, in mycological diagnostics, primary microscopy is carried out, which is an examination option when the material is obtained directly from the patient, or secondary microscopy is an examination of the culture of the pathogen obtained from a pre-taken sample [6].

Primary microscopy involves the use of colored and unpainted drugs. Direct microscopy is a valuable skill in treating skin infections and infestations; however, it is underusedin dermatology clinics. The clinical sample collection process is simple and not invasive. The samples can then be processed quickly and examined in the clinic; if properly configured, the result is available in minutes. In the context of superficial mycoses, direct microscopy provides important information about infection that other methods cannot provide. The presence of *Candida* and *Malassezia* hyphal forms, for example, can confirm active infection rather than colonization, information that cannot be obtained by culture or molecular methods [7].

Light and fluorescence microscopy are most often used to diagnose fungal infections. Fluorescence was first discovered by Frederick W. Herschel in 1845, who noted that the excitation of a quinine solution with UV rays resulted in blue emission. Later, George H. Stokes, who coined the term "fluorescence,"noted that the wavelength of the original UV radiation is shorter than that of fluorescence. The first attempts to use fluorophores to stain tissue, bacteria, and other pathogens in research were made in the early 1900s [8].

Potassium hydroxide solution, fluorescent dyes (calcofluor white, acridine orange), lactophenol cotton blue, Gram stain, etc. are used to conduct microscopy of test samples for the presence of fungi [9]. Direct microscopy with potassium hydroxide solution allows the detection and identification of fungal elements in the sample. Thus, according to the study by N. Subathra, N. Bharathi Santhose (2017), who used microscopy with KOH to diagnose onychomycosis, the method's sensitivitywas approximately 51%, which was not inferior to the culture method. To assess the diagnostic efficiency of two different microscopic methods in detecting fungal growth in affected nails with onychomycosis, *Subathra N.* et al. (2017) used the classical microscopy method with a 40% KOH solution and a modified microscopy method with 36% DMSO. The sensitivity of both methods was 50.6% and 48.2%, respectively [10]. Aroop Mohanty et al. (2021) reported that in diagnosingmucormycosis, the sensitivity and specificity of KOH microscopy are 64% and 91%, respectively [11].

Despite the venerable age of the direct microscopy method and its well-deserved advantages (simplicity, ease of implementation, etc.), it is constantly being improved. Additional use of various dyes is proposed to increase the reliability and sensitivity of this method. Schiff'sreagent is widely used as a broad-spectrum dye for detecting fungi [12]. Broadwater Devin R. et al. (2022), using a modified method of staining with the Schiff reagent, recognized this method as the most optimal for identifying fungi and not taking much time during analysis. The Schiff reagent stains the cell walls of Candida spp., Malassezia fungi, namely, polysaccharide complexes [13].

Using lactophenol blue,Vacharavel Shamly et al. (2014) noted its ability to stain thin hyphae.This finding allowed the authors to identify pathogensmore easilyduring microscopic examination [14].

Megha Tandon (2023) recognized PAS staining as a simple and rapid alternative to cultivation. As a result of the studies, the method's sensitivitywas 76.2%, and the specificity was 42.9% [15].

When comparing the staining results with rose Bengal and lactophenol blue during rapid identification of pathogens from chili leaves, I. Barman et al. (2018) found spores of "C. assamicum," "C. annuum,"and "C. Frutescen." The authors concluded that lactophenol-trypan blue is more suitable for staining spores of irregular rod shape and spores of spherical shape, pink Bengal dye [16].

Recently, the introduction of direct wool microscopy with the addition of white calcofluor luminescent substance has intensified in veterinary mycological laboratories [4].

Calcofluor is a fluorochrome dye withanaffinity for chitin and cellulose. Its peculiarity is that when a drop of dye is added to a preparation with KOH, it is absorbed by parts of the fungus [17]. The combined use of white calcofluor with potassium hydroxide (KOH) solution makes detecting both young and mature fungus hyphae possible, which increases fungal infection's detectabilityby 10% compared to the standard KOH method [18].

The use of white calcofluor dye to identify fungal pathogens showed a high sensitivity of the method. For example, according to Vishal Punjabic et al. (2020), 85% and 60% of cases, were positive when comparing the staining performance of the sensitivity of calcofluor white with acridine orange. Calcofluor white also showed a high predictive value of a positive result (63%), in contrast to acridine orange (57.1%) [19].

In the diagnosis of fungal keratitis, Dalia Moemen et al. (2019) calcofluor white showed high sensitivity and specificity (99.44% and 90.91%, respectively), not inferior to the dye methylene blue in these indicators (92.31% and 80%, respectively) [20].

Schottelius J. (2000) developed combined staining of fungal spores with calcofluor white with trichrome blue and subsequent incubation with methylene blue. Staining made it possible to distinguish microsporidium spores, spotted, bright white under the influence of calcofluor, while Candida spores had a reddish-violet color [21].

Our research aims to compare staining methods to improve the direct microscopy method, rapid and reliable detection of spores and mycelium in biomaterial, identification of pathogens, and differential diagnosis of skin mycoses.

Materials and Methods

The object of the study is a biomaterial taken from farm animals with suspected dermatomycoses in the amount of 47 samples. Biological material (hair andskin scales) was taken from the periphery of the lesions [22] andanalyzed according to the requirements for microscopic studies on mushrooms [7].

Dyes used: lactophenol cotton blue, Romanovsky dye, Giemsa reagent, Schiff reagent, methylene blue, Bengal pink, white calcofluor, andfuchsine.

In order toobtain a 10% potassium hydroxide solution, 10 g of caustic potassium wasweighed, and 50 ml of distilled water was added. The mixture was stirred until the substance was completely dissolved and the solution volume was100 ml.

Bengal pink dye was prepared by diluting 0.25 g of rose Bengal powder per 100 ml of water.

To obtain 0.1% white calcofluor dye, 0.1 g of powder was dissolved in 100 ml of distilled water under low heat [16].

KOH microscopy was performed as follows: skin scrapings and hair were fixed on a slide, 10% KOH alkali solution was added. The glass was heated over the burner flame without boiling to accelerate material clarification; then, it was covered with a slide and viewed under a microscope [7].

Direct microscopy was used to detect fungal elements in biological material. Microscopy of biomaterial samples for the detection of mycelium or spores was carried out under an increase of ×10 on an Olympus BX43 (2020) fluorescent microscope [15].

The test material was clarified with 10% KOH to detect fungal elementsand then stained with calcofluor white [23].

A cultural mycological studyinvolving isolating a pure culture of fungi from biological material was conducted to establish the species affiliation [24]. The biomaterial was cultured on Sabouraud agar to detect zoophilic fungi and Chapek agar to detect micromycetes in 28ºS. Before sowing, the material was kept in 70% alcohol for 5 minutes.

The cultures that were obtained were identified based on cultural and morphological characteristics using identifiers [25, 26, 27] and taking into account the growth characteristics of the colonies.

Staining of smears of pure cultures of micromycetes was carried out by a simple method: 20 μL of any dye was applied to the slide, fixed for 5 minutes, and microscopy was performed.

Scotch preparations were prepared from young budding culturesby placing 10 cm long self-adhesive tape (Scotch 3M®) on a glass stick;the sticky side touchedthe surface of the formed colony of the test culture. The tape was fixed on glassandexamined under a microscope under various magnifications.

Results

Morphological identification of fungi remains the foundation of any clinical laboratory. Microscopic analysis of characteristic fungal structures is one of the most important parts of fungal identification [7]. Therefore, we diagnosed dermatomycoses by the gold standard method with additional coloring with calcofluor:

- classical KOH microscopy without coloring,
- coloring of biomaterial and pure culture of pathogens with various dyes (lactophenol cotton blue, Romanovsky dye, Giemsa reagent, Schiff reagent, methylene blue, Bengal pink),
- white staining of biomaterial with calcofluor,
- cultural and morphological identification of pure culture of pathogens with staining of smears with various dyes.

A total of 47 samples from farm animals were studied, of which 28 samples revealed the presence of pathogenic micromycetes: 7 *Trichophyton* spp., 1 *Microsporum* spp., 14 *Aspergillius* spp., and 6 yeast of different species.

When studying unpainted primary biomaterial from animals by direct microscopy, structures resembling fungal mycelium were found on hair surfaces in 10 cases (21.2%). Mycelium growth inside the affected hair was also detected (Figure 1).

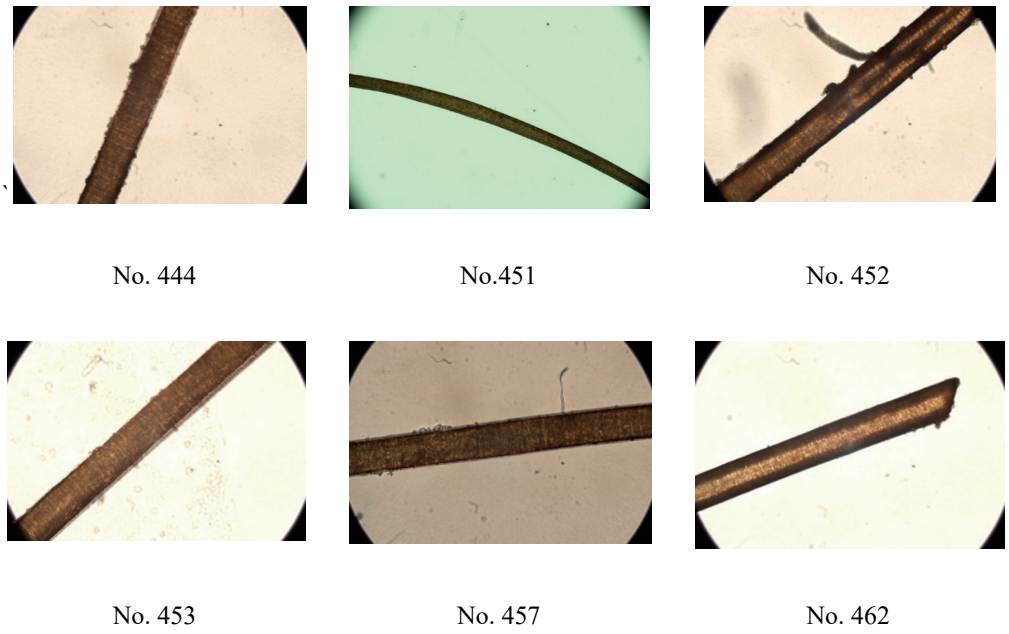


Figure 1 – Microscopy of affected animal hair

In Figure 1, external hair lesions are visible, manifested as a fluffy coating or threads in certain areas of the hair: samples No. 440, No. 452, No. 457. In some hair, the core is affected andfilled with mushroom spores. Especially strong lesions of the inner space of the hair were detected in samples No. 440, No. 444, and No. 457.

