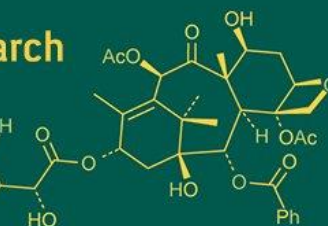
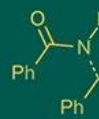
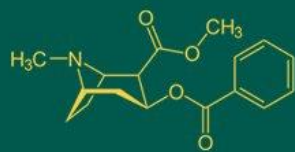


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Alarming multidrug resistance and high Multiple Antibiotic Resistance (MAR) index profiles among bovine mastitis pathogens in Andhra Pradesh, India

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Abstract

Background: Bovine mastitis causes severe economic losses in the dairy sector and poses a critical food safety risk due to the potential transmission of antimicrobial-resistant pathogens through milk.

Methods: This study evaluated the prevalence and antimicrobial resistance (AMR) profiles of mastitis pathogens around the College of Veterinary Science, Garividi, Andhra Pradesh, India. Forty-three clinical milk samples were processed using standard microbiological cultivation, biochemical identification, and *in-vitro* disk diffusion assays against 17 antimicrobials. Multiple Antibiotic Resistance (MAR) indices were calculated for all bacterial strains.

Results: The majority of samples yielded polymicrobial primary cultures, resulting in 55 distinct microbial isolates across 11 unique genera. *Escherichia coli* was the most dominant pathogen (29.09%), followed by *Staphylococci* spp. (23.63%), *Streptococci* spp. (18.18%), and *Klebsiella* spp. (9.09%). Minor environmental bacteria and opportunistic fungi (*Yeast*, *Penicillium*, and *Mucor*) were also recovered. Phenotypically, 100% of the bacterial isolates exhibited multidrug resistance (MDR), with none sensitive to more than 6 antibiotics. Severe resistance clustered against penicillins, core cephalosporins (Cefaclor, Cefixime), ciprofloxacin, and azithromycin. Conversely, Gentamicin, Cephalexin, and Ceftriaxone retained moderate therapeutic efficacy. Crucially, 100% of tested isolates exhibited an alarming MAR index strictly above the 0.20 threshold (range: 0.60–1.00), with six cases demonstrating absolute pan-resistance (MAR = 1.00).

Conclusion: The high prevalence of environmental coliforms and universal multi-drug resistance underscore severe environmental sanitation gaps and intense selective antibiotic pressure in the region. Implementing mandatory pre-treatment screening and strict herd hygiene protocols are imperative to safeguard public health and preserve remaining therapeutic options.

Keywords: Bovine mastitis, *Escherichia coli*, Bacterial pathogens, Multidrug resistance, MAR index

1. Introduction

Bovine mastitis is the most economically devastating disease affecting the dairy industry worldwide (Hogeveen *et al.*, 2019) ^[1]. This inflammatory condition of the mammary gland parenchyma compromises animal health, drastically reduces milk production, and alters milk quality (Barkema *et al.*, 2015; Cheng & Han, 2020) ^[2, 3]. The resulting economic losses stem from veterinary treatment costs, discarded milk, premature culling, and reduced processing quality (Hogeveen *et al.*, 2019) ^[1]. Beyond financial impacts, mastitis represents a significant food safety concern due to the risk of zoonotic pathogen transmission through contaminated milk (Cheng & Han, 2020) ^[3].

The disease etiology is multifactorial, involving a wide array of bacterial and fungal pathogens. These are conventionally categorized into contagious and environmental organisms: The contagious pathogens (*e.g.*, *Staphylococcus* spp. and *Streptococcus* spp.) spread primarily during milking, while the environmental pathogens (*e.g.*, coliforms like *Escherichia coli* and *Klebsiella* spp.) infect the udder between milkings via contaminated bedding, water, or soil (Gomes & Henriques, 2016; Ruegg, 2017) ^[4, 5]. Furthermore, opportunistic organisms include various *Bacilli* species, *Pseudomonas*, and mycotic agents

such as yeasts, *Penicillium*, and *Mucor*. These agents frequently complicate mastitis management under poor hygienic conditions (Gomes & Henriques, 2016)^[4].

