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Beta-Hemolytic Streptococcus Infection in Horses in Kazakhstan

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ABSTRACT

Strangles is an infectious disease of horses caused by Gram-positive bacteria *Streptococcus equi*, characterized by an abscess of the submandibular and pharyngeal lymph nodes and leading to obstruction of the respiratory tract. The disease has economic consequences for horse farms and significantly reduces their productivity. The study aimed to monitor the spread of beta-hemolytic streptococcus infection in horses in eight regions of Kazakhstan, isolate *S. equi* strains, and establish their genetic characteristics. To achieve this goal, 687 samples of swabs and blood sera from horses with clinical signs of streptococcal infection were collected. The research methods included bacteriological examination, cultivation of isolates on various nutrient media, polymerase chain reaction analysis to confirm molecular genetic characteristics, and a study of the virulence of isolates in white mice. As a result of the study, 619 positive samples out of 687 were identified, with the highest prevalence of infection in the Ulytau and Kostanay regions. Genetic analysis showed that the obtained isolates corresponded to *S. equi*. Thus, the conducted analysis contributes to regular monitoring and identification of persistent carriers of infection. Such control is key for effective monitoring and prevention of strangles, which will reduce economic losses and improve the productivity of horse farms.

Key words: Horses, Strangles, Strain, *Streptococcus equi*, Monitoring, Epizootological situation

INTRODUCTION

Strangles, caused by the bacterium *Streptococcus equi*, have a high infectivity and rapid spread in horses (Avdeyuk et al. 2022; Zhanabayev et al. 2022; Chhabra et al. 2023). The bacterium *S. equi* has the shape of cocci, is immobile, does not form spores, and is successfully stained with all aniline dyes (Barakhov et al. 2023). Its average diameter ranges from 0.4 to 1 micron, and it belongs to the serological group C and the *Streptococcus* genus (Pelkonen et al. 2013; Kim et al. 2018; Neustroev et al. 2021).

Beta-hemolytic streptococcus is transmitted from horse to horse through direct contact, with most horses becoming infected at a young age (Mussayeva et al. 2021; Sivkova and Domatsky, 2023). Vector animals not only initiate outbreaks but also make it difficult to control and prevent the disease (Shi et al. 2023; Al-Robaiee et al. 2024). It is especially important to consider that horses often cross the borders of countries and continents, transmitting the infection around the world (Libardoni et al. 2016). The disease quickly spreads to the lymph nodes of the head (Sattarova et al. 2023), where the reproduction of bacteria proceeds unhindered due to massive infiltration by polymorphonuclear leukocytes (Issabekov et al. 2022). Early lymphadenitis progresses to an abscess, then a sinus canal forms, allowing pus to flow through the nearest exit point (either through the skin or through the mucous membrane of the upper respiratory tract). This process involving one or more lymph nodes can last 2-3 weeks and is usually accompanied by fever, depression, loss of appetite, mucopurulent nasal discharge, and shortness of breath. Abscesses can also form in other organs of the

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body, the rupture of which leads to death in 10% of cases (Boyle 2017; Dauvillier et al. 2018; Neustroev et al. 2018).

The disease manifests itself sporadically, enzootically, and epizootically and is widespread in the regions of the Republic of Kazakhstan, as well as in neighboring Commonwealth of Independent States (CIS) countries. The results of epizootological studies have shown that the incidence of strangles in the adult horse population is 5.3% and in young animals 27.6% (Duran and Goehring 2021).

Diagnosis of strangles is based on analyzing nasal secretions, pus from abscesses, and lymphoid tissues using polymerase chain reaction (PCR) (Cordoni et al. 2015; Tartor et al. 2019) or enzyme immunoassay (EIA) (Mohammadi et al. 2016; Al-Gharban 2017; Štritof et al. 2021). The treatment of strangles is influenced by the stage, features, and severity of the disease (Richmond 2015).

