Original Research

Journal of Advanced Veterinary Research
(2026) Volume 16, Issue 3, 359-364

Phenotypic and genotypic characterization of bacterial isolates causing bovine mastitis with reference to MALDI-TOF-MS for isolates' identification

Nehal M. Fawzy[1], Aalaa S.A. Saad[2*], Marwa M. Fathi[3], Ghada S. Abdelhamed[3], Mona M. Osman[4]

[1]Bacteriology Department, Animal Health Research Institute (AHRI), Agricultural Research Centre (ARC), Egypt.
[2]Biotechnology Department, Animal Health Research Institute (AHRI), Agricultural Research Centre (ARC), 0000-0003-4740-4419, Egypt.
[3]Serology Unit, Animal Health Research Institute (AHRI), Agricultural Research Centre (ARC), Egypt.
[4]Mycoplasma Department, Animal Health Research Institute (AHRI), Agricultural Research Centre (ARC), Egypt.

ARTICLE INFO

Received: 03 January 2026

Accepted: 26 March 2026

*Correspondence:

Corresponding author: Aalaa S.A. Saad
E-mail address: alaa.samir87@yahoo.com

Keywords:

Mastitis, Antimicrobial Resistance, Virulence Factors, Bovine

ABSTRACT

In this investigation, we assessed the phenotypic and genotypic traits of bacterial and *Mycoplasma* species associated with bovine mastitis. We examined ninety milk samples taken from cows with mastitis by conventional bacteriological methods, testing for antimicrobial susceptibility, virulence profile, and molecular techniques to determine the presence of resistance genes. The most common pathogens identified as part of this investigation were: *E. coli*, *Enterococcus faecalis*, and *Mycoplasma* spp. Additionally, our examination of cows with mastitis revealed many cows had mixed infections, thus demonstrating the polymicrobial nature of mastitis. The antimicrobial susceptibility of the mastitis causative agents was evaluated through antimicrobial susceptibility testing and revealed that most commonly used classes of antibiotics had demonstrated high levels of resistance being present. Foundationally, the molecular analysis of the pathogens confirmed the presence of multiple critical resistance genes. Biofilm formation, as well as enzymes, were found to be elevated demonstrating the pathogens had the capacity to survive within their host's mammary tissues. This investigation document MALDI-TOF-MS as a rapid and effective tool for identification of the causative agents of bovine mastitis. The results from this study support and reinforces the need for ongoing disease monitoring and responsible antibiotic usage for the control of mastitis in dairy cattle as well as for preventing the development of antibiotic resistance in dairy herds.

Introduction

Worldwide, bovine mastitis poses a considerable challenge to sustainable dairy production, creating significant economic difficulties from decreased milk output, a decline in milk quality, increased veterinary costs, and premature culling of affected animals (Tomanić *et al*., 2025). Furthermore, beyond its financial impact, mastitis presents significant challenges pertaining to animal welfare and public health, particularly concerning the spread of antimicrobial-resistant bacteria in the dairy food supply (Paramasivam *et al*., 2023).

Historically, mastitis research has focused primarily on classical pathogens such as *Staphylococcus aureus*, *Streptococci*, and *Escherichia coli*. However, recent epidemiological studies have highlighted a noticeable shift toward opportunistic and emerging pathogens, including *Enterococcus faecalis*, members of the *Enterobacter cloacae* complex, *Serratia marcescens*, *Providencia rettgeri*, *Burkholderia cepacia* complex, and *Mycoplasma bovis* (Cobirka *et al*., 2020; Tommasoni *et al*., 2023; Guaita *et al*., 2025). These organisms are increasingly associated with subclinical and chronic mastitis, mixed infections, and poor therapeutic outcomes, largely due to their enhanced virulence traits and intrinsic or acquired antimicrobial resistance (Roșu *et al*., 2025).