Detecting lesions of the hair core is difficult with microscopy of dark-colored hair. Identifying mycelium on the hair'ssurface is also difficult, and itcan be mistaken for glass defects and other artifacts.

Subsequently, we conducted a classic KOH test, in which the hair is enlightened in an alkali solution, which facilitates the detection of spores and mycelium [28]. Figure 2 shows the results of the KOH microscopy of animal hair.

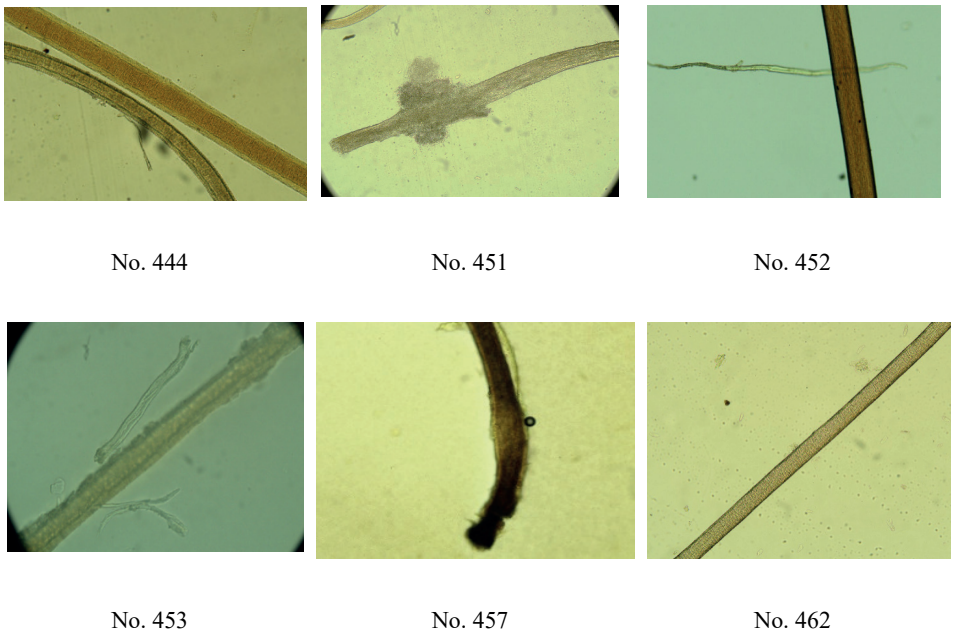


Figure 2 – KOH microscopy of affected hair, magnification ×10

Figure 2 shows various defects and lesions on the hair: in samples No. 440 and No. 457, focal thickenings and mycelium loosening were detectable. In samples No. 462 and No. 452, spore accumulation appeared inside the hair; numerous foci of hair loosening with stratification of the superficial hair membrane, and notches were also noted. Sample No. 453 revealed “pegs”characteristic wedge-shaped perforations and erosion on the hair surface, and hair thinning was observed [29]. KOH microscopy showed a positive result in 18 (38.2%) cases.

Problems in the KOH microscopic diagnosis of skin mycoses, expressed in the uniformity of mycelial lesions by pathogens of different types of dermatomycetesandthe absence of clearly visible spores and conidia, make it possible to make only an approximate preliminary diagnosis of skin mycosis or its appendages and do not allow identification ofmicromycetes to genus and species. There is also a possibility of a laboratory assistant’smistake in identifying the mycelium of dermatomycetes, which in KOH preparations looks imperceptible, has very thin, transparent, barely noticeable hyphae, and provided that there is a visible mycelium of opportunistic fungi, the novice researcher may make an erroneous diagnosis or not notice the presence of the pathogen at all.

To improve the quality of microscopy, we have developed a technology for coloring micromycete smears in biomaterial and in pure culture. The classic dyes lactophen blue, methylene blue, and Schiff’s reagent were selected for further studies to identify micromycetes in biological material from farm animals. Microscopy was performed at magnifications ×10, ×40, and ×100. When staining biomaterial samples with these dyes, the possibility of rapid detection of fungal mycelium in the biomaterial thickness (hair and scales) was detected in 11 (23.4%) cases (Figure 3).

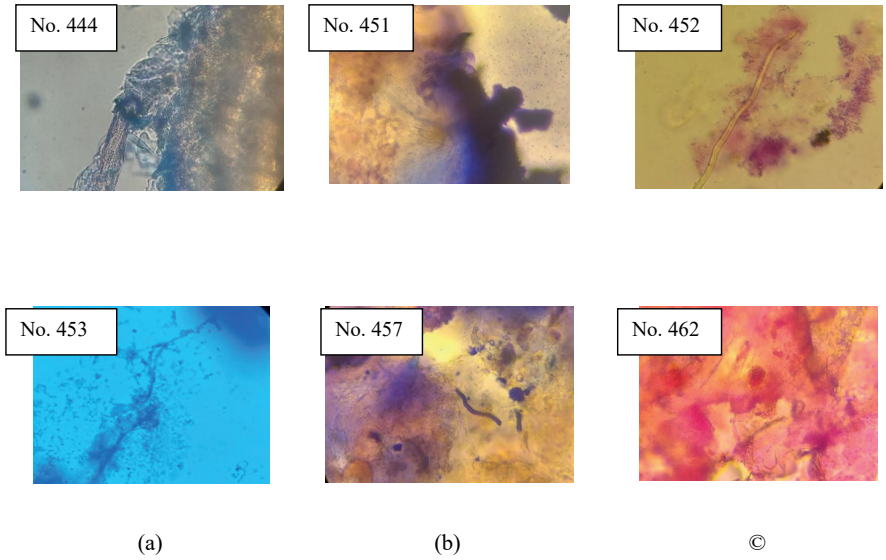


Figure 3 – Identification of fungal structures of micromycetes in the biomaterial when stained with classical dyes: a - lactophenol blue, b - methylene blue, c - Schiff'sreagent (the arrow indicates mycelium)

As can be seen from Figure 3, lactophenol blue stains mycelium well, but it also stains the general background. Methylene blue and Schiff'sreagent, along with the staining of mycelial hyphae, also stain skin scales, which complicates the diagnosis. Background staining of the tissue made it difficult to identify the morphological structures of micromycetes, which was doubtful due to the similarity in some cases with convoluted forms of bacteria, either with blood vessels or with a hair follicle. Also, in some cases, the general background of the smear was strongly colored, so the mycelium hyphae didn`t have clear boundaries.

Further searches for the optimal method of coloring served as the basis for using calcofluor white dye for coloring fungal components and conducting luminescent microscopy. 0.1% fluorescent calcofluor white dye and 10% potassium hydroxide solution were usedfor microscopy. Analysis was performed on an unfiltered fluorescent microscope (Figure 4) and with a blue filter (Figure 5).

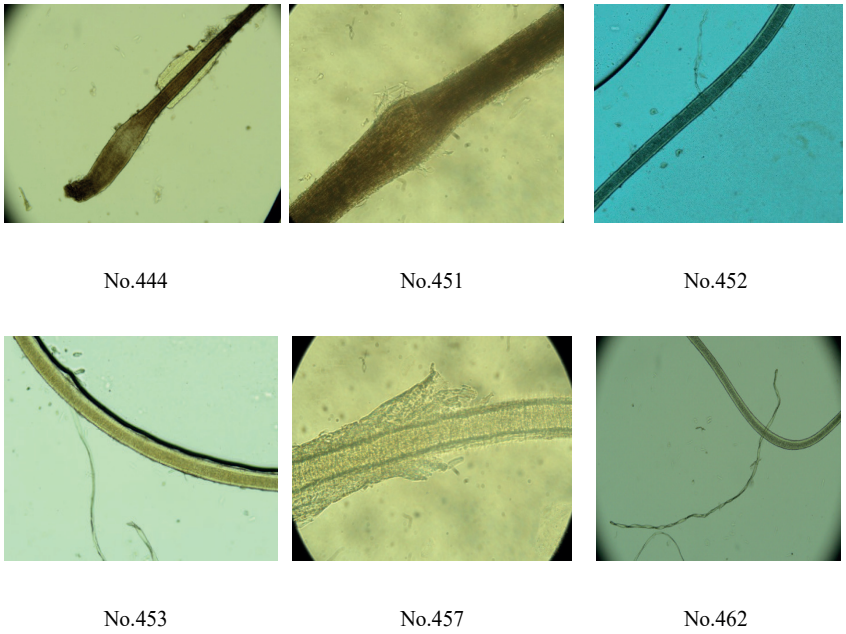


Figure 4 – Detection of spores and mycelium of micromycetes in affected hair, white color with calcofluor, magnification ×10, without filter

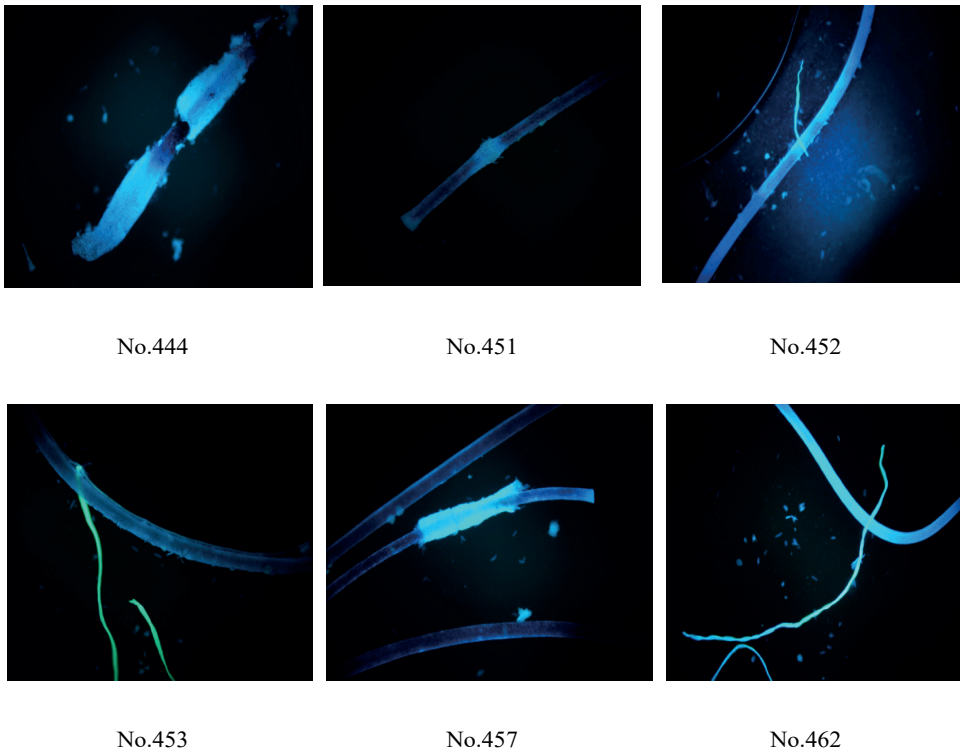


Figure 5 – Luminescence of micromycete mycelium and affected hair in biomaterial, white calcofluor staining, ×10, blue filter

A comparative analysis of images taken with a fluorescent microscope, with and without using a blue filter, found that the possibility of detecting the mycelium of fungi and affected hair is higher in the second case. Figure 5 clearlyshows luminous fungal mycelial filaments in the biological material under the blue filter of the luminescent microscope. The detection percentage was 21.3%.

