P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2024.155

# Isolation and Characterization of Bacteriophage *Streptococcus equi* for Application against Horse Strangles

Makhpal Sarmykova[1], Bolat Yespembetov[1], Aidar Sambetbayev[2], Kali Tileukhanov[1], Andrey Kaldyrkaev[3], Andrey Shestakov[3], Aibarys Melisbek[4], Yerbol Burashev[4], Bekbolat Usserbayev[4] and Nazym Syrym[1*]

[1]Laboratory of Microbiology, Research Institute for Biological Safety Problems, Gvardeysky, Kazakhstan
[2]Laboratory of Microbiology, Kazakh National Agrarian Research University, Almaty, Kazakhstan
[3]Department of Microbiology, P.A. Stolypin Ulyanovsk State Agrarian University, Ulyanovsk, Russia
[4]Laboratory for Collective Use, Research Institute for Biological Safety Problems, Gvardeysky, Kazakhstan
***Corresponding author:** nazym_syrym@gmx.com

**Article History:** 23-327 Received: 14-Nov-23 Revised: 10-Feb-24 Accepted: 18-Feb-24

## ABSTRACT

Horse strangles is an acute infectious disease that manifests itself sporadically, enzootically, and epizootically and is widespread in Kazakhstan, where it mainly affects foals and young horses. The causative agent of strangles is the bacterium *Streptococcus equi*. In conditions of global antibiotic resistance, bacteriophages can become an effective means of treating horse stranglers. The research aim is to develop a new phage therapy against horse strangles using a local test strain of *Streptococcus equi*, namely bio-material isolated from a foal with strangles. When working with bacteriophages, the following methods were used: the Appelman (serial dilutions) and Grazia (agar layers) methods were used the isolation of bacteriophages and nutrient media GRM agar and GRM broth were used for the cultivation of bacteria and their phages. As a result, 6 bacteriophages were isolated from the studied 117 samples from the collected samples, after studying their basic biological properties, it was decided in further work to select the 1 most active bacteriophage specific to *Streptococcus equi*. This 1 bacteriophage was sequenced and registered in the Streptococcus phage BV0002 GenBank "Bank It2666451 Bacteriophagum OQ319049" (https://www.ncbi.nlm.nih.gov/nuccore/OQ319049). Genome-wide sequencing was performed on an automatic genome-wide sequencer Ion Torrent (Thermo Fisher Scientific, using the Ion XpressPlus Fragment Library Kit (Waltham, MA, USA). As a result, taking into account the highest percentage of identity of the analysed sequence in the international database using the BLAST algorithm, as well as the results of phylogenetic analysis, it was found that the selected bacteriophage belongs to the family *Myoviridae*, characteristic of the species *Bacteriophagum Streptococcus equi*.

**Key words:** Horse strangles; Bacteriophage; Biological material; Test strains; *Streptococcus equi*; Environmental objects

## INTRODUCTION

Strangles is an infectious, contagious disease of Equidae characterized by abscessation of the lymphoid tissue of the upper respiratory tract. The causative organism, *Streptococcus equi*, is highly host-adapted and produces clinical disease only in horses, donkeys and mules. It is a gram-positive, capsulated β-hemolytic Lancefield group C coccus, which is an obligate parasite and a primary pathogen (Duran and Goehring 2021; Abdisa 2018; Anonymous 2021; Waśniewska-Włodarczyk et al. 2022).

Horse strangles is still an unsolved problem in veterinary practice. The economic damage caused by this disease consists of a lag in the growth and development of sick animals together with decreases in the fatness and mortality of young horses, funds spent on treatment, and the organization of economic measures aimed at combating this disease (Rendle et al. 2021; Boyle et al. 2017, 2018; Swine Health Information Center 2021).

Veterinary science has developed throughout the years and an effective method of preventing horse strangles using an inactivated vaccine together with an immunomodulator has been proposed. The disease incidence, meanwhile, is also increasing (Robinson et al. 2018; European Medicines Agency 2021; Frosth et al. 2023; Mitchell et al. 2021).

**Cite This Article as:** Sarmykova M, Yespembetov B, Sambetbayev A, Tileukhanov K, Kaldyrkaev A, Shestakov A, Melisbek A, Burashev Y, Usserbayev B and Syrym N, 2024. Isolation and characterization of bacteriophage *Streptococcus equi* for application against horse strangles. International Journal of Veterinary Science x(x): xxxx. https://doi.org/10.47278/journal.ijvs/2024.155

Of no small importance regarding the spread of horse strangles is the lack of effective treatments. The atypical form of the flow of horse strangles is extremely difficult to detect even in stationary conditions (Pringle et al. 2020; Dong et al. 2019; Morris et al. 2021; Rzabayev et al. 2022). Veterinary science and practice have established that when strangles affects horses, the existing general measures of prevention and treatment with antibiotics do not produce the desired result (British Equine Veterinary Association 2023; Jaramillo-Morales et al. 2022; Safonov 2022; Nuriyanto et al. 2019; World Health Organization 2019).

The irrational use of the latter has led to the emergence of multi-resistant, mutated strains of (*Streptococcus equi*). Passivating among non-immune livestock, these mutated strains have become stable. In cases where horses have been infected with these strains, an atypical clinical picture is observed for strangles - the absence of swelling of the submandibular lymph node with a high mortality rate of animals, reaching 55–60% (Khairullah et al. 2022; Akimbekov et al. 2023; Rotinsulu et al. 2023; Morris et al. 2022).

In this regard, the development of therapeutic agents against horse strangles that meet the needs of practice is relevant. For this purpose, new alternative approaches in therapeutic measures are required, namely the use of specific phage therapy in relation to *Streptococcus equi*. Bacteriophages have the ability to penetrate bacterial cells, reproduce in them and cause their lysis (Möller and Wöckener 2020; Domingo-Calap and Delgado-Martínez 2018; Jaramillo-Morales et al. 2023; Harvey et al. 2022).

According to numerous examples of data in the literature, phages can be used as natural antimicrobial agents to fight bacterial infections in humans, animals, and crops. The use of phage therapy in the food and agricultural industries will allow significantly reduce antibiotic use globally. Bacteriophages are modern antimicrobial drugs of natural origin. These microorganisms are capable of spot-destroying only pathogenic bacteria. Their action does not affect the beneficial microflora of the body, unlike antibiotics. They are compatible with all medications. The use of bacteriophages does not restrict the use of other drugs and does not affect their effectiveness (Tetz et al. 2017; Yespembetov et al. 2019; Ibraimova 2019; Ussenbekov et al. 2022; Yespembetov et al. 2022).

In recent years, a number of authors, in order to treat various kinds of microbial infections, such as dysentery, streptococcal, wound and other infections, recommend the use of bacteriophage preparations. The activity of therapeutic and prophylactic bacteriophages in infectious diseases of the digestive and respiratory system, musculoskeletal system, etc. is quite high - from 72 to 90%. This is often the only effective treatment. Phage therapy can also be used prophylactically to combat the spread of an infectious disease where the source is identified at an early stage (Issabekov et al. 2022; Li et al. 2021; Federal Research Center Institute 2020).