Antimicrobial therapy remains the primary strategy for treating and controlling mastitis (Melo *et al.*, 2026)^[6]. However, the widespread and often indiscriminate use of antibiotics has led to a global crisis of antimicrobial resistance (AMR) (Pretin & Devriese, 2024)^[7]. Dairy cattle have increasingly become reservoirs for multidrug-resistant (MDR) strains (Agyare *et al.*, 2024; Usman *et al.*, 2025)^[8, 9]. Of particular concern is the rising resistance to critical first-line and advanced therapies, including penicillins and cephalosporins (Melo *et al.*, 2026)^[6]. When bacterial populations exhibit a Multiple Antibiotic Resistance (MAR) index above the critical threshold of 0.2, it signals a high-risk source of contamination where antibiotics are heavily overused (Krumperman, 1983)^[10]. This creates a severe public health hazard, as resistant bacteria and their resistance genes can easily enter the human food chain via raw or poorly processed milk (Agyare *et al.*, 2024; Usman *et al.*, 2025)^[8, 9].

The distribution and resistance patterns of mastitis pathogens vary widely by geographic region, climate, and herd management. In India, local surveillance is crucial due to the unique challenges of smallholder dairy farming systems (Banyal *et al.*, 2025; Singh & Sharma, 2022)^[11, 12]. Despite this necessity, comprehensive data on microbial profiles and resistance patterns remain limited for many rural and peri-urban dairy tracts (Shome *et al.*, 2021; Singh & Sharma, 2022)^[13, 12].

This study was conducted to address this gap by investigating mastitis epidemiology around the College of Veterinary Science, Garividi. Over a one-year period, we analyzed 47 milk samples from affected cattle, yielding 55 distinct microbial isolates. We mapped the exact distribution of dominant pathogens—specifically *E. coli* (29.09%), *Staphylococci* (23.63%), and *Streptococci* (18.18%)—alongside opportunistic environmental bacteria and mycotic agents. Furthermore, we evaluated the *in-vitro* antibiotic susceptibility profiles of 43 bacterial isolates against 17 major antimicrobials to quantify MAR indices, map severe crossresistance patterns, and identify viable therapeutic options like Gentamicin.

2. Materials and methods

2.1 Study Area and Sample Collection

A cross-sectional study was conducted over a one-year period in the peri-urban dairy tracts surrounding the College of Veterinary Science, Garividi, Vizianagaram district, Andhra Pradesh, India. A total of 47 milk samples were collected from dairy cattle exhibiting clinical or subclinical mastitis. Prior to collection, the teats were thoroughly cleaned, disinfected with 70% isopropyl alcohol, and the first few strips of milk were discarded. Approximately 10–15 mL of mid-stream milk was collected aseptically into sterile tubes and transported immediately to the laboratory under refrigeration (4 °C).

2.2 Isolation and Identification of Microbial Pathogens

All milk samples were processed under strict aseptic conditions following standard microbiological laboratory protocols. Initially, a 1 mL aliquot of each milk sample was inoculated into sterile nutrient broth and incubated at 37 °C for 24 hours to enrich the microbial load. A loopful of the

resulting broth culture was subsequently streaked onto 5% sheep blood agar plates using a sterile inoculation loop. The plates were incubated aerobically at 37 °C for 24 hours. Following the incubation period, macromorphological characteristics of individual colonies were evaluated. Samples displaying multiple distinct colony types were classified as mixed cultures. To obtain pure isolates, individual distinct colonies were meticulously picked and sub-cultured onto fresh media. For fungal isolation, samples were inoculated onto Sabouraud Dextrose Agar (SDA) and incubated under appropriate ambient conditions 25 °C for up to 5–7 days.

Purified bacterial isolates were characterized using Gram staining followed by systematic microscopic examination. The presumptive phenotypic identification was based on morphological and staining characteristics of various bacterial species like: *Streptococci* spp., *Staphylococci* spp., *Bacilli* spp., *Escherichia coli*, *Pseudomonas* spp., and *Klebsiella* spp.

