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Characterization and antimicrobial susceptibility of *Escherichia coli, Salmonella enterica* isolated from Uvurkhangai province in Mongolia

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Abstract

The incidence of infectious diseases caused by the *Enterobacteriaceae* family of Uvurkhangai province in Mongolia is not decreasing and is recurring despite the implementation of preventive measures in the province. Nowadays, when the resistance of microorganisms negatively affects the health of humans and animals, food safety, the environment, and the economy, the lack of effective antibiotics for treatment is the reason for the research work on this topic. Based on the outbreak and surveillance of infectious diseases for the past 5 years, the pathological materials were received in the veterinary laboratory, two strains of *Escherichia coli* and also two strains *Salmonella enterica subsp. abortus equi* were isolated, and identified by conventional bacteriological and molecular methods. The antimicrobial susceptibility of the pathogen was determined using the disc diffusion method. The strains of *E. coli* were resistant to several antimicrobials, including vancomycin and neomycin, intermediate to colistin. The *Salmonella enterica subsp. abortus equi* showed resistance to ampicillin, vancomycin, gentamicin, and neomycin. Due to the presence of antimicrobial resistance, the results suggest a risk to public health as these pathogens. However, the four isolated bacteria in the family of *Enterobacteriaceae* showed susceptibility to groups of tetracycline and streptomycin. As a result of this study, it was determined that treatment with tetracycline and streptomycin is effective against bacterial infections caused by the *Enterobacteriaceae* group.

Keywords: Enterobacteriaceae, AMR, colibacteriosis, salmonellosis

Introduction

The members of the *Enterobacteriaceae* are geographically widespread and many are widely distributed throughout the environment in soil, water, plants, and even in the intestines of animals and humans.

According to the livestock census of 2021, 4,022,700 animals were counted in Uvurkhangai province, of which 270,000 horses, 2 million sheep, and 1,493 goats were registered [1]. Of these, since 2017, 67 outbreaks of Colibacteriosis and 6 outbreaks of Salmonellosis have been detected (Figure 1). This statistic demonstrates that the incidence of infectious diseases caused by the *Enterobacteriaceae* family is not decreasing and is recurring despite the implementation of preventive measures in the province. In African and Asian countries, *Salmonella spp* bacteria still cause abortion in mares although it has been well-controlled in many countries in recent years [2, 3, 4, 5]. Out of approximately 2600 *Salmonella* serotypes [6], most can infect several animal hosts, including humans. The rod shapes of *Salmonella spp* differ from each other in serotypes and virulence although they are morphologically indistinguishable, and *Salmonella spp* differ only in the selection and adaptation for specific hosts. Therefore, it is recommended to identify the causative agent, diagnose the infection, prevent the spread of the infection at the herd level, and immediately administer antibiotic treatment.

While antibiotics like tetracycline, sulfonamide groups, as well as gentamicin, neomycin, apramycin, ceftiofur, and others, are highly effective, there has been an increase in resistance. Hence, it is necessary to check the antibiotic's susceptibility to bacteria and select the best antibiotics for treatment.

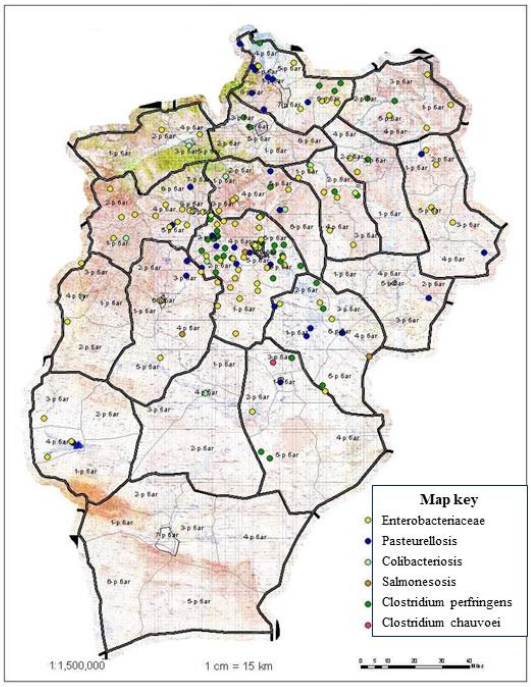


Figure 1. Map of foci of animal infectious diseases diagnosed in Uvurkhangai province between 2017 and 2022

Colibacteriosis is an acute infectious disease that manifests itself in a newborn baby animal's first days after birth. Symptoms include diarrhea, internal intoxication, dehydration, hemoptysis, and neurological symptoms. Intestinal coliforms are transmitted through the gastrointestinal tract and respiratory tract of healthy animals, through contaminated equipment and feed processing. Infected young animals usually die within 4-12 hours. In veterinary medicine, certain groups of antibiotics such as tetracycline, chloramphenicol, neomycin, and polymyxin are used to treat infectious diseases [7]. However, the selection of antibiotics based on the determination of their susceptibility has become a significant issue in today's problem of microbial resistance.