In connection with the above, improving the therapy of equine suffocation by introducing new methods of treatment using bacteriophages isolated on the territory of the Republic of Kazakhstan against epizootic strains of *Streptococcus equi*, is an urgent task and is of great interest for veterinary laboratory practice.

The novelty lies in the fact that for the first time in the Republic of Kazakhstan, a biopreparation for therapy against of horse strangles was developed.

The aim of this research is: 1) to isolate a bacteriophage specific to the strain *Streptococcus equi* for application the therapy of horse strangles; 2) to study their basic biological characteristics; 3) analysis of bacteriophage proteomes; 4) Conducting genome -wide sequencing of the bacteriophage *Streptococcus equi*.

The work was carried out in 2020-2022 within the framework of the grant project "Obtaining a bacteriophage for the therapy of horse strangles."

## MATERIALS AND METHODS

The methods of Appelman (serial dilutions) and Grazia (agar layers) were used to isolate bacteriophages (Yespembetov et al. 2019). For the cultivation of bacteria and their phages, the nutrient media HFM (hydrolysate of fishmeal) agar and broth were used.

### Sample preparation

Samples of soil and manure weighing 100g were thoroughly ground in sterile porcelain mortars and poured into flasks with broth of HFM. In addition, 120mL (milliliter) of wastewater samples and wipes applied to the surfaces of environmental objects were introduced into flasks containing 30mL of 5× concentrated nutrient broth HFM (high fish meal). The flasks were placed in a thermostat to incubate for 3 days at a temperature of 37°C (*degrees Celsius*). Incubated samples were enriched daily with 1mL *Streptococcus equi* test cultures grown in HFM broth. The flasks were periodically stirred to improve the air exchange of the incubated samples. After the incubation period, samples were poured into centrifuge tubes, centrifuged at 2500rpm for 20min (minutes) and then filtered using "Sterile Filtration System" sterilizing filters (with a pore size of 0.2μm (Micrometer) cellulose nitrate and capacity of 115mL). The filtrate obtained in this way was examined for the presence of *Streptococcus equi* bacteriophages. A total of 117 samples were examined.

### Determination of the lytic activity of bacteriophages

The lytic activity of the selected bacteriophages was determined using the Appelman and Grazia methods. The Appelman method involves the determination of liquid media by establishing the maximum dilution of bacteriophages, which causes complete lysis of bacteria in the broth culture. The Grazia method involves titration of bacteriophage by counting of the number of negative colonies formed on a dense nutrient medium.

### Specificity of bacteriophages

The specificity of the isolated phages in three different types of bacteria was characterized according to their lytic ability by instilling one drop of phagolysate on freshly prepared lawns of the studied crops (Issabekov et al. 2022).

### Characteristics of isolated bacteriophages

To study various characteristics of isolated bacteriophages, the quantitative ratio and exposure time of the phage and bacteria sensitive to them during cultivation, the development of modes of centrifugation of

phagolysates, the study of the pH of the medium, the resistance of phages to the effects of chloroform and the morphology of phage negative colonies bacteriophages to *Streptococcus equi* used appropriate methods (Kovaleva et al. 2015; Ptashnik 2017b; Ibraimova 2019; Federal Research Center Institute 2020; Li et al. 2021; Issabekov et al. 2022).

Electrophoresis for the proteomic analysis of the bacteriophage of *Streptococcus equi* bacteria was conducted by using the Laemmli method (Tamura et al. 2021).

**Sequencing of bacteriophages**

To determine the nucleotide sequence of the bacteriophage *Streptococcus equi*, the Sanger cyclic sequencing method was used using the ABI PRISM Big DyeTM Terminator v.1.1 kit ("Applied Biosystems", USA) in accordance with the manufacturer's instructions.

A set of nucleotide sequences from the GenBank International Database (NCBI) was used to determine the genotype.

Alignment of the nucleotide sequences of the conservative hyaluronidase gene (hyaluronidase) was performed using the Mega 11 program with the following parameters: Statistical Method—maximum likelihood; Test of Phylogeny—Bootstrap method; No. of Bootstrap Replicas—1000; Model/Method—Kimura 2-parameter model.

The calculation of genetic distances was performed using the Mega 11 computer program (Ptashnik 2017a; Khromov-Borisov 2015).

Statistical analysis of the research results was carried out using Microsoft Excel and GraphPad Prism 8 (Bureau of National Statistics 2023).

## RESULTS

To isolate bacteriophages, a total of 117 samples of biomaterial with suffocating foals were selected in horse breeding farms in Almaty region: 31 from the Samat farm in Enbekshikazakh district, 52 from the Sarsenbek farm and two private farmsteads in Talgar district and 34 from the Torezhan farm in Zhambyl district.

**Determination of the lytic activity of bacteriophages**

As a result of the conducted studies, six bacteriophages were isolated that were typically active against *Streptococcus equi*. The lytic activity of isolated bacteriophages was determined using the methods of Appelman and Grazia.

As can be seen from Table 1, as a result of the studies conducted against *Streptococcus equi* bacteria, six bacteriophages were isolated. We found that the studied phages caused lysis of the tested cultures. The lytic activity of phages according to Appelman was in the range of $10^5$ to $10^8$, and according to Grazia, there were $2 \times 10^8$ to $9 \times 10^8$ bodies in 1mL.

The results showed that bacteriophages had the least lytic activity: No. 10, having 2 x$10^8$ phage particles, then No. 11 – 3 ×$10^8$, No. 5 and 16 – 4×$10^8$, respectively, bacteriophage No. 6 – 5 × $10^8$. Bacteriophage No. 1, which had 9 x$10^8$ phage particles, had the greatest lytic activity.

The specificity of the isolated phages for each of three bacterial species was studied based on their lytic ability by instilling one drop of phagolysate on freshly prepared lawns of the studied bacterial cultures (Table 2).

The results presented in Table 2 show that the studied bacteriophages are strictly specific to *Streptococcus equi* and not to Escherichia coli or Staphylococcus aureus. The results of the study of the main biological properties of the strangles phage are shown in Table 3. As can be seen from Table 3, as a result of the conducted studies, it was found that the average optimal ratio of bacteriophage and sensitive bacteria is 1:2, i.e., 0.2mL of phage to 0.4mL of *Streptococcus equi* test culture, and the exposure time of 24 h was selected. The specified ratios and time do not affect the quality of phagolysates and, at the same time, do not change the technological modes of operation with phagolysates.

