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The Use of Various Tests for the Serological Diagnosis of *Salmonella* Abortion in Horses in Kazakhstan

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ABSTRACT

Abortions of horses caused by *Salmonella* cause great economic damage to horse breeding farms and private farmsteads in Kazakhstan. This infection is primarily diagnosed through bacteriology, but it is highly dependent on the state of the material under study. It is also time-consuming and lacks sensitivity. Moreover, PCR analysis is used to identify and differentiate the causative agent of *Salmonella* abortion; the main disadvantage of this is the high cost of equipment and test systems. At the same time, in our country, the effective and affordable method of enzyme-linked immunosorbent assay (ELISA) for serological diagnosis and monitoring of this infection is not used. The aim of this work is to show the use of ELISA based on outer membrane proteins (OMPs) for the serological diagnosis of *Salmonella* abortion in horses. Self-produced OMPs of *S. enterica abortus egui* were used in ELISA. Serum samples were taken from aborted mares and contact animals. According to the results of the study of blood sera of mares, the presence of specific antibodies was established in 20-43% of cases. The detection of antibodies indicates that the animal has had a disease or is a carrier of the causative agent of the infection, and, in general, an unfavourable situation for this infection in the surveyed farm. The use of ELISA and latex agglutination tests (LAT) makes it possible to determine the presence of specific antibodies in the serum of unvaccinated animals and can be used to monitor the epizootic situation for this infection.

Key words: *Salmonella enterica abortus egui*, Specific antibodies, Diagnostics, ELISA, LAT.

INTRODUCTION

Salmonella abortion in mares is an infectious disease caused by *S. enterica subsp. enterica serovar Abortus-equi*, accompanied by premature birth (abortion) and the birth of a non-viable foetus. The economic damage consists of the loss of the reproductive ability of the mares, the lack of offspring, the decrease in the productivity of mares, and the increased cost of veterinary drugs and disinfection. There is a high level of infection in horses in many countries (Marenzoni et al. 2012; Martelli et al. 2019: Bustos et al. 2020; Pavlova et al. 2020), including Kazakhstan (Sultanov et al. 2015). All horses are susceptible to the disease (Uzal et al. 2022), but it is clinically manifested more often in pregnant mares, and most abortions are recorded in young animals. New-born foals also suffer from salmonellosis (Grandolfo et al. 2018) and asymptomatic infection is noted in stallions. The source of the infectious agent is aborted mares, which excrete a large number of bacteria with the foetal membranes, amniotic fluid and outflow from the vagina, as well as sick animals without clinical signs. The bacteria can be isolated for up to 60 days. The transmission factors of the pathogen are feed, water, bedding, and horse care items. Infection of healthy animals occurs through the alimentary route and during mating. The waste products of the pathogen and its toxins cause uterine contraction and expulsion of the foetus (Robinson and Wilson 2007; Martelli et al. 2018; Grandolfo et al. 2018; Burgess 2023).

Horse breeding in the Republic of Kazakhstan is the most important branch of animal husbandry; the number of horses in the country at the beginning of 2021 amounted to more than 3 million heads (https://eldala.kz/novosti/zhivotnovodstvo/3989-po-chislennosti-loshadej-kazahstan-nahoditsya-na-shestom-meste-v-mire). According to statistics, abortions in mares ranged from 6-30% of the total livestock, with over 50% caused by *Salmonella*

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(Sultanov et al. 2015). To prevent *Salmonella* abortion of mares in Kazakhstan, vaccination of pregnant mares with a vaccine based on an attenuated strain of *S. abortus equi* E-841 (Kazakh Research Veterinary Institute LLP) is used (Mussayeva et al. 2021). However, for various reasons, not all livestock in the country are vaccinated. For example, many farmers use year-round free grazing of horses, which makes it very difficult to carry out preventive measures. In addition, the veterinary service does not provide for regular diagnostic measures for this infection.

According to the OIE guidelines, the main diagnostic method for this infection is bacteriological culture method (http://www.oie.int/). It should be noted that this method not only lacks sensitivity but is also highly dependent on the state of the material being studied and is time-consuming. The OIE also recommends the use of PCR to identify and differentiate the causative agent of *Salmonella* abortion (Amavisit et al. 2001; Kurowski et al. 2002). However, the use of PCR in veterinary laboratories is not always advisable owing to the high cost of equipment and test systems.

For the serological diagnosis of this infection, various immunological methods are used, such as enzyme-linked immunosorbent assay (ELISA), immunochromatographic assay (ICA) and latex agglutination tests (LAT) (Rajashekara et al. 1998; Gall et al. 2006; Burgess et al. 2015; Li et al. 2019).

Serological studies for the diagnosis of *Salmonella* abortion of horses in Kazakhstan are not currently carried out. The purpose of this work is to show the feasibility of using ELISA based on OMPs to determine the epizootic situation in the horse breeding farms of Kazakhstan.