Currently, strangles remain a serious problem of horse breeding, causing economic damage to the industry, especially in market conditions (Buienbayeva et al. 2024; Kabylbekova et al. 2024). The economic damage by this disease is caused by a lag in the growth and development of sick animals, a decrease in fatness, the death of young horses, funds spent on treatment, and the organization of economic measures aimed at controlling the disease (Boyle et al. 2018). Consequently, the control of strangles and the prevention of their spread are becoming a key part of efforts to provide the population of Kazakhstan with high-quality, healthy, and safe food products (Bayazitova et al. 2023; Kozhanov et al. 2023; Tkeshelashvili and Bobozhonova 2024).

A review of literature sources and practical experience shows that to control the disease, an integrated approach is needed with routine diagnostic studies and analysis of the data obtained, giving a complete picture of infection in the region. Researchers insist that the efforts of horse breeders should be aimed at preventing and controlling the disease, which consists of early detection and treatment of carrier animals and regular checks of imported horses from other countries for possible infection.

Isolation of beta-hemolytic streptococcus infection from carrier animals helps to track the spread of *S. equi* and predict subsequent outbreaks of the disease. The identification and treatment of persistent carriers are crucial for interrupting the infection cycle and thus for the eradication of strangles.

The restoration of livestock, increasing the productivity of horse breeding, and obtaining high-quality products, along with other factors, depend on the effectiveness of preventive veterinary measures, including regular monitoring studies.

The purpose of the study is to determine, through monitoring studies, the frequency of detection and the distribution zone of strangles in the regions of Kazakhstan and identify a new strangle strain, type it, and conduct its genetic analysis. The results of monitoring studies will make it possible to optimize anti-epizootic measures to prevent the spread of this disease in horses in Kazakhstan.

MATERIALS AND METHODS

Ethical approval

Authors have adhered to the rules of the regulatory legal actors of the Republic of Kazakhstan "On the Guidelines for Working with Laboratory (Experimental) Animals During Preclinical (Non-Clinical) Studies" compiled according to the Recommendations of the Board of the Eurasian Economic Commission No. 33 (November 14, 2023).

Monitoring studies of strangles were carried out based on epizootological data from veterinary reports of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan and based on the results of our research conducted during field trips in the Almaty, South Kazakhstan, Turkestan, Jambyl, Kostanay, East Kazakhstan, Jetysu and Ulytau regions.

The object of the study was samples of biological material in the form of pus, exudate of submandibular lymph nodes, and blood serum from horses with strangles. A total of 687 samples of swabs and blood serum from horses and foals with similar clinical signs of streptococcal infection were taken, and the distribution of samples in the eight regions was as follows: Almaty (15), Jambyl (18), Turkestan (119) and Kyzylorda (9), Kostanay (175), East Kazakhstan (100), Jetysu (39), and Ulytau (212).

As part of the work, we carried out bacteriological studies, including seeding from purulent masses in foals with strangles obtained from unopened fluctuating lymph nodes. Two isolates were obtained from samples of purulent exudates from submandibular lymph nodes in horses. The isolated cultures of beta-hemolytic streptococcus were subjected to laboratory examination of morphological and cultural properties after growing on solid and liquid nutrient media.

Morphological and cultural characteristics of the isolates were studied by seeding into meat-peptone broth (MPB) with the addition of 10% blood agar and meat-peptone agar (MPA) with 1% glucose and 10% blood serum. Smears from purulent exudate were recorded and stained using the Gram method. The biochemical characteristics of the isolates were studied by seeding on MPB with 40% bile, 6.5% salt MPA, agar with sodium azide, and Hiss medium with the addition of glucose, lactose, mannitol, maltose, sucrose, sorbitol, and dulcite. The cultures were incubated in a thermostat at 37°C for 18-48 hours.

The taxonomic affiliation of the isolates was determined according to Short Bergey's manual of determinative bacteriology, also adhering to the guidelines for the laboratory diagnosis of strangles, staphylococcosis, and streptococcosis.

Streptococcal isolates from semi-liquid agar were transplanted into tryptone soy broth and cultured at 37°C for 48 hours. The samples were then transplanted into Petri dishes with blood agar and a dense nutrient medium. The cups were placed in a thermostat at 37°C for 24-72 hours. Thus, cultural and morphological characteristics and the presence of catalase activity were evaluated and smears were prepared. Agar cultures were transplanted into Petri dishes with agar and cultivated at 37°C for 24 hours.

