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Identification of Causative Agent of Horse Strangles in Northern Siberia

Mikhail P. Neustroev[1*], Sargylana G. Petrova[2], Evdokia I. Elbyadova[3],Nadezhda P. Tarabukina[4], Vladislav A. Alekseev[5], Andrian A. Popov[6]

[1*]M.G. Safronov Yakut Scientific Research Institute of Agriculture (YSRIA), Yakutsk, Republic of Sakha, Yakutia, Russia. *Corresponding author. E-mail: mneyc@mail.ru

[2]M.G. Safronov Yakut Scientific Research Institute of Agriculture (YSRIA), Yakutsk, Republic of Sakha, Yakutia, Russia. E-mail: sargy1970p@mail.ru

[3]M.G. Safronov Yakut Scientific Research Institute of Agriculture (YSRIA), Yakutsk, Republic of Sakha, Yakutia, Russia. E-mail: elbyadova@bk.ru

[4]M.G. Safronov Yakut Scientific Research Institute of Agriculture (YSRIA), Yakutsk, Republic of Sakha, Yakutia, Russia. Email: hotubact@mail.ru

[5]Corporation Expel Biotex, Technopark Yakutia, Yakutsk, Republic of Sakha, Yakutia, Russia. E-mail: vldslvalekseev@gmail.com

[6]M.G. Safronov Yakut Scientific Research Institute of Agriculture (YSRIA), Yakutsk, Republic of Sakha, Yakutia, Russia. E-mail: andrian.popov.94@bk.ru

ABSTRACT

Background: Increase in the livestock and productivity of horse herd farming is limited by a number of facts that include infectious and invasive diseases.

Objectives: The aim of the present study was to isolate, study, identify by the morphological, cultural, biochemical, and molecular-genetic properties new isolates of the causative infectious agent of horse strangles for the development of vaccines.

Animals: The study included the pathological material of 2 foals died from strangles, washouts from the nasal cavity discharge of 34 foals with clinical manifestations of strangles and 18 healthy foals.

Methods: The morphological and cultural properties of the isolates of horse strangles Streptococcus were studied by the method of cultivation in selective media. The species of the isolates were identified by the biochemical properties of Streptococcus culture using the strips "API 20 Step" and the test system "API" (bioMerieux) and PCR.

Results: Nucleotide sequence of the gene 16S of rRNA of *Streptococcus equi* was revealed in 3 of the isolated strains of strangles Streptococcus. These strains can be used for the development of a vaccine. Also, *Enterococcus faecales*, *Streptococcus piogenes*, toxigenic and mold fungi *Aspergillus* and *Mucor* geni were isolated in foals with clinical manifestations of strangles. The most precise and quick method of identification of strangles Streptococcus is polymerase chain reaction with specific primers.

Conclusion and Clinical Importance: New isolates of *Streptococcus equi*were isolated and identified for the development of a vaccine against horse strangles.

KEYWORDS

Environmental Conditions, Horse Strangles, Siberia, StreptococcusStrain.

Introduction

Horse breeding is one of the main branches of animal breeding that actively develops in many

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countries.The main limitations to the development include infectious diseases of horses. Horse strangles is one of the most widespread infections, especially among foals.This acute respiratory disease is caused by *Streptococcus equi* [1]. Horse strangles is characterized by fever, nose discharge of purulent exudates and further abscess of head lymph nodes. The disease is primarily spread in Russia (Novosibirsk Oblast, Krasnoyarskiy and Altaiskiy Krai, Republic of Khakassia, Sakha (Yakutia) and Altai, Irkutsk Oblast), Kazakhstan, Kirgizia, and Mongolia [2, 3, 4, 5, 6].