Our study aimed to identify and isolate *Escherichia coli (E. coli)* and *Salmonella spp* from pathological material and swabs. Additionally, we conducted antimicrobial susceptibility testing on the isolated strains.

Material and methods

Origin of samples

Based on the outbreak and surveillance of infectious diseases in Uvurkhangai province in Mongolia for the past 5 years, the pathological material of dead kits and mares with clinical symptoms were received in the veterinary laboratory. The amniotic fluid and placenta samples from mares were taken from Bayangol, and the pathological tissue samples of the dead kits were taken from Dzunbayanulaan and Taragt sums of Uvurkhangai province. Samples were stored at -20^{0}C degrees in the freezer. Samples were obtained under approval from the Ethical Review Board for the Use of Animals in Experiments (MEBUS/23/01/05).

Salmonella spp., E. coli isolation and identification

All the samples were serially diluted by Kox's method and then cultured on Nutrient Agar (HiMedia®, India), (NA) to obtain a pure culture. *E. coli* isolates were obtained by culturing on selective media MacConkey (Biolab, Hungary) and Endo agar (Condalab, Spain) [8].

Briefly, the pathological tissue was transferred from tubes containing phosphate-buffered saline (PBS) to tubes containing 9 ml PBS and incubated at 37ºC for 24 hrs. Afterward, the samples were streaked onto MacConkey and Endo agar plates. The plates were then incubated at 37ºC for 24 hrs to observe the growth of typical *E. coli* colonies, as a metallic green reflex.

After inoculation in a selective medium, stained by Gram, biochemical characteristics were determined using trisaccharide iron agar (TSI, Biolab, Hungary) and Simmons citrate agar (Biolab, Hungary), EB20 (Nissui, Japan) test was inoculated, incubated at 37^0C for 20 hrs, and interpreted as recommended by the manufacturer [9]. To ensure standardization, we used *E. coli* ATCC 25922 as a positive control.

To identify *Salmonella* spp., pathological samples were analyzed according to the protocols described in the Technical Manual for Laboratory Diagnosis of *Salmonella* spp., [6] and MNS ISO 6579-3:2019 [10]. The steps for obtaining a pure culture are similar to those of *E. coli*. Subsequently, they were streaked onto selective xylose-lysine-deoxycholate (XLD) agar (HiMedia®, India) and Salmonella Shigella (SS) agar (HiMedia®, India) plates and incubated at 37ºC for 48 hrs. After inoculation in a selective medium, stained by Gram, biochemical characteristics were determined using trisaccharide iron agar (TSI, Biolab, Hungary) and Simmons citrate agar (Biolab, Hungary), EB20 (Nissui, Japan) test for identification of *Salmonella* spp [4, 6]. Traditionally the genera and species of the family have been distinguished biochemically, which is convenient for identifying clinical isolates. All samples were cultured on 5% sheep blood agar and incubated at 37ºC for 48 hrs.

Molecular confirmation of isolates

Salmonella spp bacteria grown in Nutrient broth (NB) and overnight cultures were used for DNA extraction according to the G-spin™ Total DNA extraction kit protocol (iNtRON, S. Korea). The gene encoding biosynthesis of outer membrane protein C of *Salmonella* genus was detected in the present study [11, 12]. The *ompC* gene was amplified using primers (provided by Bioneer Co., S. Korea) F (5'-ATCGCTGACTTATGCAATCG-3') and R (5'-CGGGTTGCGTTATAGGTCTG-3'). PCR was performed under the following conditions: 95ºC 2 min x1, 95ºC 1 min, 57ºC 1 min, 72ºC, 2 min x30, and 72ºC 5 min x1. Green master mix, 2× (Promega, USA) includes 1: Taq DNA polymerase, 2: dNTPs which include 400 μM of each dATP, dGTP, dCTP, dTTP, 3: 3 mM of $MgCl_2$, and 4: Yellow and blue dyes as the loading dye. After PCR, the profiles of amplification products were detected by gel electrophoresis. Eight μl of the total reaction mixture was loaded on a 1.5% agarose gel and electrophoresed at 100 V for 30 min. The amplified DNA fragments were visualized by UV illumination after agarose gel electrophoresis and ethidium bromide staining by standard procedures.