The investigation of revealed that the most optimal centrifugation mode is 30min exposure at 3000 rpm, which can also be used for volumes exceeding 6 L. It was found that the five isolated *Streptococcus equi* bacteriophages (phages Nos. 5, 6, 10, 11 and 16) optimally induced bacterial lysis at pH values of 5.5, 6.0, 8.0, 8.2, and 8.5, respectively. When the pH value of the culture medium was 7.0, bacterial lysis occurred in the case of only one bacteriophage.

All of the studied phages were found to be resistant to chloroform.

Regarding temperature stability studies, we found that warming up phages for 30min at 60°C did not affect their activity. A further increase in temperature to 65–75°C led to a loss of phage activity, and a temperature of 92–95°C caused complete phage inactivation. At the same time, bacteriophage No. 10 had the lowest lytic activity, and bacteriophage No. 1, which contained 9 - $10^8$ phage bodies, had the highest activity.

As a result of the obtained data, it was established during the titer of the studied bacteriophages did not change during the development of phagolysate filtration modes. After 48 h of thermosetting, the phagolysate to which *Streptococcus equi* cultures were added and not filtered became cloudy. Other phagolysates remained transparent. No growth was detected on the GRM agar, which indicates the absence of viable cells in the filtered phagolysate. No filtered cell phagolysate was detected by microscopy.

**Morphology of negative colonies of isolated bacteriophages**

The morphology of negative colonies of isolated bacteriophages to *Streptococcus equi* was determined by electron microscopy (Fig. 1). As can be seen from Fig. 1, *Streptococcus equi* bacteriophages Nos. 1 and 6 have a diameter of 3–4nm. Negative colonies of bacteriophage Nos. 5, 10, and 16 have a diameter of 1–4nm. Negative colonies of bacteriophage No. 11 have a diameter of 1.5nm and are completely transparent.

As a result, electron microscopy of the bacteriophages detected in the samples revealed that the majority of phage particles have a basal plate and a fibril. The caudal process is connected to the head by means of a characteristic structural complex, clearly visible in the shadow images. Phages attach to the bacterial cell wall by the terminal filaments of the processes, the bacterial shell then dissolves with the help of the enzyme lysozyme, the protein cover of the tail process is reduced, and nucleic

**Table 1:** Bacteriophages isolated from farms of the Almaty region and the study of their main characteristics

| *Streptococcus equi* Phages | Sources of Phage Isolation | Activity of Bacteriophages According to Appelman | Activity the Bacteriophages According to Grazia (Number of Phage Particles in 1mL) |
|---|---|---|---|
| *Streptococcus equi* phage - 1 | Water from drinkers for stable maintenance of horses | $10^8$ | $9 \times 10^8$ |
| *Streptococcus equi* phage - 5 | Manure from places where horses are kept in herds | $10^5$ | $4 \times 10^8$ |
| *Streptococcus equi* phage - 6 | Manure from places where horses are kept in herds | $10^5$ | $5 \times 10^8$ |
| *Streptococcus equi* phage - 10 | Soil near the places where horses are kept in herds | $10^7$ | $2 \times 10^8$ |
| *Streptococcus equi* phage - 11 | Soil near the places where horses are kept in herds | $10^5$ | $3 \times 10^8$ |
| *Streptococcus equi* phage - 16 | Flushing from the nasal cavity | $10^8$ | $4 \times 10^8$ |

**Table 2:** Specificity of *Streptococcus equi* bacteriophages for different types of bacteria.

| Types of Bacteriophages | Types of Bacteria | | | |
|---|---|---|---|---|
| | *Streptococcus equi* | *Streptococcus aureus* | *Escherichia coli* | Phage activity control |
| *Streptococcus equi* phage - 1 | + | - | - | - |
| *Streptococcus equi* phage - 5 | + | - | - | - |
| *Streptococcus equi* phage - 6 | + | - | - | - |
| *Streptococcus equi* phage - 10 | + | - | - | - |
| *Streptococcus equi* phage - 11 | + | - | - | - |
| *Streptococcus equi* phage - 16 | + | - | - | - |

Notes: "-" absence of lysis; "+" lysis.

**Table 3:** General characteristics of *Streptococcus equi* bacteriophages.

| Isolated Phages of *Streptococcus Equi* | Characteristics of *Streptococcus Equi* Bacteriophages | | | | |
|---|---|---|---|---|---|
| | Phage–Culture Ratio, Cultivation Exposure Time (in Hours) | Centrifuge Mode of Operation 30 rpm | pH of the Medium | Exposure to Chloroform at 1:10 for 10–40min | Effects of Temperature at 60°C |
| 1 | 1:2–24 | 3000 | 7.0 | + | $9 \times 10^8$ |
| 5 | 1:2–24 | 2000 | 5.5 | + | $4 \times 10^7$ |
| 6 | 1:2–24 | 3000 | 6.0 | + | $7 \times 10^6$ |
| 10 | 1:2–24 | 2000 | 8.0 | + | $2 \times 10^4$ |
| 11 | 1:2–24 | 2500 | 8.2 | + | $3 \times 10^8$ |
| 16 | 1:2–24 | 3000 | 8.5 | + | $6 \times 10^7$ |

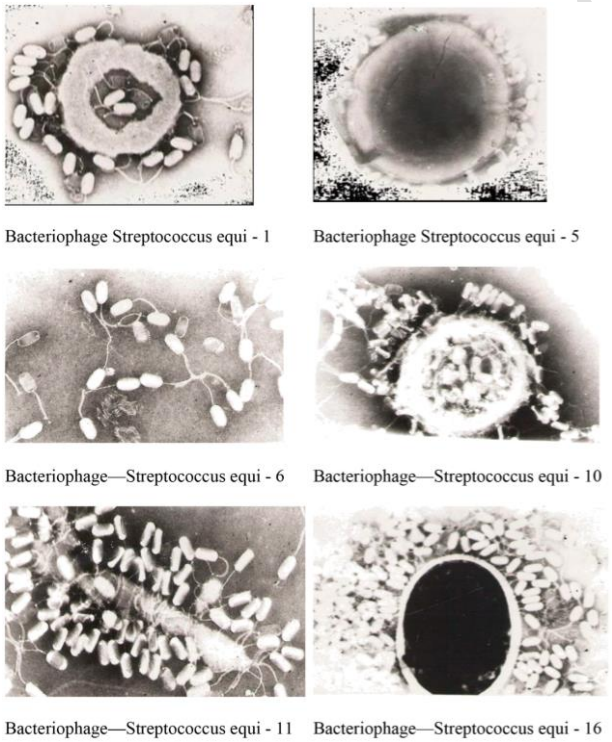



**Fig. 1:** Morphology of negative colonies of isolated bacteriophages.

acid is injected into the cyto-plasm of the cell through the channel of the tail process. The phage nucleic acid penetrates the cell by injection, while the phage shell remains on the surface of the bacterial cell. All isolated phages have an oval-shaped capsid.

Classification of Siphoviridae. The virion of *Streptococcus equi* phages consists of capsids with a rounded head with a diameter of 70nm and a long caudal process with a length of 190nm. The head is connected to the tail by means of the same structural com-plex that is characteristic of *Streptococcus equi* bacteriophages.