Mycelium in KOH smears was clear and poorly visible in the field of view. The percentage of detection was 29.9%. Samples stained with calcofluor revealed hair affected by the overgrown mycelium of fungi. A luminous mycelium wasvisible, located directly on the affected hair. The mycelium of strain No. 453 had clearly defined boundaries, was detected as bright apple-green fluorescence, and was subsequently identified as *Microsporumcanis.*

Thus, the color of calcofluor white smears allows faster and clearer detection of affected hair. The mycelium on affected hair in such smears fluoresces blue, making the fungus hyphaevisible. Therefore, direct microscopy of biomaterial smears stained with calcofluor white is a rapid and sensitive method for laboratory diagnosis of mycoses.

Myceliumdetectionserved as the basis for the initial diagnosis of skin mycosis and the continuation of work on isolating primary cultures from biomaterial and culture and morphological analysis of pure cultures of mycromycetes, the formation of colonies of which was noted from the 4th to the 12th day of incubation (Figure 6).

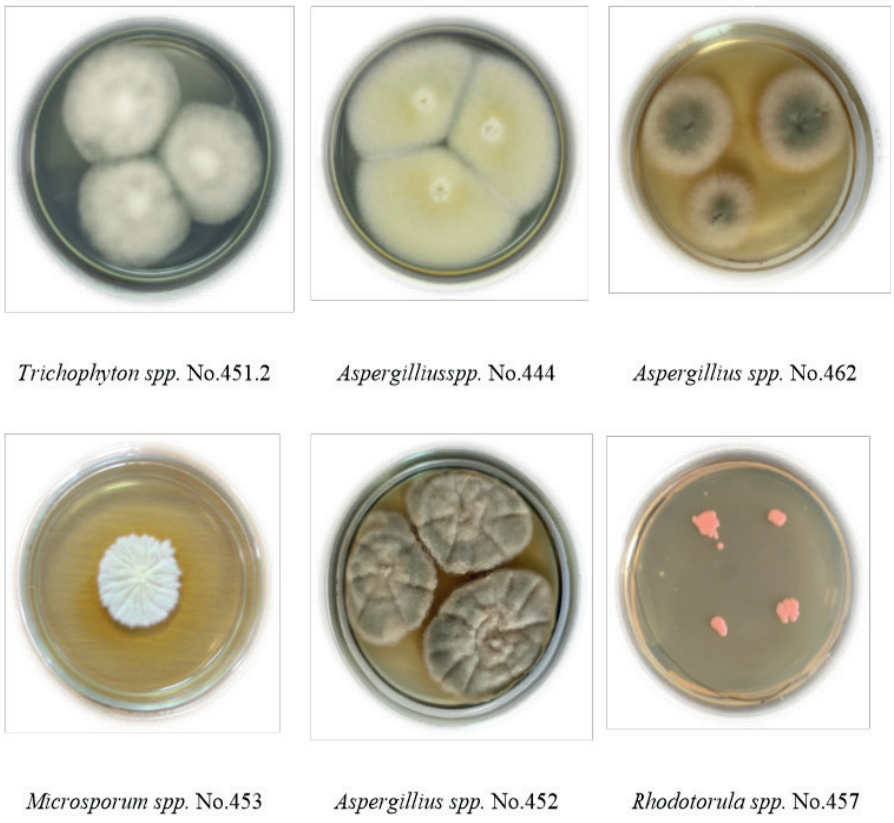
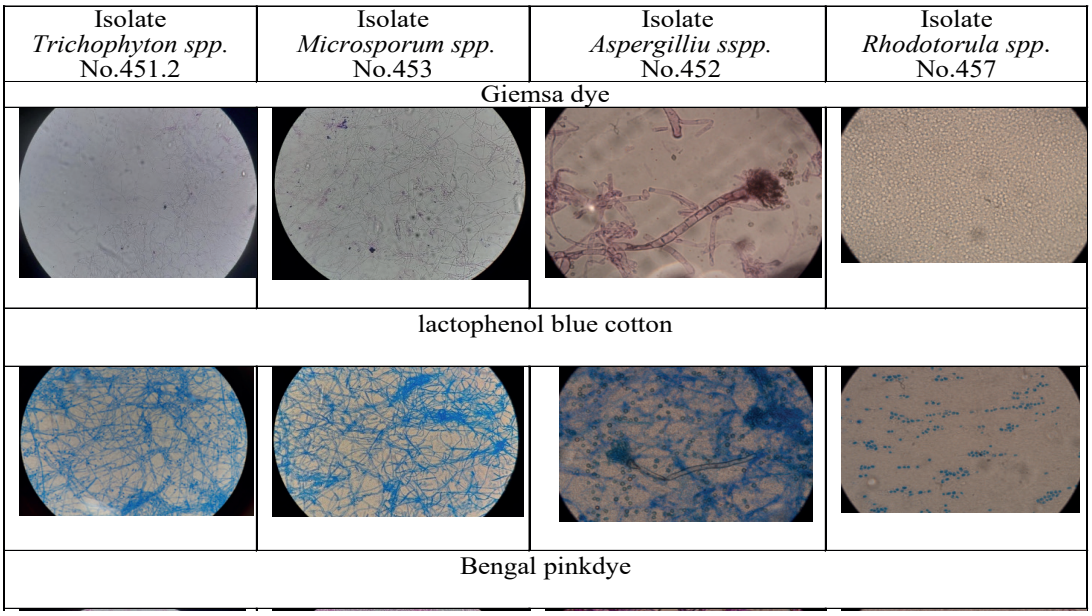


Figure 6 – Pure micromycete cultures isolated from biomaterial

Figure 6 shows colonies of primary fungal cultures of *Trichophyton spp., Microsporum spp., Aspergillius spp.* and yeast isolate *Rhodotorula spp.,* isolated from biological material of farm animals. Trichophyton spp (16.9%), less often Microsporumspp (2.1%), was detected in the culture method. Opportunistic fungi *Aspergilliusspp* were identified in 29.7% of cases, and yeast in 12.7% of cases.

The next step was comparative staining of yeast cells, spores and fungal mycelia from pure cultures of some *Rhodotorula spp.* yeast isolates. No. 457, mold fungi *Aspergillius spp.* No. 452 and *Trichophyton dermatomycetes spp.* №451.2, *Microsporum spp.* No. 453 with various dyes: methylene blue, Giemsa dye, Bengal pink, fuchsin, Schiff dye, Romanovsky dye, and lactophenol blue cotton (Figure 7).



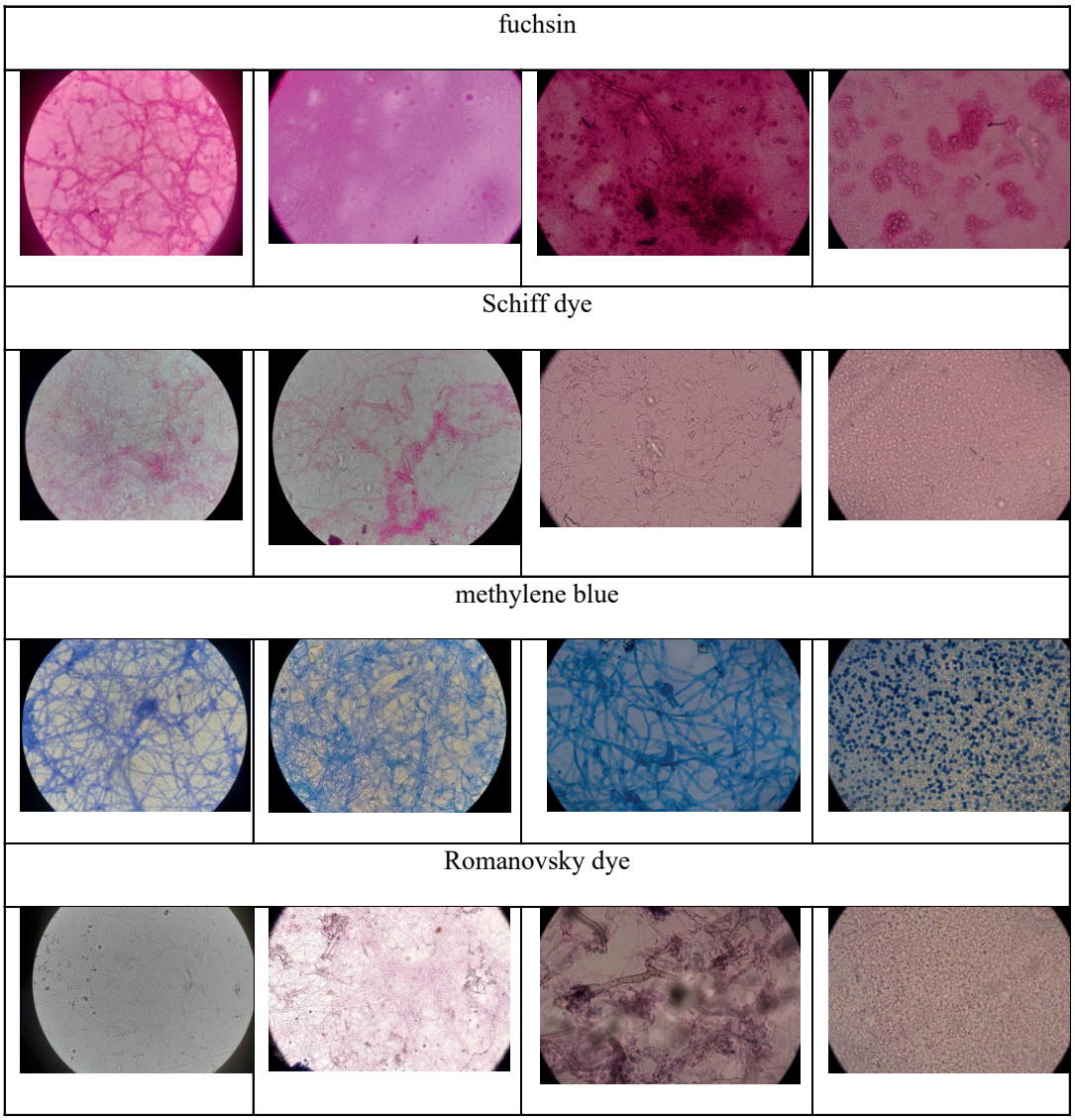


Figure 7 – Results of comparative coloration of yeast and mycelial fungi with various dyes

Figure 7 shows that when stained with methylene blue, all structural components of the mycelial fungi acquire a saturated blue tint. Romanovsky, Giemza, and Bengal pinkdyespartially stain hyphae and spores of mushrooms. Lactophenol blue cotton also stains all morphological structures of mycelial fungi of the genus Trichophyton (hyphae, mycelium, spores, conidia).