Effective disease control depends on the timely and accurate identification of microorganisms linked to mastitis (Duarte *et al*., 2015). Traditional bacteriological techniques that rely on culture traits and biochemical reactions are still commonly utilized, but they often face challenges with lengthy turnaround times and inadequate differentiation, especially for closely related species and difficult-to-culture organisms like *Mycoplasma* spp. These challenges can hinder timely treatment decisions and affect the management of mastitis. As a result, there is an increasing demand for more advanced diagnostic methods that integrate speed, precision, and consistency (Storgårds *et al*., 2006; Gaur *et al*., 2025).

Matrix-Assisted Laser Desorption/Ionization–Time of Flight Mass Spectrometry (MALDI-TOF-MS) has emerged as a transformative technology in veterinary microbiology (Elbehiry and Abalkhail, 2025). By analyzing unique protein mass spectra, MALDI-TOF-MS enables rapid and reliable species-level identification of bacterial isolates with minimal sample preparation. Recent applications in mastitis diagnostics have demonstrated its superiority over traditional methods, particularly in resolving taxonomically complex groups and detecting uncommon or emerging pathogens. Its integration into routine diagnostic workflows has significantly enhanced pathogen surveillance and epidemiological investigations in dairy herds (Clark *et al*., 2013; de Souza Ferreira *et al*., 2025).

In parallel with pathogen identification, the characterization of antimicrobial susceptibility profiles has become increasingly critical. The extensive and sometimes indiscriminate use of antimicrobials in dairy production has contributed to the global escalation of multidrug-resistant mastitis pathogens (Kiplimo *et al*., 2025). There is significant concern regarding the development of resistance mechanisms that are facilitated by transferable genetic elements, such as extended-spectrum β-lactamases, *Amp*C β-lactamases, carbapenem resistance factors, mobile colistin resistance genes (*mcr*), and vancomycin resistance genes found in enterococci. The molecular identification of these resistance genes offers important insights into the genetic foundation of phenotypic resistance and aids in the risk assessment associated with antimicrobial usage and food safety (Potter *et al*., 2016; Nasrollahian *et al*., 2024).

Moreover, virulence-associated phenotypes such as biofilm formation, proteolytic enzyme production, hemolytic activity, and oxidative stress tolerance play a pivotal role in bacterial persistence within the mammary gland (Ramírez-Larrota and Eckhard, 2022). These traits facilitate immune evasion, enhance colonization, and reduce antimicrobial efficacy, thereby contributing to treatment failure and recurrent infections. The combined evaluation of phenotypic virulence traits and genotypic resistance determinants offers a more comprehensive understanding of pathogen pathogenicity and adaptive capacity (Schroeder *et al*., 2017;

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
ISSN: 2090-6277/2090-6269/ © 2011-2026 Journal of Advanced Veterinary Research. All rights reserved.

Raymond, 2020).

The aim of this research was to provide a comprehensive description of the bacteria and *Mycoplasma* present in milk samples from cows with mastitis. It concentrated on using MALDI-TOF-MS for accurate identification of pathogens, as well as conducting tests for antimicrobial susceptibility, profiling of virulence, and molecular detection of resistance genes. This thorough investigation offers a current understanding of the pathogens associated with bovine mastitis, aiding in the development of data-informed strategies for enhanced mastitis management and antimicrobial practices.

Materials and methods

Sample collection

From March 2023 to June 2024, ninety samples of raw milk were taken from lactating cows in Fayoum and Giza, Egypt. The samples were collected aseptically from each quarter, in sterile containers, and moved in an ice box to the AHRI's Bacteriology Lab in Dokki, Giza, Egypt, for bacterial analysis.

Screening for subclinical mastitis

All collected milk samples were screened for subclinical mastitis using the California Mastitis Test (CMT) following the standard protocol described by Hogan *et al*. (1999).

Bacteriological phenotypic identification

Milk samples that tested positive for mastitis were cultured on Blood agar, MacConkey agar, and Salmonella–Shigella agar (HiMedia, India). The inoculated plates were incubated in aerobic conditions at 37°C for 24 hours.

The colonies that were recovered underwent Gram staining and initial identification through conventional biochemical tests, which include oxidase, catalase, IMViC tests (indole, methyl red, Voges–Proskauer, citrate), Triple Sugar Iron (TSI) agar, urease assay, bile esculin hydrolysis, pyrrolidonyl arylamidase (PYR) test, and growth in brain heart infusion (BHI) broth augmented with 6.5% NaCl, according to Quinn *et al*. (2002).