Thus, the following main biological properties were identified and studied: the exposure time of bacteriophages to the bacteria sensitive to them, the development of modes of centrifugation of phagolysates, the medium pH, chloroform resistance, the development of phagolysate filtration modes, the quantitative ratio of phage and culture during cultivation, and the morphology of negative colonies of isolated bacteriophages for *Streptococcus equi*.

For further investigation, the bacteriophage *Streptococcus equi* - 1 with a pH value of the cultivation medium equal to 7.0 was selected based on the results chloroform exposure and the activity parameter according to Grazia after exposure to a temperature of 60°C equal to $9 \times 10^8$.

**Analysis of the bacteriophage proteome by laemmli**

As a result of studies of the proteome of the bacteriophage *Streptococcus equi* by Laemmli using methods of purification and concentration of phage particles (ultracentrifugation in density gradients of cesium chloride and sucrose), the following results were obtained:

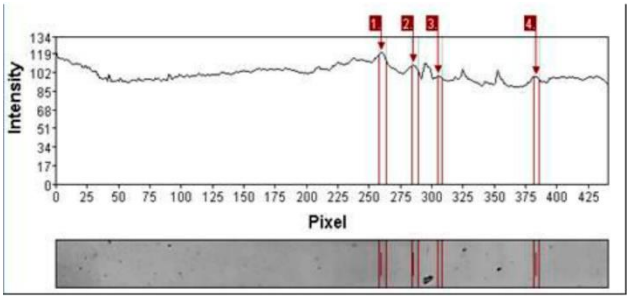



**Fig. 2:** Electropherogram of the bacteriophage proteome: (a) lanes 1–2 represent the cesium chloride gradient method for concentration and purification; (b) lanes 3–4 represent the sucrose gradient method for concentration and purification; lane 5 is the molecular weight marker 1 and the last lanes are the molecular weight marker.

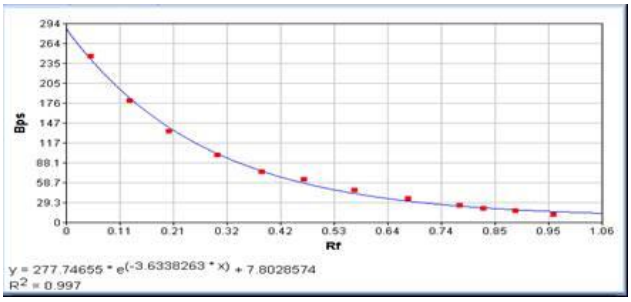



**Fig. 3:** Calibration curve of the molecular weight marker.

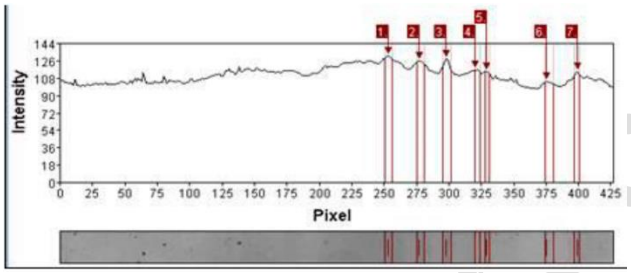
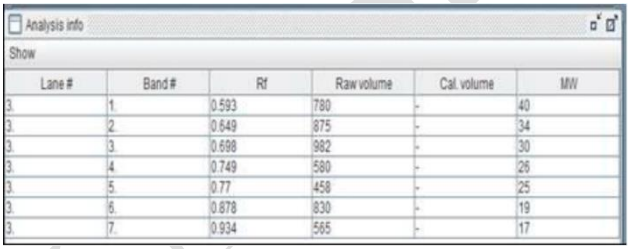



**Fig. 4:** Electropherogram—determination of molecular weights of the proteome of the bacteriophage *Streptococcus equi.*


Analysis info

Show

| Lane # | Band # | Rf | Raw volume | Cal. volume | MW |
|---|---|---|---|---|---|
| 3. | 1. | 0.593 | 780 | - | 40 |
| 3. | 2. | 0.649 | 875 | - | 34 |
| 3. | 3. | 0.698 | 982 | - | 30 |
| 3. | 4. | 0.749 | 580 | - | 26 |
| 3. | 5. | 0.77 | 458 | - | 25 |
| 3. | 6. | 0.878 | 830 | - | 19 |
| 3. | 7. | 0.934 | 565 | - | 17 |

**Fig. 5:** Structure of the molecular weight distribution of the proteome of the bacteriophage *Streptococcus equi.*

1) When using cesium chloride, four capsid proteins with molecular weights of 20, 30, 34 and 40 kDa were determined (Fig. 2 and 3) When using the sucrose gradient, seven capsid proteins with molecular weights of 17, 19, 25, 26, 30, 34, and 40 kDa were determined (Fig. 4 and 5).

Thus, the sucrose gradient method was the most accessible and resulted in the highest product yield. Based on this, for further in silico analysis via NCBI, we adopted the following proteome structure for the bacteriophage *Streptococcus equi* nucleocapsid composition: 17, 19, 25, 26, 30, 34, 40 kDa.

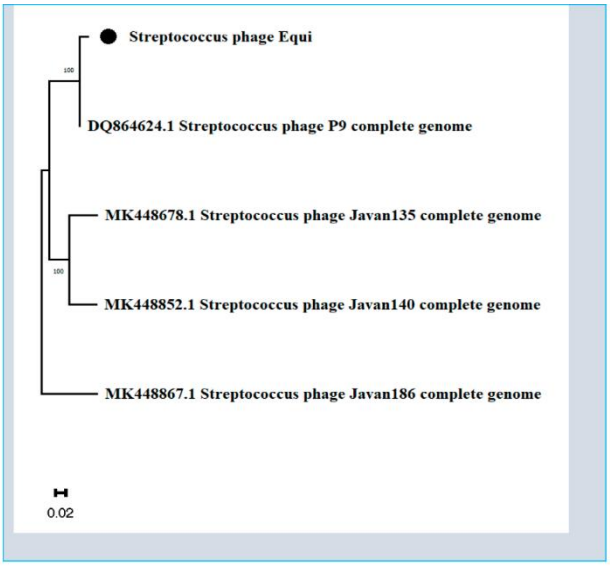



**Fig. 6:** Phylogenetic tree based on the analysis of a gene fragment of the sample of "Bacteriophagum *Streptococcus equi*/ NIIPBB/BV-0002".

**Sequencing of bacteriophages and bioinformatic data analysis: development of primers for identification of bacteriophages in the external environment**

To determine the nucleotide sequence of the bacteriophage "Bacteriophagum Str. equi/NIIPBB/BV-0002", the lysate was run through a 0.45-micron filter and then concentrated and ultra-centrifuged at 70,000 ob/min. DNA was isolated using the QIAGEN QIAamp®Viral RNAmini Kit (50) (Table 4). Next, we built a phylogenetic tree in the MEGA 11 program (Fig. 6). As can be seen in Table 6, the sample of "Bacteriophagum *Streptococcus equi RIBSP*/BV-0002" is located on the same branch with a representative of Bacteriophagum.

