Biological and molecular genetic properties of Streptococcus equi *isolated from pathological material of a foal*

Bolat A. Yespembetov[1], Nazym S. Syrym[1]*, Aidar A. Sambetbayev[2], Nadezhda N. Zinina[1], Makhpal K. Sarmykova[1], Abylai R. Sansyzbai[3], Saltanat T. Nussupova[3] and Samat I. Isabekov[2].

[1] *Laboratory of Microbiology, Research Institute for Biological Safety Problems, Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan, Guardeyskiy, Republic of Kazakhstan,*
[2] *Department of Veterinary and Sanitary Expertise and Hygiene, Kazakh National Agrarian Research University, Almaty, Republic of Kazakhstan,*
[3] *Research Institute of Veterinary Medicine, Pharmacy and Sanitation, Kazakh National Agrarian Research University, Almaty, Republic of Kazakhstan.*

* *Corresponding author at: Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan. E-mail: syrymnazyms@gmail.com*

Veterinaria Italiana 2023, **59** (3), x-xx doi: 10.12834/VetIt.2744.18370.2
Accepted: 07.07.2023 | Available on line: 31.12.2023

Keywords
Strangles,
Strain,
Antibiotic Resistance,
Therapy,
Horse Breeding.

Summary
Strangles is one of the main constraining factors in the development of horse breeding. However, the veterinary practice still does not have effective drugs designed for the prevention and treatment of this disease. To date, the development of new effective methods and means for specific prevention and therapy of strangles is of scientific interest and meet the needs of the present day. In this regard, in the summer of 2020, the staff of the microbiology laboratory of the Research Institute of Biological Safety Problems brought samples (pus, exudate of submandibular lymph nodes, a smear of the oral and nasal cavities, a smear of the mucous membrane of the eye) from a foal sick with strangles from the village of Akterek, Jambyl district of Almaty region, and the strain was isolated. The aim of this article is to study molecular biological and genetic properties of *Streptococcus equi* isolated from pathological material of a foal. Streptococci were isolated by the inoculation of samples of pathological material on GRM-broth (nutrient medium for the cultivation of microorganisms), blood and GRM agar with the addition of 1% glucose and 10% sterile horse serum (pH 7.4-7.6). The results of bacteriological and molecular genetic studies confirmed that the isolate belongs to the species *Streptococcus equi*.

Introduction

In the Republic of Kazakhstan (RK) horse breeding is a traditional branch of farming industry, which is currently developing in all major areas. According to statistics of the Ministry of Agriculture of the Republic of Kazakhstan, over the past 5 years the number of horses has increased by 350 thousand and at the beginning of 2020 amounted to more than 2.9 million units (Espembetov 2019). In Kazakhstan, strangles is the most widespread and economically damaging disease of horses' herds. At the same time, among horses, strangles disease accounts for up to 48% of possible nosological forms (Sansyzbayev 1993, Bizhanov *et al.* 1994, Kaiypbai 2003). Strangles is an acute infectious disease that proceeds with purulent-catarrhal inflammation of the mucous membrane in nose and pharynx, with suppuration of local lymph nodes. The disease manifests itself sporadically, enzootically, and epizootically and is widespread in Kazakhstan, mainly affecting foals and young horses. The causative agent of the strangles is the bacterium *Streptococcus equi* (*Str. equi)*. The source of the causative agent of infection are sick horses that secrete the pathogen with the contents of lymph node abscesses, with nasal discharge, coughing and snorting, which leads to contamination of feed, water, fomites, etc. (Isgren *et al.* 2022). The source of the causative agent of

Please refer to the forthcoming article as: Yespembetov *et al*. 2023. Biological and molecular genetic properties of the epizootic culture of *Streptococcus equi* isolated from pathological material of a foal. Vet Ital. doi: 10.12834/VetIt.2744.18370.2

infection may also be healthy horses (carriers). The carriage of beta-haemolytic streptococcus explains the spontaneous occurrence of the disease in farms. Many researchers have reported on the duration of the carriage of beta-haemolytic streptococcus for 4-10 months after the clinical illness. The transmission of beta-haemolytic streptococcus is possible by airborne, alimentary, contact routes, and during mating. Vectors and pathogens of strangles can also be blood-sucking insects. Foals can also become infected with streptococci from mothers with mastitis of glanderous aetiology. Intrauterine infection is possible, as a result of which abortions of mares are observed (Wittebole *et al.* 2014, Guliy *et al.* 2018, Pusterla *et al.* 2011).

The economic damage caused by this disease consists of a lag in the growth and development of sick animals, a decrease in the fatness, mortality of young horses, costs afforded for treatment, and the organisation of economic measures aimed at fighting this disease. The literature data on the epizootic situation of strangles indicate that the issue of studying the epizootology of the disease in horses is relevant (Jaramillo-Morales *et al.* 2022). At the same time, almost all researchers emphasise the dependence of the disease on natural and climatic conditions, horse breeding methods, the breed composition of livestock, the influence of external factors, conditions of maintenance and feeding, which contribute to a decrease in the natural resistance of the body and the manifestation of the pathogenic effect of the beta-haemolytic streptococcus. Strangles in horses is still an unsolved problem in veterinary practice, although veterinary science in different years has developed and proposed an effective method of preventing horse strangles with an inactivated vaccine with an immunomodulator. The incidence of the disease is also increasing, and there are many observations of strangles outbreaks with a relatively high lethality – up to 5%. Perhaps this indicator does not reflect the true epizootic situation. Of particular importance in the spread of strangles is the lack of effective treatment methods (Waller 2014, Antonov 1987). In Kazakhstan, due attention has not been given to the strangles in horses. Veterinary science and practice have established that with strangles in horses, the existing general measures of prevention and treatment with antibiotics do not give the desired result.

The Veterinary Department of the Ministry of Agriculture of Kazakhstan has significantly reduced the use of immunobiological drugs, including the antimycotic vaccine, since 2007. In the current situation, horse breeding farms of the country began to use antibiotics. The irrational use of them has led to the emergence of multi-resistant, mutated strains of *Str.equi*. among non-immune livestock, and these mutated strains have become stable (Newton *et al.* 2000, Yespembetov *et al.* 2020). In the case of infection with them in horses, atypical clinical aspects are observed, such as the absence of swelling of the submandibular lymph node with a high mortality rate of diseased reaching 55-60%.

The use of traditional methods of therapy does not give the desired effect, since cultural, morphological, biochemical, and, most importantly, virulent properties change due to mutations of the beta-haemolytic streptococcus (Lindahl 2013, Dzhetygenov 2016, Dzhetigenov *et al.* 2016). Based on the above, there is an urgent need to conduct comprehensive studies aimed at the diagnosis, prevention and treatment of beta-haemolytic infections, based on the study of certain biological characteristics of *Streptococcus equi* bacteria. The purpose of the study is to give a comprehensive assessment of some molecular biological and genetic properties of *Streptococcus equi* bacteria.