Isolation and recognition of Mycoplasma spp.

Milk samples were inoculated into pleuropneumonia-like organism (PPLO) broth (Oxoid, UK) and incubated at 37°C for up to two weeks. Positive cultures were subcultured repeatedly to obtain pure colonies. Differentiation between *Mycoplasma* and *Acholeplasma* genera was performed using the digitonin sensitivity test as described by Freundt (1973). Further biochemical characterization of *Mycoplasma* species was conducted using glucose fermentation and arginine deamination tests following the protocol of Ernø and Stipkovits (1973).

MALDI-TOF-MS identification

Confirmed bacterial isolates were classified at the species level through Matrix-Assisted Laser Desorption/Ionization–Time of Flight Mass Spectrometry (MALDI-TOF-MS) in accordance with the manufacturer's guidelines. The spectral profiles were evaluated using the reference database to guarantee precise identification and were contrasted with traditional phenotypic results for verification.

Testing for antimicrobial susceptibility

Antimicrobial susceptibility patterns of the isolates were assessed using the Kirby–Bauer disc diffusion technique. The analysis of inhibition zones was done based on CLSI (2020).

The antimicrobial agents tested included trimethoprim/sulfamethoxazole (25 µg), ceftazidime (30 µg), gentamicin (30 µg), piperacillin/tazobactam (100/10 µg) for both Gram-positive and Gram-negative bacteria, meropenem (10 µg) for Gram-negative isolates, and linezolid (30 µg), erythromycin (15 µg), and vancomycin (30 µg) for Gram-positive isolates. *Mycoplasma* isolates were tested against enrofloxacin (10 µg), florfenicol (30 µg), tylosin (15 µg), doxycycline (30 µg), and amoxicillin/clavulanic acid (30 µg).

Phenotypic virulence traits assays

Following species-level identification, all isolates were evaluated for selected phenotypic virulence traits.

Biofilm formation assay

All isolates, including *Mycoplasma* species, were evaluated for their capacity to produce biofilms on abiotic surfaces using a microtiter plate technique outlined by O'Toole (2011).

Hemolysin activity

To measure haemolytic activity, isolates were transferred to Blood Agar media (HiMedia, India) supplemented with 5% horse blood. After a full day of incubation at 37°C, the plates were examined for haemolysis patterns, according to Wang *et al*. (2020),

Gelatinase activity

Gelatin medium (3%) was used to measure the breakdown of gelatin. According to Balan *et al*. (2012), all isolates aside from *Mycoplasma* spp. were incubated at 37°C for 48 hours, and the existence of a distinct halo encircling the colonies suggested gelatinase activity.

Casein Hydrolysis Assay

According to Hashem *et al*. (2021), after an overnight incubation period, non-*Mycoplasma* isolates were inoculated onto BHI agar supplemented with 5% (w/v) skim milk powder and examined for clear zones indicative of casein hydrolysis.

Catalase Activity in *Mycoplasma* isolates

By applying hydrogen peroxide (H_2O_2) to bacterial cell suspensions and watching for the development of bubbles, catalase activity in *Mycoplasma* isolates was assessed, as described by Pritchard *et al*. (2014).

Identification of Genes conferring antimicrobial resistance

Genomic DNA was extracted according to the manufacturer's instructions using the COL-NA DNA Extraction Kit (REME-D, Egypt). The primers used for molecular identification and detection of antimicrobial resistance genes (Table 1).

Polymerase chain reaction (PCR) was performed using a Bio-Rad thermal cycler under appropriate cycling conditions specific for each primer set.

β-lactamase and resistance-associated genes (*bla_{TEM}*, *bla_{SHV}*, *amp*C, *bla_{IPM}*, and *mcr*-1) were screened for Gram-negative bacterial isolates. Vancomycin resistance genes (*van*A and *van*C) were detected in Gram-positive isolates. The genus-specific GPO (common *Mycoplasma*) primer and the *Mycoplasma bovis*-specific gene were used to molecularly confirm *Mycoplasma* isolates. checked for fluoroquinolone antibiotic resistance (*par*C) as well (Table 1).