In the Republic of Sakha (Yakutia), where horse herd farming is common, the morbidity rate in foals is around 57.8-62.7% of the total livestock. The lethality is around 4.0-22.0% depending on the epizootic process [7]. The morbidity with strangles in the Republic of Kazakhstan reaches 30.1-46.7%, the lethality – 16.0-28.3% [8]. In Mongolia, the spread of the infection and increased levels of the morbidity with strangles is associated with the decrease in the immune resistance of animals and mare's milk consumption by people, which deprives young livestock of sufficient amount of mare's milk [9].
For the development of effective methods of diagnostics, prevention, and treatment of strangles, it is necessary to study the infectious agents that provoke this disease. In all the countries where strangles is registered, including Kazakhstan [8, 10, 11], Kirgizstan [12], Netherlands [13], Egypt [14], Russia [7, 15], Korea [16], Brazil [17], the isolation of strangles Streptococcus is performed along with the identification of morphological, cultural, and biochemical properties of the infectious agent. During the past years, with the development of molecular-genetic studies, the reports on the isolation and identification of strangles Streptococcus by PCR method appeared [1, 16, 17; 18].

Horse strangles causative infectious agent *Streptococcus equizooepidemicus* can cause arteritis in goatlings[19] and abortions of infectious character in mares [7], which defines the role of strangles Streptococcus in other infectious diseases.

In the course of the studies performed by the authors (1989-1995), 42 isolates of strangles Streptococcus isolated from sick horses. Among them there was a strain of *Streptococcus equi* «H-34» that was deposited in the All-Russian State Scientific Research Institute (ARSRI) and was proposed to be used for the development of the vaccines against strangles and diagnostic streptococcal serum of the serogroup C [7]. Presently, this strain was removed from the depository because it lost its specific antigen properties.

The aim of the study was to isolate, to study, and to identify by the morphological, cultural, biochemical, and molecular-genetic properties of new isolates of the causative agent of horse strangles that can be used for the development of important drugs.

Materials and Methods

For the isolation of the isolates of strangles Streptococcus, the authors studied the washouts from nasal cavities and the content of abscesses of mandibular lymph nodes obtained from horses with strangles (young livestock aged 6-10 months) (Figure 1). Pathological material was collected in 2015-2017 on the farms in Namskiy, Khangalasskiy, Amginskiy, Megino-Kangalarskiy regions and in Yakutsk Republic of Sakha (Yakutia) and the Republic of Kazakhstan.

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Figure 1. Horse with strangles displaying discharge of purulent exudates

In total, 63 samples from foals aged 6-10 months were studied, 45 of them were washouts of nose discharge (27 from clinically sick foals with strangles, 18 from healthy foals), 7 samples of the content of mandibular lymph node abscesses and 11 parenchymatous organs from foals that died from strangles.

Pre-cultivation treatment preceded the bacterial studies. The washouts with probe swabs, pieces of organs and lymph nodes were placed in a sterile saline solution for 5 minutes.Further, they were treated with 70^0 ethanol and flushed 2-3 times with a saline solution.For the isolation of pure culture, bacteriological cultivation was performed on a dense nutrient medium in 3 points. The grown single colonies from the 3rd point were sampled for further identification.

Morphological and cultural properties of the isolates of strangles Streptococcus were studied by cultivation in MPB with 1% of glucose and 10% of horse serum, in MPA with 1% of glucose and 10% of blood serum or 5% of defibrinated horse blood. Smears from purulence, broth, and agar cultures were fixed and Gram stained. Biochemical properties of cultures were studied by the cultivation in MPA with 40% of bile, 6.5% of saline MPA, agar with sodium azide and Hiss medium with glucose, lactose, mannite, maltose, saccharose, sorbite, and dulcite. The cultures were incubated in a thermostat at 37°C for 18-48 hours. The cultures were conserved with SSA. The obtained samples were used for microscopic studies.

Genus and species of the isolated cultures were identified according to “Bergy’s Identifier of Bacteria” (1997) [20]. The authors also used “Methodical guidelines for laboratory diagnostics of strangles” (2007) and “Diagnostics of staphylococcosis and streptococcosis” (2013) [21].

The genus of the isolates was identified by biochemical properties of the Streptococcus culture using strips “API 20 Step” and “API” (BioMerieux) test system. The obtained results on the biochemical properties of Streptococcus culture were registered in the table “Interpretation of the reactions” of the test system “API” (BioMerieux).
For the identification of virulent activity of Streptococcus, the authors used white mice of both sexes aged 5-8 weeks and 18-20g of eight. Mice had subcutaneous injections of living bacterial

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cells of Streptococcus 0.2-0.5 cm^3 with the content from 1×10^3 to 1×10^9 CFU/per mouse.