Materials and Methods

The object of research were samples of biological material collected in the form of pus, exudate of submandibular lymph nodes, smear of the oral and nasal cavities, and smear of the mucous membrane of the eye from a foal sick with strangles, delivered from a farm in the Almaty region. Streptococci were isolated by the inoculation of samples of pathological material on GRM-broth (a nutrient medium for cultivating microorganisms), blood and GRM agar with the addition of 1% glucose and 10% sterile horse serum (pH 7.4-7.6) (Kaiypbai 2003). The identification of the isolated *Streptococcus equi* culture was carried out by studying the biochemical properties – the release of saccharolytic, proteolytic enzymes, the generation of catalase, oxidase, ammonia, hydrogen sulphide, indole (Lindahl 2013, Dzhetygenov 2016). Antimicrobial effects were determined in relation to *Streptococcus equi* strains by diffusion of active substances from the test samples into a dense nutrient medium. The method is based on the diffusion of substances from paper disks into the agar and a change in the growth pattern of microorganisms (1 billion/cm^3) inoculated in agar around these disks. The result was recorded as the diameter of the bacterial lysis zone. Confirmation of the molecular genetic properties of the *Streptococcus equi* isolate was carried out using PCR analysis (polymerase chain reaction). Isolation of bacterial deoxyribonucleic acid (DNA) of the studied isolate was carried out using a commercial QIAGEN kit. Gene Amp PCR 9700 amplifier was used for amplification of isolated DNA (Dzhetigenov *et al.* 2016). To collect samples of biomaterial from foals with strangles to isolate bacterial cultures, a field

visit was organised and conducted to farms in the Almaty region, where outbreaks of strangles were observed. The purpose of the visit was the selection of biological material from horses with strangles. The destinations were Druzhba village, Enbekshikazakh district, Uzynagash village and Akterek village, Jambyl district of Almaty region. During the field visit, washings from the mouth, nose and eyes, purulent exudate from the submandibular lymph node from horses with signs of strangles were collected, to isolate bacteria from the above-mentioned areas of the Almaty region (Figure 1).

Figure 1. *Taking biomaterial for bacteriological and molecular biological analysis*

The collection of biological material from horses with strangles was carried out from 20/11/2020 to 22/11/2020 in the farm Nurlan-D of the Enbekshikazakh district, from 23/11/2020 to 24/11/2020 in the farm Zangar of the Jambyl district of the Almaty region. In the conditions of the farm, only 75 samples from horses with strangles were taken from the Nurlan-D farm (whole blood – 18; serum – 18, washings from the mouth and nose – 36; samples from the wound – 3). Thirty-one samples were also taken from the Zangar farm in the amount of 31 samples (washings from the mouth, nose, and eyes). Six samples from horses with strangles (washings from the mouth, nose and eyes, purulent exudate from the submandibular lymph node, whole blood and blood serum) were received from the individual entrepreneur "Ali". Purulent nasal and oral discharges for examination were taken with dry sterile cotton swabs by rotational movements along the lower nasal passage, removed along the outer wall of the nose. After taking the material, the tampon was placed in a sterile disposable tube with sterile distilled water. It was delivered to the laboratory no later than 6 hours at a temperature of 18-20 °C. Pus from abscesses was delivered in a sterile test tube in thermal containers.

Pus was taken with a syringe from non-opened lymph node abscesses after treatment of the puncture site. Pus was delivered to the laboratory of the Research Institute of Biological Safety Problems in a sterile test tube, if the abscesses was already open, then the pus was taken with a cotton swab moistened with a 25% aqueous solution of glycerin. The lymph nodes for the study were delivered both fresh, without preservatives, and preserved in glycerin, being delivered to the laboratory no later than 6 hours after sampling. According to the guidelines "Collection, transportation, storage of clinical material for PCR diagnostics", for conducting studies on blood for strangles by PCR, blood plasma was collected in a test tube with an anticoagulant 6% ethylenediaminetetraacetic acid in a ratio of 1:20. Na citrate 3.8% was also used as a preservative in a ratio of 1:9, and delivered to the laboratory in a special thermal container with cooling elements or in a thermos with ice within six hours (Figure 2).

Figure 2. *Blood sampling*

As a result of this stage, a total of 112 samples were collected of biomaterial for isolation from foals with strangles (Figure 2). Beta-haemolytic streptococcus can be grown in aerobic and anaerobic conditions at a temperature of 37 °C on all nutrient media. This study utilized a nutrient medium on substrates with the addition of horse serum with glucose and glucose-blood agar to isolate a pure culture.

The susceptibility of *Streptococcus equi* to antibiotics was determined using the agar diffusion method, also known as the paper disk method. This method is quite simple to perform, allows determining the susceptibility to several antibiotics at the same time. To determine the susceptibility of *Streptococcus equi* isolates to antibiotics, 1 ml of 1 bn. suspension was injected into a sterile Petri dish on a GRM agar (with 1% glucose), followed by the application of sterile discs made of filter paper impregnated with antibiotics at a distance of 2 cm from the edge of the cup and using nine consecutive dilutions (Figure 3).

Figure 3. *Conduction of experiments using the disk method*

After being kept in the thermostat at 37 °C for 18-24 hours, the results were expressed by the size of the absence of growth zone around the disks. The result was evaluated as stable (i.e. the microorganism is resistant to the action of the drug) when the zone of the absence of growth did not exceed up to 10 mm, (other classifications were: weakly susceptible – up to 14 mm, susceptible – 15-25 mm, highly susceptible – more than 25 mm). The isolates used to investigate drug resistance were from horses of the Druzhba village of the Enbekshikazakh district (culture No.1), from the Akterek village of the Jambyl district (culture No.2) and culture No.3 from the Uzynagash village of the Jambyl district.

Results and Discussion

As of July 1st, 2021, in Kazakhstan, compared with the same date in 2020, the number of horses increased by 8.5%, amounting to 3,372.5 animals. This is reported by the Statistics Committee of the Ministry of National Economy of the Republic of Kazakhstan (Agency 2021). The majority of horses (about 96%) are concentrated in the individual households of citizens and private horse breeding farms. According to the Statistics Committee of the Ministry of National Economy of the Republic of Kazakhstan, the number of horses in the republic amounts to 2,789,100 animal units. Visual data on the number of horses are shown in the Figure 4 and in Table I.

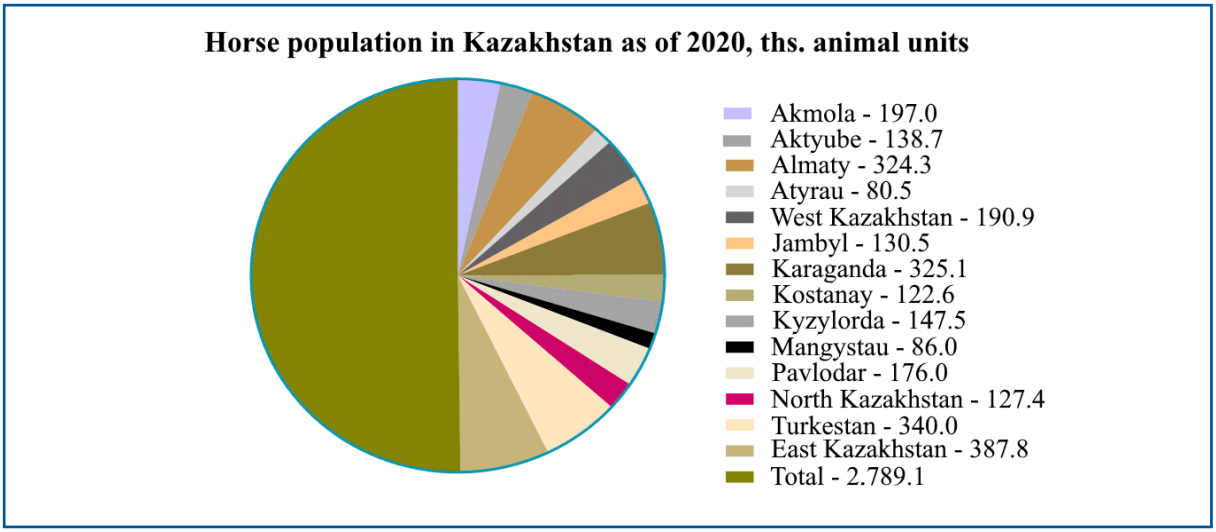

Figure 4. *The number of horses in the Republic of Kazakhstan*

Table I. *Dynamics of the number of horses in Kazakhstan in January-December 2020, ths. animal units*

