Original Research

Journal of Advanced Veterinary Research
(2026) Volume 16, Issue 4, 491-495

Antibiotic resistance profile of *Citrobacter* spp. isolated from rectal swabs of sheep in Ngrayung Village, Plumpang District, Tuban Regency

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ARTICLE INFO

Received: 01 April 2026

Accepted: 27 April 2026

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Keywords:

Antimicrobial resistance, *Citrobacter* spp., Sheep, Rectal swab, Multidrug resistance, Public health.

ABSTRACT

Antimicrobial resistance (AMR) has become a major global concern in both human and veterinary medicine, particularly due to the increasing emergence of resistant enteric bacteria in food-producing animals. This study aimed to isolate and identify *Citrobacter* spp. from rectal swabs of sheep and to determine their antibiotic resistance profile in Ngrayung Village, Plumpang District, Tuban Regency. A total of 150 rectal swab samples were collected from sheep and processed using standard bacteriological methods. Isolation was performed on MacConkey Agar, followed by Gram staining and biochemical confirmation using IMViC and Triple Sugar Iron Agar tests. Antimicrobial susceptibility testing was conducted using the Kirby–Bauer disk diffusion method on Mueller–Hinton Agar according to CLSI guidelines against five antibiotics: erythromycin, tetracycline, ampicillin, cefoxitin, and aztreonam. Out of 150 samples, 40 isolates (26.7%) showed presumptive colony morphology consistent with *Citrobacter* spp., while 6 isolates (4.0%) were biochemically confirmed. The confirmed isolates demonstrated Gram-negative rod morphology and variable biochemical characteristics. Antimicrobial susceptibility testing revealed that all isolates (100%) were resistant to erythromycin, while resistance to ampicillin and cefoxitin was observed in 66.7% and 33.3% of isolates, respectively. Lower resistance rates were detected for tetracycline and aztreonam (16.7% each). Furthermore, 2 isolates (33.3%) were classified as multidrug-resistant (MDR), including one isolate resistant to all tested antibiotics. These findings indicate that sheep may serve as reservoirs of antimicrobial-resistant *Citrobacter* spp., posing potential risks for animal health, environmental contamination, and zoonotic transmission. Routine AMR surveillance, prudent antibiotic use, and improved farm biosecurity are therefore essential to support antimicrobial stewardship within a One Health framework.

Introduction

Antimicrobial resistance (AMR) has emerged as one of the most critical global public health challenges, affecting both human and veterinary medicine (Effendi *et al*., 2018; Al-Khalaifah *et al*., 2025). The widespread and often inappropriate use of antibiotics in food-producing animals has contributed significantly to the development and dissemination of resistant bacterial strains (Abate and Birhanu, 2025). Livestock can act as important reservoirs of antimicrobial-resistant bacteria, which may spread through direct animal contact, environmental contamination, or the food chain (Khairullah *et al*., 2025). Among enteric bacteria of veterinary and zoonotic importance, *Citrobacter* spp. have increasingly attracted attention because of their opportunistic pathogenicity and capacity to acquire multiple antibiotic resistance determinants (Jabeen *et al*., 2023).

Citrobacter spp. are Gram-negative, facultatively anaerobic bacilli belonging to the family *Enterobacteriaceae* (Zhang *et al*., 2023). These bacteria are commonly found in the intestinal tract of humans and animals, as well as in soil, water, and other environmental sources (Fratini *et al*., 2026). Although often considered commensal organisms, *Citrobacter* spp. can act as opportunistic pathogens associated with urinary tract infections, septicemia, gastrointestinal disorders, and wound infections in both animals and humans (Duduveche, 2026). Certain species, such as *Citrobacter freundii* and *Citrobacter koseri*, are particularly known for their clinical relevance and their ability to harbor resistance genes, including extended-spectrum beta-lactamase (ESBL)-producing strains (Liakopoulos *et al*., 2016).

In livestock production systems, sheep represent an important source of animal protein and economic value for rural communities (Banda and Tanganyika, 2021). However, the gastrointestinal tract of sheep may serve as a reservoir for antimicrobial-resistant enteric bacteria, which can subsequently disseminate within the farm environment and potentially pose a zoonotic risk (Basnet and Kilonzo-Nthenge, 2024). Rectal swab sampling is widely used to assess intestinal bacterial colonization and to monitor the presence of resistant bacteria in apparently healthy animals (Zhang *et al*., 2018). Surveillance of bacterial resistance profiles in farm animals is therefore essential to understand the epidemiology of AMR and to support rational antibiotic use strategies (Sharma *et al*., 2018).