Yeast is well stained with dyes: lactophenol blue cotton and methylene blue. Schiff dye stains yeast pink, which is explained by the high concentration of polysaccharides in the yeast cell wall.

Discussion and Conclusion

The composition of fungal cell walls is relatively simple and includes substances not typically found in animal and plant hosts (e.g., chitin). It is assumed thatidentification of pathogen-specific molecular targets of fungal wall components becomes possible [30]. As is known, the rigid cell wall of fungi is a multilayered structure consisting of chitin microfibrils embedded in a matrix of small polysaccharides, proteins, lipids, inorganic salts, and pigments, providing skeletal support and shape to the cells enclosed in them. Chitin is a (β 1–4)-bonded polymer of N-acetyl-D-glucosamine (GlcNAc). The major polysaccharides of the cell wall matrix are composed of non-cellulosic glucans such as glycogen-like compounds, mannans (mannose polymers), chitosan (glucosamine polymers), and galactans (galactose polymers). Small amounts of fucose, rhamnose, xylose, and uronic acids may be present. Glucan belongs

to a large group of D-glucose polymers having glycosidic bonds. The most common glucans that make up the cell wall have a β configuration. Polymers with (β 1-3)- and (β 1–6)-linked glucosyl units with different bond ratios of 1-3 and 1-6 are common [31].

As a rule, the transparent unpainted mycelium of dermatomycetes is clearly visible in smears of pure culture of pathogenic fungi and practically undetected in smears of pathological material. Various staining methods have been proposed for better detection of mycelial structures. However, not all mushrooms present in hair and scrapings stain equally well. Keratinized cells are resistant to the penetration of dyes and make it difficult to stain fungi inside the hair and scales. However, colored preparations have several important advantages over potassium hydroxidetreated samples. Stained preparations reveal small morphological details of fungi more clearly [32]. Our results confirm the authors'data, although some dyes showed a better result (Figure 7).

When using methods of staining with various dyes of morphological elements of micromycetes in pure cultures, we found that when staining with methylene blue, all structural components of mycelial fungi acquire a saturated blue tint, which makes it possible to identify mycelial hyphae of various thicknesses and conduct a quick preliminary identification of dermatomycetes and opportunistic mold fungi in smears.

Staining with methylene blue mycelium of dermatomycetes makes it easy to identify in smears a characteristic thin mycelium and other morphological elements: rocket-shaped mycelium of fungi of the genus Microsporum, microconidia, macroconidia, arthrospores and other structural elements of fungi of the genus *Trichophyton*. Lactophenol blue cotton also stains all morphological structures of *Trichophyton* mycelial fungi (hyphae, mycelium, spores, conidia). In contrast, on a light background, the mycelium manifests itself as saturated blue threads, and spores in the form of dark blue morphological structures.

Romanovsky, Giemza, and Bengal pinkdyespartially stain hyphae and spores of mushrooms, the pattern in the field of view is not as clear as when staining with lactophenol blue cotton dye. However, coloring with Romanovsky, Giemza, methylene blue, Schiff, fuchsin,andBengal pink dyes allows you to quickly and reliably identify the characteristic fusiform macroconidia of the fungi of the genus *Microsporum*.

Yeast is well stained with lactophenol blue cotton, methylene blue, and Schiff dye. Given that living cells are not stained with methylene blue, and stained blue cells are considered dead [3], this stainingmethod can provide data on the age of the culture.

Schiff dye stains yeast pink, which is explained by the high concentration of polysaccharides in the cell wall of yeast. Of the disadvantages of the dye, background staining of the smear can be noted, which makes it difficult to record the results.

KOH in various concentrations is used as a keratolytic agent to detect fungi in tissues [33]. However, it does not stain fungal elements. Therefore, delicate translucent fungal elements avoid detection. Several authors reported low sensitivity and specificity of KOH preparations [34]. Saxena et al. reported 68% sensitivity and 40% specificity of KOH [35].

The combined use of white calcofluor with potassium hydroxide (KOH) solution is believed to increase the detectability of fungal infection compared to the standard KOH method [18]. The affinity of the fluorochrome dye calcofluor for chitin allows the mycelium and spores of the fungus to absorb the drug when a drop of KOH with the dye is added. Itcauses the formation of a complex of β-1.3 and β-1.4 polysaccharides and a complex of white calcofluor with caustic potassium, which gives a characteristic glow under the blue filter of a luminescent microscope [17].

Using calcofluor allowed Rasconi S. et al. (2009) to perform preliminary identification of chitin of microscopic molds stained with calcofluor in pathological material [36]. In our studies, for direct microscopic detection of fungal structures, the biomaterial was stained with calcofluor white dye, which made it possible to quickly and efficiently identify hair lesions of early diagnosis of mycoses within 30-60 seconds (Figure 8).

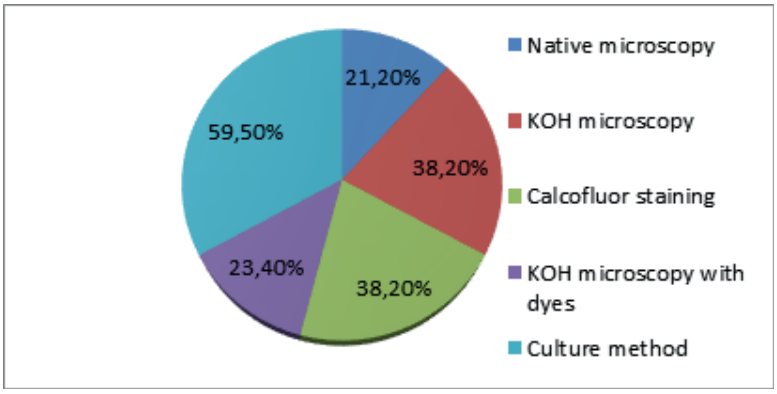


Figure 8 – Comparative results of biomaterial microscopy methods

As can be seen from Figure 8, the results of the mycological examination of 47 samples of biological material showed that during microscopy, the pathogen was detected in 10 cases (21.3%), during KOH microscopy in 15 cases (29.9%), during KOH microscopy with other dyes in 11 cases (23.4%), during KOH microscopy with calcofluor staining in 25 cases (53.2%).

The efficiency of the KOH microscopy method with white calcofluor compared with direct microscopy was 60% higher, compared with KOH microscopy with other dyes by 56%, classical KOH microscopy by 43.8%.

Identifying pathogens in samples of biological material using white calcofluor made it possible not only to quickly and reliably identify the pathogen and damage to hair or skin scales, but also to carry out differential diagnostics. This is because the mycelium of *Microsporum* spp. gives a bright apple-green fluorescence under a blue fluorescent microscope filter.

Our data are consistent with those of various authors Vishal Punjabi et al. (2020) [19], Dalia Moemen et al. (2019) [20], Schottelius J. [21], and several others. These results make it possible to recommend the use of direct microscopy with calcofluor staining for the rapid diagnosis of dermatomycoses in veterinary clinics of the Republic of Kazakhstan.

Pathogens were detected in 28 cases (59.6%) by the culture method. Compared with KOH microscopy with calcofluor white, positive results were confirmed in 89.3%. Practically similar results were obtained by Pihet M. et al. (2017) [37], who spoke of 93.8% detection of the pathogen in microscopy with calcofluor in comparison with the culture method. During the initial isolation of skin mycosis pathogens, fungal growth was obtained in 28 cases (59.6%), and growth was absent in 19 samples (40.4%).

According to the results, of the total number of pathogens, dermatomycetes were detected in 19% of cases: *Trichophyton spp.*16.9%, *Microsporum spp.* 2.1%. Opportunistic fungi *Aspergillius* spp. were detected in 29.7% of cases, yeast in 12.7% of cases. In 6.3% of cases, bacteria were detected, and in 40.4%, fungigrowthwas negative (Figure 9).

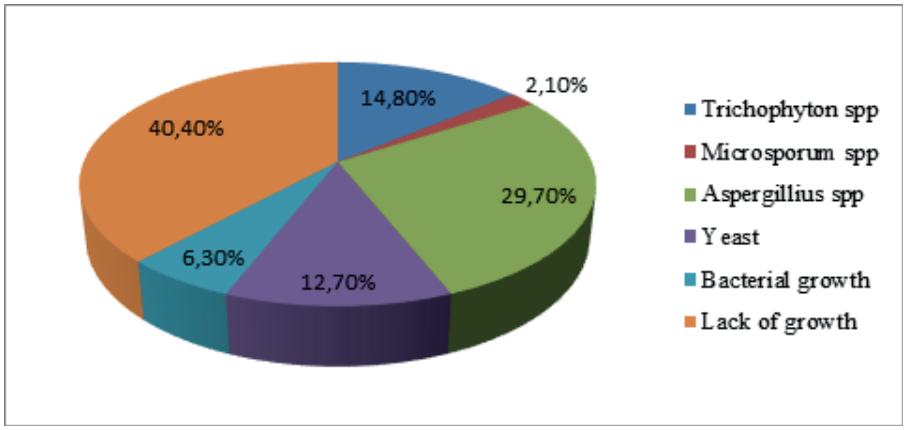


Figure 9 – Spectrum of pathogens isolated from farm animals by culture method

The staining of pure cultures of dermatomycetes, mycelial molds, and yeast also showed a higher diagnostic value when using the method of staining smears with various dyes in direct microscopy of scotch preparations.

Thus, using various dyes for coloring smears and biomaterials makes it possible to improve the direct microscopy method for the rapid and reliable detection of spores and mycelium, both in biomaterial and in pure culture. Thismethodwill make it possible to correctly identify pathogens quickly and carry out differential diagnostics of skin mycoses with high reliability.

As a result ofthemycological examination of 47 samples of biological material, it was revealed that during microscopy, the pathogen was detected in 10 cases (21.3%), during KOH microscopy in 15 cases (29.9%), during KOH microscopy with other dyes, in 11 cases (23.4%), during KOH microscopy with calcofluor staining, in 25 cases (53.2%).The efficiency of the KOH microscopy method with white calcofluor compared with direct microscopy was 60% higher, compared with KOH microscopy with other dyes, by 56%, classical KOH microscopyby 43.8%. The culture method, detected pathogensin 28a cases (59.6%), of which 7 *Trichophyton spp.*, 1 *Microsporum spp.*, 14 *Aspergillius spp*., 6 yeast. Growth was absent in 19 samples (40.4%), bacteria were detected in 3 samples (6.3%). Compared with KOH microscopy with calcofluor white, positive results were confirmed in 89.3%. Application of coloring with various dyes of morphological elements of micromycetes in pure cultures makes it possible to identify mycelial hyphae of various thicknesses, spores, conidia,andother morphological structures and to carry out rapid preliminary identification of dermatomycetes and opportunistic mold fungi in smears.