Regions of the republic	January 1, 2020	June 1 2019	December 1 2019	The difference for 1 year (+ -)
Akmola	187.3	198.1	197.0	9.7
Aktobe	144.3	140.3	138.7	-5.6
Almaty	326.4	322.1	324.3	-2.1
Atyrau	83.8	82.2	80.5	-3.3
West Kazakhstan	192.9	191.4	190.9	-2.0
Jambyl	136.0	132.4	130.5	-5.5
Karaganda	337.2	328.3	325.1	-12.1
Kostanay	122.9	122.6	122.6	-0.3
Kyzylorda	148.3	146.7	147.5	-0.8
Mangystau	74.3	86.3	86.0	11.7
Pavlodar	184.6	179.3	176.0	-8.6
North Kazakhstan	131.0	128.3	127.4	-3.6
Turkestan	346.4	342.5	340.0	-6.4
East Kazakhstan	394.5	390.4	387.8	-6.7
Total by region	2.825.9	2.805.7	2.789.1	-36.8

As illustrated in Figure 1 and Table I, during the winter months of 2020, January and February, the number of horses in Kazakhstan decreased by 1.3%. In term of absolute numbers, the number of horses in January decreased by 20.2 thousand units, and in February by another 16.6 thousand units. According to the Statistics Committee of the Republic of Kazakhstan, as of March 1, 2,789.1 thousand animal units were counted, which is 36.8 thousand less than at the beginning of the year. The horse population increased only in two regions of Kazakhstan. These are Mangystau region – 11.7,

from 74.3 to 86, and Akmola region – by 9.7, from 187.3 to 197. The number of horses decreased by more than 10 thousand heads in the Karaganda region – by 12.1 thousand heads from 337.2 to 325.1 thousand units. More than 6 thousand animal units in Pavlodar region – by 8.6 thousand, from 184.6 to 176 thousand units, in East Kazakhstan region – by 6.7 thousand, from 394.5 to 387.8 thousand animal units, and in Turkestan region – by 6.4 thousand, from 346.4 to 340 thousand units.

In recent years, the republic has pursued a strategy to increase horse breeding. To achieve this goal, significant investments have been allocated for the purchase of breeding animals from countries near and far abroad. Infectious diseases occurring in the Republic have a negative impact on this goal. In recent years, the country has seen an increase in the incidence of strangles not only among foals, but also among adult livestock.

During the examination of horse breeding farms of the Almaty region, it was found that in the conditions of the southeast of the Republic, young animals from 6 months of age to 3 years are mainly ill with strangles, and recently the disease has been observed among adult animals. During outbreaks of horse strangles on the farms Nurlan-D of the Enbekshikazakh district, Zangar and Ali of the Jambyl district of the Almaty region, the seasonality of the disease was revealed, with the peak occurring in the early winter (November-December) and spring (March-April) periods. The early-winter peak of the disease is associated with the impact of a powerful stress factor, such as the weaning of young animals from mothers and the transition to a coarser feed, and the spring peak is associated with the general depletion of the foals' body, which was confirmed by biochemical studies of animal blood. Outbreaks of strangles among foals were registered in these horse breeding farms annually for 7 years (follow-up period), which indicated the stationarity of the disease. In recent years, there have been manifestations of strangles in horses with an atypical clinical pattern among young animals, and sometimes in adults. During their studies, atypical cultures are distinguished, subsequent biochemical tests allow them to be attributed to the species *Streptococcus equi*. During analysis, *Streptococcus equi* cultures are distinguished, which, according to their cultural-morphological and biochemical properties, can be attributed to atypical strains of *Streptococcus equi* (Kutter & Sulakvelidze 2012, Kutter 2001, Newton 1999).

There is a rapid increase in cases of atypical forms of strangles. The opening of borders, the rearrangement of livestock, the lack of clear control over the movement of animals, the appearance of a large number of antibacterial agents, the deterioration of the environmental situation, and the decrease in veterinary control over the proper use of antibiotics, led to the appearance of mutant forms of beta-haemolytic streptococcus, which, in turn, may cause an increasing expansion of the distribution area of atypical forms of the disease in the region (Vasiliev *et al.* 2013, Guliy *et al.* 2016). In 2020, an outbreak of an atypical form of strangles was registered on the Zangar farm, characterised by high fever (39-40 °C), refusal of food, mucous discharge from the nasal orifices. At the same time, there was no abscess in the area of the submandibular lymph nodes characteristic of the strangles. According to the age of the animals and the location of the abscess, this case is an atypical manifestation of strangles. The disease was observed among foals under the age of one year, 2-4 months after weaning, and among young animals from one to 3 years in the winter-spring period (Figure 5).

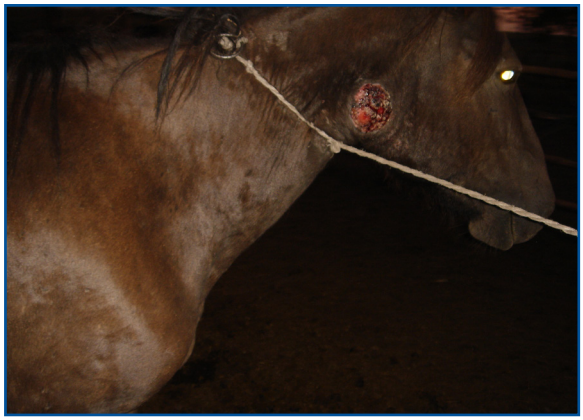

Figure 5. *Clinical features of an atypical form of strangles in adult horse*

Figure 5 shows clinical features of a mare with strangles at the age of 5 years with an abscess that has opened, from the pus of which a beta-haemolytic streptococcus has been isolated. Treatment with the use of broad-spectrum antibiotics (Bicillin-5, Catosal, and Nitox) had no effect, the lethal outcome was observed on day 4-5.

As can be seen from Figure 4 , the pathoanatomical autopsy of the fallen foals revealed a picture that was not characteristic of strangles. On the incision of the head, at the level of the submandibular lymph node, the absence of their enlargement was noted. Thus, with an outbreak of an atypical form of strangles in horse breeding farms of the Almaty region, the following details were revealed that are not characteristic of this disease:

- in the presence of some characteristic clinical aspects, sometimes there is no abscess;
- treatment with broad-spectrum durant antibiotics does not have an effect;
- the lethal outcome is observed on day 4-5;

- on the incision of the head, at the level of the submandibular lymph node, the presence of round, air-filled, hollow bubbles is observed;
- modified forms of beta-haemolytic streptococcus are very difficult to cultivate;
- gram-positive staining, arranged in the form of short chains of 2-4 cocci, elongated in the transverse direction;
- in infected white mice, death occurs within 5 days.

Based on the above, in all further cases, outbreaks of strangles with similar clinical aspects not characteristic of this nosology were called as atypical form of strangles.

At this stage of the study, washings from the mouth, nose, and eyes, and purulent exudate from the submandibular lymph node from horses with signs of strangles were collected. As a result, when sowing 112 samples of biological material in meat-peptone broth (MPB) with 10% of horse blood serum, it was possible to isolate the culture of the microbe only in 3 cases. This circumstance is conditioned by the fact that modified forms of beta-haemolytic streptococcus causing atypical cases of the disease are very difficult to cultivate. Gram-stained smears revealed gram-positive cocci, ovoid in shape, arranged in short chains of 2-4 cocci, elongated in the transverse direction. Shortening the length of the cocci chain to 2-4, while preserving the morphology of the microbe, is also not typical for *Streptococcus equi*. A picture of Gram staining is shown in Figure 6.

