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Determination of the Presence, Virulence Factors, and Antimicrobial Susceptibility of *Salmonella* spp. in Neonatal Calf Diarrhoea in the Aegean Region

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Abstract: The objective of this study was to investigate the prevalence, virulence gene profiles, and antimicrobial susceptibility of *Salmonella* spp. Isolates from neonatal calves with diarrhoea in the Aegean region of Türkiye. A total of 185 faecal samples from affected calves were collected and examined. *Salmonella* spp. were isolated from 4.3% of the samples. Molecular analysis revealed the presence of virulence genes invA, stn, mgtC, and misL in all isolates, which are associated with invasion, toxin production, intracellular survival, and host cell adhesion. Antimicrobial susceptibility testing revealed that all isolates were fully susceptible to cefepime, levofloxacin, ciprofloxacin, cefotaxime, amoxicillin/clavulanic acid, and colistin sulphate, while varying degrees of resistance to tetracycline group antibiotics were observed. These results suggest that, despite the low prevalence of *Salmonella*, the isolated strains demonstrated significant virulence potential and emerging resistance patterns. To prevent the spread of resistant strains and improve the management of neonatal calf diarrhoea, it is essential to implement continuous monitoring of antimicrobial susceptibility and establish appropriate therapeutic strategies.

Keywords: *Antibiotic Resistance, Neonatal Calf Diarrhea, PCR, Salmonella spp., Virulence.*

Introduction

Neonatal calf diarrhea (NCD) is one of the most common infectious diseases seen in animals on dairy farms. It also causes significant economic losses due to high morbidity and mortality rates (Morgan et al., 2004; Zahran and El-Behiry, 2014). The pathogenic bacteria responsible for the infection are spread to the environment through feces and can persist in water, milk, feed, and soil for long periods (Alcaine et al., 2005). It is estimated that about 75% of deaths in calves under three weeks of age are associated with diarrhea (Heinrichs and Radostits, 2001). The pathogenic bacteria responsible for the infection are spread into the environment through feces and can persist for long periods in water, milk, feed, and soil (Alcaine et al., 2005). Calves typically become infected by ingesting pathogens that are present in contaminated environments. Within just a few hours post-ingestion, the pathogen can colonise the intestinal tract and be recovered from the rectum. This situation compromises individual animal welfare and negatively impacts herd renewal rates, genetic progress, and overall farm productivity (Bentum et al., 2025). Among the bacterial agents isolated from calf diarrhoea cases, *Salmonella* spp. is particularly noteworthy due to its zoonotic potential and ability to exacerbate clinical symptoms (Hoelzer et al., 2010). *Salmonella* is a Gram-negative, facultatively anaerobic, motile pathogen of *Enterobacteriaceae*. The genus is divided into two species, *S. enterica* and *S. bongori*. *S. enterica* comprises six subspecies and over 2,700 recognised serotypes responsible for most infections in humans and domestic animals (Poppoff et al., 2001; Popoff et al., 2003; Tindall et al., 2005). *Salmonella* is a Gram-negative, facultatively anaerobic, motile pathogen of Enterobacteriaceae. Taxonomically, the genus is divided into *S.* enterica, and *S.* bongori species. *S. enterica* comprises six subspecies and over 2,700 identified serotypes and is responsible for most human and domestic animal infections (Popoff et al. 2001; Popoff et al., 2003; Tindall et al., 2005). Different *Salmonella* strains are typically identified by utilizing the Kauffmann-White classification system. This system is predicated on the differentiation of strains according to their surface antigens O (somatic), H (flagellar), and, in certain instances, Vi (capsular) types (Braden 2006; Majowicz et al., 2010). However, classification alone is not sufficient when it comes to the disease-causing capacity of this pathogen. The pathogenesis of *Salmonella* infections is primarily regulated by the bacterium's chromosomally encoded pathogenicity islands (SPIs). For example, the *invA* and *stn* genes are located on SPI-1 and SPI-2 and are associated with host cell invasion and enterotoxin production, respectively (Ramatla et al., 2024). In addition, the *mgtC* gene on SPI-3 allows the bacterium to survive in macrophages, while the *misL* gene facilitates host cell adhesion and attachment to the mucosal surface (Foley and Lynne, 2008). These genes function as critical virulence factors and molecular biomarkers, enabling diagnosis and epidemiological monitoring. Antimicrobial therapy plays a crucial role in reducing systemic complications in neonatal salmonellosis. Nevertheless, the empirical selection of antibiotics in field conditions is often a consequence of delays in obtaining culture and susceptibility testing results. This practice may contribute to the development and spread of antimicrobial resistance (Hoelzer et al., 2010). Therefore, continuous monitoring of antimicrobial susceptibility profiles is essential for designing effective and targeted treatment protocols. With proper management, the annual mortality rate of calves under one month old can be reduced to less than 35%, and the age at first calving can be optimised to about 24 months (Heinrichs and Radostits, 2001).

