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# Clinical Case of Salmonella Detected in an Aborted Mare Fetus and its Characteristics

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## ABSTRACT

In articles and reports by researchers from different countries, *Salmonella enterica* subsp. *enterica serovar Abortus equi* is often cited as the cause of abortion in mares. Here we report a clinical case of *Salmonella* isolated from an aborted mare fetus, and the results of its typing and examination of antibiotic resistance. Abortions were reported in a herd of local Jabe horses. Vaginal smears and internal organ (heart, spleen) samples of an aborted horse fetus from a farm in the Karaganda region were obtained. The mare aborted in the stage of deep pregnancy, and the fetus that formed had fur and hooves. Three different isolates were obtained and classified as Salmonella. The identical antimicrobial resistance pattern and sequencing type were seen in all three samples. The isolates were subsequently genotyped and found to be 100% identical to *S. enterica* subsp. *enterica*. Based on the results of bacteriological and molecular genetic analyses of cultures isolated from the samples, it was established that the cause of abortion in mares was an infection related to salmonella etiology and was caused by the pathogen *S. enterica subsp. enterica serovar Enteritidis*.

**Key words:** *Salmonella enterica*, Isolate, Genotyping, Antibiotic resistance.

## INTRODUCTION

*Salmonella* abortion of mares is an infectious disease caused by *Salmonella enterica subsp. Enterica serovar Abortus equi*, that results in abortions and the birth of a non-viable fetus. All horses are susceptible to *Salmonella* abortion, but it is clinically manifested more often in pregnant mares, and most abortions are recorded in young animals. Newborn foals also suffer from this disease and stallions have asymptomatic infection (Grandolfo et al. 2018). In addition, there are reports of *Salmonella* abortion in donkeys (Wang et al. 2019). Economic damage may result from the loss of the reproductive ability of mares, a decrease in their productivity, loss of offspring, and the cost of veterinary drugs and disinfection. It has been reported that there is a high level of infection in horses in many countries, including Kazakhstan (Llorente et al. 2016; Marenzoni et al. 2012; Pavlova et al. 2020; Borovikov et al. 2023). The source of the causative agent of infection is aborted mares, which secrete a large number of bacteria through the fetal membranes, amniotic fluid and vaginal discharge. In addition, the source of the pathogen can also act as a bacterial carrier, and bacterial shedding can continue for up to 60 days. The pathogen transmission factors include feed, water, bedding, and horse care items. The waste products of the pathogen and its toxins lead to contraction of the uterus and expulsion of the fetus (Robinson and Wilson 2007). To prevent *Salmonella* abortion in mares in the Republic of Kazakhstan, and certain regions of the Russian Federation, the vaccination of pregnant mares is implemented (Musaeva et al. 2018; Neustroev and Petrova 2020). However, due to the fact that the main population of horses is concentrated in the personal farmsteads of citizens and farms, not all of the horses are vaccinated. The purpose of the work was to confirm the Salmonella etiology of abortions in mares in a specific herd, and to isolate and characterize *Salmonella* isolates.

## MATERIALS AND METHODS

### Ethical Approval

The experiments related to animals were performed with the permission of the Local Ethical Committee of S. Seifullin Kazakh Agrotechnical Research University, Astana, Kazakhstan (Protocol # 1, 26.08.2020).

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**Collection of Material and Isolation of Isolates:** In the spring of 2023, we recorded the abortions of mares on a private farmstead in the Nura district of the Karaganda region. Vaginal smears and internal organ (heart, spleen) samples of an aborted horse fetus from a farm in the Karaganda region were obtained. The mare aborted in the stage of deep pregnancy, and the fetus that formed had fur and hooves.

To isolate the *Salmonella* strains, selective bismuth-sulfite-GRM agar was used (SSCAMB, Russia). Petri dishes with selective bismuth-sulfite-GRM agar were incubated for 20-24h at 37°C. The tinctorial and morphological properties of the isolated *Salmonella* cultures were studied using Gram staining and microscopy.

**Species Identification using MALDI-TOF:** Colonies with growth characteristic similar to that of *Salmonella* were subcultured onto separate Petri dishes and identified using the Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) method. Briefly, several colonies were mixed in 1µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (α-HCCA) with 50% acetonitrile and 2.5% trifluoroacetic acid (TFA), and then subsequently air-dried. The chip containing the samples was put inside a microflex LT MALDI-TOF mass spectrometer made by Bruker Daltonics. A calibration standard was used to place and calibrate the chip. Spectra were automatically collected with 40 laser pulses at a frequency of 60 Hz. The mass/charge range for analysis was 2000–20000Da. The Bruker MALDI-TOF Biotyper v4.0 software was used to analyze the spectra, and the minimum score for species identification was 1.8.