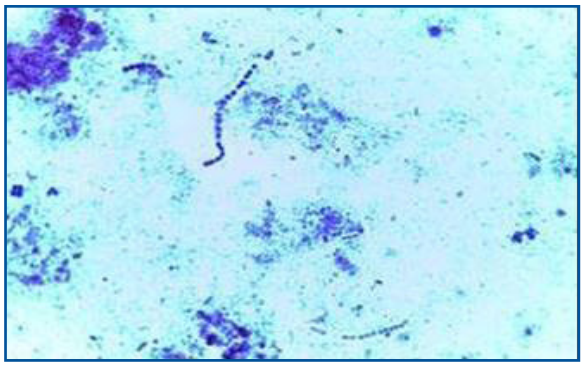

Figure 6. *Streptococci in MPB with blood serum; Gram staining. Magnification: 1000*

To clarify the strangles diagnosis, a biological test was performed on white mice. White mice were taken for a bioassay, their infection was carried out by washings from agar at a dose of 0.2 cm^3 subcutaneously, where 5 mice were left as controls. In the mice under experiment, and in the first days after infection, depression, fever, mucous discharge from the nasal cavity were observed. Pyemia developed for 5 days, then all mice died (100% lethality). The control mice remained alive. When blood from the heart of fallen white mice was seeded on MPB, with the addition of 10% inactivated equine blood serum, the inoculated culture of beta-haemolytic streptococcus was re-isolated. The fallen mice were dissected, cultures were inoculated from their organs on meat-peptone agar, the growth of smooth, small, rounded, transparent colonies were observed, when examined against the light – with a bluish tinge. The growth in the broth was characterised by its turbidity and the appearance of tender, fluffy flakes, which settled to the bottom after 3-5 days, and the broth brig\htened. On agar, both individual and merged colonies appeared at first in the form of tiny dewdrops, but later they became grey-white, opaque, streptococcus is detected by haemolysis on blood agar and broth.

For the purpose of biochemical identification, transplants were made to Hiss media with glucose, mannitol, sorbitol, sucrose, maltose, lactose. Meat-peptone gelatin, MPB with 40% bile, with milk, samples for indole, ammonia, hydrogen sulphide. Enzymes play an important role in the vital activity of bacteria. They are mandatory participants in a variety of biochemical reactions underlying the functions of nutrition, respiration, reproduction. Each type of bacteria produces a constant set of enzymes for it.

The stability of bacterial enzymatic systems allows the use of biochemical properties in combination with morphological and cultural characteristics to determine the genus and species of bacteria. To investigate the biochemical properties and identification of beta-haemolytic streptococci, bacterial cultures were inoculated on differential diagnostic nutrient media. The results are shown in Table II.

Table II. *Enzymatic and biochemical properties of 3 isolates of* Str.equi

Strain Str. equi	Lactose	Glucose	Sucrose	Dulcite	Mannitol	Sorbitol	Raffinose	Xylose	Fructose	Bile	H 2 S 2	Inositol	Production of catalase	Production of oxidase
Str.equi No.1	n/f	f	n/f	n/f	n/f	n/f	n/f	n/f	n/d	n/d	n/p	n/d	n/p	n/p
Str.equi No.2	n/f	f	f	f	n/f	n/f	n/f	n/f	n/d	n/d	n/p	n/d	n/d	n/d
Str.equi No.3	n/f	f	f	f	n/f	n/f	n/f	n/f	n/d	n/d	n/p	n/d	n/d	n/d

Note: f – ferments; n/f – does not ferment; n/d – does not dilute; n/p – does not produce.

As can be seen from Table II, when differentiating streptococci, great importance is attached to determining their biochemical activity. It was found that the most pathogenic streptococci exhibit minimal biochemical activity. Isolated cultures: ferments glucose with the generation of acid without gas, does not ferment lactose, sucrose, dulcite, mannitol, sorbitol, does not produce indole, ammonia, hydrogen sulphide, does not dilute fructose, bile, inositol, does not produce catalase and oxidase. The isolated strain of *Str.equi* No.1, unlike the previously known ones, does not ferment maltose, sucrose, dulcite. The virulence of *Str.equi* cultures was studied in white mice by determining the lethal dose (LD_{50}) of isolates – No.1, No.2, No.3 in intraperitoneal infection of animals with a suspension of bacteria. LD_{50} was determined by the Kerber method in the Ashmarin modification. The results are shown in Table III.

Table III. *Virulence of* Str.equi *isolates*

Str.equi	The death of mice when they were injected with culture (in billion microbial cells)						LD50 (bn. m. c.)
	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	1/4	0/4	0/4	0/4	0.8521
No.3	4/4	3/4	1/4	0/4	0/4	0/4	0.7244

Note: in the numerator – the number of fallen mice, in the denominator – the number of mice in the group.

As can be seen from Table III, the strain of *Str.equi* No.1 turned out to be the most virulent, its LD_{50} has amounted to 0.5129 billion m. c. Molecular identification of *Str.equi* strains.

In the future, to confirm the genus, DNA was isolated from an isolated colony and PCR was performed, DNA isolation of *Streptococcus equi* was carried out with a QIAGEN kit.

A 1.7% agarose gel was prepared on a tris-acetate buffer with ethidium bromide.

The results of electrophoretic analysis are shown in Figure 7.

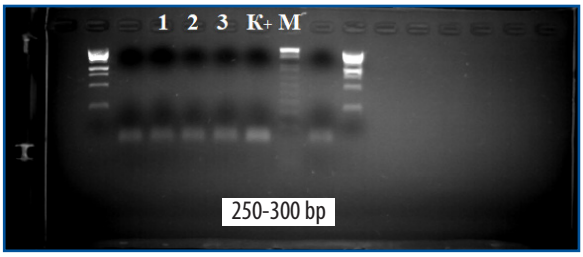

Figure 7. *Results of PCR analysis*

Note: Sample No.1 – culture isolated from the Druzhba village, Enbekshikazakh district; Sample No.2 – culture isolated from the Akterek village, Jambyl district; Sample No.3 – culture isolated from the Uzynagash village, Jambyl district; K+ – positive control of Str. equi DNA.

As can be seen from the electropherogram, the size of all the tested cultures coincides with the positive control, which indicates that all 3 cultures are *Str. equi*. Given the uniqueness of the isolated culture, which consists in high virulence, the development of short chains, the ability to quickly cause the death of laboratory animals, the goal was set to choose the *Str.equi* No.1 as an object for molecular biological research.

The resulting PCR product was analysed using electrophoresis in 1.5% agarose gel: 1-2 µl of isolated DNA was added to 3-5 µl of xylincyanoline. After that, the DNA with the dye was applied to the wells of the agarose gel. Electrophoresis was performed in a tris-borate buffer. A DNA marker with a size of 100 np (Invitrogen) was used. Visualised in a transilluminator.

The presence of *Str.equi* DNA was determined by the presence in the electrophoretic PCR tracks of products with sizes of 250 and 350 nucleotide pairs (np). The result of the analysis is shown in Figure 8.

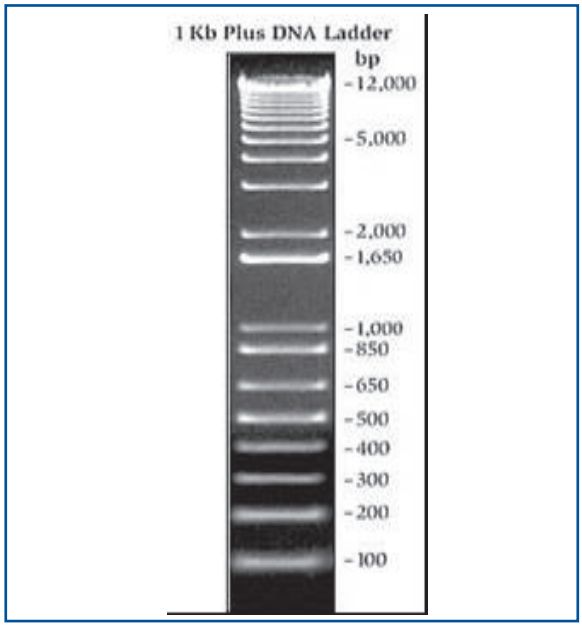

Figure 8. *Electrophoretic analysis of PCR products*

Subsequently, the PCR product was cloned into a pGEM-T vector and sequenced using M13 primers. The resulting sequence is analysed in the Basic Local Alignment Search Tool (BLAST) (Figure 9).