Taking into account the highest percentage of identity of the analyzed sequence in the international database using the BLAST algorithm, as well as the results of phylogenetic analysis, it was found that the sample of "Bacteriophagum *Streptococcus equi*/ *RIBSP* /BV-0002" belongs to the family Myoviridae, part of the species Bacteriophagum *Streptococcus equi*. Next, primers were designed to identify the bacteriophage *Streptococcus equi* using the Pick Primer (BLAST) program and the following steps.

(1) Choose a suitable organism.

(2) Set certain parameters for PCR (primer annealing temperature).

(3) Design one pair of primers for the conservative hyaluronidase gene (hyaluronidase).

## DISCUSSION

In recent years, a decision has been taken to increase the number of horses in Kazakhstan. To achieve this goal, an increase in the number of cases of horse strangulation has a negative impact not only on foals but also on adult livestock (Jung and Möller 2022).

Strangles is a contagious purulent disease of the upper respiratory tract of horses, caused by the bacterium *Streptococcus equi*, which penetrates through the mucous membrane of the nose and mouth, affecting the lymph nodes, where abscesses are caused (Young 2020; DeNotta and House 2023).

**Table 4:** Results of identification by analysis of the nucleotide sequence of the Hylauronidase (hylauronidase) gene of the strain "Bacteriophagum *Streptococcus equi*/NIIPBB/BV-0002".

| Name of the strain | The nucleotide sequence of the gene responsible for the protein Hyaluronidase (hyaluronidase) | Identification of nucleotide sequences in an international database (http://www.ncbi.nlm.nih.gov /) the BLAST algorithm | | |
|---|---|---|---|---|
| | | Inventory Genbank number (Access number) | Name of the strain | % matches |
| "*Bacterio-phagum Str. equi / NIIPBB/BV-0002*" | ATGTCAGAGACTATATCAGCTATAGTTGTGCATAAA<br>AGCATGACAAAAAACGAGTGGGAGTCTAGTGACA<br>TCATTTTGCCACAAGGCCAGCTCGTCTATGAGTCTG<br>ACACAGGCCATAGTAAATTTGGCGACGGTAAAAA<br>TAGATATGCAGATTTGATCTATCAAGGTGGGCCACC<br>AGGTCCCCAAGGCCCACCAGGTAAAACAGGGGAG<br>CAGGGCCCGCCAGGCCCTGCTGGCAAGCCTGGAAC<br>GACAGATTATAATCAACTCCAAAATAAACCAAATC<br>TAGATGCGTTTGCACAAAAAAAAGAAACTGATAGT<br>AAAATCGCCAAATTAGTATCAAGCAAAGCAGATAA<br>AAGCGCTGTTTACTTAAAAGCAGAGTCAAAAATAG<br>AGCTAGACAAAAAATTGAGTTTGACAGGTGGCGTT<br>ATGACAGGCCAACTAAAATTTAAGCCAGCCGCCAC<br>TGTTGCTTATTCCTCGTCAACGGGTGGAGCGGTCA<br>ATATTGACTTGTCGTCTAGCAGAGGTGCTGGTGTTG<br>TTGTCTATTCTAACAATGATACCAGTGATGGGCC<br>GTTAATGAGCTTGCGGACGGGTAAAGAGACCTTCA<br>ATCAATCGGCGCTTTTTGTCGATTATAAGGGAACA<br>ACAAATGCCGTTAATATTGCGATGCGTCAGCCAACC<br>ACCCCCAATTTTTCATCGGCGCTTAATATTACTA<br>GCGGCAATGAAAATGGTAGTGCGATGCAAATTAGA<br>GGCGTTGAAAAAGCATTGGGAACGCTCAAAATCAC<br>GCACGAAAACCCAAACGTTAAGGCAAGTTACGATA<br>AAAACGCTGCAGCGTTATCTATTGATATTGTCAAA<br>AAGGCAAACGGTGTAGGAACAGCCGCTCAGGGAAT<br>CTACATTAACTCAACCTCAGGCACAACTGGTAAAA<br>TGCTCAGAATCAGAAACCTTAATGATGATAAGTTCT<br>ACGTCAAGCCTGACGGTGGTTTTTATGCCAAGGA<br>AACTTCGCAGATTGATGGCAACCTGAAACTCAAGG<br>ATCCCATAGCGAATGATCATGCGGCAACCAAAGCT<br>TATGTTGATGGTGAAATCAAAAAAATAAAAGCACT<br>CTTAACGGCTAAGTAA | OQ319049 | *Streptoc occus equi* | 98 |

**Table 5:** Amino acid sequence of hyaluronidase protein (hyaluronidase) strain "Bacteriophagum *Streptococcus equi*/NIIPBB/BV-0002".

| Name of Strain | Amino Acid Sequence of the Gene Responsible for the Protein Hyaluronidase (Hyaluronidase) | Identification of Nucleotide Sequences in an International Database BLAST Algorithm | | |
|---|---|---|---|---|
| | | Inventory GenBank Number (Access Number) | Name of Strain | % Matches |
| *Bacteriophagum Streptococcus equi RIBSP BV-0002* | MSETISAIVVHKSMTKNEWESSDIILPQGQLVYESDTGHSKFGD<br>GKNRYADLIYQGGPPGPQGPPGKTGEQGPPGPAGKPGTTDYNQ<br>LQNKPNLDAFAQKKE<br>TDSKIAKLVSSKADKSAVYLKAESKIELDKKLSLTGGVMTGQL<br>KFKPAATVAYSSSTG<br>GAVNIDLSSSRGAGVVVYSNNDTSDGPLMSLRTGKETFNQSAL<br>FVDYKGTTNAVNIAM<br>RQPTTPNFSSALNITSGNENGSAMQIRGVEKALGTLKITHENPN<br>VKASYDKNAAALSI<br>DIVKKANGVGTAAQGIYINSTSGTTGKMLRIRNLNDDKFYVKP<br>DGGFYAKETSQIDGN<br>LKLKDPIANDHAATKAYVDGEIKKIKALLTAK | OQ319049 | *Streptococcus equi* | 100 |

Bacterial cells of the *Streptococcus equi* subspecies have a massive capsule that gives them the appearance of honeydew. The hyaluronic acid capsule is constitutively expressed and is an important virulence factor that protects bacteria from phagocytic destruction (Vyatka State University 2019; Penziner et al. 2021; Rao et al. 2021; Shikina et al. 2022).

As a result of the analysis of the spread of *Streptococcus equi* strains pathogenic to horses it was found that outbreaks of horse strangles periodically of the Zhambyl district of the Almaty region and the seasonality of the disease was revealed as corresponding to the early winter (November–December) and spring (March–April) periods.