When assessing the virulence of streptococci, white mongrel mice (n = 70) of both sexes at the age of 5-8 weeks with a weight of 18-20g were used. The mice were subcutaneously injected with a suspension of a daily culture of live bacterial streptococcal cells in a volume of 0.2-0.5cm³ (from 1×10^3 to 1×10^9 colony-forming units (CFU)/g). The virulent activity of isolates (LD50) was

determined using the Kerber method modified by Ashmarin and Vorobyov (1962).

The blood serum of animals was studied in the prolonged complement fixation test (PCFT). The studied positive and negative serums were heated in a water bath at a temperature of 63-64°C for 30min. An antigen with a titer of 1:20 was used to detect antibodies in the PCFT.

To confirm the molecular genetic characteristics of the *S. equi* isolate, a PCR analysis was performed. The extraction of bacterial DNA from the studied isolate was carried out using a commercial DNA extraction kit, namely PureLink Genomic DNA MiniKit manufactured by Invitrogen (USA), following the manufacturer's recommendations with the preparation of 1.7% agarose gel on a Tris/ethylenediaminetetraacetic acid (TE) buffer with ethidium bromide. The StepOne real-time PCR machine manufactured by Thermo Scientific was used to perform the PCR reaction.

In further studies, the genetic material of the test sample containing *S. equi* was sequenced. The quality control of the reeds was performed using the Fast QC program. The obtained data were then processed in Trimomatic v. 0.36, sequences shorter than 50 nucleotides were excluded from the analysis; adapters were removed. After removing low-quality reads and trimming adapters, the sequences were analyzed by the Kaiju program using a database of non-redundant proteins: bacteria, archaea, viruses, fungi, and microbial eukaryotes (NCBI BLAST nr + euk) with default parameters. The complete genome of *S. equi* was collected using the Geneious software package using default settings. Phylogenetic analysis of microbial sequences was also performed using the Geneious software package.

RESULTS AND DISCUSSION

Epizootological studies on strangles showed the presence of seasonality and stationarity of the disease in horse farms. We observed that in those farms, strangles occurred in the early spring and early winter periods. The incidence of strangles in the adult horse population was 5.3% and in young animals 27.6%.

As can be seen from Table 1, based on the results of blood serum studies, 200 positive samples were detected in PCFT of horses from the Ulytau region, 173 in the Kostanay region, 206 in the Turkestan region, 80 in the East Kazakhstan region, 23 in the Jetysu region, 14 in the Almaty and Jambyl regions respectively, and nine in the Kyzylorda region. The visualization of the Table data is shown in Fig. 1.

Table 1: Results of the study of isolates in the following areas

Sampling location (regions)	Number of samples taken	Number of positive samples	Number of negative samples
Almaty	15	1	14
Jambyl	18	1	14
Turkestan	119	13	106
Kyzylorda	9	0	9
Kostanay	175	2	173
East Kazakhstan	100	13	80
Jetisu	39	16	23
Ulytau	212	12	200
Total	687	619	68

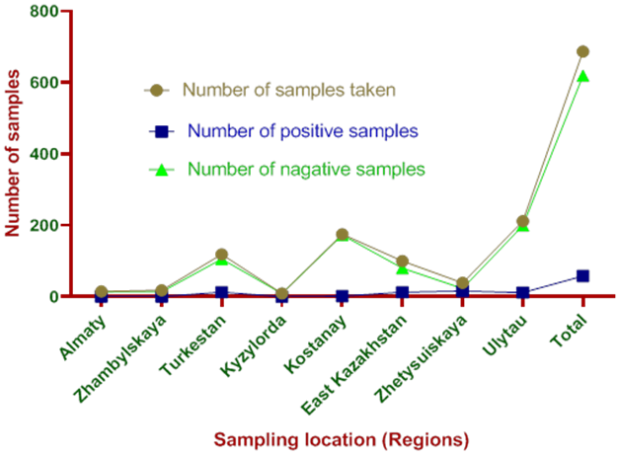

Fig. 1: Results of the study of obtained isolates in the following areas

This data aligns with the findings from a recent study in Kazakhstan where Seitkamzina et al. (2023) reported higher rates of infections in regions with colder climates, suggesting that environmental stressors could predispose to infections. This aspect could explain the lower infection rates in the relatively warmer Kyzylorda region.