Antimicrobial susceptibility testing

The antibiotic susceptibility of the pathogen was determined by the disc diffusion method [13]. Briefly, the test used a sterile cotton swab to pick colonies from an overnight culture of non-selective media. Then, the colonies were suspended in saline and mixed to adjust the density to a 0.5 MacFarland standard. The culture was spread on Mueller-Hinton agar (HiMedia®, India) and incubated at 36^0C for 20 hrs. Inoculum into petri dishes was done according to the technique by Kirby-Bauer [14]. The antibiotics tested were amoxicillin (10 mcg), penicillin G (10 unit), ampicillin (10 mcg), tetracycline (10 mcg), oxytetracycline (30 μg), doxycycline (10 mcg), vancomycin (10 mcg), streptomycin (25 mcg), novobiocin (10 mcg), gentamicin (10 mcg), colistin (10 mcg), neomycin (10 mcg) (Biolab, Hungary). After incubation, the diameters of the zones of incubation were measured to the nearest millimeter with a ruler. Interpretation of zones has been found from EUCAST. Accordingly, clinical results obtained were either evaluated as susceptible (S), intermediate (I), or resistant (R) for clinical application. A reference strain of *E. coli* ATCC 25922 was used for quality control of antimicrobial susceptibility tests.

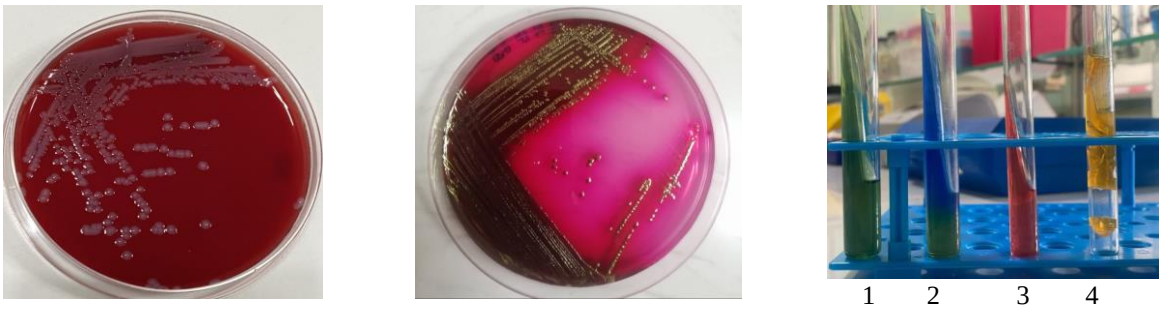
Results

E. coli and Salmonella spp detection and isolation

A total of 4 microbial strains were isolated from pathological samples. In the *E. coli* isolation, medium-sized, light yellow, "S" colonies with rounded surfaces were found on NA, and NB was highly turbid,

Bacteria were stained by Gram method and microscopic examination of the smear at 100X magnification revealed Gram-negative, non-sporulating, blunt-ended, bipolarly stained bacilli. However, using TSI and Simmons citrate agar to determine biochemical characteristics, the sucrose and glucose were oxidized to form a gas, and the waxy, and disintegrated on shaking, and cultured without hemolysis on blood agar. *E. coli* bacteria were isolated by cultured shiny, golden yellow, S-shaped, dome-shaped colonies on Endo agar, which is a selective medium, and a light pink S-shaped colony on MacConkey agar (Fig. 2A).

citrate test showed positive results (Fig. 2B). The ID-EB20 test showed positive results for indole, lysine, ornithine, raffinose, sorbitol, mannitol, and arabinose, and the correlation was 99% for *E. coli*. Lactose fermenting Gram-negative coccobacilli were obtained by conventional bacteriological methods.



(A) (B) (C)

Figure 2. Characterization of isolated *E. coli*.