The use of antibiotics in animal husbandry, whether for therapeutic, prophylactic, or growth-promotion purposes, may exert selective pressure that promotes the emergence of resistant bacterial populations (Abate and Birhanu, 2025). In rural farming systems, antibiotic administration is sometimes carried out without prior bacteriological examination or antimicrobial susceptibility testing, which may accelerate the development of resistance (Manyi-Loh *et al*., 2018). This situation is particularly concerning for enteric bacteria such as *Citrobacter* spp., which possess intrinsic and acquired resistance mechanisms, including efflux pumps, beta-lactamase production, and plasmid-mediated resistance genes (Jabeen *et al*., 2023).

Previous studies have reported the presence of antimicrobial-resistant *Enterobacteriaceae* in ruminants, but information specifically related to *Citrobacter* spp. isolated from sheep in Indonesia remains limited (Ejeh *et al*., 2025). In particular, there is a lack of published data regarding

ISSN: 2090-6277/2090-6269/

the resistance profile of *Citrobacter* spp. from sheep farms in Ngrayung Village, Plumpang District, Tuban Regency, an area with active small ruminant farming activities. Local surveillance data are important to provide baseline information for veterinary public health interventions and to evaluate the potential role of sheep as reservoirs of resistant bacteria (Engdawork and Negussie, 2025).

Therefore, this study aimed to isolate and identify *Citrobacter* spp. from rectal swabs of sheep and to determine their antibiotic resistance profile using the Kirby–Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) standards. The antibiotics tested included ampicillin, cefoxitin, aztreonam, tetracycline, and erythromycin, representing commonly used antimicrobial classes in veterinary practice. In addition, the study assessed the occurrence of multidrug-resistant (MDR) isolates, defined as resistance to three or more antimicrobial classes.

Materials and methods

Study location and period

Sample collection was conducted at sheep farms in Ngrayung Village, Plumpang District, Tuban Regency. Laboratory procedures, including bacterial isolation, identification, and antimicrobial susceptibility testing, were carried out from April to May 2025 at the Veterinary Public Health Division Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga.

Sample collection and sample size

A total of 150 rectal swab samples were collected from sheep raised in farms located in Ngrayung Village, Plumpang District, Tuban Regency, East Java, Indonesia. Samples were collected using a random sampling technique with sterile cotton swabs.

The minimum sample size was initially estimated using the Slovin formula, which yielded a minimum requirement of 92 animals. To improve the representativeness of the study population and strengthen the robustness of the findings for publication purposes, the total number of samples was increased to 150 sheep.

Isolation and identification of Citrobacter spp.

Rectal swab samples were directly streaked onto MacConkey Agar (MCA) using the quadrant streak method and incubated at 37°C for 24 h. Colonies suspected of belonging to *Citrobacter* spp. were purified by subculturing to obtain single colonies for further analysis (Ezeh *et al*., 2024).

Presumptive colonies were subjected to Gram staining for microscopic identification. Smears were prepared on glass slides, heat-fixed, and sequentially stained with crystal violet, Lugol's iodine, 96% alcohol, and 0.25% safranin. The stained slides were examined under 1000× magnification using immersion oil. Gram-negative bacilli appearing red or pink rod-shaped cells were considered presumptive *Citrobacter* isolates. Further confirmation of *Citrobacter* spp. was performed using Indole, Methyl Red, Voges–Proskauer, and Citrate (IMViC) tests and Triple Sugar Iron Agar (TSIA) (Arbab *et al*., 2025).

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller-Hinton Agar (MHA) following Clinical and Laboratory Standards Institute (CLSI) guidelines 2024.

Pure bacterial colonies were suspended in physiological saline and adjusted to 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/mL). The bacterial suspension was evenly swabbed onto the entire surface of MHA plates.

After allowing the inoculated plates to stand for 15 min, five antibiotic disks were placed on the agar surface: Ampicillin (10 µg), Cefoxitin (30 µg), Aztreonam (30 µg), Tetracycline (30 µg), and Erythromycin (15 µg). The plates were incubated at 37°C for 18–24 h.

After incubation, the diameter of the inhibition zones was measured using a vernier caliper and interpreted as resistant, intermediate, or susceptible according to Clinical and Laboratory Standards (CLSI, 2024).