The evaluation of the virulent activity of LD_{50} of isolates was performed according to the method of Kerber in the modification of Ashmarin and Vorobiev[22].

Genetic typing of the isolates of Streptococcus was performed with PCR method with the following strain-specific primers: Seel-F: 5′-CGGATACGGTGATGTTAAAGA-3′ and Seel-R: 5′-TTCCTTCCTCAAAGCCAGA-3′ earlier described by Boyle et al. [17, 18]. For the amplification of DNA, the authors used qPCRmix from Evrogen (Russia). The conditions of PCR were set according to the manufacturer's instructions.

The isolates of strangles Streptococcus were confirmed molecular-genetically in the All-Russian State Center of quality and standardization of drugs and feed for animals by the "Genetic identification of bacteria based on the analysis of nucleotide sequence of the gene 16S rRNA".

Results

As a result of primary bacteriological studies, 40 cultures were selected that corresponded to strangles staphylococcus by the cultural, tinctorial, enzymatic, and hemolytic properties, in 11 of them, contamination with toxigenic and mold *Aspergillus* and *Mucor* fungi was revealed.

The isolated cultures were identified as *Streptococcus*. They grew well in MPB with 10% blood serum, in 1% MPA with 10% blood serum. In 1% MPB with 10% horse blood serum, the cultures grew as an even medium opacity with white precipitation that lifted after shaking.In 1% of MPA with 10% horse blood serum, some cultures grew as small mildew-like colonies. The growth of glittering colonies with even edges and opaque merged colonies was also observed.

In further study, 7 isolates grew in MPA with 1% of glucose and 40% of bovine bile and in MPA with 6.5% of sodium chloride. They fermented glucose, lactose, maltose, mannite, sorbate, and dulcite with the synthesis of acid without gas. These isolates were characterized by near bottom growth as a dense white precipitate in liquid nutrient media, on dense nutrient media, they grew as white mucoid colonies. Microscopy of the culture grown in broth revealed short chains of gram-positive cocci. The smears taken from dense medium contained cocci in the form of a bunch of grapes. Based on the results of biochemical, cultural morphological and molecular-genetic properties, the isolates "4g", "MK1/1", "UG", "SM", "N-34" and "M" were classified as *Enterococcus faecalis*, and the isolate «Khatas-3» – as *Streptococcus piogenes*.

In October-November 2017, new isolates of Streptococcus were additionally isolated from foals with strangles. These isolates grew in liquid nutrient medium near the walls with the formation of a white flocculent precipitate (Figure 2). On dense nutrient medium, they grow as mildew-like, semi-transparent, small colonies (Figure 3). In blood agar, they exert ß-hemolysis (Figure 4). They do not grow in nutrient medium with bile and sodium chloride; ferment glucose and lactose with the synthesis of acid without gas; do not ferment mannite, sorbate, and dulcite. Smears from one-day-old broth culture contained Gram stained long coiled chains of gram-positive cocci. Smears from agar culture contained short-chains or double and single cocci. Morphological and cultural biochemical properties of these isolates are typical for strangles Streptococcus. Molecular-genetic typing of 40 isolates was performed by the methods of extraction of DNA,

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PCR and electrophoresis in agarose gel.