MATERIALS AND METHODS

Ethical Approval

All experiments related to animals were carried out with the permission of the Local Ethical Committee of S. Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan (Protocol No. 1 dated 26.08.2020).

Research Design

The present study was a test of serum samples from aborted mares, as well as animals that had contact with them, using our developed ELISA and commercial LAT. Positively responding animals were established and the level of specific antibody titers was determined.

Study Period and Sample Collection

The study was carried out in private horse breeding farms and private farmsteads of farmers in Akmola, Karaganda, Kostanay and Zhambyl regions, Kazakhstan. Serum samples were collected from horses that had previously experienced foetal abortion or had direct contact with such mares. Control sera were kindly provided by the National Veterinary Reference Center, Astana, Kazakhstan.

Antigen Preparation

As an antigen, OMPs from an attenuated vaccine strain of *S. abortus equi* E-841 (Kazakh Research Veterinary Institute LLP) were used. OMPs were obtained from the Laboratory of the Research platform of Agricultural Biotechnology, S. Seifullin Kazakh Agrotechnical University, according to the previously described method (Padmanabhan et al. 2018).

Antigen-ELISA

The study of serum samples was carried out in an indirect ELISA. The 96 well plates were sensitised with antigen at a concentration of 0.01mg/mL and incubated for 14-16h at 4°C. The plate wells were washed with PBS×1 after each incubation step. Free sites were blocked with 0.1% solution of bovine serum albumin (Amresco, Solon, OH, United States) and incubated for 1 hour at 37°C. The studied blood sera were added in two dilutions: 1:100 and 1:200, and incubated at 37°C. As a positive control, the blood serum of a mare with a diagnosis of *Salmonella* abortion confirmed by bacteriological and PCR methods was used. The anti-species conjugate Rabbit Anti-horse IgG (Cusabio Technology, Houston, TX, USA) was added at a working dilution of 1:5000. 3,3',5,5'-Tetramethylbenzidine (AppliChem, Darmstadt, Germany) was used as a substrate, and the reaction was terminated with a 0.02M sulfuric acid solution (Sigma Aldrich, Louis, MO, USA). The results were read on a spectrophotometer HiPo MPP-96 (BioSan, Riga, Latvia) at a wavelength of 450nm. Commercial latex diagnosticum "Salmonella Latex Kit" (Liofilchem, Roseto d. Abruzzi, Italy), which is designed to detect specific antibodies in blood serum, was used according to the instructions.

Statistical Analysis

GraphPad Prism 9.4.1 software (https://www.graphpad.com/updates/prism-941-release-notes) was used for statistical processing and comparison of the obtained results (Fig. 1).

RESULTS

The results of testing horse serum samples in ELISA are presented in Table 1. As can be seen from Table 1, specific antibodies to *S. abortus egui* antigens were detected in 11 out of 36 blood serum samples from animals from the Karaganda region. In the study of 50 blood serum samples from animals from the Kostanay region, 10 positive samples were identified. In serum samples from the Zerenda district of the Akmola region, the presence of antibodies was found in 12 out of 31 samples. In the samples from Korgalzhin district of Akmola region, seven samples contained specific antibodies. The highest percentage of positive samples was found in samples from farms in the Zhambyl region (43.75%).

Positive sera according to the results of ELISA were additionally tested by LAT (Table 2). In the study of sera using the Salmonella Latex Kit, a positive reaction, according to the instructions, was estimated at +++ and ++++; doubtful results (less than three crosses) were not considered. In the blood serum samples of animals from the Karaganda region, three positive samples were detected in the reaction of latex agglutination. In the study of blood sera from animals from the Kostanay region, four positive samples were confirmed in the lat. In sera samples from the Zerenda district of the Akmola region, the presence of antibodies to *S. abortus egui* antigens was found in seven cases.

Table 1: Results of testing horse serum samples in ELISA

Study region of Kazakhstan	Samples tested	Positive (%)	Negative (%)
Karaganda region	36	11 (30.5)	25 (69.5)
Kostanay region	50	10 (20)	40 (80)
Jambyl region	16	7 (43.75)	9 (56.25)
Akmola region			
Zerenda district	31	12 (38.7)	19 (90)
Atbasar district	6	0	6 (100)
Korgalzhyn district	27	7 (25.9)	20 (74.1)
Total	166	47 (28.3)	119 (71.7)

Table 2: Results of testing horse sera with the commercial Salmonella Latex Kit test

Study region of Kazakhstan	Samples tested	Positive (%)	Negative (%)
Karaganda region	11	3 (27.3)	8 (72.7)
Kostanay region	10	4 (40)	6 (60)
Jambyl region	7	4 (57.1)	3 (42.9)
Akmola region			
Zerenda district	12	7(58.3)	5 (41.7)
Atbasar district	6	0	6 (100)
Korgalzhyn district	7	0	7 (100)
Total	53	18 (34)	35 (66)

Similar studies were carried out with serum samples from the farms of the Zhambyl region. It was found that out of seven positive samples according to the results of ELISA, four were positive in the latex agglutination test.