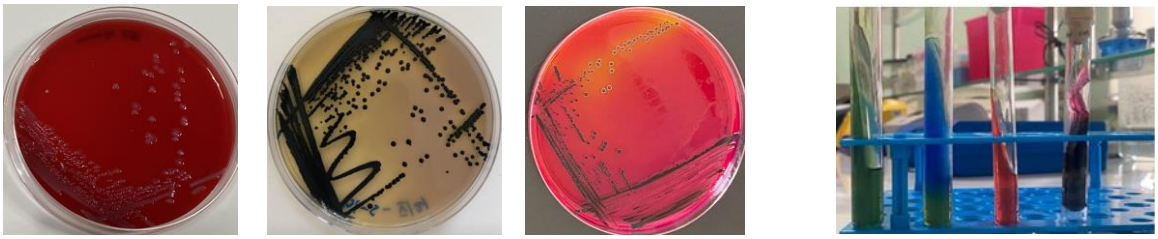
The left (A) showed no hemolysis on blood agar, and the right (B) showed metallic green colony growth on Endo agar. (C) showed 1-uninoculated, 2-reaction of Simmons citrate agar and TSI agar.

In the *Salmonella spp* isolation, medium-sized, pale yellow, S-shaped colonies were observed on NA, uniformly turbid on NB, and cultured without hemolysis on blood agar. However, in MacConkey agar, which is a selective nutrient medium, lactose-negative or pale-yellow colonies were formed, and in SS and XLD agar, colonies with a black center and a clear circle were cultured, and *Salmonella spp* bacteria were isolated. It was stained with Gram and microscopic examination of the smear at 100X magnification revealed gram-negative, non-sporulating, blunt-edged bacilli. Colonies were identified with large black centers with a clear perimeter growth on a selective medium for differentiation of *Enterobacteriaceae* and *Proteus*. Additionally, using TSI and Simmons citrate agar to determine biochemical characteristics, glucose was oxidized, H_2S was released, and the citrate test showed positive results. Also, biochemical characteristics were determined by ID-EB20 test, with 99% detection of *Salmonella enterica subsp. abortus equi*. These findings were also confirmed by VITEK® 2 system.

Molecular confirmation of isolated strains

The PCR amplification results of S*almonella enterica subsp. abortus equi* were confirmed by electrophoresis analysis. The amplified DNA was 204 bp for the omp C gene, as observed with the 1000 bp DNA ladder marker (data not shown). For differential diagnosis, Equine herpesvirus-1, the cause of the mare's abortion, was determined by PCR and the results were negative.

Based on our resources, we performed a PCR to identify Enterohemorrhagic *E. coli* (EHEC), but it was negative. We could not identify any of the other eight pathovars of *E. coli* through molecular biology studies. It will be discussed in our next publication.



(A) (B)

Figure 3. Characterization of isolated *Salmonella enterica subsp. abortus equi.*
The left (A) showed no hemolysis on blood agar, and the right middle showed growth characterization on SS and XLD agar. (B) showed 1-uninoculated, 2-reaction of Simmons citrate agar and TSI agar.

Antimicrobial susceptibility testing

In the study of antimicrobial susceptibility testing by disc diffusion method, *E. coli* is more sensitive. Results are shown for streptomycin (17-19 mm) doxycycline (~14 mm), and colistin (10 mm). However, high resistances to vancomycin and neomycin were determined experimentally (Fig. 4). Zone diameter breakpoints for antimicrobial agents are taken from CLSI document M100 [14, 15]