**Species Identification using 16S rRNA analysis:** Qiagen's QIAamp DNA Mini Kit was used to isolate genomic DNA. Genotyping of isolates was performed by analyzing a fragment of the 16S rRNA gene (Srinivasan et al. 2015). The PCR reaction was run in a total volume of 30µL using the universal primers 8f 5' - AgAgTTTgATCCTggCTCAg-3 and 806R- 5' ggACTACCAgggTATCTAAT. The BigDye® Terminator v3.1 Cycle Sequencing Kit was used for the sequencing procedure, and the 3730xl DNA Analyzer was used to separate the sequenced fragments. SeqScape 2.6.0 software was used to evaluate and integrate nucleotide sequences obtained using forward and reverse measurements. Using the BLAST method, the acquired 16S rRNA gene nucleotide sequences were compared to existing nucleotide sequences stored in the GenBank database (www.ncbi.nih.gov). Sequences deposited in the global GenBank database were also used to build phylogenetic trees. Phylogenetic trees were created using the Mega 6 program. The Muscle algorithm was used to align nucleotide sequences, and the neighbor-joining NJ method was used to build trees.

**Determination of Sensitivity to Antimicrobial Drugs:** A determination of the sensitivity of isolates to antimicrobial drugs was conducted using the disk diffusion method according to EUCAST standards. Briefly, a bacterial suspension equivalent to the McFarland turbidity standard (No. 0.5) was added to the surface of Mueller-Hinton agar (TM Media, India) in a Petri dish using a sterile cotton swab. Inoculation was performed manually by applying the inoculum using streak movements over the entire surface of the agar in three directions. Discs containing antimicrobial agents (TM Media, India) were applied to the agar surface no later than 15 min after inoculation. The dishes were incubated at 35°C for 18h (Testing TECoAS 2020).

## RESULTS

*Salmonella* isolates were only obtained from samples of the parenchymal organs of the aborted fetus. Individual colonies with growth characteristic similar to that of *Salmonella* were identified on a MALDI Biotyper. The MALDI-TOF method facilitated identification at the generic level. Three isolates were identified as *Salmonella spp*, with a high score value (2.1-2.5).

Next, genetic identification was performed using 16S rRNA analysis. Nucleotide sequences were analyzed and combined into a common sequence using SeqMan software (DNA Star). Thereafter, the terminal fragments (primer nucleotide sequences and low-quality score fragments) were eliminated. The BLAST method was used to find the retrieved sequences in GenBank. The outcomes are displayed in Table 1.

As follows from Table 1, all three isolates belong to the species *S. enterica subsp. enterica*. After considering the literature data indicating the presence of errors in the international banks of nucleotide sequences GenBank, and Ribosomal Database Project (RDP-II), we further constructed phylogenetic trees using nucleotide sequences of the 16S rRNA gene of reference *S. enterica subsp. enterica* strains (http://www.bacterio.net). The analysis includes the 16S rRNA gene nucleotide sequences of the bacteria that are most closely related phylogenetically (Fig. 1).

As displayed in Fig. 1, samples 1, 2 and 3 are located on the same phylogenetic branch as that of *S. enterica subsp. enterica serovar Enteritidis*. One of the important characteristics of these isolates is their antibiotic resistance indicators. Discs containing 18 types of drugs (TM Media, India) were used to determine antibiotic resistance. After measuring the diameters of the zones of growth inhibition, which was dependent on the data in the table of breakpoints, the isolates were categorized as Sensitive or Resistant to certain types of antimicrobial drugs. If the data fell within the range of uncertain interpretation, according to EUCAST recommendations, the situation was defined as an “Area of Technical Uncertainty (ATU)” (Table 2).

According to the results shown in Table 2, all three isolates of *S. enterica subsp. enterica* were resistant to Ampicillin + Sulbactam and Cefuroxime; however, all three isolates were sensitive to the remaining 16 drugs.