2.3. In-vitro Antibiotic Susceptibility Testing (ABST)

The antimicrobial resistance profiles of the bacterial isolates ($n=43$) were determined using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA) in accordance with the Clinical and Laboratory Standards Institute guidelines. The bacterial turbidity was standardized to match a 0.5 McFarland standard. A panel of 17 commercial antibiotic discs representing major antimicrobial classes was deployed: Penicillins/Combinations: Penicillin (P), Ampicillin/Sulbactam (AMS), Amoxicillin/Clavulanate (AMC); Cephalosporins: Cephalothin/Cefazolin (CF), Ceftriaxone (CTR), Cefoperazone (CPZ), Cefixime (CFM); Aminoglycosides: Amikacin (AK), Kanamycin (K), Gentamicin (GEN), Cephalixin/Gentamicin variant (CN); Fluoroquinolones/Macrolides: Ciprofloxacin (CIP), Enrofloxacin (EX), Azithromycin (AZM); Others: Co-trimoxazole (COT), Doxycycline (DO), Tetracycline (TE). Zones of inhibition were measured after 24 hours of incubation at 37 °C and interpreted as Susceptible or Resistant.

2.4. Calculation of the Multiple Antibiotic Resistance (MAR) Index

The MAR index for the isolates was calculated using the standardized formula: $MAR\ Index = a/b$; Where, a:- represents the total number of antibiotics to which a specific isolate exhibited resistance. b:- represents the total number of antibiotics evaluated (17 in this study).

3. Results

3.1 Microbial Isolation and Genotypic Distribution Profile

Out of the 43 milk samples collected from clinical mastitis cases, only a minority yielded monomicrobial primary cultures. The vast majority of the samples exhibited polymicrobial primary cultures, indicating a high prevalence of mixed infections in field conditions. Following the systematic separation and subculturing of individual colonies from these polymicrobial culture plates, 55 distinct microbial isolates spanning 11 different genera were successfully recovered. Among these, bacterial pathogens heavily dominated the microflora landscape, while a smaller proportion comprised opportunistic fungal entities. The

detailed prevalence and percentage distribution of these 11 microbial genera are illustrated in Figure 1 and quantified in

Table 2.

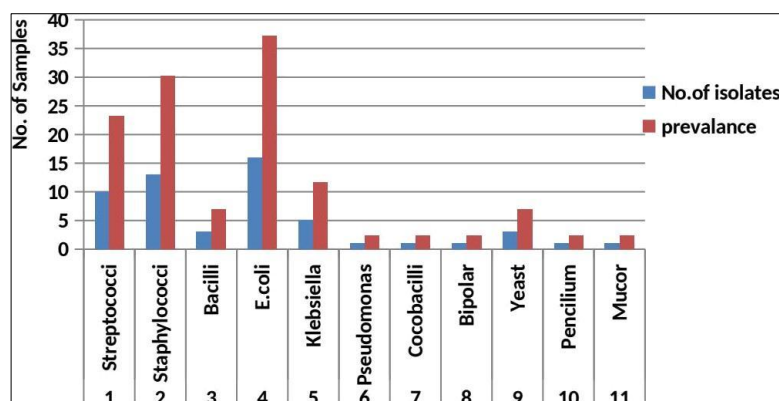


Fig 1: Prevalance of Mastitis bacterial pathogens

Gram-negative coliforms represented the single largest group of isolated pathogens, led prominently by *Escherichia coli* as the most frequent organism, accounting for 16 isolates (29.09%). This was followed by *Klebsiella* spp., which contributed 5 isolates (9.09%). Grampositive cocci also maintained a substantial clinical footprint, spearheaded by *Staphylococci* spp. with 13 isolates (23.63%) and *Streptococci* spp. with 10 isolates (18.18%). Together, these four core pathogens accounted for (80.00%) of the entire microbial population recovered.

Among the remaining bacterial isolates, *Bacilli* species were recovered in 3 instances (5.45%). Minor environmental and morphological variants including *Pseudomonas* spp., *Cocobacilli*, and bacteria displaying distinct *Bipolar* rods were rare, each accounting for a single isolate (1.81%). Mycotic agents accounted for the final portion of the microbial distribution, led by *Yeast* isolates at 3 instances (5.45%), alongside filamentous molds belonging to the genera *Penicillium* (1.81%) and *Mucor* (1.81%).