Table 1 shows that out of the total number of 687 samples of equine blood serum taken, 619 samples showed a positive result in the PCFT study, where the antibody titer was 1:40. This indicates a widespread exposure to *Streptococcus equi* and possibly high circulation of *Streptococcus equi* among the horse population in the surveyed regions.

Furthermore, the high prevalence we observed could also be reflective of persistent carriers within the population, a phenomenon noted by Tartor et al. (2020) in their study on equine populations in Egypt. These carriers, often asymptomatic, can shed the pathogen and serve as a reservoir for new infections, complicating control efforts and necessitating the identification and management of such carriers as part of disease control strategies.

Fig. 2 and 3 show isolates from samples of purulent exudates from submandibular lymph nodes in horses grown on solid and liquid nutrient media. The results of the morphological and cultural analysis of the obtained samples are presented in Table 2.

As can be seen from Table 2, streptococcus isolated from purulent samples of submandibular lymph nodes in horses did not show fermentation of lactose, sorbitol, and mannitol. It also did not produce indole or ammonia and did not form hydrogen sulfide (H_2S).

The biochemical profile reported in our study supports the use of these tests as reliable methods for confirming *S. equi* in suspected cases of strangles.

The specificity of these biochemical markers also highlights the potential for developing targeted therapies and interventions. As suggested by Khan et al. (2022) and Muteeb et al. (2023), understanding the metabolic pathways that differ among streptococcal species could lead to the development of more effective treatments that are less likely to affect non-target bacterial flora.

The study of the virulence of *S. equi* cultures by determining LD50 isolates No. 1, 2, and 3 yielded the following results (Table 3). As can be seen from Table 3, *S. equi* strain No. 1 turned out to be the most virulent, with its LD50 of 0.5129 billion microbial cells.

Table 2: Enzymatic and biochemical properties of *S. equi* culture

Name of the culture	Enzymatic and biochemical characteristics of bacterial culture											
	Fermentation of lactose	Fermentation of sorbitol	Fermentation of mannitol	Dilution of gelatin	Formation of indole	Formation of ammonia	Formation of hydrogen sulfide	Litmus	Methylene blue	Curdling of milk	Formation of oxidase	Formation of catalase
Streptococcus equi	-	-	-	-	-	-	-	-	-	-	-	+

Table 3: Virulence of *S. equi* isolates

Str. equi	The death of mice injected with culture (in billion microbial cells)						LD50 (billion microbial cells)
	2	1	0.5	0.25	0.125	0.0625	
No. 1	4/4	3/4	2/4	1/4	0/4	0/4	0.5129
No. 2	4/4	2/4	¼	0/4	0/4	0/4	0.8521
No. 3	4/4	3/4	¼	0/4	0/4	0/4	0.7244

Note: the numerator is the number of dead mice, the denominator is the number of mice in the group

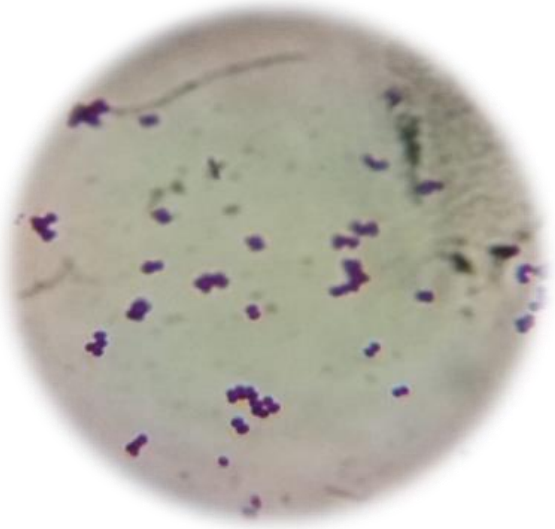

Fig. 2: *S. equi* culture, Gram staining.

