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Antibiotic Resistance Profile and Biofilm-forming Ability of *E. coli* and Non-Typhoidal *Salmonella* spp. from Dairy Cattle Farm settings: A Public Health Concern

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ABSTRACT: The present study was envisaged to analyse a total of 164 samples (feed: 60, water: 60 and handwash: 44) for the presence of *E. coli* and non-typhoidal *Salmonella* (NTS), by microbiological and molecular methods. Overall occurrence rates of 64.63%, 19.47% and 10.36% were observed for *E. coli*, *Salmonella* spp. and NTS, respectively. The antibiotic susceptibility testing confirmed that 21.69% of *E. coli* isolates were multidrug-resistant (MDR), with 19.81% being ESBL producers, among which the highest MDR isolates were recovered from trough water (28.8%), followed by feed (21.21%) and handwash (10.71%). Among the NTS isolates, 20.41% showed MDR pattern, and 11.76% were ESBL producers. In this study, a moderate biofilm-forming ability was exhibited by MDR test strains of *E. coli* at 24 and 48 h when compared with MDR-NTS strain which revealed weak biofilm formers at 48 h. Hence, further studies need to be pursued to address the challenges of AMR in the dairy industry.

Keywords: Antimicrobial resistance, Biofilm, Organised dairy cattle farm, *E. coli*, Non-typhoidal *Salmonella*, Multi-drug-resistance.

INTRODUCTION

Antimicrobial resistance (AMR) remains a global public health threat (Founou *et al.,* 2021; Rayanoothala *et al.,* 2021). The use of antibiotics in livestock farming for therapeutics and metaphylaxis is a fundamental practice (Van *et al.,* 2020). However, the excessive and inappropriate use of antimicrobials in livestock rearing is a key factor driving the development of AMR (Silva *et al.,* 2023), potentially leading to the transmission of drug-resistant pathogens to humans and the environment (Teng *et al.,* 2023). India, with nearly 193.50 million cattle and 109.90 million buffaloes, faces substantial challenges in managing antibiotic use in livestock. By 2030, antibiotic usage among livestock in developing countries is projected to double due to the expansion of food animal production. It has been half a century since the initial approval of antibiotic-infused feeds for livestock, aiming to enhance their overall health and boost animal productivity (Afema *et al.,* 2018).

On a global scale, *Salmonella* ranks as the third most frequently encountered bacterial pathogen identified in food-borne illnesses in humans, following *Escherichia coli* and *Campylobacter* (WHO, 2015). Dairy cattle can serve as significant reservoirs for *Salmonella* and *E. coli*, with contaminated milk and dairy products often implicated in outbreaks in humans (Wang *et al*., 2023). Strains of *E. coli* and *Salmonella* found in cow feces on dairy farms have the potential to contaminate the farm environment (Sobur *et al.,* 2019). Intensive livestock operations significantly contribute to AMR transmission among humans, animals, and the ecosystem. Factors such as infection control, animal husbandry practices, animal movement, and biosecurity measures can also influence the emergence of AMR pathogens in dairy farms. Identifying these risk factors is crucial for preventing the emergence and potential transmission of AMR pathogens from dairy farm settings to humans.

WHO estimated that in 2010 food borne STEC caused more than 1.2 million illnesses, 128 deaths and nearly 13,000 Disability Adjusted Life Years (DALYs) (Darshan *et al.,* 2023). Despite the high percentage of human infections with enteric pathogens, the systematic studies on antibiotic-resistant enteric bacterial pathogens in the livestock farming system, particularly in dairy cattle and the farm environment, have not yet been adequately explored. As far as our current understanding goes, there is a noticeable scarcity of comprehensive studies examining the presence of drug-resistant enteric bacterial pathogens within dairy farm environments in India. In addition, studies evaluating the factors leading to the emergence of AMR pathogens in dairy farm settings are also limited in the Indian

context. Hence, this study aims to fill this gap by investigating the presence of drug-resistant *E. coli* and *Salmonella* spp. among dairy cattle farm settings and associated risk for the emergence of AMR in one health perspective.