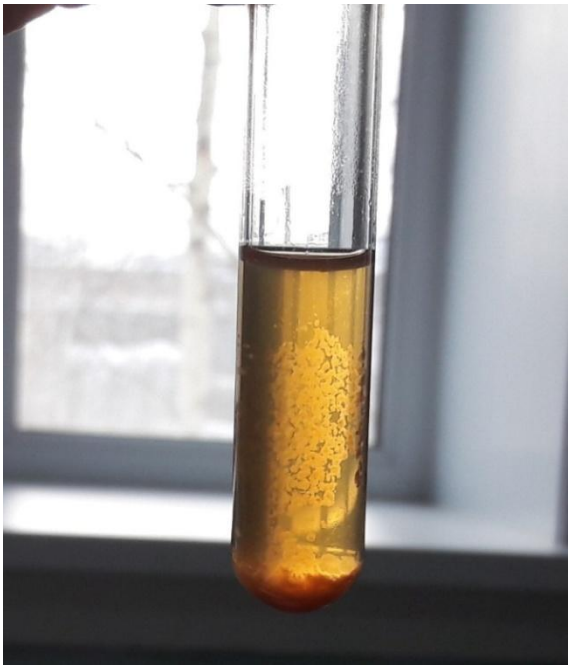

Figure 2. Growth of *S. equi* in 1% MPB

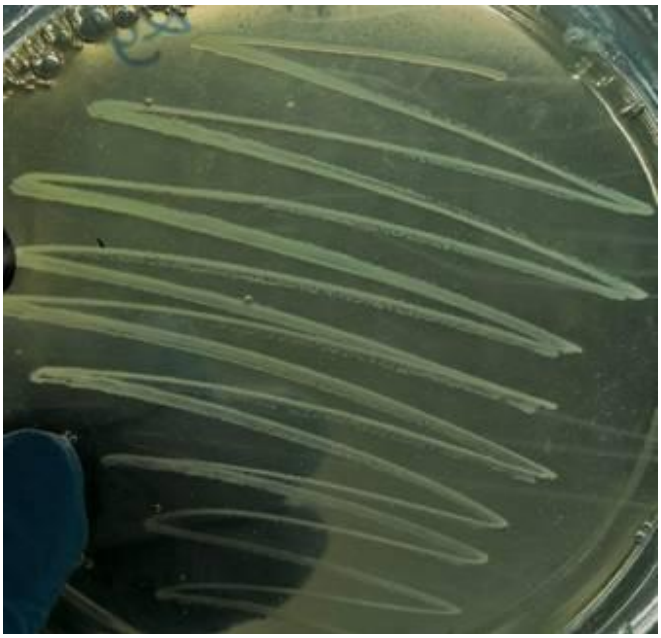

Figure 3. Growth of *S. equi* on 1% MPA

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Figure 4. Growth of *S. equi* on 5% blood agar media showing beta-hemolytic grow colonies

Nucleotide sequence of the gene 16S rRNA of *Streptococcus equi* was established in 6 isolates ("P1", "N-1 caz", "N-5/1", "N-12-3", "1-3", "7-3") isolated from foals with strangles, 3 of them ("N-1 caz", "N-5/1", "N-12-3") were selected for the development of a vaccine.

Further molecular-genetic typing in the All-Russian State Scientific Research Institute (ARSRI) confirmed that the analyzed nucleotide sequence of the studied strains of*Streptococcus equi* "N-5/1", "N-12-3", "N-1 caz" was 100% identical to the nucleotide sequence of the fragment of the gene 16S rRNA of *Streptococcus equi*.
The strain "N-5/1" of *Streptococcus equi* was considered the most promising and underwent commission trials at the department of All-Russian collection of microorganism strains in the department of quality evaluation and standardization of probiotic drugs of the All-Russian State Scientific Research Institute (ARSRI).

The strain "N-5/1" of *Streptococcus equi* produces such enzymes as ß-glycosidase, β-glucuronidase, and leucinearylamidase. It ferments esculin, starch, and glycogen with the synthesis of acid without gas, and does not ferment arginine, ribose, arabinose, mannite, sorbate, lactose, tregalose, inulin, and raffinose. It does not form acetoin and does not hydrolyze hippurate. The identification of the strain "N-5/1" was performed with a strip "API 20 step" and the tests system "APIR" (bioMerieux).

Virulent activity LD_{50} of the strain "N-5/1" of *Streptococcus equi* for white mice was $1x10^2$ CFU/mouse after parenteral introduction.

Discussion

Isolation and study of the causative agent of horse strangles allowed the authors to expand the knowledge on the microbiological peculiarities of this bacteria, and thus, to use them for the development of preventive and therapeutic drugs. In 2015-2017, based on the results of bacteriological studies of 63 samples taken from sick, healthy and dead foals, the authors isolated 40 cultures similar to strangles Streptococcus by cultural, tinctorial, enzymatic, and hemolytic properties.