Authors' Contributions

EK and GB: Concept development, design and planning of the study, data collection and analysis, critical review of the article and final approval, research, statistical analysis. AN and GB: Conducted a comprehensive literature search and conducting research. All the authors have read, reviewed and approved the final version of the manuscript.

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Analysis of heavy metals content in domestic and imported poultry meat

Olzhas M. Omirzakov[1], Vitaliy V. Krivets[1], Maxat A. Berdikulov[1], Almira E. Kuanysh[1], Aigul S. Omarova[1], Kazbek A. Dyussembayev[2,3]

[1]Republican State Enterprise on the Right of Economic Use "National Reference Center for Veteri-nary Medicine" of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan, Astana, Kazakhstan
[2]L.N. Gumilyov Eurasian National University, Astana, Kazakhstan
[3]National Center for Biotechnology, Astana, Kazakhstan

Corresponding author: Maxat A. Berdikulov: berdikulov.ma@mail.ru
Co-authors: (2:OO) omirzakov_olzhas@mail.ru; (3:VK) vitaliy.krivets89@gmail.com; (4:AK) almira.kuanysh00@gmail.com; (5:AO) omarovaac@mail.ru; (6:KD) dyussembayev@biocenter.kz
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Abstract

Background and Aim. Heavy metal contamination of the environment and food has attracted intense public attention as it poses a serious threat to the ecological system and human health. Heavy metals are chemical elements that have a toxic effect on the human body. The expansion of various industries has led to a significant increase in levels of these components within envi-ronment.

Materials and Methods. Current work represents diagnostics and comparison of the content of heavy metals in poultry meat of domestic production and imported origin studied in 2023 using inductively coupled plasma mass spectrometry (ICP-MS).

Results. Large concentrations of arsenic and lead were found in the samples, not exceeding the maximum permissible concentrations, while the content of mercury and cadmium was at trace levels. The obtained data allows to assess the level of heavy metal contamination and compare the safety of poultry meat produced in the Republic of Kazakhstan and abroad.

Conclusion. A clear understanding of the ways and mechanisms by which heavy metals pose a risk to human health when consuming contaminated food products makes it possible to adopt appropriate strategies for managing and mitigating their negative effects. In addition, development of hand-held portable devices is needed for timely on-site diagnostics of HMs to ensure quality of meat products is sufficient for human consumption.

Key words: food monitoring; heavy metals; ICP-MS; poultry meat.

Introduction

Ensuring the safety and quality of food products is an important task of the state policy of healthy nutrition and acts as a key link in preserving the food independence of Kazakhstan. A person must consume the elements necessary for the body, but along with this, a large number of potentially dangerous chemicals can be consumed [1]. This is due to the fact that environmental pollution is aggravated by the industrial revolution and human anthropological activity [2]. Thus, toxic elements are found in 90% of the studied food products [3].

Heavy metals (HMs) are a group of pollutants that have recently become widespread, be-longing to the groups of trace and ultratrace elements according to the biological classification [4, 5]. In the environment, HMs manifest themselves as toxicants and ecotoxicants, where the toxicants are elements that have a harmful effect on an organism or group of organisms, whereas the ecotoxicants are elements

or compounds that negatively affect ecosystem as a whole [6]. Toxic metals such as cadmium (Cd), arsenic (As), mercury (Hg) and lead (Pb) are among the most dangerous for the environment, human and animal health [7]. Generally, the main sources of HM-based environmental pollution are heavy industry, motor vehicles, boiler houses and incinerators, as well as production and use of agricultural fertilizers and pesticides [8, 9]. HM compounds enter the body mainly through the gastrointestinal tract with concentrated foods and water, to a lesser extent through the respiratory organs [10].

Today, great attention is paid worldwide to the protection of the habitat and the internal environment from the increasing action of chemical substances (in particular, HMs and soluble forms of their toxic compounds) of an anthropogenic and original nature [11]. In this regard, monitoring of food products in Kazakhstan is the most important state task. The monitoring is based on the content control of pollutants, including HMs in food, which are regulated according to sanitary and hygienic standards. One of the main organizations that are responsible for food monitoring in Kazakhstan is the Republican State Enterprise on the Right of Economic Use "National Reference Center for Veterinary Medicine" of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan (NRCV).

The objects of monitoring are raw materials of animal origin, such as meat, milk, fish, honey and chicken eggs. Among them, one of the most important food products is chicken meat (poultry meat), since it is a source of animal protein at an affordable price [12]. The era of globalization requires competitive products. Current domestic poultry meat producers must ensure not only accessibility, but also products that are safe to eat. The safety of poultry meat starts from the farm, from the processing process to the moment of consumption.

Since its introduction in the 1980s, ICP-MS has become, perhaps, the most versatile method for detecting specific elements. Unlike other popular methods of mass spectrometric ionization, ICP-MS uses a high-temperature plasma discharge as a source of predominantly single positively charged ions. As a result, ICP-MS has become a powerful and reliable method for detecting most of the elements present in the periodic table [13]. In ICP-MS, noble gases such as argon are mainly used as plasma gas, in which effective evaporation, dissociation or atomization, excitation and final ionization of the analyzed components of the sample occur. In addition, this high-temperature process leads to complete fragmentation of each molecule of the sample, leaving only their detectable atomic components, namely metals, metalloids or heteroatoms [14].

The purpose of this work was to detect and compare the level of contamination of poultry meat of domestic production and imported origin with such HMs as arsenic, cadmium, mercury and lead. As a result, no HM was found above the permissible level, however, it is worth mentioning that in the samples from one of the studied farms, the «Izhevsky PC» in the Akmola region, a sufficiently high number of HMs was detected compared with others. As this was the first study in Kazakhstan to conduct a comparative analysis of the heavy metal content in poultry meat from domestic and imported sources, this research provides valuable data.

Materials and Methods

Samples of poultry meat, namely broiler chickens, were taken as test objects. A total of 41 samples were examined including 25 domestic production and 16 imported origins .The samples were collected by the Territorial Inspections of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan in accordance with the Rules for sampling of transported objects and biological material approved by the Order of the Minister of Agriculture of the Republic of Kazakhstan dated April 30, 2015, No. 7-1/393 [15] and deliv-ered to NRCV. The prefrozen samples were transported in thermal containers with gel and liquid refrigerants.The samples were collected from the following enterprises of the Republic of Ka-zakhstan: LLP «Poultry-Agro» (Kostanay region) – 1 sample; LLP «Turkey PVL» (Pavlodar re-gion) – 1 sample; LLP «PTF Yessil Poultry Farm» (North Kazakhstan region) – 1 sample; LLP «Zapad K» (West Kazakhstan region) – 1 sample; Astana markets – 2 samples; LLP «Aknar PF» (Karaganda region) – 1 sample; LLP «Makinskaya Poultry Farm» (Akmola region) – 9 samples; LLP «Izhevskiy» (Akmola region) – 2 samples; LLP «CAPITAL PROJECTS LTD» (Akmola region) – 7 samples (Fig. 1). Number of samples by importing countries: United States of America – 6, Russian Federation – 4, Republic of Belarus – 3 and Ukraine – 3 samples.

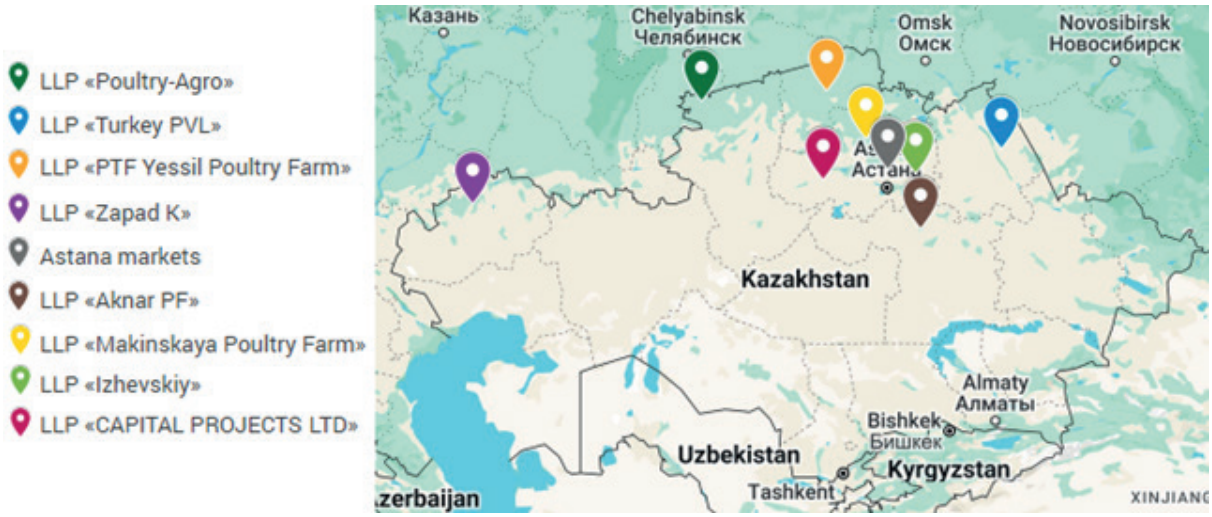

Figure 1 – Sampling sites of the studied poultry meat samples on the map of the Republic of Kazakhstan (Google Maps)

Sample preparation was carried out by the method of decomposition under pressure. This physico-chemical method is designed for the detection of trace elements in food products by mineralizing samples using a microwave system. When preparing the samples, the requirements of the EN 13805-2012 standard were followed [16]. The Multiwave GO Plus (Anton Paar GmbH, Germany) microwave decomposition system was used for decomposition.

Arsenic, cadmium, mercury and lead were included in the range of defined HMs. The studies were performed on the basis of a quadrupole mass spectrometer with inductively coupled argon plasma ICP MS Agilent Technologies 7500 (Agilent Technologies, USA), with an operating mass range from 5 to 240 am. The analysis was carried out in accordance with the requirements of the standard ST RK EN 15763-2017 [17]. The data were analyzed using the MassHunter quantitative calculation software, which supports efficient data collection and management of the mass spectrum library.

Calibration standards were prepared with a multi-element solution IV-ICPMS-71A containing arsenic, lead and cadmium, as well as a single-element solution of mercury MSHGN-10PPM. These standards of the Inorganic Ventures company (USA) are produced in accordance with ISO 17034, ISO 17025 and are traceable in NIST (National Institute of Standards and Technology, USA). Solutions of various concentrations (5 μg/L, 10 μg/L, 20 μg/L, 50 μg/L and 100 μg/L) were used for calibration of ICP-MS, taking into account the linear dynamic range. As well as a blank (control) sample, deionized water with the addition of nitric acid was used, which was subjected to the same sample preparation procedure described above. The error bars represent the standard deviation obtained using the automatic function of three-fold repetition of measurements. Moreover, the Student's t-test for independent populations was used for determination of statistical significance of differences in the average values of HM content.