MATERIALS AND METHODS

The study area comprised three taluks of Wayanad district (Fig. 1) in Kerala state of India *viz*., Vythiri, Sulthan Bathery and Manathavady (110 26' 28" - 110 58' 22" N latitude and 750 46'38" - 760 26'11' E) at an average altitude of 700 and 2100 m above the mean sea level. The animals used in this study were apparently healthy dairy cattle from small holder dairy farms. A total of 60 privately owned dairy cattle farms (with a herd size of 5-10 dairy cattle), 20 each from 3 taluks of Wayanad district, were selected for the study. The study was approved by the Institutional Research Committee, and all norms and standard protocols for animal welfare were followed. A longitudinal study was conducted to generate the desired data from August 2022 to August 2023. A total of 164 samples, comprising pooled samples of feed (n=60), trough water (n=60) and human handwashes (n=44) were aseptically collected, labelled and immediately transported under pre-chilled insulated storage boxes to the food quality assurance laboratory of the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode for isolation of the bacterial pathogens. The milkers' hand washes were collected in a sterile phosphate-buffered saline (PBS; HiMedia Laboratories Pvt. Ltd., India). The samples were immediately processed for isolation and identification of *E. coli* and *Salmonella* spp. by cultural methods and polymerase chain reaction (PCR) assays.

The isolation and identification of *E. coli* from the samples were performed as per ISO 16649: 2001, while ISO 6579-1: 2001 was employed for *Salmonella* spp. The samples were enriched at a rate of 1:10 dilution in buffered peptone water for *E. coli*. The enriched samples were streaked onto EMB agar for the isolation of *E. coli* and incubated at 37°C for 24 hr. Three to five representative colonies that showed typical metallic sheen of *E. coli* on EMB agar were picked and confirmed by molecular assay. The samples were enriched at a rate of 1:10 dilution in BPW for *Salmonella* spp. This was followed by selective enrichment in Rappaport- Vassiliadis (RV) broth followed by selective plating on xylose lysine deoxycholate (XLD) agar and incubated at 37°C for 24 h. Three to five representative colonies that showed red colonies with the black center of *Salmonella* spp. on XLD agar were picked and confirmed by molecular assay.

The antibiotic susceptibility testing for all the recovered bacterial isolates was carried out by the Kirby-Bauer disc diffusion method (Bauer *et al.,* 1996) on Mueller-Hinton agar (HiMedia) according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2019). The commercial antibiotic discs (HiMedia) were selected based on the information provided by practicing field veterinarians *i.e*., gentamicin (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), amoxicillin-clavulanic acid (10 µg), oxytetracycline (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ceftazidime/ clavulanic acid (30/10 µg). *E. coli* ATCC 25922 served as the quality control strain.

The biofilm-forming ability of the recovered isolates was qualitatively assessed using Congo red agar (CRA) assay (Freeman *et al.,* 1989). The production of black streaks or colonies with a dry crystalline consistency indicated biofilm-forming ability, whereas pink or red colonies were observed with weak or moderate biofilm formers.

The biofilm biomass of the recovered isolates was estimated based on the crystal violet staining in the 96-well microtiter plate (Wakimoto *et al.,* 2004). In brief, 96-well flat-bottom microtiter polystyrene plates (Tarsons, India) were inoculated with individual cultures of each test isolate @ 200 µL/well in nutrient broth in quadruplicate. After incubation at 37 °C for both 24 h and 48 h, the 'planktonic' cells were removed, and the biofilm formation was assessed by staining with 0.10% crystal violet for 30 min, followed by washing thrice with PBS (pH 7.20). Finally, the stain acquired by adherent (biofilm forming) bacteria was resolved in 200 µL of 95% ethanol, and biofilm-forming ability was quantified by measuring the optical density of each test isolate at 595 nm by a microplate reader (iMark microplate reader, Bio-Rad, USA). The bacterial isolates were classified into four categories based on their biofilm-forming ability: non-biofilm producers, weak, moderate, and strong biofilm producers. The cutoff value (OD_c) for this classification was calculated as three standard deviations above the mean OD_{595} of the negative control (*E. coli* DH5α). Further, the isolates were classified as non-biofilm former, $OD_{595} \leq OD_c$; weak biofilm former, $OD_{595} > OD_c$ and $\leq 2\times OD_c$; moderate biofilm former, $OD_{595} > 2\times Od_c$ and $\leq 4\times Od_c$; strong biofilm former, $OD_{595} > 4\times Od_c$ (Stepanovic *et al.,* 2000).

RESULTS AND DISCUSSION

A. Occurrence of E. coli and NTS

Out of the total 164 samples tested, 106 (64.63%) samples detected the presence of *E. coli* both by isolation and PCR assays, that includes 45 (75.0 %) trough water samples, 33 (55 %) feed samples and 28 (63.63 %) handwash samples (Table 1). Also, 37 (19.47%) samples detected *Salmonella* spp. that comprised 13 (21.66%) trough water, 15 (25%) feed and 9 (20.45%) handwash samples.