Table 1: Distribution of isolated genera in milk samples of clinical mastitis (n=55) total isolates

S.No.	Isolated Organism	No. of Isolates	Distribution (%)
1.	<i>Escherichia coli</i>	16	29.09%
2.	<i>Staphylococci</i> spp.	13	23.63%
3.	<i>Streptococci</i> spp.	10	18.18%
4.	<i>Klebsiella</i> spp.	5	9.09%
5.	<i>Bacilli</i> spp.	3	5.45%
6.	<i>Yeast</i>	3	5.45%
7.	<i>Pseudomonas</i> spp.	1	1.81%
8.	<i>Cocobacilli</i>	1	1.81%
9.	<i>Bipolar</i> rods	1	1.81%
10.	<i>Penicillium</i> spp.	1	1.81%
11.	<i>Mucor</i> spp.	1	1.81%
	Total	55	100.00%

The *in-vitro* antibiotic susceptibility assay performed against a panel of 17 commercial antimicrobials revealed an alarming therapeutic landscape. Every single tested bacterial isolate exhibited multidrug resistance (MDR), demonstrating phenotypic resistance across multiple antibiotic classes. The extreme nature of this resistance profiles is underscored by two critical findings: A small subset of hyper-resistant isolates demonstrated complete resistance across all 17 evaluated antimicrobials. None of the isolates were susceptible to more than 6 out of the 17 tested drugs. The susceptibility and resistance patterns of the isolates stratified clearly into two primary therapeutic performance tiers:

- **Severe Resistance Profiles:** Widespread phenotypic resistance was systematically documented against key first-line, broad-spectrum, and combination therapies. The highest failure rates were observed against: Penicillins & Combinations: Penicillin and Amoxicillin-Sulbactam; Cephalosporins: Cefaclor and Cefixime; Fluoroquinolones & Macrolides:

Ciprofloxacin and Azithromycin; Other Classes: Kanamycin and Co-trimoxazole. The severe resistance across these diverse drug classes indicates an almost complete loss of their empirical value for clinical mastitis management in the study region.

- **Relatively Moderated Resistance & Viable Therapeutics:** Conversely, a small cluster of antimicrobials retained a degree of relative efficacy, showing noticeably lower resistance rates and greater susceptibility among the isolates. These remaining therapeutic assets included: Aminoglycosides: Gentamicin; Cephalosporins: Cephalexin and Ceftriaxone; Fluoroquinolones: Enrofloxacin; Tetracyclines: Tetracycline. Among these, Gentamicin and specialized cephalosporins like Cephalexin exhibited the most favorable residual sensitivity profiles, representing the narrow remaining therapeutic window available to practitioners for targeted veterinary intervention.