It was found that when of horses strangles, the existing general measures of prevention and treatment with antibiotics do not give the desired result. The incidence is also increasing.

**Table 6:** Design of one pair of primers for the conservative gene hyaluronidase (hyaluronidase) *Streptococcus equi*.

| Sequence (5′->3′) | Template Strand | Length | Start | Stop | Tm | GC % | Self-Complementarity | Self - 3′ Complementarity |
|---|---|---|---|---|---|---|---|---|
| Forward primer GGCCAGCTCGTCTATGAGTC | Plus | 20 | 31,737 | 31,756 | 59.97 | 60.00 | 4.00 | 3.00 |
| Reverse primer GCGCCGATTGATTGAAGGTC | Minus | 20 | 32,259 | 32,240 | 59.97 | 55.00 | 4.00 | 1.00 |
| Product length 523 | | | | | | | | |

| Parameters | Forward primer | Reverse primer |
|---|---|---|
| Sequence (5′->3′) | GGCCAGCTCGTCTATGAGTC | GCGCCGATTGATTGAAGGTC |
| Template Strand | | |

Product length=523bp

Meanwhile, in recent years, a number of authors, in order to treat various kinds of microbial infections, such as dysentery, streptococcal, wound and other infections, recommend using various preparations of bacteriophages.

Based on the accumulated experience, they are increasingly convinced of the effectiveness of bacteriophage, because. in a number of cases, the superiority of the therapeutic efficacy of phages in comparison with antibiotics has been directly shown (Jung and Möller 2022). Perhaps because we were faced with the task of developing a preventive drug based on the bacteriophage *Streptococcus equi* against horse strangles.

When studying bacteriophages, their molecular organization is important, necessitating the analysis of phage proteomes and genomes. Currently, the main methods of analyzing bacterial and viral proteomes are based on the principles of their fractionation according to molecular weight, charge, density, etc. (Vyatka State University 2019; Andreeva 2019; Safonov et al. 2021; Zhu et al. 2022; Rao et al. 2023).

The main aspect of conducting research on bacteriophages is their concentration and purification from ballast proteins. In this regard, we used the Laemmli method, which involves electrophoretic separation of proteins in polyacrylamide gel depending on the molecular weight (Vyatka State University 2019; Andreeva 2019; Fang et al. 2020; Ventsova and Safonov 2021; Rao et al. 2023).

The molecular weight of proteins that are potential structural components of the bacteriophage *Streptococcus equi* capsid ranges 8 to 140kDa. The results obtained by us in comparison with foreign researchers by definition of the analysis of proteomes coincide. Thus, a biopreparation based on the bacteriophage *Streptococcus equi* was created for the therapy of strangles horse.

Comparison of the obtained results with similar results of domestic and foreign works gives grounds to assert that the manufactured therapeutic biopreparation based on virulent streptococcal phage will improve the of farms from the disease of strangles horse of the republic faster and more effectively.

## Conclusion

Environmental samples were collected by flushing them from the nasal cavity, mouth, and feces of foals from horse breeding farms to isolate *Streptococcus equi* bacteriophages. As a result, phages of six bacteriophages with respect to *Streptococcus equi* were isolated and characterized from the studied 117 samples from collected samples specific to the causative agent of horse strangles. As a result of studying the basic biological properties of bacteriophages, one virulent strain of bacteriophage of *Streptococcus equi* was selected from the isolated six bacteriophages. The proteome of the bacteriophage of Streptococcus bacteria was analyzed in silico and compared with analogs in NCBI, to search for virulent determinants.

As a result of studying the basic biological properties and proteome of bacteriophages from the isolated bacteriophage by Laemmli using sucrose gradient purification methods, one highly purified and virulent strain of bacteriophage of *Streptococcus equi* was selected, which had no virulent determinants.

Genome-wide sequencing was performed on an automatic genome-wide sequencer Ion Torrent (Thermo Fisher Scientific) using the Ion XpressPlus Fragment Library Kit (Thermo Fisher Scientific). As a result, taking into account the highest identity of the analyzed sequence in the international database using the BLAST algorithm, as well as the results of phylogenetic analysis, it was found that the sample "Bacteriophagum *Streptococcus equi*/NIIPBB/BV-0002" belongs to the family Myoviridae.

The technical and economic efficiency of the implementation of the research results will make it possible to obtain highly sensitive therapeutic biological products necessary for implementing measures against strangles.

## Funding

We express our gratitude to the Science Committee of theministry of Education and Science of the Republic of Kazakhstan for financing the grant project IRN AR 08855635, "Obtaining a bacteriophage for the therapy of horse strangles" 2020–2022.

## Institutional review board statement

The design of the article was carried out in compliance with the principles of scientific ethics, including maintaining high standards of intellectual integrity and preventing the fabrication of scientific data, falsification, plagiarism, false co-authorship, use by individual participants of collective research, data and conclusions obtained in research, without the consent of other participants, taking into account the declaration on ethical principles of scientific activity of the Antiparliamentary Assembly of the Commonwealth of Independent States.

## Conflicts of interest

This research has no conflict of interest.

## Acknowledgment

Not applicable.

## Authors' contribution

M.S., B.Y., A.S., K.T., A.K., A.S., A.M., Y.B., B.U., and N.S. contributed equally to the experimentation. M.S.,

B.Y., A.S., and K.T. wrote and edited the article. A.K., A.S., A.M., and Y.B. equally designed and conducted the experiment. B.U., and N.S. studied scientific literature about the topic. All authors read and approved the final manuscript.

## REFERENCES


Abdisa T, 2018. Systematic Review: Effect of Strangle on Health of Equine Animals and its Prevention. Current Trends in Biomedical Engineering & Biosciences 16(1): 1-10. http://dx.doi.org/10.19080/CTBEB.2018.16.555927

Akimbekov AR, Uskenov RB, Iskhan KZ, Assanbayev TS, Sharapatov TS and Baimukanov DA, 2023. Creation of smart farms in the herd horse breeding of Kazakhstan (results of using trackers). OnLine Journal of Biological Sciences 23(1): 44-49. https://doi.org/10.3844/ojbsci.2023.44.49

Andreeva AM, 2019. Prospects of proteom researches of water organisms (review). Transactions of IBIW (Institute of Biology of Inside Water) 8(91): 7-18. https://doi.org/10.24411/0320-3557-2019-10023

Anonymous 2021. All About Strangles (*Streptococcus equi*). Ballinger Equine. https://www.ballingerequine.com/all-about-strangles-streptococcus-equi/. Accessed 12 August 2023.