Among the 37 *Salmonella* spp., 17isolates were detected as NTS and of which 6.66 % of trough water samples, 15% of feed and 9.09% of human handwash samples were found to be positive to NTS.

It's well established that dairy cattle can serve as reservoirs for both *E. coli* (Eldesoukey *et al.,* 2022) and *Salmonella* spp. (Eguale *et al*., 2016), often carrying these bacteria asymptomatically. These pathogens are known to play a critical role in the transmission of drug-resistant genes within and between species (Manishimwe *et al.,* 2021). The growth and survival of *E. coli* are typically influenced by factors such as

nutrient and energy availability in various environmental conditions. Moreover, *E. coli* can enter a 'dormant' state, where cells cannot be easily recovered on standard laboratory media, and *E. coli* populations under complex natural conditions are often not accurately predictable (Semenov *et al.,* 2007). These factors, along with differences in environmental and management factors, could be the likely reasons for the variation in the occurrence of *E coli* from different sources in our study. The detection of non-typhoidal *Salmonella* (NTS) in dung and slurry samples is highly significant for public health. Cattle manure is frequently utilized in organic farming, posing a potential risk of infection due to contamination with enteric bacteria. This could lead to adverse health outcomes for individuals exposed to such bacteria.

This study's findings indicate that the drinking water provided to cattle is microbiologically inadequate. The daily exposure of animals to *E. coli* and Salmonella spp. from this water source alone can be significant. Once these bacteria are introduced, they have the potential to persist over the long term, acting as a reservoir and a potential source of infection for cattle. Various other factors like nutrient availability in water, water trough design and farm location, biofilm-forming ability of the pathogens, exposure of trough to sunlight, ambient atmospheric temperature, competition with and predation by other microflora (Aditya *et al.,* 2023), absence of effective periodic disinfection, contamination of groundwater by manure and slurry may also affect bacterial load of trough water.

In the present study, The *E coli* isolates recovered from trough water were strong biofilm formers. Biofilms in water troughs typically consist of various species of microorganisms and when multiple species collaborate to form biofilms, it can increase the resistance of foodborne pathogens to sanitizers. *E. coli* can create biofilms in conjunction with other bacterial types, potentially boosting the survival of its pathogenic variants within the biofilm community.

The present study revealed higher levels of *E. coli* and NTS in feed samples compared to data reported by the US Food and Drug Administration (FDA) animal food surveillance program, with *Salmonella* spp. and *E. coli* rates standing at 12% and 12.5%, respectively (Ge *et al.,* 2020). Feed stuffs may become contaminated with *E. coli* if they come in contact with contaminated agricultural produce or any surface that harbours the bacteria either during harvesting, storage, or transportation. The presence of non-typhoidal *Salmonella* (NTS) in feed could be attributed to several factors, including the ability of *Salmonella* spp. to survive in dry environments such as feed mills and bins, the access of rodents and birds to feed storage areas, the survivability of *Salmonella* in the farm environment, and the ability of *Salmonella* to multiply in warm, moist conditions during feed storage and under certain climatic conditions (Shahbazi *et al.,* 2023). Furthermore, *E. coli* and NTS strains originating from contaminated feed can potentially be transferred to milk, raising significant health concerns for consumers.

The present study revealed that 63.6% of human handwash samples tested positive for *E. coli*, 20.45% of human handwash samples with *Salmonella* spp. and 9.09% of handwash samples with NTS (*S.* Typhimurium). However, all the NTS isolates exhibited only a weak biofilm-forming ability. The handwashing practices of farm workers have a significant impact on the occurrence of *E. coli* in hand wash samples of milkers and due to a lack of hygiene awareness, they usually contaminate their hands with their stool. Washing udders before and after milking with unclean water, cleaning milking equipment without using detergents, and milking with dirty hands can contaminate *Salmonella*.

A. Antimicrobial susceptibility testing among E. coli and NTS isolates

In this study, among the recovered *E. coli* isolates (n= 106), the decreasing trends in resistance were observed (Table 2) in the order: amoxicillin-clavulanate (84.90%; 90/106), followed by oxytetracycline (39.62%; 42/106), gentamicin (30.18%; 32/106), ciprofloxacin (22.64%; 22/106), ceftriaxone (13.20%; 14/106) and meropenem (0.94%; 1/106). Among the 106 *E. coli* isolates, 19.81% were extended-spectrum beta-lactamase (ESBL) producers and 23 *E. coli* isolates were MDR of which 28.88%, 21.22% and 10.71% of trough water, feed and handwash samples were MDR.