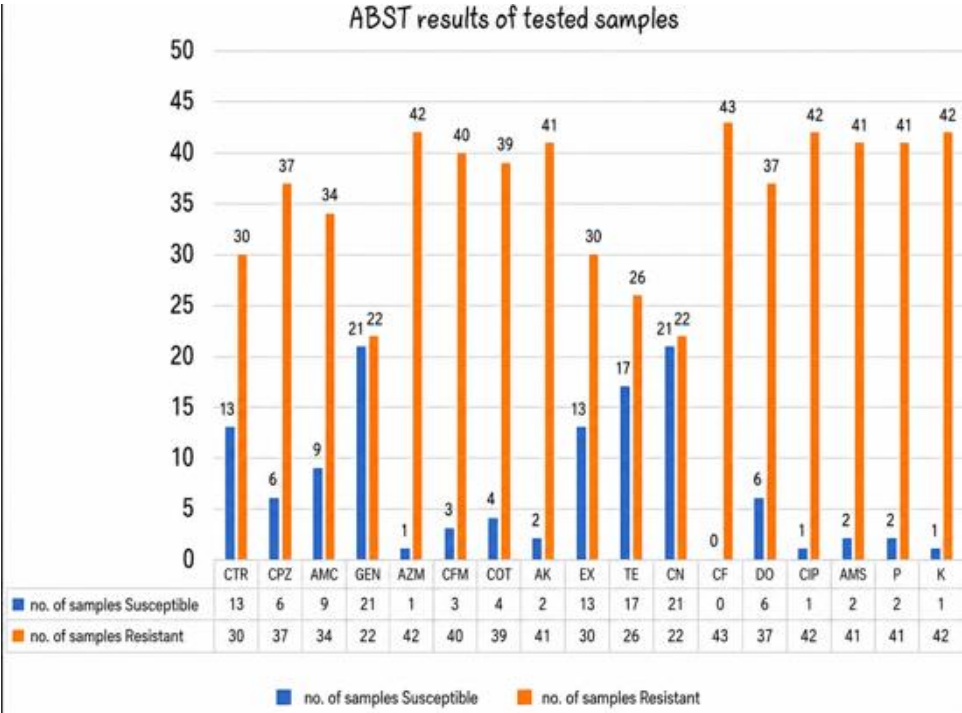


Fig 2: Antibiogram of mastitis bacterial pathogens

3.3 Multiple Antibiotic Resistance (MAR) Index Profiles of Isolated Bacteria

To evaluate the extent of health risks and the degree of selective antibiotic pressure in the study area, the Multiple

Antibiotic Resistance (MAR) index was calculated individually for all 43 isolated bacterial case strains across the 17 tested antimicrobials. The comprehensive distribution of MAR indices among the specific clinical cases is illustrated in Figure 3.

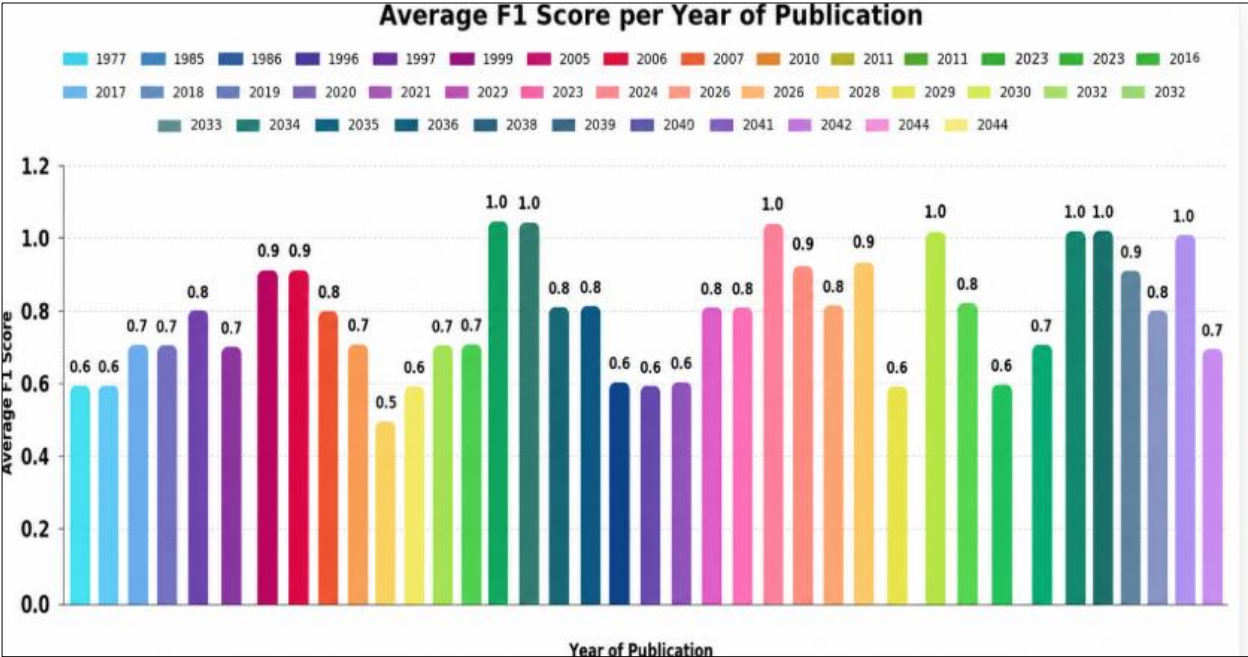


Fig 3: Multiple Antibiotic Resistance (MAR) index profiles of bacterial pathogens isolated from clinical bovine mastitis cases.