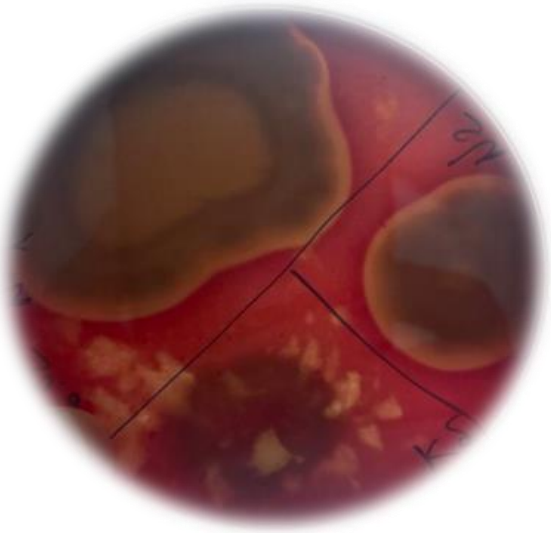

Fig. 3: *S. equi* isolated from pus and grown on 10% blood agar.

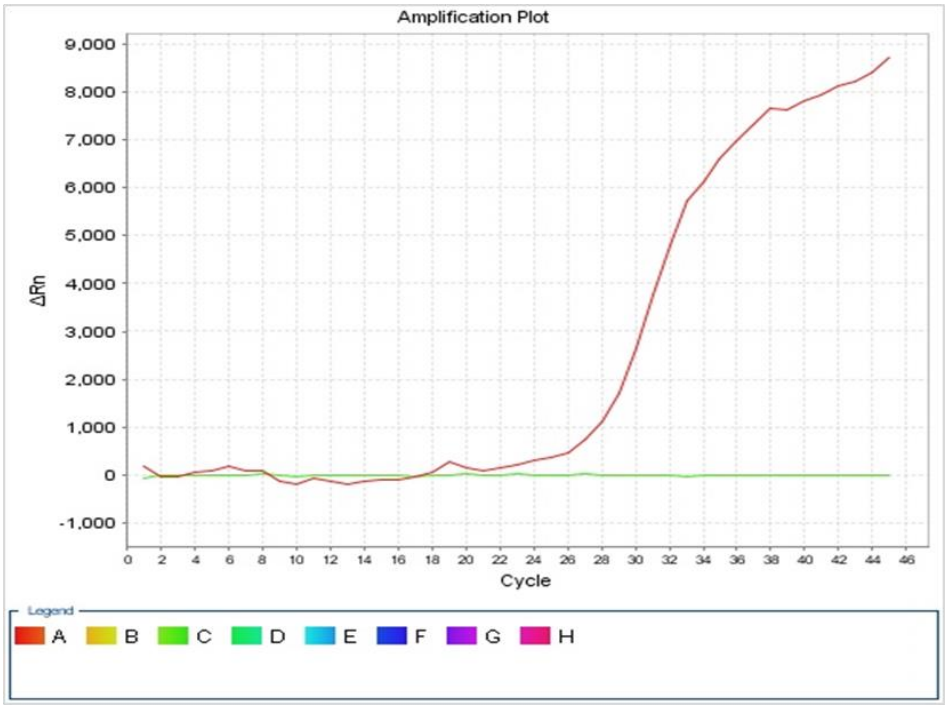

Fig. 4: PCR amplification results; Note No. 1 (biomaterial); No. 2 (negative control).

DNA was isolated from an isolated colony to confirm the genus. The results of real-time PCR analysis confirmed the presence of *S. equi* genetic material in the studied samples (Fig. 4). In the course of molecular biological studies, a draft version of the *S. equi* genome with a length of 2,253,416 nucleotide pairs was collected (Fig. 5). The genome contains at least 1,500 genes, including several prophages located evenly along the entire length of the genome and several sequences of transport RNAs (Fig. 6).