## DISCUSSION

In Kazakhstan, cases of abortions in mares are recorded often, sometimes resulting in widespread infections and leading to significant economic damage to farms (Sultanov et al. 2015). In this regard, it is very important to determine the exact cause of abortions, as this will allow veterinary specialists to take appropriate measures. According to the results of studies performed in

**Table 1:** Results of identification of isolates using 16S rRNA analysis

| Sample | Sequence | GeneBank Accession number | Strain name | Match (%) |
|---|---|---|---|---|
| 1 | AGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAA CTGCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAA CGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGC CCAGATGGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATC CCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC CTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTAC TTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTA CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC | OQ915464.1 | *Salmonella enterica subsp. enterica* | 100 |
| 2 | TACACATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGA GTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATA ACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGG GGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGT TGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGA TGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC GTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGG TGTTGTGGTTAATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATC GGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGA AATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTTG | ON764807.1 | *Salmonella enterica subsp. enterica* | 100 |
| 3 | TACACATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGA GTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATA ACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACCAAAGAGG GGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGT TGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGA TGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC GTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGG TGTTGTGGTTAATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCC | ON764807.1 | *Salmonella enterica subsp. enterica* | 100 |

**Table 2:** Results of determining the sensitivity of isolates of *S. enterica subsp. enterica* to antimicrobials

| # | Name of the drug | Substance concentration (µg/mL) | Number of isolates by sensitivity | | |
|---|---|---|---|---|---|
| | | | Sensitive | ATU | Resistant |
| 1. | CO-Trimoxazole | 25 | 3/3 | 0/3 | 0/3 |
| 2. | Amikacin | 30 | 3/3 | 0/3 | 0/3 |
| 3. | Amoksiklav | 30 | 3/3 | 0/3 | 0/3 |
| 4. | Ampicillin | 10 | 3/3 | 0/3 | 0/3 |
| 5. | Azithromycin | 15 | 3/3 | 0/3 | 0/3 |
| 6. | Cefotaxime | 30 | 3/3 | 0/3 | 0/3 |
| 7. | Ceftriaxone | 30 | 3/3 | 0/3 | 0/3 |
| 8. | Gentamicin | 10 | 3/3 | 0/3 | 0/3 |
| 9. | Cefadroxil | 30 | 3/3 | 0/3 | 0/3 |
| 10. | Ampicillin + Sulbactam | 20 | 0/3 | 0/3 | 3/3 |
| 11. | Cefuroxime | 30 | 0/3 | 0/3 | 3/3 |
| 12. | Tobramycin | 10 | 3/3 | 0/3 | 0/3 |
| 13. | Amoxicillin | 10 | 3/3 | 0/3 | 0/3 |
| 14. | Ofloxacin | 5 | 3/3 | 0/3 | 0/3 |
| 15. | Chloramphenicol | 30 | 3/3 | 0/3 | 0/3 |
| 16. | Nitrofurantoin | 300 | 3/3 | 0/3 | 0/3 |
| 17. | Piperacillin | 100 | 3/3 | 0/3 | 0/3 |
| 18. | Ciprofloxacin | 5 | 3/3 | 0/3 | 0/3 |

Canada (Ricard et al. 2022), abortions of mares are not always associated with infectious diseases. Marenzoni et al. (2012) analyzed 107 cases of equine abortion in Brazil and found that 28.6% of the total cases were attributed to infectious diseases.

In contrast, Bustos et al. (2020) reported the isolation of 27 isolates of *S. abortus equi* from horses in Argentina between 2011 and 2016, which were studied using virulence gene profiling and pulsed-field gel electrophoresis. Nine pulsotypes from pulsed-field gel electrophoresis and four pathogenicity profiles were found. Various strains were discovered in the same holding, indicating the existence of various infection origins or the possibility of isolate mutation.

In a herd of Murgese horses, an epidemic of *S. Abortus equi* was reported by Grandolfo et al. (2018). Of the 34 newborn foals, ten died at birth, and seven more died within the first ten days of life after developing acute clinical symptoms. Four sick foals' synovial fluid samples, tissue samples from two dead foals' organs, and vaginal and rectal