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In glucose-serum broth, the isolated cultures grew with general medium opacity with near bottom and sometimes near walls flocculent precipitate that lifted as a braid after shaking. In smears taken from one-day-old broth, theS cultures looked like long threads, which is typical for strangles Streptococcus (Figure 5). In glucose-serum agar, all the isolated isolates of strangles Streptococcus grew as thin, small, glass-like, mildew-like mucoid colonies. In smears taken from agar cultures, streptococci looked like coupled or single short chains (Figure 6). They exerted β-hemolysis in blood agar. The study of biochemical properties of 40 isolated strains on the ferment activity with glucose, saccharose, lactose, maltose, sorbate, dulcite, and mannite showed that 6 of them fermented glucose and lactose with the synthesis of acid without gas, and did not ferment mannite, sorbate, and dulcite, and did not grow in media with bile and sodium chloride.

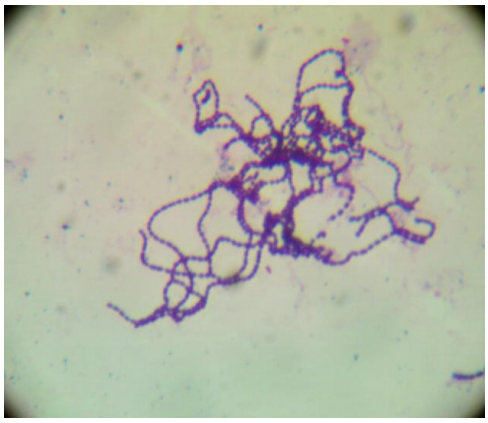

Figure 5. Smear from daily broth culture

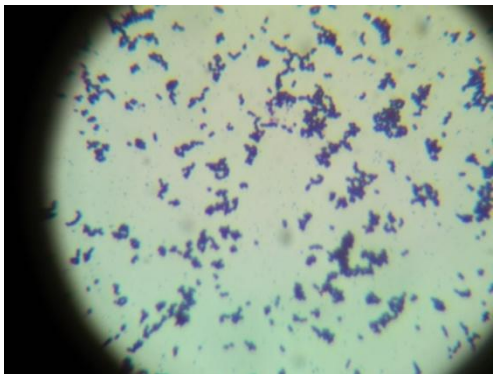

Figure 6. Smear from daily agar culture

Pre-cultivation treatment of biological material was performed before bacteriological studies. It was placed in a 0.9% saline solution for 5 minutes and, then, in 70% ethanol. In the present study, the washouts were effective for the inhibition of the growth of concomitant microflora, which facilitated and accelerated the time of isolation of pure culture. Lifetime diagnostics of horse strangles, especially in the conditions of the Far North, is a complicated task because this disease is often accompanied by other infections that develop due to the influence of unfavorable factors. Thus, low temperatures and formation of icing on the grass and hay that is the main source of feed for tebenevka and stable keeping of horses in winter can become the main cause of a reduction of horse immune system (Figure 7). It was established that low-quality and poor diet contributes to the contamination of horse respiratory tract with fungi, which accompanies horse

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strangles. In 11 untreated samples, microbiological studies revealed toxigenic and mold fungi *Aspergillus* and*Mucor*. It should be mentioned that pre-cultivation treatment of biological material is an important moment in the isolation of a pure culture of Streptococcus.

Figure 7. Foals on winter pasture

In 2015-2016, it was impossible to isolate strangles Streptococcus from clinically sick and dead foals because of a high rate of contamination with toxigenic and mold fungi and the association of *Streptococcus* bacteria. The obtained results revealed a certain role of *Enterococcus faecales* and *Streptococcus piogenes* in the development of the infectious process in the respiratory tract, including strangles.

From October to November 2017, all 40 isolates of Streptococcus isolated from foals with strangles had genetic typing by PCR with further electrophoresis in agarose gel.

Nucleotide sequence of the gene 16S of rRNA of *Streptococcus equi* was revealed in the isolates № "P1", "N-1 caz", "N-5/1", "N-12-3", "1-3", "7-3". Thus, the authors isolated 6 isolates of strangles Streptococcus, which corresponded to the results of morphological and cultural studies (Figure 8).