Table 1. The target genes, primer sequences, that are used for genomic analysis of *E. coli* and *E. faecalis* isolates and *M. bovis*.

	Sequence of Primer	Annealing temperature	Product Size (bp)	Reference
bla_{TEM}	F: TTGCTCACCCAGAAACGCTGGTG R: TACGATACGGGAGGGCTTACC	63°C	708	(Mataseje *et al*., 2012)
bla_{SHV}	F: CGCCGGGTTATTCTTATTTGTCGC R: TCTTTCCGATGCCGCCGCCAGTCA		1016	
*amp*C	F: GATCGTTCTGCCGCTGTG R: GGGCAGCAAATGTGGAGCAA	56°C	271	(Oliver *et al*., 2002)
bla_{IPM}	F: TCGTTTGAAGAAGTTAACG R: ATGTAAGTTTCAAGAGTGATGC	60°C	568	(Mahmoud *et al*., 2020
*mcr*1	F: CGGTCAGTCCGTTTGTTC R: CTTGGTCGGTCTGTAGGG		308	(Newton-Foot *et al*., 2017)
*van*A	F: GGG AAA ACG ACA ATT GC R: GTA CAA TGCG GCC GTT	59°C	732	(Karsidani *et al*., 2010)
VanC1,2	F: ATGGATTGGTAYTKGTAT R:TAGCGGGAGTGMCYMGTAA	54°C	815/827	Depardieu *et al*., 2004
GPO	F: TGGGGAGCAAACAGGATTAGATACC R: TGCACCATCTGTCACTCTGTTAACCTC	53°C	275	(Cetinkaya *et al*., 2009)
M. bovis	F: CCT TTT AGA TTGGGATAGCGGATG R: CCGTCAAGGTAGCGTCAT TTCCTAC	60°C	360	(Yleana *et al*., 1995)
parC	F: GAGCA ACAGTTAAACGATTTG R: GGCATAAC AACTGGCTCTT	54ºC	488	(Lysnyansky *et al*., 2009)

Results

Bacteria isolated from mastitic milk samples

50 milk samples were confirmed as mastitic milk by CMT. *Escherichia coli* was the most frequently detected pathogen, being isolated from 43 samples, which represents a prevalence of 86%. *M. bovis* were identified in 19 samples (38%). *Enterococcus faecalis* was recovered from 14 samples (28%), while *Enterobacter hormaechei* and *Providencia rettgeri* were each detected in 7 samples, corresponding to a prevalence of 14% for each pathogen. *Serratia marcescens* was isolated from 5 samples (10%), whereas members of the *Burkholderia cepacia* complex occurred in 3 samples (6%).

Patterns of single and mixed bacteria existence in mastitic milk

Single-pathogen infections were detected in 13 samples (26%), with *Escherichia coli* being the most frequently isolated single pathogen (16%), followed by *Enterococcus faecalis* (10%) (Table 2).

Mixed bacterial infections were identified in the majority of samples. Mixed infections involving two different pathogens were the most prevalent, accounting for 31 cases (62%). Among these, co-infection with *E. coli* and *Mycoplasma bovis* was the most common pattern, detected in 30% of samples. The other two-pathogen combinations included *E. faecalis* + *E. coli* (10%), *E. coli* + *Providencia rettgeri* (14%), *E. coli* + *Enterobacter hormaechei* (4%), and *E. hormaechei* + *Burkholderia cepacia* complex (4%).

Mixed infections involving three bacterial pathogens were identified in 8%. These included co-infections of *E. coli* + *S. marcescens* + *E. faecalis* (4%) and *E. coli* + *E. faecalis* + *M. bovis* (4%). Infections involving four different bacterial pathogens were the least frequent, detected in 2 samples (4%), characterised by the simultaneous presence of *E. coli*, *S. marcescens*, *E. hormaechei*, and *M. bovis*.

Antimicrobial susceptibility patterns

Overall, marked variability in antimicrobial response was observed among the different bacterial species.