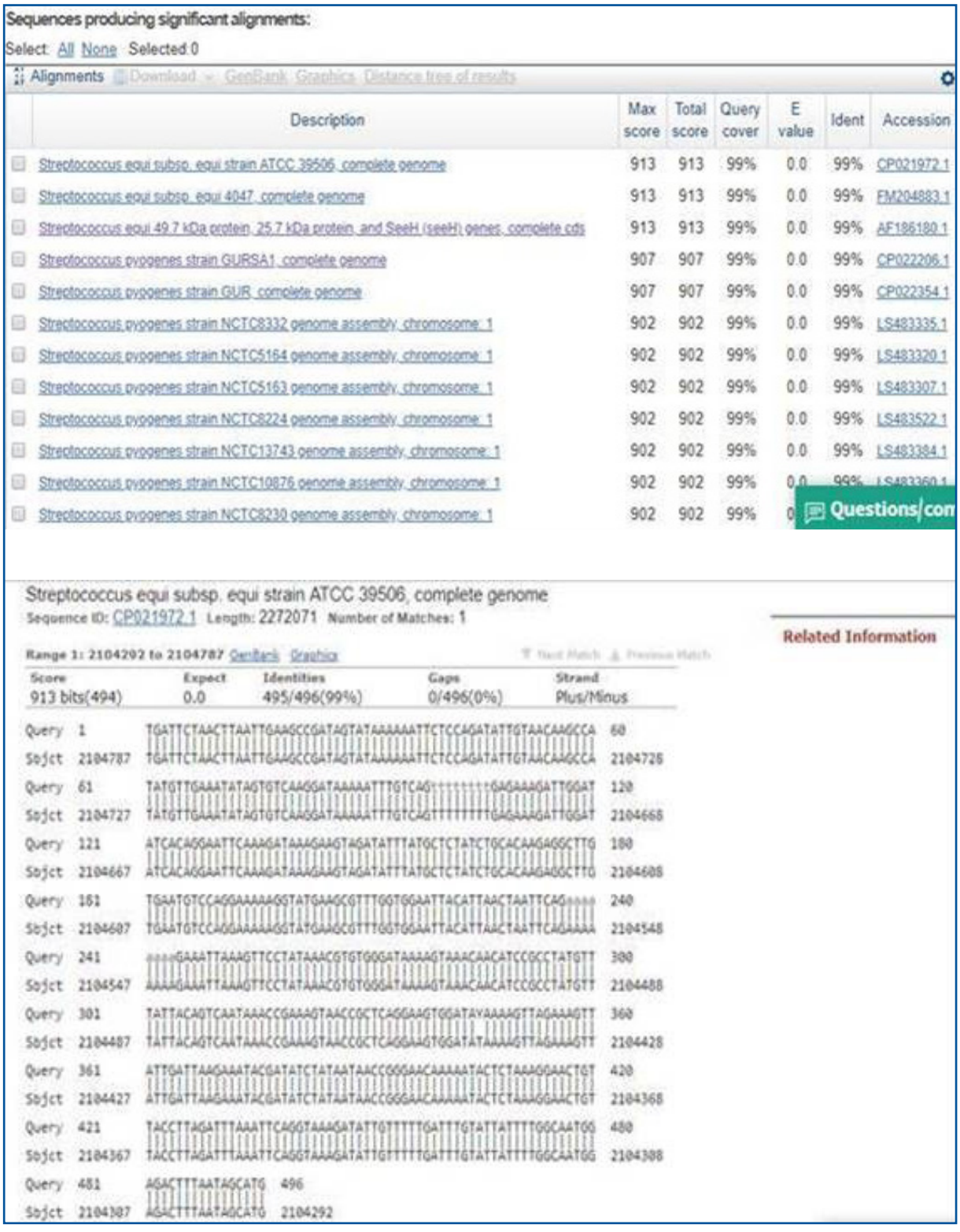

Figure 9. *Analysis of the resulting sequence in BLAST*

The nucleotide sequence of the *Streptococcus equi* bacterial strain is 99% identical to the nucleotide sequence of the *Str.equi* 16 S rRNA gene fragment. The resulting bacterial isolate belongs to the type *Str.equi*.

Determination of the susceptibility of microorganisms to antibacterial drugs is becoming increasingly important due to the emergence and widespread antibiotic resistance in bacteria. Standard methods for determining the susceptibility

of microorganisms to antibiotics (disco-diffusion and serial dilutions) were developed in the second half of the 1960s and, since then, from a methodological standpoint, have not undergone fundamental changes. The susceptibility of isolates to antibiotics was determined using the agar diffusion method (paper disk method). The method is quite simple to perform, allows determining the susceptibility to several antibiotics at the same time. To determine the susceptibility of the isolate to antibiotics, *Str.equi* was inoculated on GRM-agar (with 1% glucose), 1 ml per 1 billion of suspension was introduced into sterile Petri dishes, followed by the imposition of sterile discs made of filter paper impregnated with preparations at a distance of 2 cm from the edge of the cup with 9 consecutive dilutions.

This study investigated 22 drugs: Vetbicin-3; Vetbicin-5; benzylpenicillin; Bicillin-3; Bicillin-5; oxytetracycline hydrochloride; streptomycin sulphate; ceftriaxone (generation III); cefazolin (generation I); cefepime (generation IV); cefuroxime (generation II); azithromycin; streptocid; lincomycin hydrochloride; Amacin (amoxicillin); ampicillin; ciprofloxacin; Inofol (ofloxacin); rifampicin; Biseptol; erythromycin; gentamicin (Figures 10-12).