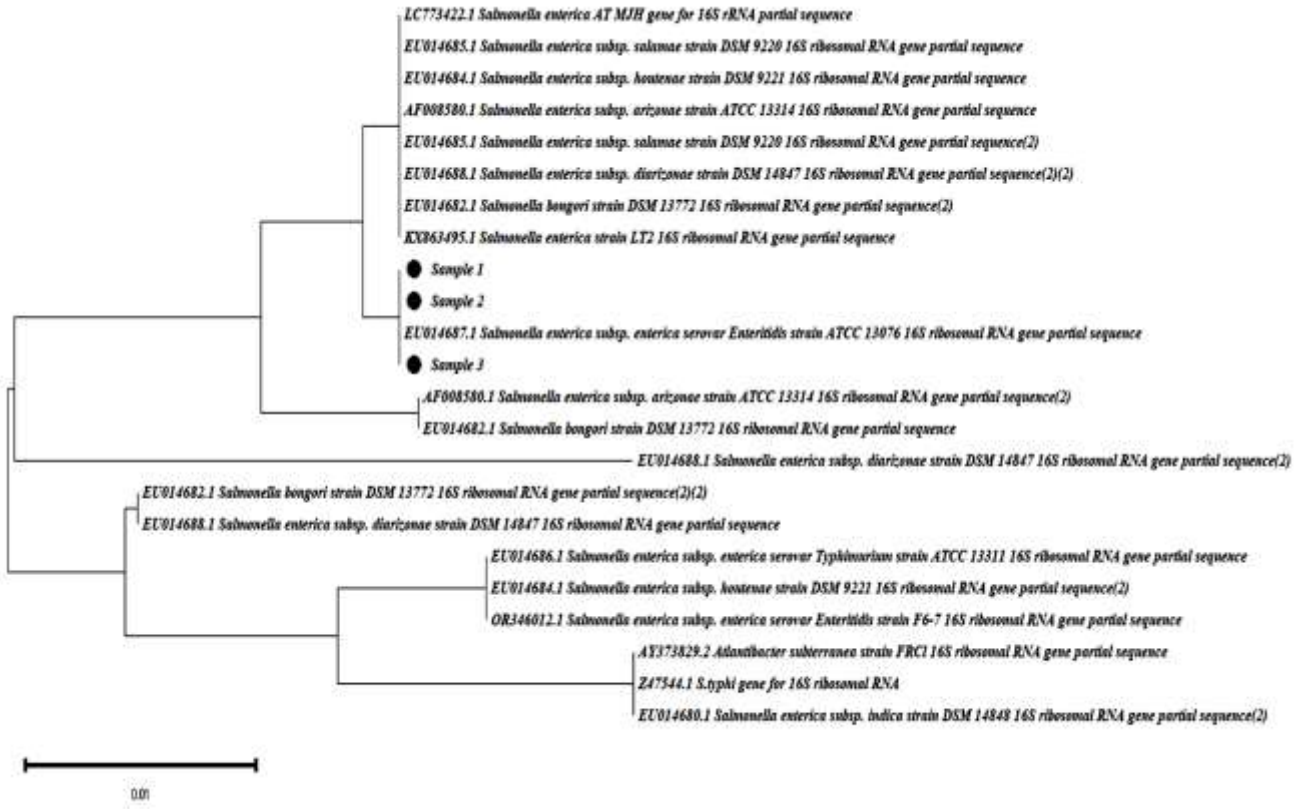


**Fig. 1:** Phylogenetic tree constructed based on analysis of the 16S rRNA gene fragment of samples 1, 2 and 3.

swabs from mares were all used for the culture. *Salmonella* was found in a total of 16 isolates, all of which were isolated as pure cultures. The samples had a similar pattern of antibiotic resistance and the *S. Abortus equi*-related ST251 sequence type. Serotyping revealed that six of the 16 isolates were *S. Abortus equi* 4,12:-:e,n,x.

In the present study, three *Salmonella* isolates from samples of parenchymal organs of an aborted mare fetus were assigned to the species *S. enterica* subsp. *enterica* via genetic identification using 16S rRNA analysis. The construction of phylogenetic trees using nucleotide sequences of the 16S rRNA gene of reference strains made it possible to classify the isolates as *S. enterica* subsp. *enterica serovar Enteritidis.*

Also noteworthy is the limitation of this study that the Salmonella isolates were obtained from a single aborted fetus. For a more reliable picture, studies with a larger number of samples are necessary.

## Conclusion

In our study, we demonstrated that the cause of abortion on an individual horse farm was the spread of an infection of *Salmonella* etiology. The pathogenic isolates were identified as *S. enterica* subsp. *enterica serovar Enteritidis*. The sensitivity of the isolates to antibiotics was studied. All three isolates of *S. enterica* subsp. *enterica* were resistant to the drugs Ampicillin + Sulbactam and Cefuroxime but were sensitive to the remaining 16 drugs. Based on the results, we can recommend the use of this protocol to determine the causes of abortion in mares and a list of appropriate antibiotics for the treatment of these animals.

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**Author Contributions:** SB: Conceptualized and designed the study, wrote the manuscript. AM and OA: Collected samples, conducted the experiments, analyzed data. MK: Revised and finalized the manuscript.

**Conflicts of Interest:** The authors declare that they have no competing interests.

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