Among *E. coli* isolates, moderate susceptibility was observed to trimethoprim/sulfamethoxazole, azithromycin, and gentamicin, with 60% of isolates showing sensitivity to each agent. In contrast, low susceptibility was detected to meropenem (21%), while complete resistance (100%) was observed against ceftazidime, piperacillin/tazobactam, and enrofloxacin.

Enterococcus faecalis isolates exhibited moderate susceptibility to gentamicin (67%), linezolid (60%), and vancomycin (67.7%). However, 100% of the isolates were resistant to trimethoprim/sulfamethoxazole, ceftazidime, piperacillin/tazobactam, and erythromycin.

Enterobacter hormaechei isolates showed high susceptibility to gentamicin (100%) and trimethoprim/sulfamethoxazole (71%), while complete resistance was recorded against meropenem, azithromycin, ceftazidime, piperacillin/tazobactam, and enrofloxacin.

Members of the *Burkholderia cepacia* complex demonstrated moderate susceptibility to trimethoprim/sulfamethoxazole (67%) and meropenem (67%), but were resistant to ceftazidime. *Serratia marcescens* isolates were fully susceptible to gentamicin (100%) but exhibited complete resis-

Table 2. Patterns of single and mixed bacterial infections detected in bovine mastitis.

n= of detected bacteria in samples	Type of bacteria	Frequency	%
1	*E. faecalis*	5	10
	E. coli	8	16
2	*E. faecalis* + *E. coli*	5	10
	E. coli+ *E. hormaechei*	2	4
	E. coli + *Mycoplasma species*	15	30
	E. hormaechei + *Burkholderia cepacia complex*	2	4
	E. coli + *Providencia rettgeri*	7	14
3	*E. coli* + *Serratia marcescens* + *E. faecalis*	2	4
	E. coli + *E. faecalis* +*Mycoplasma species*	2	4
4	*E. coli* + *Serratia marcescens* + *Enterobacter hormaechei* + *Mycoplasma bovis*	2	4

tance to meropenem, ceftazidime, and piperacillin/tazobactam.

Providencia rettgeri isolates showed variable susceptibility, with moderate sensitivity to trimethoprim/sulfamethoxazole (57%) and piperacillin/tazobactam (57%), while complete resistance was observed against gentamicin, meropenem, ceftazidime, and azithromycin.

All *Mycoplasma* isolates demonstrated complete susceptibility to enrofloxacin (100%) and florfenicol (100%). Moderate susceptibility was observed to tylosin (63%) and doxycycline (37.3%), whereas low susceptibility was detected against amoxicillin/clavulanic acid (21%).

Phenotypic virulence factors

The distribution of phenotypic virulence factors among bacterial and *Mycoplasma* isolates recovered from mastitic milk samples is presented in Figure 1. Considerable variation in virulence-associated traits was observed among the different bacterial species.

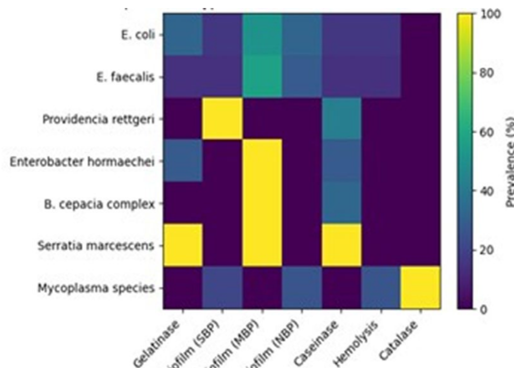

Figure 1. Heatmap of phenotypic virulence factors among bacterial pathogens isolated from bovine mastitic milk. Rows represent bacterial species, while columns indicate virulence traits, including gelatinase, biofilm formation (strong, moderate, and non-biofilm producers), caseinase, hemolysis, and catalase activity. Color intensity corresponds to the percentage of positive isolates, with higher intensities indicating greater prevalence.

Among *Escherichia coli* isolates, gelatinase activity was detected in 32.5% of isolates. Biofilm formation was highly prevalent, with 51.2% classified as moderate biofilm producers (MBP), 32.5% as non-biofilm producers (NBP), and 16.3% as strong biofilm producers (SBP). Caseinase activity and hemolytic activity were each recorded in 16.3% of *E. coli* isolates.