The present study investigated the presence of virulence genes (*invA*, *stn*, *mgtC,* and *misL*) in *Salmonella* spp. Isolates obtained from calves with neonatal diarrhoea. Polymerase chain reaction (PCR) was utilised to detect these genes, and antimicrobial susceptibility models were evaluated using phenotypic methods. It is anticipated that the findings of this study will provide veterinarians with the necessary knowledge to select the most suitable antimicrobial agents, contribute to the updating of treatment protocols, and support the development of herd-level management strategies against *Salmonella* infections.

Material and Methods

This study was conducted on dairy cattle farms in the Aegean region of Türkiye between 2021 and 2024. The farms were particularly focused on in the provinces of İzmir, Manisa, Aydın, Kütahya, Uşak, and Muğla. One hundred eighty-five neonatal calves, aged 21 days or younger, were sampled on private and commercial farms. Sampling was conducted on subjects presenting with symptoms consistent with neonatal diarrhoea, including watery faeces, dehydration, anorexia, depression and a weakened sucking reflex. Faecal samples were collected directly from the rectum in a meticulous manner, under aseptic conditions, with the utilisation of sterile gloves and containers. The samples comprised approximately 10-15 grams of material each. The samples were then transported to the Izmir Bornova Veterinary Control Institute, Bacteriology Department laboratory in ice-filled containers, where they were analysed within 24 hours of arrival. Permission was obtained from Bornova Veterinary Control Institute HADYEK E-71705440-550-18501575 for this study. Additionally, the authors declare that they have complied with Research and Publishing Ethics.

Isolation and Identification of *Salmonella* spp.

For microbiological analysis, faecal samples were initially subjected to pre-enrichment in Peptone Buffered Water at 37 °C for 18–24 hours. Subsequently, selective enrichment was performed using Müller-Kauffmann tetrathionate broth and Rappaport-Vassiliadis broth, which were then incubated for 24 hours at 37 °C and 42 °C, respectively. Following this, cultures obtained from these enrichment steps were streaked onto MacConkey agar and Xylose Lysine Deoxycholate (XLD) agar plates, which were then incubated at 37 °C for 24–48 hours (WHO, 2010). Colonies manifesting lactose-negative and H_2S-positive

morphology were then sub-cultured for purification, and biochemical identification of the isolates was conducted using the VITEK® 2 Compact Automated Identification System (bioMérieux, France).