Boyle AG, Smith MA, Boston RC and Stefanovski D, 2017. A case-control study developing a model for predicting risk factors for high SeM-specific antibody titers after natural outbreaks of *Streptococcus equi* subsp *equi* infection in horses. Journal of the American Veterinary Medical Association 250(12): 1432–1439. https://doi.org/10.2460/javma.250.12.1432

Boyle AG, Timoney JF, Newton JR, Hines MT, Waller AS and Buchanan BR, 2018. *Streptococcus equi* infections in horses: guidelines for treatment, control, and prevention of strangles—revised consensus statement. Journal of Veterinary Internal Medicine 32(2): 633-647. https://doi.org/10.1111/jvim.15043

British Equine Veterinary Association, 2023. Practice Policy: Dose and Routes of Administration of Common Antimicrobials (Protect Me). https://www.beva.org.uk/Protect-Me (accessed 12 August 2023).

Bureau of National Statistics, 2023. Agency for strategic planning and reforms of the Republic of Kazakhstan. https://www.stat.gov.kz/official/industry (accessed 12 August 2023).

DeNotta S and House A, 2023. Equine Strangles: Management and Prevention: VM172/VM134, Rev. 2/2023. EDIS (Emergency Department information systems) 1: 1-3. https://doi.org/10.32473/edis-vm134-2008

Domingo-Calap P and Delgado-Martínez J, 2018. Bacteriophages: Protagonists of a post-antibiotic era. Antibiotics 7(3): 66. https://doi.org/10.3390/antibiotics7030066

Dong J, Gao N, Waller AS, Cook FR, Fan S, Yuan D, Du Y, Li F, Norimine J and Zhu W, 2019. An outbreak of strangles associated with a novel genotype of *Streptococcus equi* subspecies equi in donkeys in China during 2018. Equine Veterinary Journal 51(6): 743–748. https://doi.org/10.1111/evj.13114

Duran MC and Goehring SL, 2021. Equine strangles: An update on disease control and prevention. Austral Journal of Veterinary Sciences 53(1): 23-31.

European Medicines Agency, 2021. CVMP Assessment Report for Strangvac (EMEA/V/C/005309/0000): Vaccine Common Name: *Streptococcus equi* Vaccine (Recombinant Proteins). https://www.ema.europa.eu/en/documents/assessment-report/strangvac-epar-public-assessment-report_en.pdf (accessed 12 August 2023).

Fang Q, Tang WC, Tao P, Mahalingam M, Fokine A, Rossman MG and Rao VB, 2020. Structural morphing in a symmetry mismatched viral vertex. Nature Communications 11: 1713. https://doi.org/10.1038/s41467-020-15575-4

Federal Research Center Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, 2020. Virus Concentration and Electron Microscopy. https://www.icgbio.ru/ (accessed 12 August 2023).

Frosth S, Morris ER, Wilson H, Frykberg L, Jacobsson K, Parkhill J, Flock J-I, Wood T, Guss B, Aanensen DM, Boyle AG, Riihimäki M, Cohen ND and Waller AS, 2023. Conservation of vaccine antigen sequences encoded by sequenced strains of *Streptococcus equi* subsp. equi. Equine Veterinary Journal 55(1): 92-101. https://doi.org/10.1111/evj.13552

Harvey AM, Ramp D and Mellor DJ, 2022. Review of the Foundational Knowledge Required for Assessing Horse Welfare. Animals 12(23): 3385. https://doi.org/10.3390/ani12233385

Ibraimova S, 2019. Not a single antibiotic: The "Killers" of bacteria are bacteriophages. https://www.klinikaexpert.ru/articles/10 (accessed 12 August 2023).

Issabekov SS, Syrym NS, Sambetbayev AA, Alikhanov KD and Yespembetov BA, 2022. Prospects of bacteriophage collections in disinfectant applications. Veterinary World 15(1): 220. https://doi.org/10.14202/vetworld.2022.220-231

Jaramillo-Morales C, Gomez DE, Renaud D and Arroyo LG, 2022. *Streptococcus equi* culture prevalence, associated risk factors and antimicrobial susceptibility in a horse population from Colombia. Journal of Equine Veterinary Science 111: 103890. https://doi.org/10.1016/j.jevs.2022.103890

Jaramillo-Morales C, James K, Barnum S, Vaala W, Chappell DE, Schneider C, Craig B, Bain F, Barnett DC, Gaughan E and Pusterla N, 2023. Voluntary Biosurveillance of *Streptococcus equi* Subsp. equi in Nasal Secretions of 9409 Equids with Upper Airway Infection in the USA. Veterinary Sciences 10(2): 78. https://doi.org/10.3390/vetsci10020078

Jung C and Möller S, 2022. Update on the diagnosis of strangles. https://laboklin.com/en/update-on-the-diagnosis-of-strangles/ (accessed 12 August 2023).

Khairullah AR, Sudjarwo SA, Effendi MH, Ramandinianto SC, Widodo A and Riwu KH, 2022. A review of horses as a source of spreading livestock-associated methicillin-resistant *Staphylococcus aureus* to human health. KHP. Veterinary World 15(8): 1906-1915.

Khromov-Borisov NN, 2015. Biostatistical programs of free access. Journal of Traumatology AND Orthopedics of Russia 21(4): 154-159.

Kovaleva EN, Zolotukhin SN and Vasiliev DA, 2015. Electron microscopic examination of enterococcal bacteriophages. Vestnik OrelGAU 2(53): 68-71.

Li P, Zhang Y, Yan F and Zhou X, 2021. Characteristics of a bacteriophage, vB_Kox_ZX8, isolated from clinical Klebsiella oxytoca and its therapeutic effect on mice bacteremia. Frontiers in Microbiology 12: 3628. https://doi.org/10.3389/fmicb.2021.763136

Mitchell C, Steward KF, Charbonneau ARL, Walsh S, Wilson H, Timoney JF and Waller AS, 2021. Globetrotting strangles: The unbridled national and international transmission of *Streptococcus equi* between horses. Microbial Genomics 7(3): 000528. https://doi.org/10.1099/mgen.0.000528

Möller S and Wöckener A, 2020. Druse as a "new" old problem in horse health - tips for diagnostics. Pferdespiegel 23(2): 82-85. https://doi.org/10.1055/a-1127-4095

Morris ERA, Boyle AG, Riihimäki M, Aspán A, Anis E, Hillhouse AE, Ivanov I, Bordin AI, Pringle J and Cohen ND, 2021. Differences in the genome, methylome and transcriptome do not differentiate isolates of *Streptococcus equi* subsp. equi from horses with acute clinical signs from isolates of inapparent carriers. PLoS ONE 16(6): e0252804. https://doi.org/10.1371/journal.pone.0252804

Morris ER, Wu J, Bordin AI, Lawhon SD and Cohen ND, 2022.