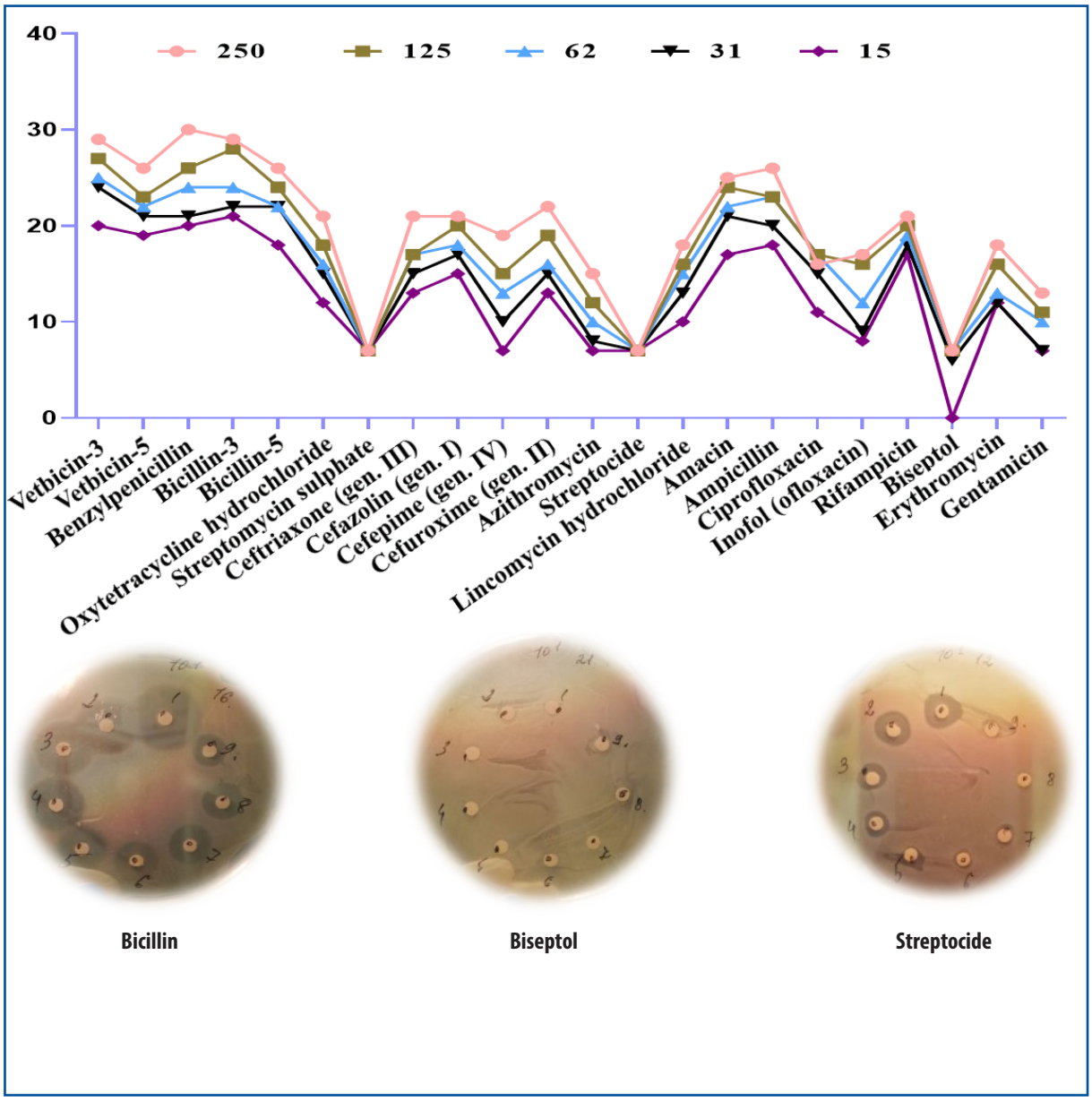

Figure 10. Streptococcus equi *No. 1*

As can be seen from Figure 10, the results of the zone of inhibition growth of *Streptococcus equi* No.1 in a concentration of 250 was highly susceptible to Vetbicin-3. The diameters of lysis zones were 29 mm for Vetbicin-3, Vetbicin-5 – 26 mm Penicillin – 30 mm, Bicillin-3 – 29 mm, Bicillin-5 – 26 mm. At a concentration of 125, the susceptibility to these drugs was slightly lower, and the diameter of the lysis zone was Vetbicin-3 – 27 mm, Vetbicin-5 – 23 mm, Benzylpenicillin – 26 mm, Bicillin-3 – 28 mm, Bicillin-5 – 24 mm. In further concentrations 62, 31, and 15, the diameter of the lysis zone was below 25 mm. *Streptococcus equi* No. 1 was resistant to streptomycin sulphate, streptocide, and Biseptol in all concentrations.

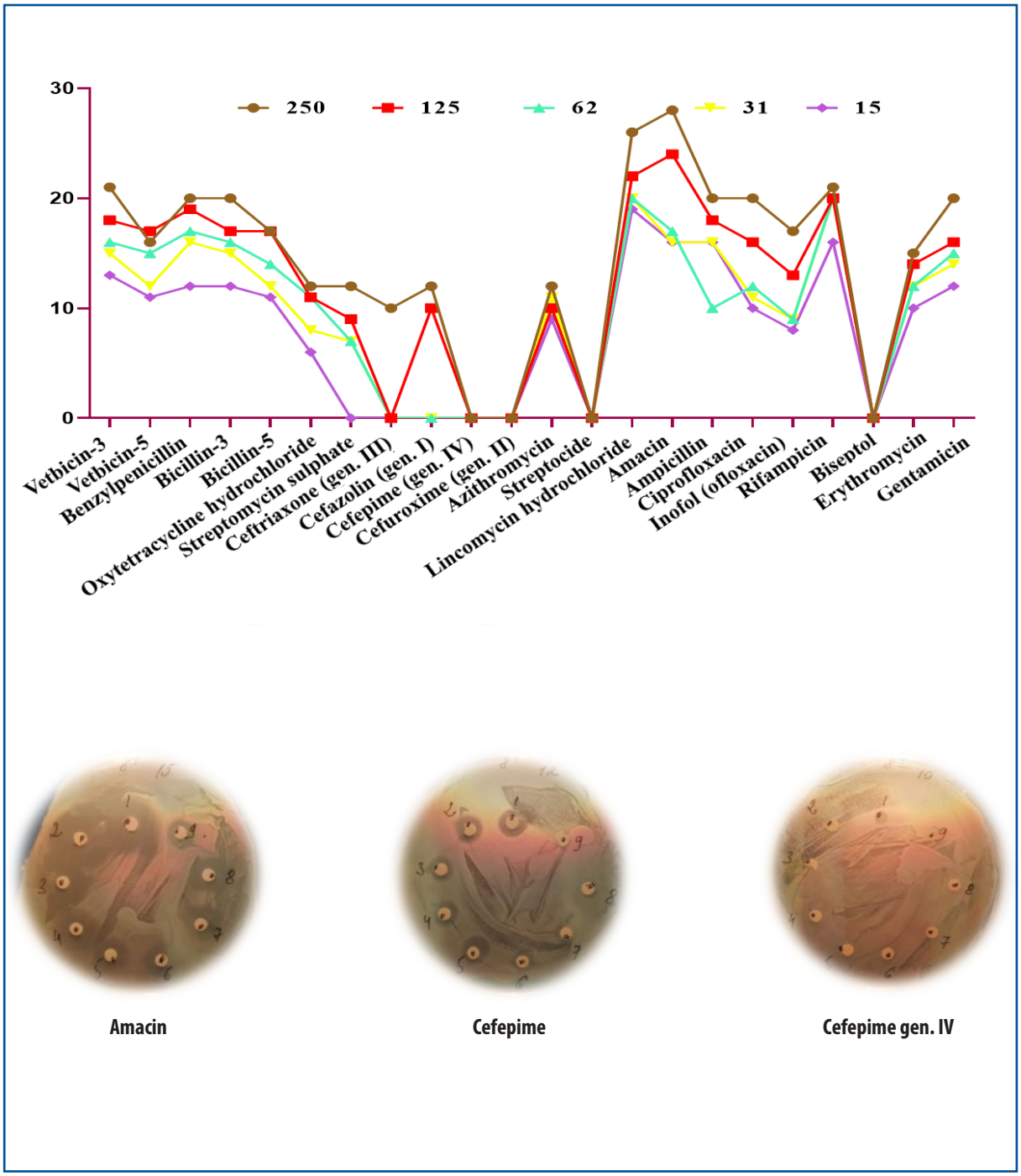

Figure 11. Streptococcus equi *No. 2*

As can be seen from Figure 11, *Streptococcus equi* No.2 at a concentration of 250 was susceptible to lincomycin and Amacin, the diameter of their lysis zone was 26 and 28 mm, respectively, at a concentration of 125 – from 22 and 24, respectively. At a concentration of 62, the diameter of their lysis zone in relation to the drug Lincomycin was 20 mm. In the concentrations of 31 and 15, the lysis zones were insignificant. *Streptococcus equi* No. 2 was resistant to the drugs cefepime gen. IV, cefuroxime gen. II, streptocide, and Biseptol in all tested concentrations.