Enterococcus faecalis isolates demonstrated lower overall virulence factor prevalence. Gelatinase production was detected in 14.3% of isolates. Biofilm formation was common, with 57.1% identified as moderate biofilm producers, 28.6% (4/14) as non-biofilm producers, and 14.3% as strong biofilm producers. Caseinase and hemolytic activities were each detected in 14.3% of isolates.

Providencia rettgeri isolates showed no gelatinase activity. However, all isolates (100%) were founded as strong biofilm producers. Caseinase activity was detected in 42.8% of isolates, while no hemolytic activity was observed.

In *Enterobacter hormaechei*, gelatinase and caseinase activities were each detected in 28.6% (2/7) of isolates. All isolates (100%) were identified as moderate biofilm producers, while hemolytic activity was not detected.

All *Mycoplasma* isolates exhibited catalase activity (100%). Biofilm formation was detected in a subset of isolates, with 21.1% classified as strong biofilm producers and 26.3% as non-biofilm producers. Hemolytic activity was observed in 26.3% of *Mycoplasma* isolates.

Members of the *Burkholderia cepacia* complex did not exhibit gelatinase or hemolytic activity. However, all isolates (100%) demonstrated moderate biofilm formation, and caseinase activity was detected in 33.3% of isolates.

Serratia marcescens isolates showed the highest virulence profile, with gelatinase and caseinase activities detected in all isolates (100%). All isolates were also detected to be moderate biofilm producers, while hemolytic activity was not observed.

Identification of Genes conferring antimicrobial resistance

The bla_{TEM} and Bla_{IPM} genes had been detected in 20% of *E. coli* isolates and *Providencia rettgeri* isolates, respectively. However, these genes were not found in isolates of Serratia, *Enterobacter hormaechei*, or the *Burkholderia cepacia* complex. The *amp*C gene was present in all isolates, whereas the bla_{SHV} gene was not detected. The *mcr*1 gene was found in 20% of the *Providencia rettgeri* strains only. Additionally, the *van*A and *van*C genes were detected in 40% and 20% of *E. faecalis* isolates, respectively. The *par*C was detected in 10% in *M. bovis* isolates.

Discussion

The present study highlights the diverse bacterial etiology of bovine mastitis, with notable differences in the prevalence of isolated pathogens. *E. coli* was found to be the most prevalent bacterium in bovine mastitic milk. These results align with earlier research Abed *et al*. (2021; Ahmed *et al*. (2023).

The detection of *M. bovis* in 38% of mastitic milk samples confirms its substantial contribution to mastitis cases and supports growing evidence that *M. bovis* mastitis is becoming more common, especially in intensive dairy systems, according to recent studies (Nicholas *et al*, 2008; Gelgie *et al*., 2024). Recent studies have emphasized the increasing prevalence of *M. bovis* mastitis, particularly in intensive dairy systems, where Li *et al*. (2021) and Ahmed *et al*. (2023) found *Mycoplasma* species in milk samples at rates of 42.3% and 51.5%, respectively.

The relatively high prevalence of *Enterococcus faecalis* (28%) observed in this study aligns with recent reports identifying enterococci as emerging mastitis pathogens of environmental origin. *E. faecalis* is increasingly recognized for its ability to persist in the dairy farm environment (Abed *et al*., 2021; Liu *et al*., 2024).

Enterobacter hormaechei and *Providencia rettgeri* have been detected at equal prevalence levels (14% each) highlights the importance of opportunistic Gram-negative bacteria in the aetiology of bovine mastitis. Members of both species have been increasingly reported in mastitis cases and are often associated with environmental contamination and inadequate milking hygiene (Ayyal, 2020; Dubey *et al*., 2025).

Serratia marcescens, isolated from 10% of the samples, is a well-established environmental mastitis pathogen frequently linked to contaminated disinfectants, teat dips, and milking equipment. Recent outbreaks of *S. marcescens* mastitis have been reported in dairy herds (Moreira *et al*., 2025).