Differences in the Accessory Genomes and Methylomes of Strains of *Streptococcus equi* subsp. equi and of *Streptococcus equi* subsp. zooepidemicus Obtained from the Respiratory Tract of Horses from Texas. Microbiology Spectrum 10(1): e00764-21. https://doi.org/10.1128/spectrum.00764-21

Nuriyanto R, Uli E, Hardjopangarso S, Unang U, Kurniawan W and Prasetyo A, 2019. Indeks Obat Hewan Indonesia Edisi XII (Indonesian Veterinary Drug Index). 12th ed. Asosiasi Obat Hewan Indonesia, Jakarta, Indonesia, pp: 10-12.

Penziner S, Schooley RT and Pride DT, 2021. Animal models of phage therapy. Frontiers in Microbiology 12: 631794. https://doi.org/10.3389/fmicb.2021.631794

Pringle J, Venner M, Tscheschlok L, Waller AS and Riihimäki M, 2020. Markers of long term silent carriers of *Streptococcus equi* ssp. equi in horses. Journal of Veterinary Internal Medicine 34: 2751–2757. https://doi.org/10.1111/jvim.15939

Ptashnik O, 2017a. 12 Methods in Pictures: Nucleic Acid Sequencing. https://biomolecula.ru/articles/metody-v-kartinkakh-sekvenirovanie-nukleinovykh-kislot (accessed 12 August 2023).

Ptashnik O, 2017b. 12 Methods in Pictures: Proteomics. https://biomolecula.ru/articles/12-metodov-v-kartinkakh-proteomika (accessed 12 August 2023).

Rao VB, Fokine A, Fang Q and Shao Q, 2023. Bacteriophage T4 head: Structure, assembly and genome packaging. Viruses 15(2): 527. https://doi.org/10.3390/v15020527

Rao VB, Fokine A and Fang Q, 2021. The remarkable viral portal vertex: Structure and a plausible model for mechanism. Current Opinion in Virology 51: 65–73. https://doi.org/10.1016/j.coviro.2021.09.004

Rendle D, de Brauwere N, Hallowell G, Ivens P, McGlennon A, Newton R, White J and Waller A, 2021. *Streptococcus equi* infections: current best practice in the diagnosis and management of 'strangles'. UK-Vet Equine 5(Sup2): S3-15. https://doi.org/10.12968/ukve.2021.5.2.S.3

Robinson C, Frykberg L, Flock M, Guss B, Waller AS and Flock JI, 2018. Strangvac: A recombinant fusion protein vaccine that protects against strangles, caused by *Streptococcus equi*. Vaccine 36(11): 1484-1490. https://doi.org/10.1016/j.vaccine.2018.01.030

Rotinsulu DA, Ewers C, Kerner K, Amrozi A, Soejoedono RD, Semmler T and Bauerfeind R, 2023. Molecular Features and Antimicrobial Susceptibilities of *Streptococcus equi* ssp. *Equi* Isolates from Strangles Cases in Indonesia. Veterinary Sciences 10(1): 49. https://doi.org/10.3390/vetsci10010049

Rzabayev TS, Assanbayev TS, Rzabayev S, Bazargaliyev A and Rzabayev KS, 2022. Linebreeding as a system of stock breeding to improve the productive qualities of horses of the Kushum breed. Reproduction in Domestic Animals 57(12): 1584-1592. https://doi.org/10.1111/rda.14235

Safonov V, Ermakov V, Danilova V and Yakimenko V, 2021. Relationship between blood superoxide dismutase activity and zinc, copper, glutathione and metallothioneines concentrations in calves. Biomath 10(2): 2111247. https://doi.org/10.11145/j.biomath.2021.11.247

Safonov V, 2022. Dependence of antioxidant and biochemical status on selenium content in the blood of animals. Advances in Animal and Veterinary Sciences 10(2): 263-269. https://doi.org/10.17582/journal.aavs/2022/10.2.263.269

Shikina EV, Kovalevsky RA, Shirkovskaya AI and Toukach PV, 2022. Prospective bacterial and fungal sources of hyaluronic acid: A review. Computational and Structural Biotechnology Journal 20: 6214-6236. https://doi.org/10.1016/j.csbj.2022.11.013

Swine Health Information Center, 2021. *Streptococcus Equi* subsp. Zooepidemicus. https://www.swinehealth.org/wp-content/uploads/2021/03/S.-Zoo-Fact-Sheet.pdf (accessed 12 August 2023).

Tamura K, Stecher G and Kumar S, 2021. MEGA 11: Molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution 38(7): 3022-3027. https://doi.org/10.1093/molbev/msab120

Tetz GV, Ruggles KV, Zhou H, Heguy A, Tsirigos A and Tetz V, 2017. Bacteriophages as potential new mammalian pathogens. Scientific Reports 7(1): 7043. https://doi.org/10.1038/s41598-017-07278-6

Ussenbekov Y, Bagdat A, Bimenova Z, Orynkhanov K, Sobiech P, Samardžija M, Pareek CS and Dobos A, 2022. Identification of monomorphic and polymorphic genes associated with recessive fertility defects in Holstein cows reared in Kazakhstan. Veterinarski Arhiv 92(1): 27-35.

Ventsova I and Safonov V, 2021. Biochemical criteria for the development mechanisms of various reproduction disorders in dairy cows. Biodiversitas Journal of Biological Diversity 22(11): 4997-5002. https://doi.org/10.13057/biodiv/d221135

Vyatka State University, 2019. Protein Analysis by Electrophoresis in Polyacrylamide Gel under Denaturing Conditions (SDS-PAGE). https://e.vyatsu.ru/mod/page/view.php?id=39350 (accessed 12 August 2023).

Waśniewska-Włodarczyk A, Pepaś R, Janowicz R and Konopka W, 2022. *Streptococcus equi* subsp. *equi* in retropharyngeal abscess: Case report and review of literature. Microorganisms 10(10): 2032. https://doi.org/10.3390/microorganisms10102032

World Health Organization, 2019. Critically Important Antimicrobials for Human Medicine: Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non Human Use. 6th Revision 2018. World Health Organization, Geneva, Switzerland, pp: 9-11.

Yespembetov BA, Sarmykova MK and Sambetbayev AA, 2022. The structure of *Streptococcus equi* bacteriophages according to molecular microscopy data. Nature and Technical Science 6: 126-131.

Yespembetov BA, Syrym NS, Shestakov AG and Vasiliev DA, 2019. Monograph: Practical application of bacteriophages on the territory of the Republic of Kazakhstan. Nauka Ulyanovsk, Russia, pp: 12-20.

Young A, 2020. Strangles. https://ceh.vetmed.ucdavis.edu/health-topics/strangles (accessed 12 August 2023).

Zhu J, Ananthaswamy N, Jain S, Batra H, Tang WC and Rao VB, 2022. CRISPR Engineering of Bacteriophage T4 to Design Vaccines Against SARS-CoV-2 and Emerging Pathogens. In: Thomas S (ed), Vaccine Design. Methods in Molecular Biology, vol 2410. Humana, New York, NY, USA. pp: 209–228. https://doi.org/10.1007/978-1-0716-1884-4_10