Molecular Analysis of Virulence Genes

Deoxyribonucleic acid (DNA) was extracted following the manufacturer's protocol (QIAamp DNA Mini Kit, Qiagen, Germany). The present study focused on four virulence genes: *invA* (244 base pairs), *stn* (617 base pairs), *mgtC* (655 base pairs), and *misL* (986 base pairs). The *invA* gene is located on *Salmonella* Pathogenicity Island 1 (SPI-1) and is associated with epithelial cell invasion. The *stn* gene has been identified as the responsible locus for encoding an enterotoxin. The *mgtC* gene, located on SPI-3, modulates intracellular survival and magnesium transport, while the misL gene, situated on SPI-4, contributes to host epithelial cell adhesion. The PCR amplifications used the Techne TC-412 thermal cycler (Keison Products) and Xpert Fast Hotstart Mastermix (2×, GRiSP). The thermal cycling conditions were as follows: initial denaturation at 94°C for 3 minutes; 40 cycles consisting of denaturation at 94 °C for 15 seconds, annealing at primer-specific temperatures for 30 seconds, and extension at 72 °C for 15 seconds; followed by a final extension at 72 °C for 3 minutes. The GRS Universal DNA Ladder (GRiSP) was used as the molecular weight marker. The amplification products were separated on a 1.5% agarose gel, stained with Xpert Green DNA Stain Direct (GRiSP), and visualized under UV illumination using the Vilber E-Box gel documentation system. The confirmation process yielded results indicating the four targeted virulence genes in all eight isolates.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was conducted on Mueller-Hinton agar utilizing the Kirby-Bauer disc diffusion method. The bacterial suspensions were prepared following the 0.5 McFarland standard and uniformly inoculated onto the agar surface. After the antibiotic discs had been applied to the plates, these were then placed in an incubator set to a temperature of 37 °C for 18 to 24 hours. The inhibition zone was interpreted using the standards outlined by the Clinical and Laboratory Standards Institute (CLSI, 2022). The panel of antibiotics tested in this study included cefepime, levofloxacin, ciprofloxacin, cefotaxime, amoxicillin combined with clavulanic acid, colistin sulphate, ampicillin, amoxicillin, doxycycline, and oxytetracycline. It was found that all isolates were 100% susceptible to cefepime, levofloxacin, ciprofloxacin, cefotaxime, amoxicillin/clavulanic acid, and colistin sulphate. Conversely, resistance was observed in 16.7% of isolates against ampicillin and amoxicillin, and 33.3% against doxycycline and oxytetracycline.

Results

In this study, 185 faecal samples collected from neonatal calves in the Aegean Region between 2021 and 2024 were bacteriologically examined, and *Salmonella* spp. was isolated in 4.3% of cases (n = 8). Positive samples originated from the provinces of İzmir (n = 3), Manisa (n = 2), Aydın (n = 1), Muğla (n = 1), and Uşak (n = 1). All isolates were obtained from calves exhibiting clinical signs of diarrhoea; no *Salmonella* was detected in samples taken from clinically healthy animals. The low number of infected calves, the limited diversity of detected serotypes, and the absence of other enteric pathogens in *Salmonella* positive cases may suggest that salmonellosis was not the sole cause of diarrhoea observed in this study.

The distribution of virulence genes

The distribution of virulence genes among the isolates and the presence of four key virulence genes (*invA, stn*, *mgtC*, and *misL*) were determined through a Polymerase Chain Reaction (PCR) analysis. The target gene regions, primer sequences, amplicon sizes, and annealing temperatures utilised in this analysis are presented in Table 1.

Table 1. Virulence genes targeted by PCR in *Salmonella* spp. Isolates.

GENE REGION	PRIMER SEQUENCES	ANNEALING (°C)	AMPLICON SIZE (BP)	REFERENCE
invA	5'- ACAGTGCTCGTTTACGACCTGAAT -3' 5'- AGACGACTGGTACTGATCCGATAAT -3'	56	244	Chiu and Ou (1996)
stn	5'- TTGTGTCGCTATCACTGGCAACC -3' 5'- ATTCGTAACCCGCTCTCGTCC -3'	59	617	Murugkar et al. (2003)
mgtC	5'- TGACTATCAATGCTCCAGTGAAT -3' 5'- ATTTACTGGCCGCTATGCTGTTG -3'	60	655	Soto et al. (2006)
misL	5'- GACGTTGATAGTCTGCCATCCAG -3' 5'- CAATGCCGCCAGTCTCCGTGC -3'	60	986	Soto et al. (2006)

All tested *Salmonella* isolates were confirmed by PCR to carry the virulence genes *invA* (244 bp), *stn* (617 bp), *mgtC* (655 bp), and *misL* (986 bp). The resulting amplicon products for the four genes mentioned above were then analysed by agarose gel electrophoresis (Figures 1-4). This analysis yielded visible bands corresponding to the expected sizes.