Although the *Burkholderia cepacia* complex members were the least frequently detected (6%), their presence remains epidemiologically significant. Recent studies have documented their involvement in persistent and difficult-to-control mastitis cases, highlighting their clinical relevance despite low prevalence (Zhang *et al*., 2023).

The moderate susceptibility of *Escherichia coli* isolates to trimethoprim/sulfamethoxazole, azithromycin, and gentamicin contrasts sharply with the complete resistance observed against ceftazidime, piperacillin/tazobactam, and enrofloxacin. These phenotypic resistance patterns are partly explained by the detection of bla_{TEM} and bla_{IPM} genes in 20% of *E. coli* isolates. The presence of these β-lactamase genes has been increasingly reported in mastitis-associated *E. coli* and is strongly associated with resistance to extended-spectrum β-lactams and reduced susceptibility to carbapenems (Różańska *et al*., 2021). The universal detection of the *amp*C gene among isolates further supports the widespread cephalosporin resistance observed phenotypically and aligns with recent reports highlighting the dominance of *Amp*C-producing Enterobacterales in bovine mastitis (Subhi *et al*., 2023). In contrast, the absence of the bla_{SHV} gene suggests that resistance in the present isolates is primarily driven

by bla_{TEM}-, bla_{IPM}-, and *amp*C-mediated mechanisms rather than classical ESBLs.

The 20% of the *Providencia rettgeri* isolates harboured the *mcr*-1 gene, despite phenotypic resistance to multiple antimicrobials, which is particularly concerning. Recent studies have emphasised the emergence of *mcr*-1 in food-producing animals as a critical public health threat because of its potential for horizontal transfer and its association with multidrug-resistant Gram-negative bacteria (Ayyal, 2020).

In *Enterococcus faecalis*, the moderate susceptibility to gentamicin, linezolid, and vancomycin observed phenotypically corresponds with the molecular detection of vancomycin resistance genes. The presence of *van*A in 40% and *van*C in 20% of isolates provides a molecular explanation for the reduced susceptibility to vancomycin and highlights the increasing occurrence of vancomycin-resistant enterococci (VRE) in dairy environments (Massella *et al*., 2025; Babacan, 2025). The coexistence of these resistance genes with biofilm-forming ability, as demonstrated in this study, further exacerbates treatment challenges and enhances the persistence of *E. faecalis* in the mammary gland.

The phenotypic resistance profiles of *Enterobacter hormaechei*, *Serratia marcescens*, and members of the *Burkholderia cepacia* complex—characterized by extensive resistance to β-lactams and variable susceptibility to other agents—are consistent with their known intrinsic resistance mechanisms. These species are well recognized for harbouring chromosomal *amp*C β-lactamases, efflux pumps, and reduced outer membrane permeability, which collectively limit antimicrobial efficacy (Rhodes and Schweizer, 2016; Yeh *et al*., 2022). The absence of bla_{TEM}, bla_{SHV}, and bla_{IPM} genes in these species suggests that resistance is predominantly chromosomally encoded rather than plasmid-mediated, a finding supported by recent genomic studies (Liakopoulos *et al*., 2016).

In contrast to bacterial pathogens, *Mycoplasma* isolates exhibited complete susceptibility to enrofloxacin and florfenicol, consistent with the absence of a cell wall and the intrinsic resistance of *Mycoplasma* spp. to β-lactam antibiotics. Recent investigations have similarly reported fluoroquinolones and phenicols as the most effective therapeutic options for *Mycoplasma bovis* mastitis (Nicholas and Ayling, 2022; Zhang *et al*., 2023). However, the reduced susceptibility to doxycycline and amoxicillin/clavulanic acid underscores the narrowing spectrum of effective antimicrobials and raises concerns regarding the emergence of resistance mediated by target-site mutations and efflux mechanisms. The findings of *par*C were comparable with recent studies from different geographic regions, where *par*C mutations were detected at variable frequencies among *M. bovis* isolates, reflecting localized antimicrobial usage patterns and selective pressure within dairy herds (Gautier-Bouchardon, 2014; Khalil *et al*., 2017).