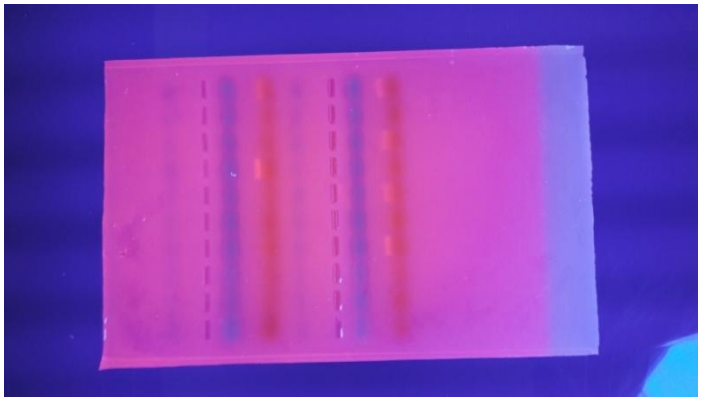

Figure 8. Results of genetic typing by a PCR method with further electrophoresis in agarose gel

Further molecular-genetic typing in the Russian State Center for Animal Food and Drug Standardization and Quality) confirmed that the analyzed nucleotide sequence of the studied 3 trains of *Streptococcus equi* "N-5/1", "N-12-3", "N-1 caz" was 100% identical to the nucleotide sequence of the fragment of the gene 16S rRNA of *Streptococcus equi*. Thus, 3 promising strains

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of Streptococcus equi were isolated and identified that can be used for the development of immune biological drugs.

"N-5/1" strain of *Streptococcus equi* was the most effective and underwent commission trials at the department of All-Russian collection of microorganism strains in the department of quality evaluation and standardization of probiotic drugs of the All-Russian State Scientific Research Institute (ARSRI).

It was established that *Streptococcus equi* "N-5/1" belonged to the family *Streptococcaceae*, genus*Streptococcus*, species*Streptococcus equissp*by the cultural, morphological, and biochemical properties and corresponded with the typical characteristics of the representatives of the species. Based on the results of the conducted studies, this strain was deposited to the All-Russian state collection of the strains of microorganisms used in veterinary and animal breeding of the ARSRI (registration number ВКШМ-Б-141П, record on the deposit dated May 22nd 2018). "N-5/1" strain of *Streptococcus equi* is used as a derivative for the production of immune biological drugs for the prevention of horse strangles in the Russian Federation. Patent application was submitted.

"N-1 caz" strain of *Streptococcus equi* isolated from a sick horse in the Republic of Kazakhstan can be used for the development of a vaccine used in horse herd farming in Kazakhstan.
The authors isolated the causative agent of horse strangles (strangles Streptococcus), as well as *Enterococcus faecales*, *Streptococcus piogenes* and toxicogenic and mold fungi *Aspergillus* and *Mucor*, in foals with respiratory diseases with clinical manifestations of strangles.

The isolation of toxicogenic and mold fungi from the nasal cavity of foals with respiratory diseases can be explained by a significant spread of microscopic fungi Aspergillus and Mucor in the flora of tebenevka pastures in Yakutia, especially in rainy years [23; 24].

The obtained results agree with the study of Danvillier et all (2018) [25], who revealed the presence of different fungi in the respiratory tract of horses and indicated the necessity of the further study of fungi elements in the etiology of the diseases of the respiratory tract in horses and humans. The studies of the interrelation between bacterial and fungal infection are also important.

The available data indicates that "Sakhabactisubtil" (probiotic) can be successfully used for the treatment of mycotoxicosis [24].

Thus, during the development of the means of prevention of horse strangles, substances against the effect of toxigenic fungi and immune modulators should be used for the enhancement of immune biological reactivity.
The obtained results confirm the suggestion that the identification of the carriers of strangles Streptococcus by standard methods of cultivation is complicated because of insufficient sensitivity and significant labor input [26]. The most sensitive, specific, and quick method of diagnostics of strangles and identification of strangles Streptococcus is PCR test.

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Conflict of Interests. The authors declare no conflict of interest.

Off-Label Antimicrobial Declaration

Authors declare no off-label use of antimicrobials.

Institutional Animal Care and Use Committee (IACUC) or Other Approval Declaration

Authors declare no IACUC or other approval was needed.

Human Ethics Approval Declaration

Authors declare human ethics approval was not needed for this study.

Abbreviations

MPA - meat-and-peptone agar, MPB - meat-and-peptone broth, SSA - semisolid agar, LD_{50} – 50 % of lethal dose, PCR – polymerase chain reaction, rRNA – ribosomal ribonucleic acid, CFU – colony forming unit.

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