The images obtained demonstrated the presence of positive bands corresponding to the expected sizes, thereby confirming the molecular identification. Additionally, the presence of all four gene regions in each isolate suggests the potential for the expression of virulence characteristics such

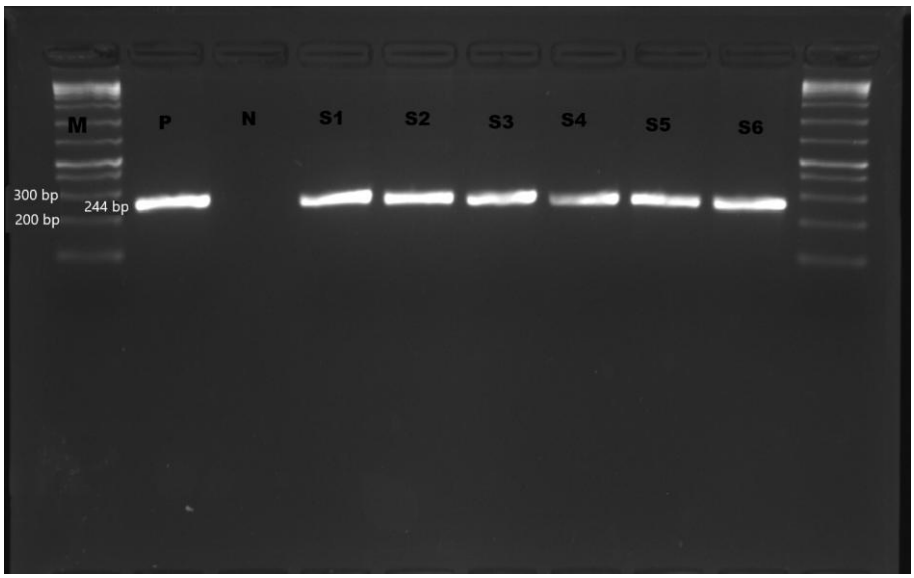

Figure 1. Electrophoresis result of the PCR product of the invA gene (244 bp).

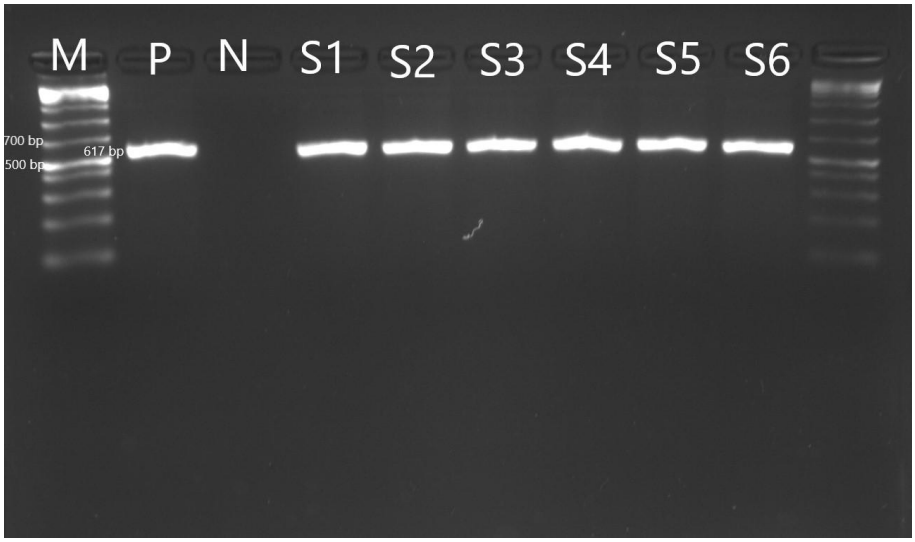

Figure 2. Electrophoresis result of the PCR product of the stn gene (617 bp).