Data Analysis

Data obtained from bacterial identification and antimicrobial susceptibility testing were analyzed descriptively and presented in the form of tables and figures. The inhibition zone diameters were compared with CLSI interpretative standards to determine resistance profiles.

Results

Isolation and identification of Citrobacter spp.

A total of 150 rectal swab samples were collected from sheep raised in Ngrayung Village, Plumpang District, Tuban Regency. Following primary isolation on MacConkey Agar (MCA), 40 isolates (26.7%) showed colony morphology suspected to be *Citrobacter* spp., characterized by pink colonies, slightly mucoid texture, circular shape, and convex elevation (Figure 1).

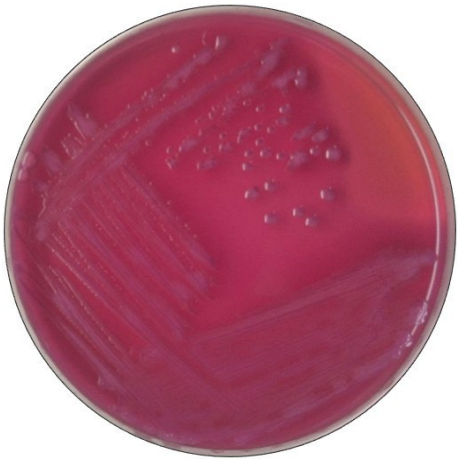

Figure 1. Colony morphology of presumptive *Citrobacter* spp. on MacConkey Agar showing pink, slightly mucoid, circular, and convex colonies after incubation at 37°C for 24 h.

These presumptive colonies were subsequently subjected to Gram staining, which revealed Gram-negative rod-shaped bacteria, appearing as red bacilli under 1000× magnification (Figure 2).

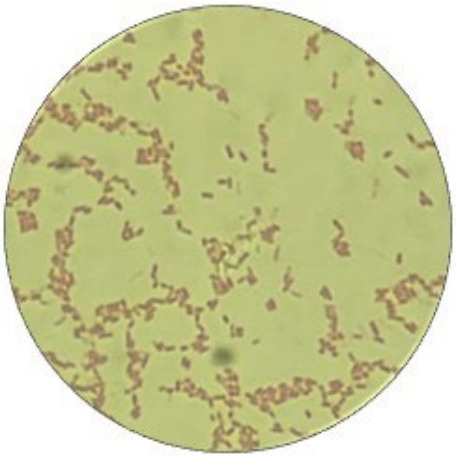

Figure 2. Gram staining of presumptive *Citrobacter* spp. isolates showing Gram-negative rod-shaped bacteria under oil immersion microscopy (1000×).

The 40 presumptive Gram-negative rod isolates were then further examined using biochemical tests, including Indole (SIM), MR-VP, Simmons Citrate Agar (SCA), and Triple Sugar Iron Agar (TSIA). Of these, 6 isolates (15.0% of presumptive isolates; 4.0% of total samples) were biochemically confirmed as *Citrobacter* spp.

Among the six confirmed isolates, one isolate (S53) showed an indole-negative, H_2S-positive, and motile profile on SIM medium, with MR-positive, VP-negative, citrate-positive, and A/A reaction with H_2S production on TSIA (Figure 3). In contrast, the remaining five isolates (S3, S17, S32, S63, and S106) demonstrated indole-positive, H_2S-negative, and motile characteristics, together with MR-positive, VP-negative, citrate-positive, and A/A reaction without H_2S production on TSIA (Figure 4). Detailed biochemical characteristics of the confirmed isolates are presented in Table 1.

Figure 3. Representative biochemical reaction of isolate S53 showing SIM (Indole–, H_2S+, motile), MR+, VP–, citrate positive, and TSIA A/A with H_2S production.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the six confirmed isolates was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton Agar (MHA) against five antibiotics: erythromycin, tetracycline, ampicillin, cefoxitin, and aztreonam.

The results showed that all isolates (100%) were resistant to erythromycin, as evidenced by the absence or very small inhibition zones around the erythromycin disks. Resistance to ampicillin was observed in 4 out of 6 isolates (66.7%), while resistance to cefoxitin was found in 2 isolates (33.3%). Only one isolate each (16.7%) was resistant to tetracycline and aztreonam. Representative susceptibility results for isolate S32 are shown in Figure 5. The detailed inhibition zone diameters and interpretations are presented in Table 2.