All chemical reagents and materials used in this study were of high analytical purity. In the laboratory, the accuracy and reliability of the results were achieved through participation in interlaboratory comparison analyses, including with foreign qualification verification providers such as FAPAS, England. All studies were conducted in the laboratory "Analysis of food products" of the NRCV, which is accredited according to GOST ISO IEC 17025-2019 for the detection of HMs by the ICP MS method.

Results

Data on the content of HMs in poultry meat samples studied in 2023 are shown in Figure 2. The maximum permissible levels regulated by the Customs Union are for arsenic 0.1 mg/kg (100 μg/kg), cadmium 0.05 mg/kg (50 μg/kg), mercury 0.03 mg/kg (30 μg/kg) and 0.5 mg/kg (500 μg/kg) of lead were not exceeded in the studied poultry meat samples [18].

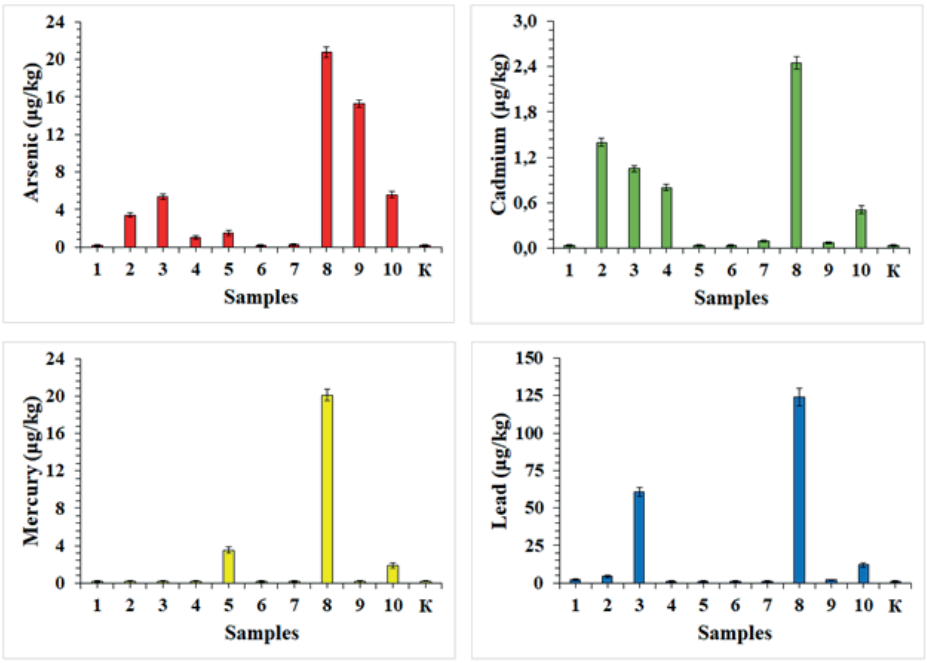

Figure 2 – Average content of heavy metals (µg/kg) in poultry meat studied in 2023: 1-LLP «Poultry-Agro»; 2 - LLP «Turkey PVL»; 3 - LLP «PTF Yessil Poultry Farm»; 4 - LLP «Zapad K»; 5 - Astana markets; 6 - LLP «Aknar PF»; 7 - LLP «Makinskaya Poul-try Farm»; 8 - LLP «Izhevskiy»; 9 - LLP «CAPITAL PROJECTS LTD»; 10 - Imported poultry meat. As well as a blank (control) sample, deionized water with the addition of nitric acid was used, which was subjected to the same sample preparation procedure de-scribed above – K. The error bars are the standard deviation obtained using the automat-ic three-fold measurement repetition function

According to Figure 2, the average arsenic content in poultry meat ranged from 0.26 µg/kg to 20.7 µg/kg. The lowest concentration of arsenic was found in poultry meat produced by LLP «Makinskaya Poultry Farm», while the highest amount was detected in samples from LLP «Izhevskiy». In contrast, no residual amount of arsenic was detected in poultry meat produced by LLP «Poultry-Agro» and LLP «Aknar PF». The average arsenic content for all samples of poultry meat produced in Kazakhstan was - 5.28 µg/kg and in imported - 5.59 µg/kg, which in turn is much lower than the permissible amount of arsenic (Fig. 3). These indicators are extremely im-portant because arsenic has genotoxicity that causes cognitive and reproductive dysfunction. The toxic effect of arsenic can lead to bladder and lung cancer [19].

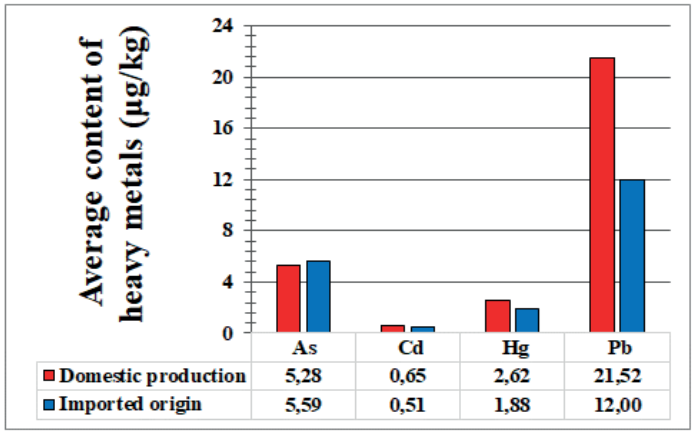

Figure 3 – Average content of arsenic, cadmium, mercury and lead for poultry meat samples of domestic producers and imported origin

Cadmium, a heavy metal vapor and a compound of which are toxic to the human body. Cadmium poisoning leads to damage to the kidneys, central nervous system and immune system. It causes damage to bones and the reproductive system [20].

The following minimum and maximum values of cadmium were found in the studied poultry meat samples: 0.06 μg/kg in poultry meat produced by LLP «CAPITAL PROJECTS LTD» and 2.45 μg/kg by LLP «Izhevskiy». Cadmium concentrations were not detected in the samples obtained at the markets of Astana, LLP «Poultry-Agro» and LLP «Aknar PF» (Figure 2). It was established that the average concentration of cadmium in domestic (0.65 μg/kg) and imported (0.51 μg/kg) poultry is 100 times less than the permissible level.

Mercury is a neurotoxin that is a global pollutant, and its organic form, methylmercury, is associated with neurocognitive changes in human fetuses and cardiovascular diseases in adults [20]. As a result of the conducted research, mercury was detected only in poultry meat selected in the markets of Astana – 3.48 μg/kg, LLP «Izhevskiy» – 20.0 μg/kg and in imported products – 1.88 μg/kg (Figure 2). The detected mercury value for imported and domestic poultry meat is 1.88 μg/kg and 2.62 μg/kg, respectively (Figure 3).

An increased concentration of lead in the body affects behavior, cognitive functions, post-natal growth, delays puberty and reduces hearing in infants and children. In adults, lead causes problems of the cardiovascular, central nervous system, kidneys and fertility. During pregnancy, lead can inhibit fetal growth at an early stage [22].

A high concentration (123.8 μg/kg) of lead was detected in poultry meat produced by LLP «Izhevskiy», while the lowest concentration (0.13 μg/kg) was diagnosed in LLP «Makinskaya Poultry Farm». Notably, no lead was detected in the samples of LLP «Zapad K» and LLP «Aknar PF» (Figure 2). The average lead content in domestic poultry turned out to be much higher than in the imported ones (Figure 3).

In some of the testing samples, there was quite high concentration of lead, arsenic and mer-cury. This could be due to the content of HMs in feed, feed additives [23], water [24] and bird droppings [25]. In addition, birds can be treated with antiparasitic baths, sprayers or aerosol generators using pistils, which in turn contain HMs [26]. The cadmium content in poultry meat samples of domestic production and imported origin is at a trace level.

Overall, in poultry meat of domestic production, the lead content was higher by 44.2%, cadmium by 21.5%, mercury by 28.2% than in imported ones, while the arsenic content was higher by 5.5% in the imported products.

To determine the statistical significance of differences in the average values of HM content in domestic and imported poultry meat, Student's t-test for independent populations was used (Table 1).

Table 1 - Calculated data using Student’s t-test

Data	Comparable populations	As	Cd	Hg	Pb
Number of measurements (n)	Domestic	9	9	9	9
	Import	16	16	16	16
Average value (M)	Domestic	5.28	0.65	2.62	21.51
	Import	5.59	0.51	1.88	12
Average error of the arithmetic mean (m)	Domestic	2.66	0.3	2.34	15.24
	Import	1.63	0.22	1.62	9.94
Number of degrees of freedom (f)		23	23	23	23
Level of significance (p)		0.05	0.05	0.05	0.05
Critical value of Student's t-test		2.069	2.069	2.069	2.069
The meaning of the Student's t-test		0.10	0.38	0.26	0.52

We compared the obtained values of the Student's t-test with the critical value of the Stu-dent's t-test, which, with the number of degrees of freedom (f) equal to 23 and the significance level (p) = 0,05, is 2,069. The obtained values were greater than the critical value, according to which we concluded that the differences between the content of heavy metals in domestic and imported poultry meat were statistically insignificant.

According to the results of this study, it was determined that the poultry meat of imported origin and domestic production meet the hygienic requirements of food safety in terms of HM content and are safe in accordance with regulations. The risk assessment based on the obtained data did not reveal any harm to the health of the population.

However, due to the ability of HMs to accumulate in the body, it is worth considering that, having accumulated and exceeded the established norms, HMs can cause great harm to human health [27]. Regular consumption of products, even with a low content of HMs, leads to disruption of the body.

Discussion and Conclusion

Due to the obtained data and their statistical analysis in this study, it can be concluded that the difference in the HM content in poultry meat samples of domestic and imported origin is not significant. We assume that this could be due to approximately the same conditions of maintenance and growing technology, identical methods of treatment and processing, as well as a similar diet of broiler chickens in poultry farms of all countries. Notably, no heavy metal exceeding standards was detected in both domestic and imported meat samples. However, even small amounts of HMs can potentially affect human health.

In fact, environmental pollution, food safety and human health are inextricably linked. Concentrations of HMs in the environment have been significantly increased in recent decades due to global industrial progress. A reliable diagnostics, accurate analysis and assessment of potential risks caused by HMs allow an application of timely management strategies for reducing and/or preventing the HM contaminations. Moreover, development of portable devices is needed for on-site diagnostics of HMs for constant timely tests provided and regulated by government to ensure highquality food products for daily consumption.