According to our findings, phenotypic screening of antimicrobial resistance among *E. coli* and *Salmonella* spp. from dairy cattle farms displayed an alarming MDR pattern to indispensable antibiotics in human and veterinary medicine. The MDR- *E. coli* strains pose a public health concern because they indicate potential drug resistance in Gram-negative bacteria. These results suggest that these isolates originated from high-risk sources where multiple antibiotics were used. While examining AMR patterns, we consistently identified resistance against β-lactams, fluoroquinolones, and tetracyclines, pointing to the extensive utilization of penicillins/beta-lactamase inhibitors (Amoxicillin-clavulanic acid), fluoroquinolones (Ciprofloxacin), and tetracyclines (Oxytetracycline) within these farms for treatment and/or prophylaxis. This heightened resistance underscores the significant selective pressure exerted by the widespread use of these antibiotic classes in the dairy farms we investigated.

NTS strains were given importance as they bear significant zoonotic importance. The NTS isolates recovered from this study exhibited comparatively higher resistance towards amoxicillin-clavulanate (64.70%), oxytetracycline (64.70%), gentamicin (29.41%), ciprofloxacin (29.41%) and ceftriaxone (23.52%); however, were sensitive to meropenem (100%). All the MDR-NTS isolates (n= 5) detected as *S.* Typhimurium were found to be resistant to amoxicillin-clavulanate, gentamicin, oxytetracycline, and ciprofloxacin. Nonetheless, meropenem was found to be comparatively better with 100% susceptibility to the tested MDR-NTS isolates, respectively (Table 2).

It is important to note that the occurrence of carbapenemase-producing (CP) bacteria in food-producing animals and their surrounding environment has not been sufficiently investigated in countries characterized by a high prevalence of CP bacterial

infections in humans (Bonardi and Pitino 2019). Even though the occurrence of CP microbes in food-producing animals is relatively rare, the potential transmission of CP bacteria from these animals to products derived from them poses a significant risk to consumers, with severe consequences. Unprocessed foods such as raw milk have the potential to promote the dissemination of carbapenem resistance, and the presence of mobile genes that encode carbapenemase in foods of animal origin represents a possible hazard to human health. The MDR pattern observed in this study suggests that viable therapeutic options for treating common infections like mastitis in dairy cattle farms could be limited and emphasize the urgent need for antimicrobial stewardship practices in Indian dairy farm settings. The correlation between the occurrence of CP bacteria and the use of antimicrobials on the farm should also be better investigated.

C. Biofilm forming ability of the MDR isolates

(i) Congo Red Assay. Among the 23 MDR-*E. coli* isolates, 18 exhibited moderate to strong biofilm production, while 5 were weak biofilm formers. Of the 5 MDR-NTS isolates, 1 isolate was a moderate to strong biofilm-former, while the remaining 4 exhibited a weak biofilm-producing ability on congo red assay.

(ii) Microtitre plate assay. In this study, the biofilm-forming ability of the recovered MDR strains was estimated and graded (Table 3). The cutoff values of the absorbance for the negative control were calculated to be 0.087432 and 0.12418 for 24 h and 48 h, respectively. Based on the formula, the MDR strains were graded as non-biofilm formers, weak, moderate, or strong biofilm formers. It was also estimated that

At 24 h, $OD_c \times 2 = 0.174862$ and $OD_c \times 4 = 0.349724$

At 48 h, $OD_c \times 2 = 0.24836$ and $OD_c \times 4 = 0.49672$

In this study, pronounced biofilm-forming ability was exhibited by the MDR- *E. coli* strains at 48 h than 24 h in contrast to the MDR-NTS (Fig. 2) strains (Table 3). Hence, among the 23 MDR- *E. coli* (Fig. 3) isolates, at 48 h, 82.60% were found to be moderate biofilm formers (n= 19), whereas 17.39% were strong biofilm formers (n= 4). Among the strong biofilm formers of MDR-*E. coli*, 2 isolates each were recovered from trough water and feed.