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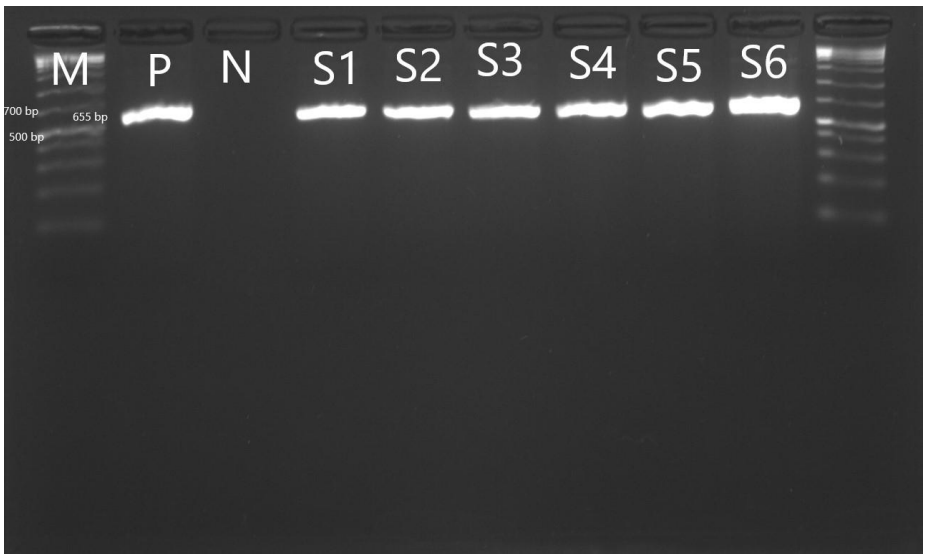

Figure 3. Electrophoresis result of the PCR product of the *mgtC* gene (655 bp).

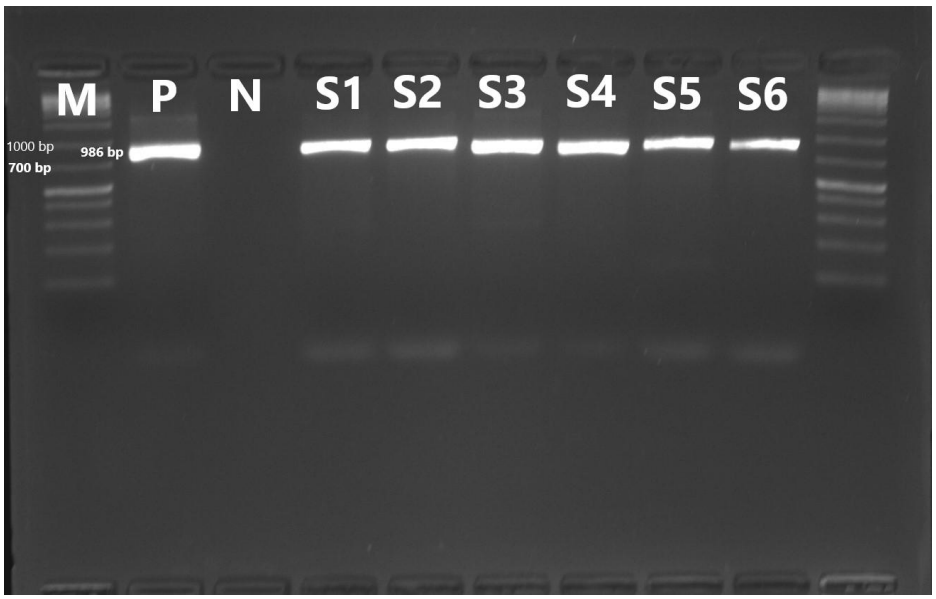

Figure 4. Electrophoresis result of the PCR product of the *misL* gene (986 bp)

as invasiveness, toxigenicity, adhesion, and intracellular survival.

Antimicrobial Susceptibility Profile

Antimicrobial susceptibility testing of all eight *Salmonella* isolates was performed using the Kirby–Bauer disc diffusion method according to CLSI (2022) guidelines. The antibiotics tested and the corresponding susceptibility/resistance rates are presented below (Table 2).

The detection of all four gene regions in each isolate confirmed that these strains possess fundamental virulence characteristics, including invasiveness, toxin production, adhesion, and intracellular survival. The efficacy of colistin sulphate, fluoroquinolones (levofloxacin and ciprofloxacin), and third-generation cephalosporins (cefepime and cefotaxime) was found to be 100% effective. This finding supports their continued applicability as therapeutic options in clinical settings. However, the observed 33.3% resistance to tetracycline-class antibiotics underscores the imperative for their judicious and well-regulated utilisation in clinical practice.

Table 2. Antimicrobial susceptibility profile of *Salmonella* spp. isolates (n=8).