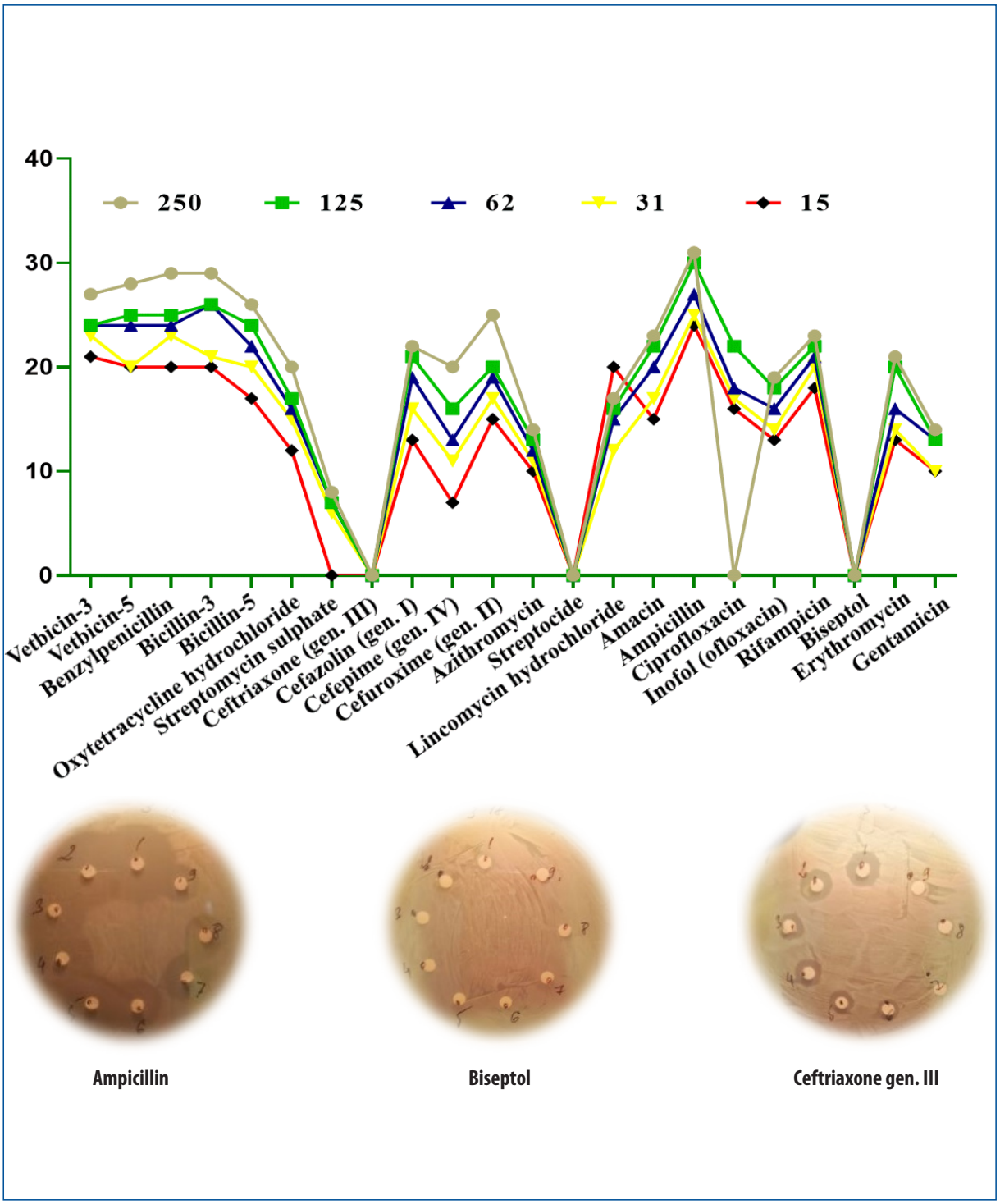

Figure 12. Streptococcus equi *No. 3*

As can be seen from Figure 12, *Streptococcus equi* No.3 at a concentration of 250 was highly susceptible to Vetbicin-3, Vetbicin-5, benzylpenicillin, Bicillin-3, the diameter of the lysis zone was from 29 to 30 mm, respectively. In concentrations 125 and 62 – from 24 to 26, respectively. Ampicillin in the indicated concentrations – 30 and 27 mm, respectively. At concentrations of 31 and 15, Vetbicin-3, Vetbicin-5, benzylpenicillin, and Bicillin, the diameter of the lysis zone was below 25. Ampicillin in these concentrations showed 25 and 24 mm, respectively. *Streptococcus equi* No. 3 was resistant to streptomycin sulphate, ceftriaxone gen. III, streptocide, and Biseptol in all concentrations.

The above preparations on the disk were diffused into agar, causing the death of 3 susceptible Streptococcus bacteria isolated from horses of the Almaty region. Zones of growth delay (absence) were formed around the disks with drugs. The diameter of the growth retardation zone of 3 epizootic cultures *Str.equi* was measured using a ruler.

If there is no growth retardation zone of epizootic cultures *Str.equi* around the discs, the cultures are resistant to the antibiotic. If it is less than 14 mm – weakly susceptible, from 15 to 25 mm – susceptible, more than 25 mm – highly susceptible. Active growth of *Streptococcus* cultures in the vicinity of disks with the drug indicates that bacteria are resistant.

When considering the results, susceptible drugs were those in which the diameter of the growth retardation zone of microorganisms was more than 15-25 mm. Above 25 mm – highly susceptible, less than 14 mm – weakly susceptible. The visualisation of drug susceptibility of all 3 isolated epizootic strains of *Streptococcus equi* is shown in Figure 13.

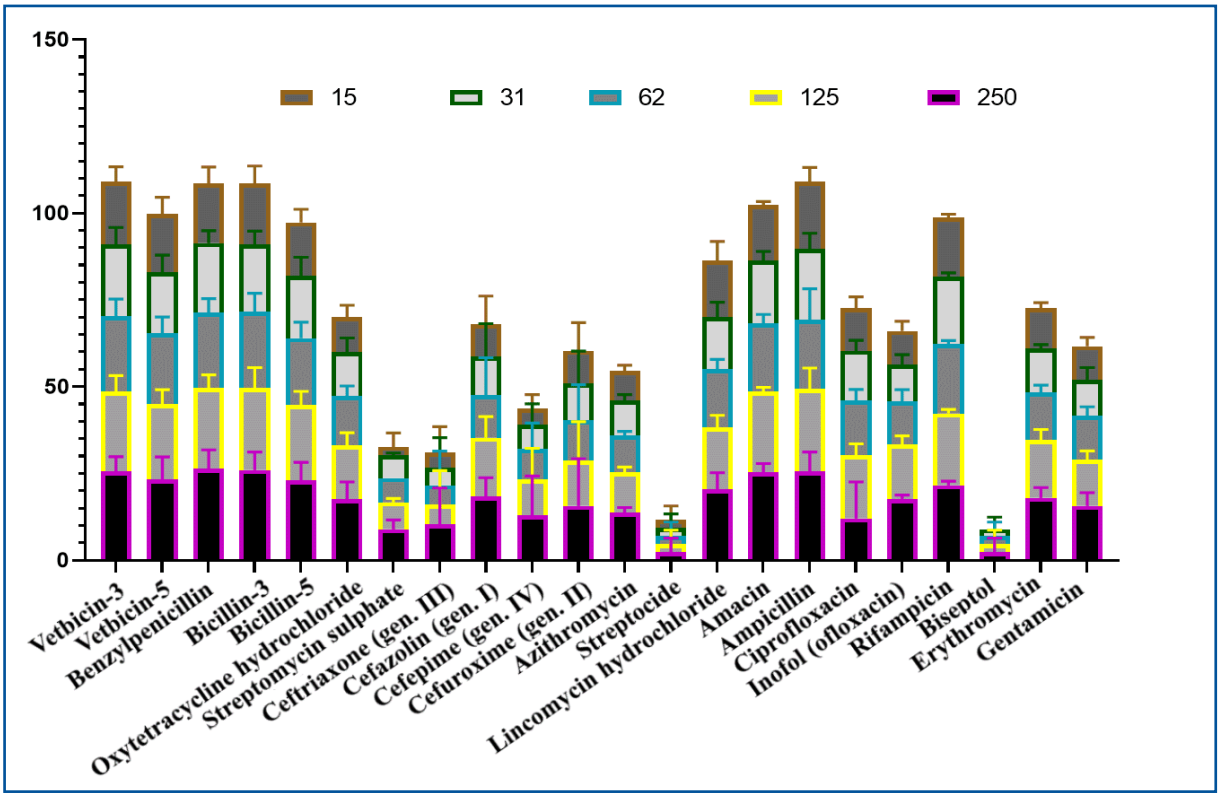

Figure 13. *Diameter of the growth inhibition zone of isolated Streptococcus equi cultures to preparations (mm)*