Authors' Contributions

M.B., O.O. and K.D. conceived and planned the research. O.O., V.K. and A.K. carried out the experiments. A.K. contributed to sample preparation. O.O., V.K. and A.O. contributed to the interpretation of the results. O.O. took the lead in writing the manuscript. M.B. and K.D. corrected the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Study of allergic reaction to somatic antigen made from a live plague vaccine of the EV strain

Alim B. Bizhanov[1], Bolat Sh. Karatayev[1], Batyrbek D. Aitzhanov[1], Fatima E. Sembina[1], Begali Kanatov[1], Malik R. Yusupov[2]

[1]Department of bacteriology, Kazakh Scientific Research Veterinary Institute,
[2]Production department, Kazakh Scientific Research Veterinary Institute, Almaty, Kazakhstan

Corresponding author: Alim B. Bizhanov: alimakyntai@mail.ru
Co-authors: (2: BK) bolatkaratai@gmail.com; (3:BA) batirdos@mail.ru;
(4: FS) fatimsem@mail.ru; (5: BK) kanat_bek59@mail.ru; (6: MY) malik_imhana@mail.ru
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Abstract

Background and Aim. This article presents the results of experiments conducted to study delayed-type hypersensitivity to somatic plague antigen derived from a live plague vaccine from strain EB. This study aims to investigate delayed-type hypersensitivity to a somatic plague antigen derived from a live plague vaccine of the EV strain.

Materials and Methods. Methods included the following: extraction of the main somatic antigen from the vaccine strain *Yersinia pestis EV*, determination of the Lowry protein concentration by superimposing the results obtained from a spectrophotometer's digital optical density values of the test preparation on a calibration line; testing of the allergen for sterility (on nutrient media), toxicity (on healthy white mice and guinea pigs), pyrogenicity (on rabbits), absence of sensitizing properties and specificity (on healthy guinea pigs); and determination of the allergenic activity of the main somatic antigen by detecting its hyperergic reactivity in in vivo experiments on guinea pigs.

Results. Further experiments conducted to study delayed-type hypersensitivity to somatic plague antigen showed that the obtained preparation is sterile, harmless, apyrogenic, has no sensitizing properties, and is specific.

Additionally, evaluating the allergenic activity of different doses of the investigated preparation revealed that a dose of 50 mcg is optimal for intradermal administration.

Conclusion. The conducted studies will be used for quality control of somatic plague antigen-allergen, which will allow detection of the absence of sensitizing properties or immunological harmlessness of the allergen at the site of injection in healthy people and animals, thereby avoiding positive responses in both infected and immunized individuals, as well as minimizing the occurrence of false reactions.

Key words: Delayed-Type Hypersensitivity; Plague Dry Live Vaccine from EV Strain; Somatic Plague Antigen.

Introduction

Allergic diagnostic tests are biological reactions used to diagnose various diseases, primarily infectious diseases, based on hypersensitivity of the organism caused by an allergen. In the context of infectious diseases, the allergen is typically the causative agent or its toxin. When the corresponding allergen is introduced, the body responds with a local or general reaction, which can be used to diagnose the presence of a specific disease. The diagnostic value of allergic diagnostic tests is determined by their specificity, sensitivity, and safety for humans or animals. Examples of these tests include the Pirke and Mantoux reactions for tuberculosis [1], the Byrne reaction for brucellosis [2], and the Schick reaction for diphtheria [3]. These tests can also detect hypersensitivity to substances causing bronchial asthma attacks [4] and other allergic diseases.

In veterinary medicine, allergic diagnostic tests are used to diagnose diseases such as glanders [5], tuberculosis [6], brucellosis [2], paratuberculosis enteritis [7], tularemia [8], toxoplasmosis [9], and other infectious and invasive diseases. The primary advantage of these tests in veterinary practice is their ease of use for both individual and mass examinations of animals. These tests can identify infected animals even in the absence of clinically expressed disease. Specific allergens used in veterinary diagnostics include mallein for glanders, tuberculin for tuberculosis, abortin, brucellisate, and brucellohydrolizate for brucellosis. The tests are conducted by applying preparations to the conjunctiva of the eye (e.g., mallein and tuberculin) or intradermally.

Scientists at the Kazakh Scientific Research Veterinary Institute, in collaboration with scientists at the Kazakh Center for Quarantine and Zoonotic Infections named after M. Aikimbayev, have developed a method for obtaining somatic plague antigen-allergen from a live plague vaccine derived from the EV strain [10]. Despite the advantages of many recently developed allergodiagnostics, such as precise dosing, ease of application and result recording, and low cost, these preparations often contain a large number of antigens, leading to several disadvantages. The most significant of these is the occurrence of positive responses in both infected and immunized individuals, as well as a certain number of false reactions.

Materials and Methods

A dry live plague vaccine from the Yersinia pestis EV strain, deposited in the museum of live cultures of microorganisms at the State Enterprise "Kazakh Scientific Center for Quarantine and Zoonotic Infections named after M. Aikimbayev," was used as the source material.

Ethical approval

Ethical approval for the animal studies was obtained from the Ethical Commission of the Kazakh Scientific Research Veterinary Institute (Approval No. 2 of 01.02.2005).

The sterility, toxicity, pyrogenicity, absence of sensitizing properties, and specificity of the allergen were tested.

Protein Concentration Determination. After extracting the main somatic antigen from the vaccine strain Yersinia pestis EV, the protein concentration was determined using the Lowry method. This involved superimposing the spectrophotometric results of digital optical density values of the test preparation onto a calibration line, with the abscissa axis representing the protein concentration. The protein content of the sample at pH 7.0-7.2 was 2.7-3.0 mg per ml.

Sterility Testing. Sterility was assessed by culturing the allergen on meat-peptone agar and meat-peptone broth. For each medium (five tubes each), 0.25 ml of the preparation dissolved in physiological solution to a concentration of 1 mg/ ml was sown. The cultures were incubated at 37 °C and 28 °C. After 10 days, the presence or absence of growth in the tubes was checked to determine the sterility of the preparation.

Toxicity Testing. The toxicity of the preparation was studied in healthy white mice (10 individuals) weighing 20-25 g and guinea pigs (6 individuals) weighing 300 g. The allergen was administered to white mice intravenously at a dose of 0.02 mg and to guinea pigs intracardiacally at a dose of 0.3 mg. The animals were monitored for 48 hours and then autopsied.

Apyrogenicity testing. For apyrogenicity testing, the allergen was administered to rabbits at a dose of 1 mg intramuscularly, and body temperature was monitored overnight.

Determination the absence of sensitizing properties. The absence of sensitizing properties or immunological harmlessness of the allergen was tested in healthy guinea pigs (6 individuals). Three guinea pigs were injected three times with 50 mcg of the drug in a volume of 0.1 ml of sterile physiological solution at 5-day intervals. Three guinea pigs served as controls. After 15 days, all animals were injected intradermally with 50 mcg of allergen in 0.1 ml of sterile physiological solution. After 24 hours, the reaction was read by assessing the presence of redness and its size at the injection site.

Specificity testing. The specificity of the intradermal test with the proposed allergen was studied in 10 guinea pigs immunized with live vaccines against plague and tularemia at a dose of 1 million microbial cells. Each guinea pig was simultaneously tested with 0.01 mg of intradermal samples containing specific and nonspecific allergens in a 0.1 ml volume on both flanks.

Determination the allergenic activity. To determine the allergenic activity of the main somatic antigen, its hyperergic reactivity was detected in vivo. Guinea pigs previously immunized with the live plague vaccine EV were used in the experiments. Vaccination was carried out subcutaneously at a dose of 1 million microbial cells. One month after immunization, an intradermal test was performed on the lateral surface of the guinea pigs' torso with a preparation of somatic antigen from the plague microbe containing various doses-30, 50, or 70 mcg-in 0.1 ml of physiological solution. A needleless injector, adjusted for intradermal administration, was used.

The depth of drug penetration was assessed by preliminary experiments involving intradermal injection of physiological solution with a 1% carcass to guinea pigs. After dissection of the tissues at the injection site, the ink infiltrated only the skin if the apparatus was properly adjusted. Allergic reactions were assessed 24 hours after the sample was administered. A positive reaction was characterized by hyperemia and skin thickening. Hyperemia alone was not considered a positive reaction. The reaction was deemed positive if the thickening was at least 10 mm in diameter. In negative reactions, redness and slight thickening disappeared after 24 hours, while in positive reactions, thickening and redness persisted for 3-4 days.

Statistical processing of the obtained data was performed in Statistica 6.0 programme. Student's t-criterion was used to identify the statistical significance of the differences between the results. Differences were considered reliable at $p \leq 0.05$.

Results

Sterility Testing. The results of the study of the sterility of the plague antigen-allergen samples are shown in Table 1.

Table 1 - Results of the Sterility Study of the Plague Antigen-Allergen Samples

Name of Nutrient Medium	Addition of Antigen-Allergen	Test Tube Number	Presence of Growth
Meat-peptone agar	+	1	Absent
	+	2	Absent
	+	3	Absent
	+	4	Absent
	+	5	Absent
Meat-peptone broth	+	1	Absent
	+	2	Absent
	+	3	Absent
	+	4	Absent
	+	5	Absent

As shown in Table 1, after seeding 0.25 ml of the preparation dissolved in physiological solution to a concentration of 1 mg/ml in all 10 tubes, after 10 days of incubation in the thermostat (at 37 °C and 28 °C), no postrenal growth was detected, which indicated the sterility of the preparation.

Toxicity Testing. The results of the toxicity study of the plague antigen-allergen samples are shown in Table 2.

Table 2 - Results of the Toxicity Study of the Plague Antigen-Allergen Samples

Name of Animal	Number of Animals	Introduced Allergen		Death within 48 Hours	Autopsy Results after 48 Hours
		Method	Quantity		
White mouse	1	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	2	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	3	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	4	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	5	Intravenously	0.02 mg	No	No change
	6	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	7	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	8	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	9	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
	10	Intravenously	0.02 mg	No	Hyperaemia of the subcutaneous tissue vessels
Guinea pig	1	Intracardiacally	0.3 mg	No	Hyperaemia of the subcutaneous tissue vessels
	2	Intracardiacally	0.3 mg	No	Hyperaemia of the subcutaneous tissue vessels
	3	Intracardiacally	0.3 mg	No	Hyperaemia of the subcutaneous tissue vessels
	4	Intracardiacally	0.3 mg	No	Hyperaemia of the subcutaneous tissue vessels
	5	Intracardiacally	0.3 mg	No	No change
		Intracardiacally	0.3 mg	No	Hyperaemia of the subcutaneous tissue vessels

As shown in Table 2, intravenous injection of the allergen at a dose of 0.02 mg did not cause the death of white mice within 48 hours and at autopsy after the expiry of this period no changes were registered in mouse no. 5, and in the other 9 white mice only hyperaemia of subcutaneous tissue vessels was detected, which indicated that the plague antigen-allergen was harmless. Experimental, intracardiac injection of the allergen at a dose of 0.3 mg did not cause the death of guinea pigs within 48 hours and at autopsy after the expiry of this period no changes were registered in guinea pig no. 5, and in the other 5 guinea pigs only hyperaemia of subcutaneous tissue vessels was detected, which also indicated the harmlessness of the plague antigen-allergen.