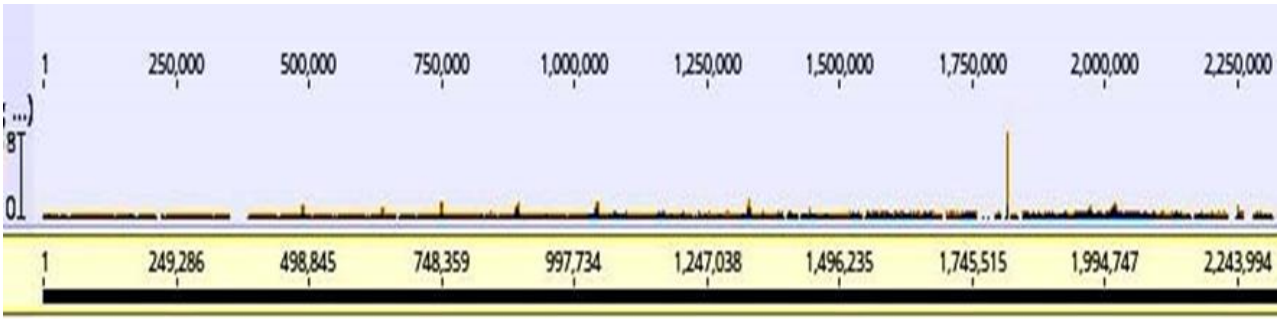

Fig. 5: The genome *of S. equi*, collected from the obtained reads and contigs after sequencing.

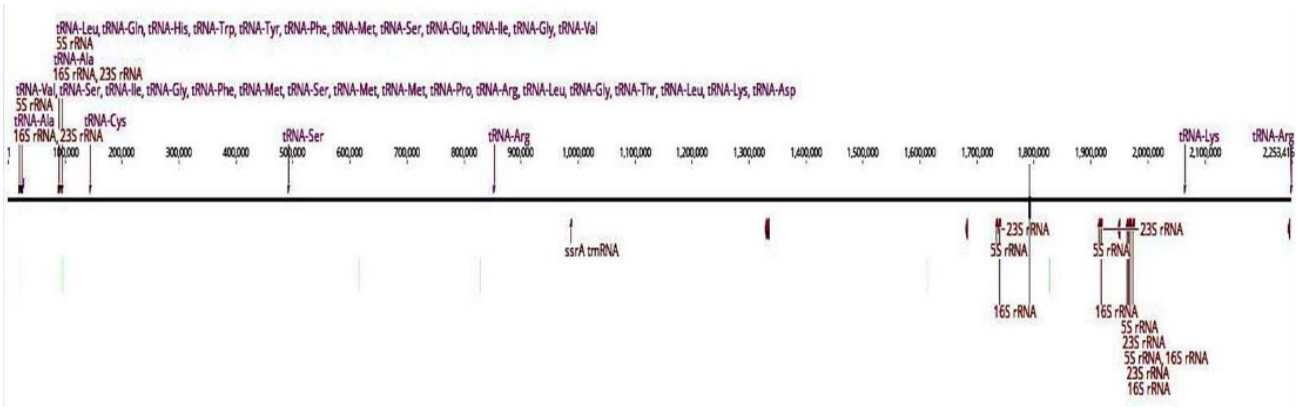

Fig. 6: Diagram of the location of transport RNAs in the *S. equi* chromosome.