The perpendicular streak method is used for rapid screening of microbial antagonism. To determine the susceptibility of *Streptococcus equi* to antibacterial preparations by the method of perpendicular streaks on the surface of the agar GRM medium in Petri dishes were inoculated with a streak of *Str. equi* with a bacteriological loop with a diameter of 2 mm and left at room temperature until the liquid was completely absorbed, then perpendicular from the edge of the cup to the streak of the inoculated culture *Streptococcus equi* the bacteria were applied with a streak of the studied antibacterial drugs in various dilutions. After that, they were incubated at an optimal temperature for 24 hours for the diffusion of inhibitory compounds into the agar.

As a result, if the studied antagonist microorganism forms a substance that has an antimicrobial effect on test cultures, the growth of the latter will begin at some distance from the growth of the antagonist itself. The greater this distance, the more susceptible the test culture is to the produced antibiotic substance. Resistant microorganisms will develop in the immediate vicinity of the stroke.

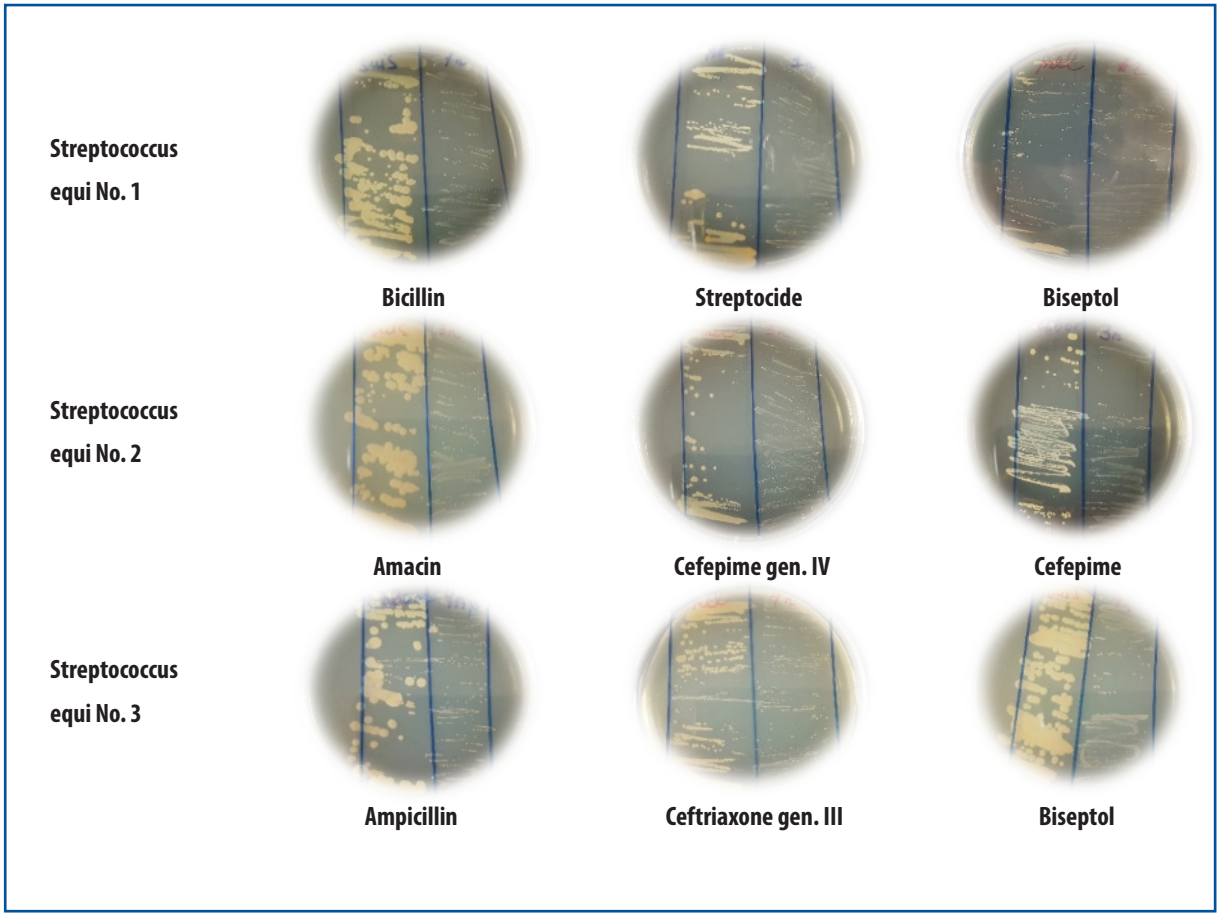

Figure 14. *Results of the perpendicular streaks method*

As can be seen from Figure 14, the presence and degree of susceptibility of *Streptococcus equi* were assessed by the size of the inhibition zones at the border with the growth streak in the tested drugs. The free parts of the spots served to control the viability of cultures.

Growth retardation zones of epizootic cultures of *Streptococcus equi* gave the following results: *Streptococcus equi* No.1 is resistant to Biseptol, susceptible to ampicillin and not susceptible to the streptocide.

Streptococcus equi No.2 is resistant to cefepime and is weakly susceptible to gen. IV cefepime, and susceptible to Amacin. *Streptococcus equi* No. 3 are weakly susceptible to ceftriaxone gen. III, resistant to Biseptol, and susceptible to ampicillin.

Conclusions

Throughout many years of research, the mixed forms of strangles with pasteurellosis, salmonella abortion, and atypical forms with additional symptoms uncharacteristic of strangles have not been subjected to molecular biological differentiation.

In the result of the analysis of the spread of strains of *Str.equi*, pathogenic for horses on the farms in Almaty region, it was established that outbreaks of strangles in horses periodically occur on farms Nurlan-D, Enbekshikazakh district, Zangar and Ali of the Jambyl district of Almaty region, identified the seasonality of the disease – early winter (November-December) and spring (March-April) periods.In the course of the study, 112 samples of biomaterial were collected from foals with strangles for the isolation of bacterial cultures.

As a result, 3 bacterial cultures were isolated, which were previously assigned to the genus *Streptococcus* based on standard bacteriological tests. As a result of biochemical identification of isolated bacterial cultures, it was found that with a probability of up to 85% all isolates belonged to the species *Streptococcus equi*.

The results of molecular identification of phylogenetic groups indicated that the studied isolate belongs to the phylogenetic group of microorganisms *Streptococcus equi subsp.* equi by 99%.

Based on the data obtained during the study

of antibiotic susceptibility of bacterial isolates, the isolates exhibited: *Str.equi* No.1 – resistant to the drug Biseptol, susceptible to ampicillin, and not susceptible to azithromycin; *Str.equi* No.2 – resistant to cefepime, weakly susceptible to azithromycin, and susceptible to Amacin; *Str.equi* No.3 – weakly susceptible to gentamycin, resistant to metronidazole, and susceptible to ampicillin.

Acknowledgements

The authors express gratitude to the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan: for financing the grant project IRN AR 08855635 "Obtaining a bacteriophage for the therapy of strangles in horses" 2020-2022

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