Antibiotic	Number of Resistant Strains (%)	Number of Susceptible Strains (%)
Cefepime	0 (0.0%)	8(100.0%)
Levofloxacin	0 (0.0%)	8 (100.0%)
Ciprofloxacin	0 (0.0%)	8 (100.0%)
Cefotaxime	0 (0.0%)	8 (100.0%)
Amoxicillin/Clavulanic Acid	0 (0.0%)	8 (100.0%)
Colistin Sulfate	0 (0.0%)	8 (100.0%)
Ampicillin	2 (25.0%)	6 (75.0%)
Amoxicillin	2 (25.0%)	6 (75.0%)
Doxycycline	3 (33.3%)	6 (66.7%)
Oxytetracycline	3 (33.3%)	6 (66.7%)

Discussion

Diarrhoea in newborn calves is one of the major health problems causing significant economic losses in the livestock sector. Calves are highly susceptible to diarrhoea, especially in the first month of life, and the incidence of diarrhoea cases tends to decrease with age. Epidemiological studies have revealed that diarrhoea in neonatal calves is primarily due to infectious agents (El-Seedy et al., 2016). In this study, the prevalence, virulence gene profiles, and antimicrobial susceptibility levels of *Salmonella* spp. strains isolated from calves with neonatal diarrhoea symptoms in the Aegean Region were evaluated. *Salmonella* spp. were isolated from 8 (4.3%) of 185 faecal samples collected by a random sampling method. This rate is low compared to the high prevalence values reported by Moussa et al. (2010), 43.53%, Abdullah et al. (2013), 21.9%, and Ibrahim et al. (2025), 9.7%. However, the findings of our study are in line with Haggag and Khaliel (2002) (4%) and Younis et al. (2009) (4.09%). In the United States of America 3.8% (Fossler et al., 2005), in

India 5% (Joon and Kaura, 1993), in Mozambique 5% (Achá et al., 2004), in Australia 23.8% (Izzo et al., 2011), in Algeria 66.6% (Akam et al, 2004), 17.5% in Egypt (Seleim et al., 2004) and 16.4% in Brazil (Coura et al., 2015), the results reported in international studies show that there are significant differences in *Salmonella* prevalence.

This variation is reported to be caused by geographical differences and differences in herd management, animal welfare levels, sampling methods, and laboratory techniques (Younis et al., 2009). Some studies have stated that *Salmonella* infections are not always directly associated with clinical symptoms and that clinical symptoms are mild or almost absent. However, high carrier rates are observed in specific herds. Cummings et al. (2009a) reported that only 17% of positive cattle herds were responsible for 70% of clinical salmonellosis cases. This suggests that *Salmonella* infections may be highly severe on certain farms while remaining subclinical on others. It is also known that there are differences in pathogenicity between different *Salmonella* serotypes. Although the most frequently isolated serotypes in calves are *S.* Typhimurium and *S.* Dublin, serotypes such as *S.* enteritidis and *S.* Agona can also be associated with clinical cases (Ambrosin et al., 2002; Younis et al., 2009; Barrow et al., 2010). Although serotyping was not performed in our study, prospective serotyping of *Salmonella* strains and their association with clinical symptoms is important for both animal health and prevention of zoonotic risks.

In this study, *Salmonella* spp. The positivity rate was 4.3% in faecal samples taken from calves with neonatal diarrhoea symptoms. This rate is generally similar to 4.8% reported by Fossler et al. (2004), Wells (5.4%), and Huston (5.9%). In the study of Fossler et al., 92.7% of the 110 farms sampled had at least one positive sample. However, it was stated that negative results were also found in sampling at different times in the same farms. Positive positivity rates were also reported in farms with large herds. The fact that data were collected only from individuals with clinical symptoms and one-time sampling in this study may provide a limited perspective on the dynamics of intra-herd spread of *Salmonella*. However, the fact that the results obtained were similar to those of larger studies suggests that the finding generally aligns with the trends reported in the literature. In order to evaluate the prevalence more accurately, it may be helpful to prefer sampling strategies that are spread over time and include asymptomatic individuals in future studies.