Each individual bar represents a unique bacterial isolate plotted against its corresponding sample identification (ID) number. The dashed horizontal line denotes the critical threshold of 0.20, above which isolates originate from high-risk environments characterized by intensive antimicrobial contamination and selective pressure. The mathematical analysis revealed an exceptionally critical landscape of resistance. Every single one of the 43

tested clinical isolates (100%) exhibited a MAR index strictly greater than 0.20, which represents the universally accepted threshold indicating high-risk environments with intense, unscientific antimicrobial abuse. The calculated MAR values ranged from a minimum of 0.60 to a maximum of 1.00, yielding a profoundly elevated overall mean index. 1. Pan-Resistant Profiles (MAR = 1.0): An alarming proportion of the clinical cases exhibited absolute

phenotypical resistance to every drug evaluated. Specifically, six case isolates (Case numbers: 1487, 1493, 1351 [pink variant], 1132, 1237, and 1599) demonstrated a MAR index of 1.00, indicating absolute multi-drug resistance against all 17 tested antibiotic discs. 2. Severe Resistance Profiles (MAR = 0.8 to 0.9): The vast majority of the remaining isolates clustered within highly severe resistance thresholds: MAR Index of 0.90: Documented in 8 case strains, including cases 4178, 4179, 4610, 1351 (peach variant), 1183, 1436, 1480, and 1540. These isolates retained susceptibility to only a single drug class or compound. MAR Index of 0.80: Exhibited by 13 case strains, comprising cases 1626, 4609, 984, 1069, 4634, 1110, 1141, 1549, 1578, and others. 3. High-Risk Baseline Profiles (MAR = 0.6 to 0.7): Even the least resistant bacterial strains isolated in this study presented remarkably high baseline values. A MAR index of 0.70 was recorded for 13 cases (including 1385, 1386, 1657, 3106, 1765, 2289, and 1228), while a MAR index of 0.60 was restricted to only 3 cases (Cases 1377, 1385, and 4611). No single isolate fell below the 0.60 threshold.

This distribution underlines a widespread, systemic contamination pattern where multi-drug resistant strains dominate the local dairy microflora, severely threatening clinical resolution and confirming a massive risk of antimicrobial resistance transmission through the raw milk supply.

4. Discussion

The primary microbiological objective of this investigation was to evaluate the current prevalence and distribution of mastitis-causing pathogens in the peri-urban dairy tracts surrounding the College of Veterinary Science, Garividi. Simultaneously, the study characterized the structural antibiotic resistance phenotypes and Multiple Antibiotic Resistance (MAR) indices of these strains.

Pathogen Prevalence and Polymicrobial Context

Our results reveal an extreme burden of polymicrobial primary cultures, with 43 clinical milk samples giving rise to 55 separate microbial isolates spanning 11 unique genera. The low recovery of monomicrobial primary cultures mirrors findings by contemporary epidemiological reviews highlighting that high-producing dairy animals managed under smallholder conditions are highly susceptible to mixed udder infections (Abebe *et al.*, 2022; Bailly *et al.*, 2025; Shittu *et al.*, 2026) [14, 15, 16].

Escherichia coli was identified as the absolute dominant pathogen at 29.09%, followed by *Klebsiella* spp. at 9.09% (El-Sayed *et al.*, 2023; Ruegg, 2022) [17, 18]. The high prevalence of environmental coliforms is directly connected to significant sanitation challenges in local housing facilities. When calving areas are muddy, bedding changes are infrequent, or pre-milking teat preparation practices are skipped, environmental coliforms can easily multiply and colonize the teat canal (Gleeson & Mary, 2021; Hogan *et al.*, 1982; Patel *et al.*, 2019; Rowbotham & Ruegg, 2016) [19, 20, 21, 22].

Concurrently, classic contagious pathogens retained a large clinical footprint, with *Staphylococci* spp. (23.63%) and *Streptococci* spp. (18.18%) combining for more than 40% of the total microbial burden. These Gram-positive cocci reside on mucosal surfaces and are primarily spread horizontally cow-to-cow via milkers' hands and shared washing cloths

during milking routines (Gao *et al.*, 2020; Ndahetuye *et al.*, 2024) [23, 24]. The isolation of *Bacilli* (5.45%), *Pseudomonas* (1.81%), and fungal pathogens including *Yeast* (5.45%), *Penicillium* (1.81%), and *Mucor* (1.81%) highlights a severely compromised microenvironment (Ndahetuye *et al.*, 2024) [24]. This mycotic proliferation is typically accelerated by poor environmental hygiene or prior, unscientific intramammary antibiotic infusions that eliminate normal bacterial microflora, allowing opportunistic fungi to fill the niche (Abadi *et al.*, 2025; Saini *et al.*, 2023) [25, 26].