Table 4: Results of genetic identification of the test sample

Name of the strain	Sequence of the M protein gene	Identification of nucleotide sequences in an international database, the BLAST algorithm		
		Inventory number	Name of the strain	% of identity
Qargaly-2022	ATGTTTTaGAGAAAyAACAAGCAAAAATTTAGCATCAGAAAACTAAGTGCCGGTGC AGCATCAGTATTAGTTGCAACAAGTGTGTTGGGAGGGACAACTGyAAAAGCGAAC TCTGAGGTTAGTCGTACGGCGACTCCAAGATTATCGCGTGATTTAAAAAATAGATT AAGCGAGATAGCCATAAGTAGAGATGCCTCATCAGCCCAAAAAGTTCGAAATCTT CTAAAAGGCGCCTCTGTTGGGGATTTACAGGCATTATTGAGAGGTCTTGATTCAGC	KF859902	*S. equi* strain Blue SeM (sem) gene	99.52
	AAGGGCTGCGTATGGTAGAGATGATTATTACAATTTATTGATGCACCTTTCATCGA TGTTAAATGATAAACCTGATGGGGATAGAAGACAATTAAGTTTGGCTTCATTACTT GTAGATGAAATTGAAAAGCGGATTGCTGATGGAGATAGTTATGCAAAACTTCTTG	FM204883	*S. equi* subsp. equi 4047	99.44
	AGGCTAAACTTGCAGCTATTAAATCTCAACAAGAAATGCTTAGAGAAAGAGATTC CCAACTTCGAAATCTAGAGAAGGAAAAAGAACAAGAACTACAAAAAGCTAAAGA TGAGCGTCAAGCTCTTACCGAATCATTCAACAAAACTTTATCAAGATCAACAAAA GAGTATAATAAACTAAAAACAGAACTTGCAAAAGAAAAAGAAAAAGCAGCTAAG ATGACTAAGGAATTAGCAGAyAAGCTAAGCAATGCTGAAGCAAGTCGTGATAAAG CCTTTGCAGTATCAAAAGATTTAGCAGATAAACTAAGTAGTGCTGAAGCAAGTCG TGATAAAGCTTTTGCAGTATCAAAAGATTTAGCAGATAAATTGGCAGCTAAAACA GCAGAAGCTGAAAAGTTAATGGAAAACGTTGGTAGTCTAGACCGCTTGGTAGAGT CTGCAAAACGTGAAATGGCTCAAAAATTAGCAGAAATTGATCAATTAACTGCTGA TAAGGCTAAGGCTGATGCAGAGCTTGCAGCTGCAAATGACACCATTGCATCACTT CAAACAGAGCTAGAAAAAGCTAAGACAGAGCTTGCTGTTTCAGAGCGTTTGATTG AATCAGGCAAACGTGAAATTGCTGAGCTACAAAAACAAAAAGATGCTTCTGATAA GGCTTTAGTAGAATCACAAGCTAATGTAGCAGAGCTTGAAAAACAAAAAGCAGCA TCAGATGCTAAGGTAGCAGAGCTTGAAAAAGAAGTTGAAGCTGCTAAAGCTGAGG TTGCAGATCTTAAAGCACAATTAGCTAAGAAAGAAGAAGAGCTTGAA	SEU73162	*S. equi* M-protein (seM) gene	99.05

To assess the differences between circulating *S. equi* variants and vaccine variants, a phylogenetic analysis was performed on the M-protein gene responsible for the virulence of the strain. We found that the circulating variant of *S. equi* was a group of variants combining microorganisms that caused epizootic outbreaks in Russia and China (Table 4, Fig. 7). Besides that, the correspondence of circulating strains with the antigenic specificity of the protective M protein of the microorganism was shown. Similarly noted by Zarreen Simnani et al. (2023), who reported that antigenic drift in pathogenic bacteria can lead to vaccine escape, necessitating continual updates to vaccine formulations to maintain their effectiveness.

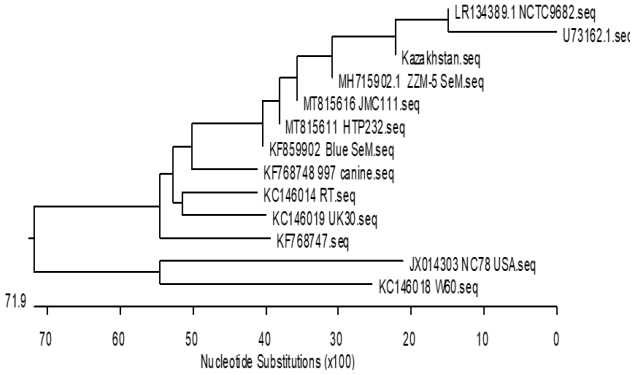

Fig. 7: Phylogenetic analysis of *S. equi* variants on an M-protein model.