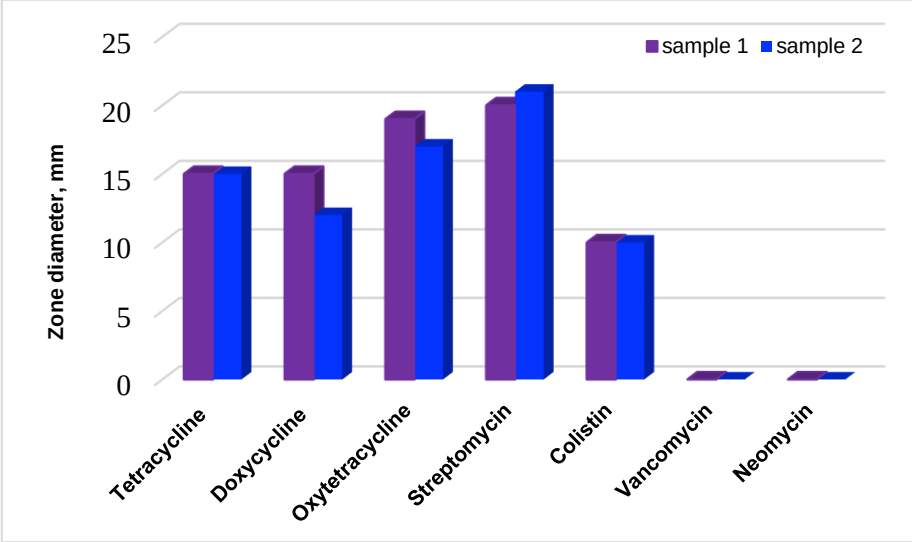


Figure 4. Antimicrobial susceptibility pattern of *Escherichia coli* isolates from dead kits in Uvurkhangai, Mongolia

Antibiotic sensitivity studies of *Salmonella enterica subsp. abortus equi* showed high sensitivity to streptomycin (>15 mm), oxytetracycline (>15 mm), tetracycline (>15 mm), and doxycycline (>15 mm) antibiotics in 4 groups of 10 antibiotics. The intermediate zones for antibiotics such as amoxicillin and penicillin G were found to be between 14-16 mm. However, resistance to ampicillin (<13 mm), vancomycin, gentamicin (<12 mm), and neomycin has been determined (Fig. 5).

Discussion

Salmonellosis is one of the most widespread infectious diseases in the world that affects humans, mammals, reptiles, and poultry [3, 5, 17]. It is caused by *Salmonella*-type gram-negative bacteria and infects the animal body with the clinical symptoms of fever, lethargy, abortion, and diarrhea. Humans and animals can be infected through their food products, the environment, animal feed, and contaminated soil and water.

Since 2017 in Uvurkhangai province, 6 outbreaks of Salmonellosis have been detected, which shows that despite the implementation of preventive measures in the province, the incidence of infectious diseases caused by *Salmonella spp* has not decreased and occurs repeatedly.

There were significant economic losses due to the high number of foals that were lost and the cost of treatment. Salmonella differ from each other in serotypes and virulence but are morphologically indistinguishable, and Salmonella differ only concerning the specific host species they can infect. It is recommended to identify the causative agent, diagnose the infection, prevent its spread at the herd level, and immediately administer antibiotic treatment [6, 7].

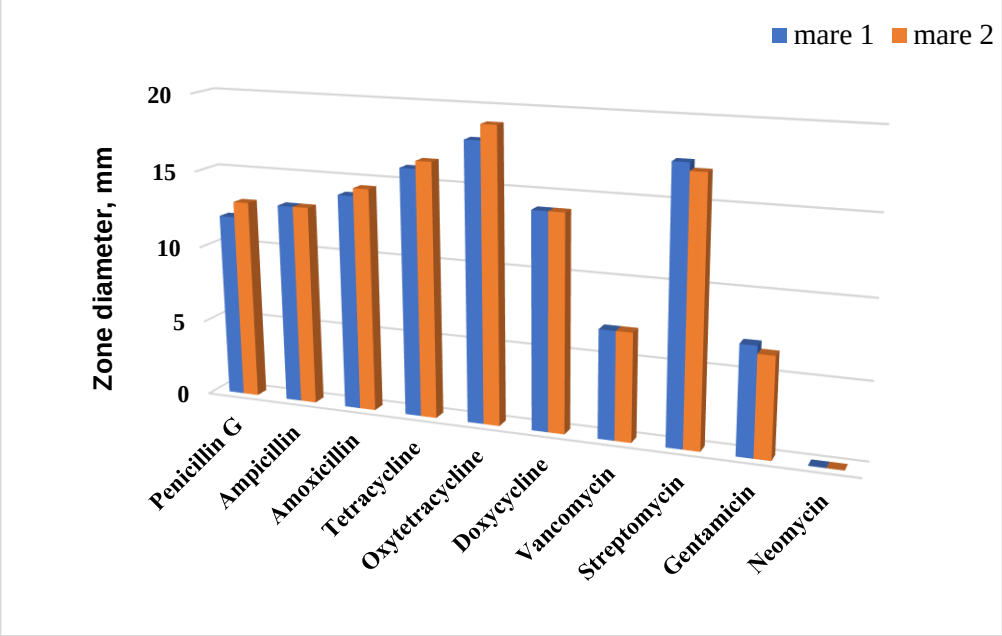


Figure 5. Antimicrobial susceptibility pattern of *Salmonella enterica subsp. abortus equi* isolates from mares in Uvurkhangai, Mongolia

In our study, epidemiological characteristics, bacteriological, and molecular detection of *Salmonella enterica subsp. abortus equi* in pure isolation from all samples allowed the identification of *Salmonella* outbreaks. This finding was similar to that reported for equine abortion by Madic *et al.* [3] negative for lactose, H_2S was released, and the citrate test showed it was positive. Diagnosis of infectious abortions was associated with bacterial etiology as Equine Salmonellosis. All of the samples from the mares were negative to the virologic screening and no other bacteria were isolated.