For instance, Wells et al. (2004) reported that at least one positive sample was detected in 100 of the 110 farms examined (90.9%). However, the researchers emphasised that positivity can vary over time and that negative results can be obtained in samples taken at different times on the same farms. The intermittent shedding of Salmonella may explain this via faeces. This situation can be explained by the fact that *Salmonella* has the characteristic of intermittent shedding via faeces. The 4.3% positivity rate detected in our study is consistent with the 4.8–5.9% range reported by studies such as Huston et al. (2002). Cummings et al. (2009a) emphasized that Salmonella infections cause severe clinical symptoms only in a limited subset of farms, stating that only 17% (16/93) of positive cattle herds account for 70% of clinical salmonellosis cases. This finding suggests that clinical observations may not support the low prevalence rate in our study and that there may be differences in the intensity of the disease spreading on a herd basis.

In our study, the *invA*, *stn*, *mgtC*, and *misL* genes were positive in all isolates, suggesting that these strains have high invasiveness and enterotoxigenic potential. The *invA* and stn genes are located in the SPI-1 and SPI-2 pathogenicity islands, respectively, and regulate host cell invasion and enterotoxin production at the onset of infection. In contrast, the mgtC (SPI-3) gene ensures survival in phagocytic cells, and the misL (SPI-4) gene ensures binding to epithelial cells (Han et al., 2023; Pico-Rodriguez et al., 2024). The fact that these genes were detected in all isolates suggests that the isolated strains may have a high potential to cause clinical infection. Since *Salmonella* spp. are facultative intracellular pathogens, choosing an antimicrobial with good tissue penetration and the ability to reach intracellular therapeutic drug concentrations in macrophages is preferable. It has been reported to show resistance at different rates to broad-spectrum agents such as ampicillin, amoxicillin, amoxicillin-clavulanic acid, ceftiofur, florfenicol, neomycin, sulfonamides, tetracycline, and trimethoprim-sulfamethoxazole (Mohler et al., 2009).

Antimicrobial susceptibility analyses performed in this study revealed that all isolates were 100% susceptible to cefepime, levofloxacin, ciprofloxacin, cefotaxime, amoxicillin/clavulanic acid, and colistin sulfate. This finding indicates that fluoroquinolones and third-generation cephalosporins are effective treatment options. On the other hand, 37.5 % of the isolates were resistant to doxycycline and oxytetracycline, and 25.0 % were resistant to ampicillin and amoxicillin, supporting the variable resistance patterns developed against beta-lactam and tetracycline group antibiotics. These results agree with Abdullah et al. (2013) and Ahmed et al. (2009). *Salmonella* infections represent a significant global public health concern due to widespread zoonotic transmission, antimicrobial resistance, and associated morbidity and mortality. Infections caused by antimicrobial resistance reduce treatment efficacy and increase the risk of spread of resistant bacteria within herds and zoonotically to humans (Nazir et al., 2025). This is particularly associated with plasmid-mediated gene transfer, chromosomal mutations, and inappropriate antibiotic use. In addition, the mutations or genes responsible for resistance accumulate in specific strains or clones of pathogenic bacteria and can persist even in many drugs (Endale et al., 2023). It should not be ignored that antibiotic usage habits in veterinary practices in the region play a decisive role in resistance patterns in the field.

Conclusion

Salmonella spp. continues to be an important bacterial agent in neonatal calf diarrhea. In this study, although *Salmonella* spp. positivity was relatively low (4.3 %), significant virulence genes (*invA*, *stn*, *mgtC*, and *misL*) and

multiple antibiotic resistance were detected in all isolated strains. The resistance observed against tetracycline group antibiotics (37.5 %) and beta-lactams (25.0 %) is especially remarkable, highlighting the importance of regular antimicrobial susceptibility surveillance. The coexistence of virulence and resistance genes indicates that these strains may increase the potential for persistence and spread under unfavourable environmental conditions. Therefore, targeted antibiotic applications based on susceptibility tests are crucial for effectively controlling *Salmonella* infections. Furthermore, strict hygiene measures supported by regular and effective disinfection practices should be taken on farms to reduce the risk of infection, especially during rainy and neonatal periods.

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Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Bornova Veterinary Control Institute Animal Experiments Local Ethics Committee (HADYEK E-71705440-550-18501575 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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