Apyrogenicity testing. The apyrogenicity test results of the plague antigen-allergen samples are summarized in Table 3.

Table 3 - Results of Apyrogenicity Tests of Plague Antigen-Allergen Samples

Body Temperature of Rabbits During the Day					
Experimental Rabbits Injected with the Preparation			Control Rabbits Injected with Distilled Water		
Numbers by n/a	Readings		Numbers by n/a	Readings	
	Before Experiment	During Experiment		Before Experiment	During Experiment
1	38.8 °C	38.8 °C	1	38.7 °C	38.7 °C
2	38.9 °C	38.9 °C	2	38.6 °C	38.6 °C
3	38.7 °C	38.7 °C	3	38.9 °C	38.9 °C
4	38.9 °C	38.9 °C	4	38.7 °C	38.7 °C
5	39.0 °C	39.0 °C	5	38.9 °C	38.9 °C
6	38.8 °C	38.8 °C	6	39.0 °C	39.0 °C
7	38.6 °C	38.6 °C	7	38.8 °C	38.8 °C
8	38.7 °C	38.7 °C	8	38.6 °C	38.6 °C
9	38.8 °C	38.8 °C	9	38.7 °C	38.7 °C
10	38.7 °C	38.7 °C	10	38.9 °C	38.9 °C

A test conducted overnight on 10 rabbits injected intramuscularly with the allergen at a dose of 1 mg showed that the body temperature of the experimental animals remained within normal limits (38.6-39.0 °C). No increase in body temperature was detected in control animals administered distilled water (38.6-39.0 °C). The test demonstrated the absence of pyrogenic properties in the plague antigen-allergen samples.

Determination the absence of sensitizing properties. Experiments conducted on six healthy guinea pigs (3 experimental and 3 control) to determine the absence of sensitizing properties or immunological harmlessness of the allergen at the site of administration involved three administrations of the drug at a dose of 50 mcg at 5-day intervals to the experimental animals. A control intradermal injection of the preparation was given to all 6 animals at a dose of 50 mcg after 15 days. The presence of redness no more than 5 mm in size indicated the absence of sensitizing properties of the proposed preparation.

Specificity testing. The study of the specificity of the intradermal test with the proposed allergen, conducted on guinea pigs immunized with live vaccines against plague and tularemia at a dose of 1 million microbial cells, showed that only the pigs immunized against plague reacted positively to the intradermal injection of the allergen. Animals immunized against tularemia reacted positively only to specific allergens. These data confirmed the specificity of the preparation obtained.

The results of the study of the biological properties of plague allergens are summarized in Table 4.

Table 4 - Results of the Study of the Biological Properties of Plague Allergens

Name of Indicators	Characteristics
Appearance, color, solubility	Amorphous powder, light cream color, soluble in distilled water and physiological solution
Indicator pH	7.0-7.2
Sterility	Sterile
Toxicity	Harmless
Pyrogenicity	Apirogenic
Sensitizing properties	None
Specificity	Specific

From the data in Table 4, it follows that the obtained preparation is sterile, harmless, apirogenic, has no sensitizing properties, and is specific.

Determination the allergenic activity. The results of the intensity of the skin reaction in guinea pigs to allergen administration are shown in Table 5.

Table 5 - Intensity of Skin Reactions to Allergen Injection in Guinea Pigs

Immunization Dose	Animal Number	Papule Size after 24 h (mm)
30 mcg	1	3
30 mcg	2	3
30 mcg	3	-
30 mcg	4	3
30 mcg	5	2
30 mcg	6	2.2
50 mcg	1	5
50 mcg	2	6
50 mcg	3	5
50 mcg	4	7
50 mcg	5	6
50 mcg	6	5.8
70 mcg	1	5
70 mcg	2	6
70 mcg	3	7
70 mcg	4	6
70 mcg	5	5
70 mcg	6	5.8

From the data in Table 5, it follows that when the drug was administered at doses of 50 and 70 mcg, the intensity of the skin reaction was similar, averaging 5.8 mm. When a dose of 30 mcg was administered, the size of the papules was approximately half as large.

The results of the activity of recording the allergic state in plague-immune guinea pigs with somatic antigen preparations are summarized in Table 6.

Table 6 - Activity of recording the Allergic State in Plague-Immune Guinea Pigs with Somatic Antigen Preparations

Animal Group		Allergen Dose (mcg)	Effectiveness of Intradermal Test with Allergen
Immunized	1	30	4/5
	2	50	5/5
	3	70	5/5
Controls	4	30	0/5
	5	50	0/5
	6	70	0/5

*Note: The numerator indicates the number of positive responses, and the denominator indicates the number of animals in the experiment.

Table 6 shows that of the guinea pigs inoculated with the plague vaccine, 4 guinea pigs reacted positively to the allergen administered at a dose of 30 mcg, and 5 reacted positively at doses of 50 mcg and 70 mcg. In contrast, the control (unimmunized) guinea pigs showed 100% negative reactions. Evaluating the allergenic activity of different doses of the drug under study, it becomes apparent that the dose of 50 mcg is optimal for intradermal administration.

Discussion and Conclusion

Modern medicine and veterinary medicine have made significant progress in the use of allergic diagnostic tests to detect infectious and invasive diseases in humans and animals, even in the absence of clinically expressed disease. The diagnostic value of allergic diagnostic tests is determined by their specificity, sensitivity, and safety for humans and animals. Experiments conducted at the Kazakh Center of Quarantine and Zoonotic Infections named after M. Aikimbayev and the Kazakh Scientific Research Veterinary Institute led to the development of a method for obtaining somatic plague antigen-allergen from a live plague vaccine of strain EV.

Despite the many advantages of recent allergodiagnostics, such as clear dosing, ease of staging and result recording, and low preparation costs, these preparations have notable disadvantages due to the presence of numerous antigens. The most significant issue is the occurrence of positive responses in both infected and immunized humans and animals, as well as a certain number of false reactions.

Further experiments to study delayed-type hypersensitivity to somatic plague antigen showed that the obtained preparation is sterile (after seeding 0.25 ml of the preparation dissolved in physiological solution in all 10 tubes, after 10 days of incubation in the thermostat (at 37 °C and 28 °C), no postrenal growth was detected), harmless (intravenous and intracardiac injections of the allergen did not cause the death of laboratory animals within 48 hours and at autopsy after the expiry of this period no changes were registered in laboratory animals), apyrogenic (a test conducted on rabbits injected intramuscularly with the allergen showed that the body temperature of the experimental animals remained within normal limits), has no sensitizing properties (the presence of redness no more than 5 mm in size indicated the absence of sensitizing properties of the proposed preparation), and is specific (the study of the specificity of the intradermal test with the proposed allergen, conducted on guinea pigs immunized with live vaccines against plague and tularemia, showed that only the pigs immunized against plague reacted positively to the intradermal injection of the allergen. Animals immunized against tularemia reacted positively only to specific allergens).

The data obtained by us correlate with the data of Gostischev et al. (2018), where the authors report the verification of specificity and allergenic activity of the pestin allergen obtained by them from the vaccine strain of the plague microbe Yersinia pestis EV. In the preparation obtained, the pH and the protein concentration were determined. To check the specificity, the samples were subjected to spectrophotometric and chromatographic analysis. To assess specific activity, blood samples of 17 people immunized with the plague live vaccine from the Yersinia pestis EV strain of the NIIEG line were used for epidemic indications. The contingent was examined before vaccination on days 7, 21 and 3 months after immunization by evaluating the expression intensity of basophils CD63. Biochemical analysis of the obtained by the modified procedure of the pestin and derivatives allowed to judge the qualitative composition, to show the absence of impurities of the protein nature, as well as to determine the carbohydrate profile. The use of the drug as an allergen to assess the formation of antiplague immunity in the vaccinated contingent confirmed its specificity [11].

After the intradermal injection of guinea pigs, previously subcutaneously immunized with live plague vaccine EB or somatic antigen-allergen at doses of 50 and 70 mcg, the intensity of the skin reaction was the same, averaging 5.8 mm. When a dose of 30 mcg was administered, the size of the papules was approximately half as large. Of the laboratory animals inoculated with the plague vaccine, 4 guinea pigs reacted positively to the allergen injected at a dose of 30 mcg, 5 at a dose of 50 mcg, and 5 at a dose of 70 mcg, while the control unimmunized guinea pigs showed 100% negative reactions. Evaluating the allergenic activity of different doses of the drug under study reveals that the dose of 50 mcg is optimal for intradermal administration.

The somatic plague antigen made from the live plague vaccine of strain EV is sterile, harmless, apyrogenic, has no sensitizing properties, and is specific. The optimal dose of somatic plague antigen-allergen for intradermal injection is 50 mcg. The developed preparation is suitable for practical application in the early diagnosis of plague in humans and animals.

Born et al. (2020) point out the need for highly specific, rapid and easy-to-use confirmatory diagnostic methods for reliable pathogen (Yersinia pestis) identification independent of PCR or immunological detection methods based on F1 antigens. The authors used the host specificity provided by phage

receptor-binding (or tail fibre/spike) proteins (RBPs) to develop a specific, rapid and simple method for the detection of Y. pestis based on fluorescence microscopy [12].

The conducted studies will be used for quality control of the somatic plague antigen-allergen, allowing the detection of the absence of sensitizing properties or immunological harmlessness of the allergen at the site of administration in healthy people and animals. This will help avoid positive responses in both infected and immunized individuals, as well as reduce the occurrence of false reactions.

Authors' Contributions

AB and BK: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. BA, FS, BK and MY: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript".

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Using indirect hemagglutination assay for the diagnosis of cattle brucellosis

Mikail M. Mikailov[1], Shahrudin A. Gunashev[1],
Elmira A. Yanikova[1], Ahmed A. Halikov[1], Aitbay. K. Bulashev[2]

[1]Laboratory of Infectious Pathology of Farm Animals, Caspian Zonal Research Veterinary Institute, Republic of Dagestan, Makhachkala, Russia;
[2]Department of Microbiology and Biotechnology,
Faculty of Veterinary Medicine and Livestock Technology,
Saken Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan.

Corresponding author: Aitbay K. Bulashev: aytbay57@mail.ru
Co-authors: (1:MM)* mikail.mikailov1981@mail.ru; (2:ShG) sgunashev@mail.ru;
(3:EY) nvetmedservis@mail.ru; (4:AH) axmedx93@mail.ru

ORCIDs:
1st Author: https://orcid.org/0000-0002-9620-431X
2nd Author: https://orcid.org/0000-0003-4804-2755
3rd Author: https://orcid.org/ 0000-0002-5561-2499
4th Author: https://orcid.org/ 0000-0002-9765-008X
5th Author: https:// orcid.org 0000-0002-8427-509X

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