At 48 h, of the 5 tested MDR-NTS isolates, 80.0% were weak biofilm formers, while 20% were moderate biofilm formers. However, none of the MDR-NTS isolates exhibited strong biofilm-forming ability at 48 h. The source-wise analysis of 23 MDR- *E. coli* isolates revealed significant biofilm-producing abilities from those isolates recovered from trough water ($P< 0.001$) and feed ($P< 0.01$). However, the isolates recovered from handwash revealed significant ($P< 0.05$) biofilm-forming ability both at 24 h and 48 h.

A similar study was conducted in large-scale Chinese dairy farms to explore the biofilm ability of Staphylococcus aureus by Liu *et al.* (2020) and the rates of weak, moderate, and strong biofilm producers were 59.7% (37/62), 22.6% (14/62), and 17.7% (11/62) respectively. The formation of biofilms is linked to heightened tolerance for stressful conditions and increased pathogenicity. The capacity of E. coli strains to form biofilms serves as a survival strategy, enabling these microorganisms to persist in the environment for extended periods (Madani *et al.,* 2022).

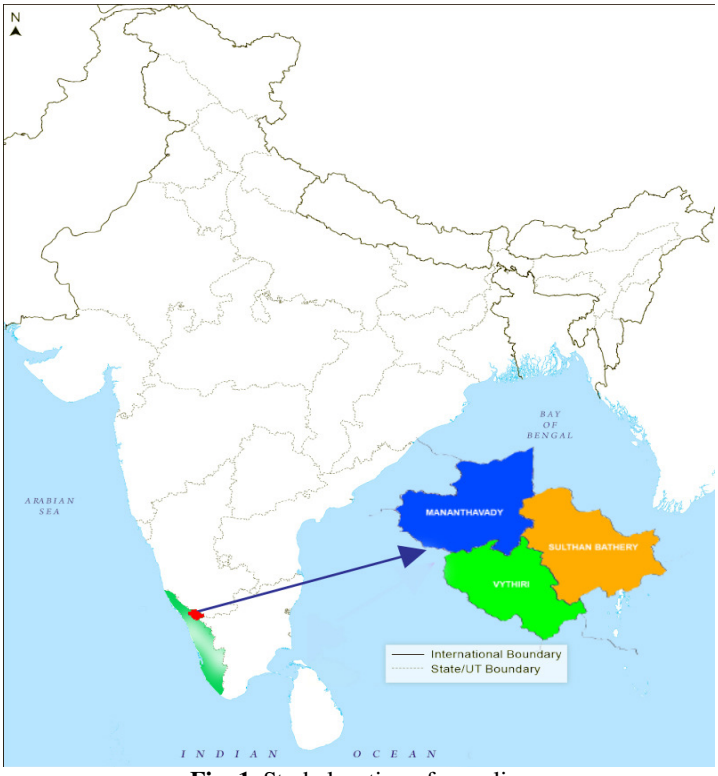
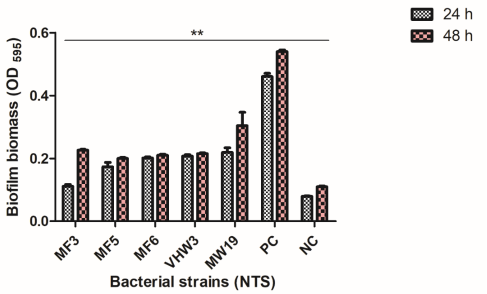
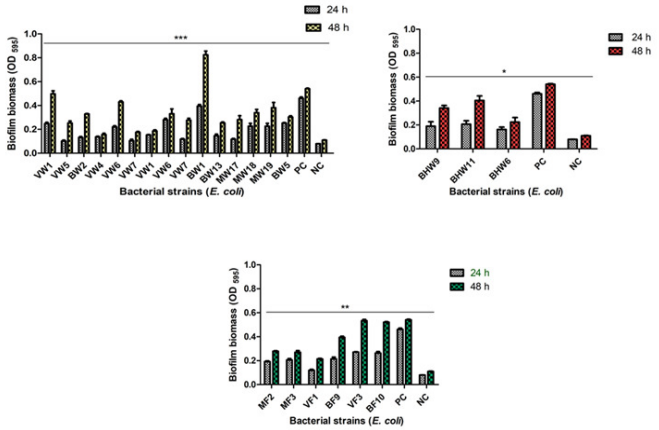


Fig. 1. Study location of sampling.



The isolate prefix V, M, B represents Vythiri, Mananthavady, and Sulthan Bathery, respectively, while isolate suffix F, W, HW represents feed, trough water, and handwash, respectively, whereas the numericals represent laboratory isolate numbers. PC represents positive control (*E. coli* ATCC 25922), while NC represents negative control (*E. coli* DH5α).