In the study of positive samples from the Korgalzhinsky district of the Akmola region, according to the results of LAT, no positive samples were found. Samples of six blood sera from a farm in the Atbasar district of the Akmola region were negative according to the results of both tests.

DISCUSSION

To conduct a mass screening of the horse population to determine the presence of antibodies against *S. abortus equi*, we developed an ELISA protocol using outer membrane proteins from the *S. abortus equi* strain bacteria.

In our opinion, this disease is not given due attention in the Republic of Kazakhstan generally. For example, in terms of research on the number of horses for *Salmonella* abortion, the purchase of tests for serological diagnostics in the Republican Veterinary Laboratory is not provided. Meanwhile, in case of abortions in mares, it is crucial to establish the exact cause since this fact will determine the further actions of veterinarians and animal owners. Several research results suggest that abortions of mares are not always associated with infectious diseases. Thus, Ricard et al. (2022) report that they have been studying cases of mare abortions in Canada for 13 years and have concluded that most abortions were non-infectious.

Along with the bacteriological method and PCR analysis (Heymans et al. 2018; Yang et al. 2021), serological methods are successfully used to diagnose salmonellosis in horses (Martelli et al. 2018). Thus, Gall et al. (2006) proposed an indirect ELISA for the detection of antibodies in the blood serum of horses to the lipopolysaccharide (LPS) of *S. enterica subsp. Enterica serovar Abortus equi*, which was a more sensitive and stable test for the detection of serum antibodies.

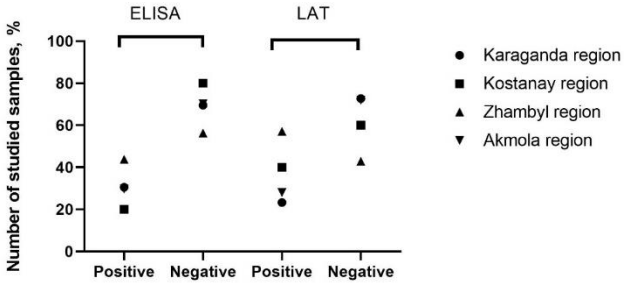

Fig. 1: The results of the study of serum samples from the regions of Kazakhstan by ELISA and LAT

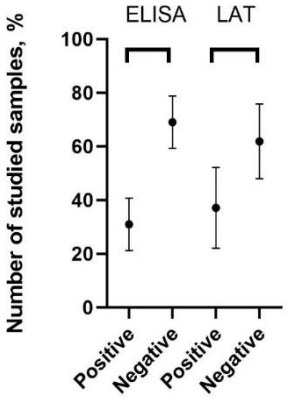

Fig. 2: Comparison of the results of the study of serum samples by ELISA and LAT

It is well known from the literature that the antigen obtained by ultrasonic disintegration retains the nativeness of its chemical structure but contains a large number of protein and biopolymer complexes, which reduce their specificity (Padmanabhan et al. 2018). LPS is a thermostable component of the outer part of the cell membrane of all gram-negative microorganisms; hence, the likelihood of cross-reactions is high. At the same time, outer membrane proteins are a very valuable component of the cell wall, consisting of diagnostically important proteins that have the necessary specificity and can be used in serological diagnostic tests (Ricard et al. 2022). Thus, when developing an ELISA protocol, it is advisable to use outer membrane proteins as antigens (Sova and Kusaykin 2006; Bulashev et al. 2016).

As a result of studies of mare serum samples from several regions of Kazakhstan, it was found that specific antibodies were detected in 15 samples (11%) from the total number of those examined. The conducted studies show that specific antibodies to the pathogen *S. abortus egui* can be detected in animals at any time of the year, although abortions in mares are observed, as a rule, in winter and early spring (from December to April). Thus, considering the obtained results, both tests can be used to study the epizootic situation with *Salmonella* abortion in horses and control the spread of infection among horses. It should be noted that the results of the study can only be used for the serological diagnosis of *Salmonella* abortion in horses.

Conclusion

As a result of the studies, the expediency of using an indirect ELISA for the serological diagnosis of *Salmonella* abortion in mares has been shown. It has been established that in the serum samples of horses from farms where the

livestock was not vaccinated, specific antibodies were found in a significant number of individuals. The obtained data correlated with the results of the commercial Salmonella Latex Kit test.

Given the above, we can recommend using ELISA and LAT for the serological diagnosis of *Salmonella* abortion of mares in horse breeding farms to establish the epizootic situation for this infection.

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Author's Contribution

SB: Conceptualized and designed the study, wrote the manuscript. AS and OA: Collected samples, conducted the experiments, analyzed data. KT: Revised and finalized the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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