Antimicrobial Susceptibility and Resistance Tiers

The *in-vitro* antibiotic susceptibility assay results paint a highly concerning therapeutic picture, as every single tested bacterial isolate (100%) exhibited multidrug resistance (MDR). The baseline severity is underscored by the fact that no isolate was sensitive to more than 6 of the 17 tested compounds, with a subset showing absolute resistance to all 17 antibiotic discs.

The severe resistance profiles against Penicillin, Amoxicillin-Sulbactam, Cefaclor, and Cefixime confirm that conventional β -lactams and core cephalosporins have effectively lost their empirical therapeutic value in this region. This complete loss of efficacy is a known outcome of long-term selective pressure caused by empirical, over-the-counter medication purchases and sub-therapeutic dosing protocols deployed by dairy farmers without veterinary oversight (Biolife Editorial Board, 2017; Van Boeckel *et al.*, 2019) [27, 28]. Furthermore, extensive cross-resistance was documented for Ciprofloxacin, Azithromycin, Kanamycin, and Cotrimoxazole, showing that resistance spans across fluoroquinolones, macrolides, aminoglycosides, and sulfonamides. This limits the options for frontline field treatments.

Conversely, the lower resistance and better retention of efficacy observed for Gentamicin, Cephalexin, Ceftriaxone, and Enrofloxacin indicates a narrow but vital remaining therapeutic window. Gentamicin and specialized combinations like

Cephalexin/Gentamicin variants were the top performers, maintaining nearly 50% clinical susceptibility (Biolife Editorial Board, 2017; Mohammed & Haytham, 2019) [27, 29]. This suggests that aminoglycosides are less frequently abused by local farmers in the Garividi tract compared to heavily overused β -lactams (Abadi *et al.*, 2025; Van Boeckel *et al.*, 2019) [25, 28].

Multiple Antibiotic Resistance (MAR) Index Realities

The patient-by-patient MAR index analysis provides direct evidence of severe public health risks. 100% of the tested bacterial isolates exhibited a MAR index strictly greater than 0.20, ranging from 0.60 to a maximum of 1.00. A MAR value exceeding the critical threshold of 0.20 indicates that the isolates originate from high-risk environments where antibiotic classes are heavily overused (Krumperman, 1983; Manyi-Loh *et al.*, 2021) [10, 30].

The fact that six separate isolates (including strains from cases 1487, 1493, 1351, 1132, 1237, and 1599) achieved a maximum MAR index of 1.00 signals total therapeutic exhaustion against standard testing groups. When dairy cattle become reservoirs for pathogens with MAR profiles between 0.60 and 1.00, these resistant strains and their residual determinants enter the raw milk supply (Davis *et al.*, 2024; Oliver *et al.*, 2020) [31, 32]. This represents a

significant food safety concern, as these resistant pathogens can transfer resistance genes horizontally to human pathogens via the food chain, compounding the global crisis of antimicrobial resistance (Manyi-Loh *et al.*, 2021; Oliver *et al.*, 2020) [30, 32].

Our findings emphasize that managing bovine mastitis in Andhra Pradesh requires a shift away from empirical antibiotic treatments. To preserve the remaining utility of drugs like Gentamicin, it is essential to implement regular herd sanitation reviews, strict premilking hygiene practices, and mandatory pre-treatment antibiotic sensitivity testing.

5. Conclusion

This study reveals a high burden of environmental and contagious mastitis pathogens among dairy cattle in the Garividi region, spearheaded by *Escherichia coli* (29.09%) and *Staphylococci* spp. (23.63%). The isolated bacterial populations demonstrated universal multidrug resistance (100%), completely exhausting standard penicillins and core cephalosporins, while leaving Gentamicin and Cephalexin as the narrow remaining options for targeted treatment. Crucially, 100% of the isolates exhibited an alarming Multiple Antibiotic Resistance (MAR) index far exceeding the 0.20 threshold (range: 0.60–1.00), signaling intense selective antibiotic pressure in the region. These findings emphasize an urgent public health need to transition away from empirical antibiotic therapies and implement routine pre-treatment sensitivity screening alongside strict herd sanitation protocols to prevent the risk of transmitting resistant pathogens to consumers through the milk supply chain.

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