To the best of our knowledge, this is the first study to investigate the antimicrobial susceptibility of *Salmonella enterica subsp. abortus equi* in mares in Mongolia. Clinical and Laboratory Standards Institute (CLSI) breakpoints were used to interpret disk diffusion results for bacteria associated with animal infections. All of the samples displayed a high level of sensitivity to antibiotics from the tetracycline groups and streptomycin [15, 16]. Grandolfo *et al.* [2] discussed similar results except for streptomycin and doxycycline. The Journal of Equine Veterinary Science published a paper on equine abortion in 2016 [3] regarding antibiotic susceptibility testing. The isolates from the study exhibited susceptibility to tetracycline, ampicillin, and gentamicin, but resistance to doxycycline and streptomycin. Some antibiotics from the penicillin group showed only intermediate sensitivity for the bacteria. An interesting finding was that the isolated *Salmonella enterica subsp. abortus equi* bacteria were resistant to gentamicin. The detection of resistant strains can be explained by the maintenance of genetic mutations through spontaneity or bacterial species via horizontal gene transfer.

Escherichia coli, a gram-negative, rod-shaped bacteria belonging to *Enterobacteriaceae*, causes the infectious disease Colibacteriosis in animals [19]. Infected young animals usually die within half a day.

In our study, we analyzed the intestinal enteric *E. coli* among dead kits with infectious diarrhea in Uvurkhangai province in Mongolia.

The epidemiological characteristics and bacteriological analysis detected E. *coli* in pure isolation from all samples allowing the identification of *E. coli* outbreaks. These results were consistent with studies by Oscar et al., published in 2010 [20], and Kodaka et al., published in 2004 [9]. Further investigation of pathotypes of *E. coli* for the animal origin is necessary.

Recently, the resistance of bacteria to antibiotics has been increasing; it is necessary to check the antibiotic sensitivity in the laboratory along with the diagnosis and carefully select the antibiotic to be used for treatment. Through the CLSI breakpoint data, *E. coli* is more sensitive to the antibiotics of oxytetracycline and streptomycin; however, resistance to vancomycin, neomycin, and colistin for intermediate was determined experimentally. Gram-negative bacteria have an outer membrane and cell-surface modifications that act as a barrier to certain antibiotics. The glycopeptide antibiotics, including vancomycin, are clinically effective only against Gram-positive bacteria. The D-Ala-D-Ala binding site of peptidoglycan for vancomycin inhibits cell growth and binary division. However, those termini of Gram-negative bacteria are located in the periplasm and are consequently inaccessible to vancomycin due to their inability to breach the outer membrane barrier [21, 22].

Colistin (polymyxin E), a last resort antibiotic against multidrug-resistant Gram-negative bacterial infections in the WHO AWARE reserve group, is available for the treatment of animals. Colistin is commonly used in animal husbandry for treatment and as a growth promoter in food supplements, either in normal or excessively high doses, to increase the selection for colistin resistance in animals [23]. In veterinary medicine, the excessive use of antibiotics, including colistin, is threatening their effectiveness. On average, the levels of colistin in food animals were more than 600 times higher than in humans in some countries in 2012 [24]. There is a tendency for an increase in colistin-resistant members of the *Enterobacteriaceae* bacteria in the future.

Conclusion

In conclusion, the study concluded that a highly effective antibiotic treatment of tetracycline and streptomycin can be used to treat bacterial infections caused by the Enterobacteriaceae group in Uvurkhangai province of Mongolia.

The authors declare no conflict of interest.

Conflict of Interests

Authors' Contribution

S.S., U.T. designed the concept of this study. S.S., Ts.B., and Ch.B. equally conducted laboratory work. N.G., Eo.To., U.T. analyzed data and validated. Writing including original draft preparation, review, and editing was performed by U.T. All authors read and approved the final manuscript.

Acknowledgment

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