Fig. 2. Biofilm-forming ability among MDR-NTS isolates.



Images (a, b, c) denote biofilm-forming abilities among MDR- *E. coli* isolated from, trough water, feed, and human hand wash, respectively. The isolate prefix V, M, B represents Vythiri, Mananthavady, and Sulthan Bathery, respectively, while isolate suffix F, W, HW represents feed, trough water, and handwash, respectively, whereas the numericals represent laboratory isolate numbers. PC represents positive control (*E. coli* ATCC 25922), while NC represents negative control (*E. coli* DH5α).

Fig. 3. Biofilm-forming ability among MDR- *E. coli* isolates.

Table 1: Proportion of *E. coli* and NTS isolates recovered from dairy cattle farm settings under study.

Samples	No. of Samples	*E. coli* isolates (%)	*Salmonella* isolates (%)	NTS isolates (%)
Water	60	75	21.66	6.66
Feed	60	55	25.0	15.0
Human handwash	44	63.63	20.45	9.09
TOTAL	**164**	**74.71**	**19.47**	**8.13**

Table 2: **Antimicrobial susceptibility of *E. coli* and NTS isolates.**

Antibiotics	*E. coli* isolates (n=106)		MDR- *E. coli* isolates (n=23)		NTS isolates (n=17)		MDR- NTS isolates (n=5)	
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
Amoxclav	84.90	15.09	100	00	64.70	35.71	100	00
Gentamicin	30.18	69.81	8.69	91.30	29.41	85.14	100	00
Oxytetracycline	39.62	60.37	91.30	8.69	64.70	46.42	100	00
Meropenem	0.94	99.05	4.34	95.65	00	100	00	100
Ciprofloxacin	22.64	79.24	78.26	21..73	29.41	75	100	00
Ceftriaxone	13.20	86.79	52.17	47.82	23.52	82.14	80.0	20.0
ESBL-producers	19.81		21.73		11.76		40.00	

R: Resistant; S: Sensitive

Table 3: Gradation of biofilm-forming abilities of MDR isolates of *E. coli* and NTS recovered from dairy cattle farm settings.

MDR-bacterial strains	Time (h)	Non-biofilm formers (%)	Weak biofilm formers (%)	Moderate biofilm formers (%)	Strong biofilm formers (%)
E. coli (n=23)	24	13.04	34.78	43.47	8.69
	48	0	40.32	82.60	17.39
NTS (n=5)	24	0	40	40	00
	48	0	80.0	20.0	00

CONCLUSIONS

The study reveals the widespread occurrence of antibiotic-resistant *E. coli* in dairy farm settings. Most *E. coli* isolates in this study were recovered from trough water, whereas NTS was isolated mainly from feed samples, with alarming drug-resistance patterns. These pathogens can spread from animals to people via the food chain either directly or indirectly. The findings of this study demonstrate the need for a thorough surveillance system for antimicrobial usage as well as an AMR in livestock with a holistic approach to navigate these issues successfully; the key components being access to clean water, safe feed, and hygiene. The judicious use of antibiotics in dairy cattle, good farm management practices such as the personal hygiene of farm workers and manure treatment must also be implemented to minimize the risk of transmission of antibiotic-resistant microorganisms to humans.

Large herds are an important predictor of resistance to multiple types of antimicrobials as they suffer from more disease problems and use antimicrobials more frequently than small herds. In addition, antibiotic-resistant gene transmission pathways are more complex than those on small farms. One potential risk with the present study design was that conclusions were drawn based on the susceptibility testing of samples obtained from small dairy farms. So, further research using samples from large dairy herds is essential to identify and thoroughly understand the key risk factors significantly influencing the development of MDR enteric pathogens. A One Health philosophy with a holistic mindset can address these issues through effective farm management systems.

FUTURE SCOPE

A significant gap in knowledge and practice among farmers in this study area regarding animal biosecurity and management. Strengthening extension efforts seems crucial to raise awareness and promote improved practices. Providing education, training sessions, and resources can help empower farmers to make informed decisions and enhance the health and productivity of their animals. Also, identifying the risk factors for the emergence and spread of antibiotic-resistant bacteria is important to identify effective interventions to contain this menace. Furthermore, formulating a comprehensive method for the continued surveillance of Biosecurity and antimicrobial resistance in dairy farms in Indian settings- Biosecurity is a key element in the fight against antibiotic resistance.

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Conflict of Interest. None.

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