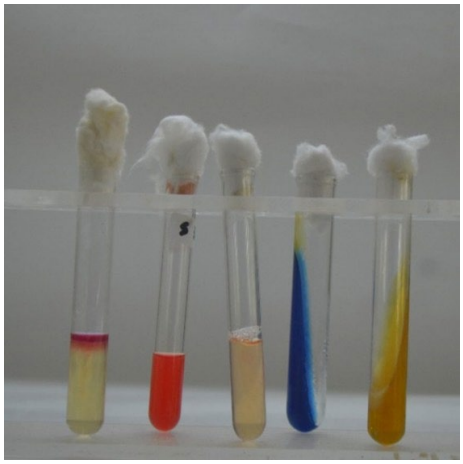

Figure 4. Representative biochemical reaction of isolate S32 showing SIM (Indole+, H_2S–, motile), MR+, VP–, citrate positive, and TSIA A/A without H_2S production.

Figure 5. Representative Kirby–Bauer disk diffusion result of isolate S32 on Mueller–Hinton Agar.

Table 1. Biochemical characteristics of confirmed *Citrobacter* spp. isolates.

Sample	Indole	H_2S (SIM)	Motility	MR	VP	Citrate	TSIA (slant/butt)	H_2S (TSIA)
S3	+	–	+	+	–	+	A/A	–
S17	+	–	+	+	–	+	A/A	–
S32	+	–	+	+	–	+	A/A	–
S53	–	+	+	+	–	+	A/A	+
S63	+	–	+	+	–	+	A/A	–
S106	+	–	+	+	–	+	A/A	–

Note: A = acid; (+) = positive; (–) = negative.

Table 2. Antibiotic susceptibility test results of *Citrobacter* spp. isolates.

Sample	Erythromycin	Tetracycline	Ampicillin	Cefoxitin	Aztreonam
S3	8.0 (R)	28.3 (S)	9.5 (R)	31.0 (S)	32.7 (S)
S17	6.0 (R)	9.4 (R)	6.0 (R)	7.8 (R)	12.6 (R)
S32	6.6 (R)	30.4 (S)	9.4 (R)	31.4 (S)	35.1 (S)
S53	6.0 (R)	27.0 (S)	7.1 (R)	11.0 (R)	31.9 (S)
S63	12.3 (R)	27.9 (S)	26.2 (S)	30.3 (S)	34.4 (S)
S106	6.0 (R)	15.8 (S)	25.5 (S)	33.8 (S)	34.0 (S)

Note: R = resistant; S = susceptible.

Multidrug resistance profile

Based on the resistance patterns, two isolates (33.3%) were classified as multidrug-resistant (MDR), defined as resistance to three or more antimicrobial classes. Isolate S53 was resistant to erythromycin, ampicillin, and cefoxitin, while isolate S17 showed resistance to all five tested antibiotics (Table 3).

The highest resistance rate was observed for erythromycin (100%), followed by ampicillin (66.7%), whereas the highest susceptibility rates were found for tetracycline (83.3%) and aztreonam (83.3%).

Table 3. Antibiotic resistance profiles of confirmed isolates.

Sample code	Resistant antibiotics
S63, S106	E
S3, S32	E, AMP
S53	E, AMP, FOX
S17	E, TE, AMP, FOX, ATM

Note: E = erythromycin; TE = tetracycline; AMP = ampicillin; FOX = cefoxitin; ATM = aztreonam.

Discussion

The present study demonstrated the presence of *Citrobacter* spp. in rectal swab samples collected from sheep in Ngrayung Village, Plumpang District, Tuban Regency, with a confirmed prevalence of 4.0% (6/150) based on biochemical identification. This finding confirms that the gastrointestinal tract of apparently healthy sheep may serve as a reservoir for opportunistic enteric bacteria belonging to the family *Enterobacteriaceae* (Irimaso *et al*., 2024). Similar observations have been reported in small ruminants, where enteric Gram-negative bacteria are frequently isolated from fecal and rectal samples, reflecting their role as part of the intestinal microbiota and potential sources of zoonotic transmission (Al-Asmari *et al*., 2023).