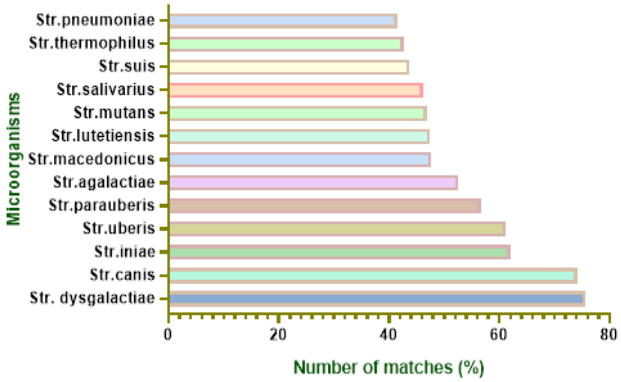

Fig. 8: Comparative analysis of conservative *S. equi Qargaly-22* sites using the BLAST+ software.

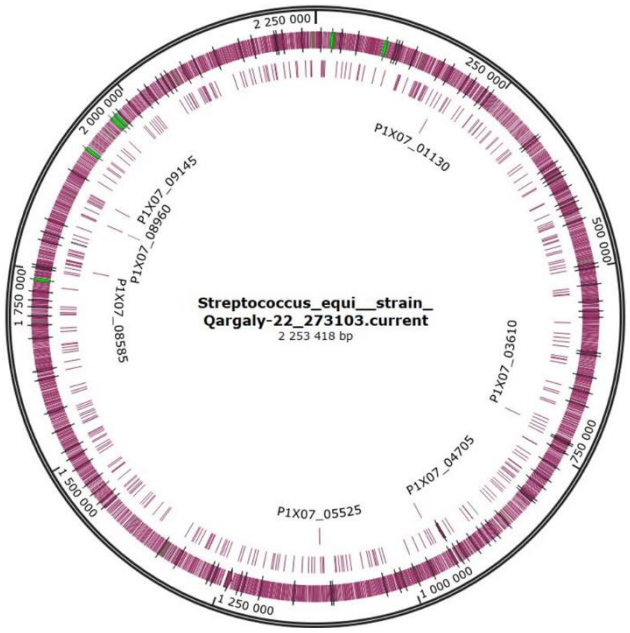

Fig. 9: Phylogenetic tree of complete genomes of *S. equi* Qargaly-22.273103.

As can be seen from the data presented in Fig. 8 and 9, the closest to *S. equi* are *S. dysgalactiae* and *S. canis*, where 74-75% of the 726 conservative sites are present. The comparison between circulating strains and vaccine strains in terms of antigenic specificity of the M-protein further highlights the challenges in vaccine development and effectiveness. The variability in the M-protein gene among circulating strains can lead to reduced efficacy of existing vaccines, which are typically formulated based on historical strains.

Conclusions

We monitored the spread of beta-hemolytic streptococcus infection in horses in eight regions of Kazakhstan. The results showed the presence of seasonal and constant morbidity, with peaks in the early spring and early winter periods. Of 687 samples of swabs and blood sera from horses and foals with similar clinical signs of streptococcal infection, 619 samples showed a positive result in PCFT, which indicated the presence of many *S. equi* vector animals in horses of Kazakhstan. The analysis also showed the high values of virulence of *S. equi* cultures. Moreover, according to the results of the study, the first strain of the three isolated ones had the highest virulence, where LD50 was 0.5129 billion microbial cells.

Sequencing of the genetic material of the studied sample confirmed the presence of *Streptococcus equi* and also showed the relation of circulating variants to a group of microorganisms causing epizootic outbreaks in Russia and China. According to the results of a comparative analysis of conservative sites, the bacteria *S. dysgalactiae* and *S. canis* were the closest to *S. equi*. The results of the genetic identification can be used as a molecular biological characteristic of the strain.

The data obtained are important for the development and optimization of measures to control and prevent strangles, including early diagnosis, treatment of infected animals and prevention of the spread of the disease. An integrated approach to infection monitoring and control will help reduce economic losses and increase the productivity of horse breeding farms, providing the population with high-quality and stable horse breeding products.

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Authors' Contribution

ZU, SA, and SN – coordination of material collection and work in the field; DK and BY and NS – coordination of manuscript preparation; SA, BY, and ZU - DNA extraction and sequencing; AS, SN, and NS - data processing and analysis and manuscript preparation; ZU, SA, AS, SN, BY, and NS – project supervision. All authors have read and agreed to the published version of the manuscript.

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