The initial isolation on MacConkey Agar yielded 40 presumptive isolates (26.7%), but only six were biochemically confirmed as *Citrobacter* spp. This difference highlights the importance of confirmatory biochemical testing because several members of *Enterobacteriaceae*, including *Escherichia coli*, *Klebsiella*, and *Enterobacter* spp., may exhibit similar lactose-fermenting colony morphology on MacConkey Agar (Cheng *et al*., 2012; Faridah *et al*., 2023). The observed pink, slightly mucoid colonies were consistent with the typical appearance of lactose-fermenting *Citrobacter* isolates (Ejeh *et al*., 2025).

Biochemical characterization showed heterogeneity among the confirmed isolates. One isolate (S53) exhibited an indole-negative and H_2S-positive profile, whereas the remaining five isolates were indole-positive and H_2S-negative. This variation is consistent with the known biochemical diversity within *Citrobacter* species, particularly among *C. freundii*, *C. koseri*, and related species (Janda *et al*., 1994). Such diversity suggests that more than one *Citrobacter* species may have been present in the sheep population sampled. However, because species-level molecular identification was not performed, the exact species distribution could not be determined (Brenner *et al*., 1999). This limitation should be considered when interpreting the results.

The antimicrobial susceptibility findings are particularly important from a veterinary public health perspective. The study revealed 100% resistance to erythromycin among all confirmed isolates. This result is not unexpected, as Gram-negative enteric bacteria often exhibit intrinsic or low-level susceptibility to macrolides due to the permeability barrier of the outer membrane and active efflux mechanisms (Gomes *et al*., 2017). Therefore, erythromycin is generally considered less effective against *Enterobacteriaceae*, including *Citrobacter* spp. (Li *et al*., 2015).

A high resistance rate was also observed for ampicillin (66.7%), which is consistent with the known presence of AmpC β-lactamase genes in *Citrobacter* spp., especially *C. freundii*. These enzymes confer resistance to aminopenicillins and certain cephalosporins and may be either chromosomally encoded or plasmid-mediated (Chen *et al*., 2018). Similar findings have been reported in livestock-associated *Citrobacter* isolates, where resistance to β-lactam antibiotics is common (Barlow and Hall, 2002).

In contrast, the relatively high susceptibility observed for tetracycline (83.3%) and aztreonam (83.3%) suggests that these antibiotics may still retain therapeutic effectiveness against *Citrobacter* spp. in this setting (Zhou *et al*., 2019). However, the detection of resistance in one isolate for each of these drugs indicates the early presence of resistant subpopulations, which may increase if antibiotic selection pressure persists (Nawaz *et al*., 2008).

Of particular concern is the identification of multidrug-resistant (MDR) isolates in 33.3% (2/6) of confirmed samples. Isolate S17 demonstrated resistance to all five tested antibiotics, indicating a broad resistance phenotype that may involve multiple mechanisms, including β-lactamase production, efflux pumps, and horizontal gene transfer via plasmids (Phuadraksa *et al*., 2023). The presence of MDR *Citrobacter* spp. in apparently healthy sheep highlights the potential role of livestock as reservoirs of resistant bacteria and resistance genes that can spread to other animals, humans, and the farm environment (Herawati *et al*., 2023). This observation is in line with global reports indicating increasing AMR in sheep and goats.

The findings emphasize the need for routine antimicrobial resistance surveillance and prudent antibiotic use in sheep farming systems. Improved farm biosecurity, rational prescription practices, and periodic bacteriological monitoring are essential to minimize the dissemination of resistant enteric bacteria and to support antimicrobial stewardship within the One Health framework (Dhaka *et al*., 2023).

Conclusion

In conclusion, *Citrobacter* spp. was successfully isolated from rectal swabs of sheep in Ngrayung Village, with a confirmed prevalence of 4.0% (6/150). The isolates showed a notable antibiotic resistance pattern, particularly 100% resistance to erythromycin and 66.7% resistance to ampicillin. Additionally, 33.3% of isolates were classified as multidrug-resistant (MDR). These findings indicate that sheep may act as reservoirs of antimicrobial-resistant *Citrobacter* spp. and highlight the importance of routine AMR surveillance, prudent antibiotic use, and improved farm biosecurity to reduce the spread of resistant bacteria in livestock systems.

Acknowledgments

The authors would like to express their sincere gratitude to Prof. Dr. Mustofa Helmi Effendi, DTAPH for his generous financial support and valuable contribution to the completion of this research.

Conflict of interest

The authors have no conflict of interest to declare.

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