Interestingly, despite the presence of *par*C in a subset of isolates, phenotypic susceptibility to enrofloxacin remained high in this study, suggesting that the detected *par*C variants may represent early or partial resistance determinants rather than fully established resistance phenotypes. This observation aligns with recent reports indicating that high-level fluoroquinolone resistance in *M. bovis* typically requires the accumulation of multiple mutations in both *par*C and gyrA genes (Maunsell *et al*., 2011; Abd El Tawab *et al*., 2019).

Among *Escherichia coli* isolates, the high prevalence of biofilm formation particularly moderate biofilm producers corroborates recent studies identifying biofilm-associated *E. coli* as a major contributor to chronic and recurrent mastitis cases (Zuo *et al*., 2025). Biofilm formation in *E. coli* enhances resistance to host immune mechanisms and antimicrobials, while the concurrent detection of gelatinase, caseinase, and hemolytic activities in a subset of isolates suggests enhanced tissue damage potential and nutrient acquisition within the mammary gland (Zaatout *et al*., 2022). These combined virulence traits may explain the frequent involvement of *E. coli* in severe clinical and persistent mastitis.

The relatively lower virulence factor expression observed in *Enterococcus faecalis* isolates aligns with recent reports describing enterococci as opportunistic mastitis pathogens with moderate pathogenicity but strong persistence capabilities (Lui *et al*., 2024). Despite limited gelatinase, caseinase, and hemolytic activity, the high proportion of biofilm-producing *E. faecalis* isolates supports their role in subclinical and chronic infections. Biofilm-associated enterococci are particularly problematic due to their ability to act as reservoirs of antimicrobial resistance genes, posing both animal health and public health risks.

Providencia rettgeri demonstrated a striking virulence profile characterized by universal strong biofilm formation despite the absence of gelatinase and hemolytic activity. This finding supports emerging evidence that Providencia species rely primarily on biofilm-mediated persistence rather than classical enzymatic virulence mechanisms (Silva *et al*, 2022). The detection of caseinase activity in nearly half of the isolates further suggests an ability to exploit milk proteins, facilitating survival and colonization within the mammary environment.

Similarly, *Enterobacter hormaechei* isolates exhibited consistent moderate biofilm formation, with limited gelatinase and caseinase activity and no detectable hemolysis. Members of the *Enterobacter cloacae* complex are increasingly recognized as mastitis-associated pathogens, particularly in environments with suboptimal hygiene (Annavajhala *et al*., 2019). Their pathogenic success appears to be driven more by biofilm formation and antimicrobial resistance than by aggressive tissue-destructive virulence factors.

All *Mycoplasma* isolates displayed universal catalase activity, consistent with previous studies indicating the role of oxidative stress resistance in *Mycoplasma* survival within the host (Pritchard *et al*., 2015; Nicholas and Ayling, 2022). The detection of biofilm formation and hemolytic activity in a subset of isolates further supports recent evidence that *Mycoplasma bovis* can form biofilm-like structures, contributing to chronicity and poor therapeutic outcomes in mastitis (Zhang *et al*., 2023; Bürki *et al*., 2015). These traits partly explain the difficulty in eradicating *Mycoplasma*-associated mastitis from affected herds.

Members of the *Burkholderia cepacia* complex exhibited moderate biofilm forming ability in all isolates, despite lacking gelatinase and hemolytic activity. This is consistent with the well-documented ability of B. cepacia complex organisms to persist in moist environments and resist disinfectants through biofilm-based survival strategies (Murphy and Caraher, 2015; Tavares *et al*., 2020). Even at low prevalence, their biofilm-forming capacity makes them epidemiologically significant in mastitis control programs.

Serratia marcescens exhibited the most pronounced virulence profile, characterised by universal gelatinase and caseinase production and consistent biofilm formation. These findings align with recent reports identifying *S. marcescens* as a highly virulent environmental mastitis pathogen capable of causing outbreaks linked to contaminated milking equipment and disinfectants (Friman *et al*., 2019; Moreira *et al*., 2025). The strong proteolytic activity observed may contribute to extensive mammary tissue damage and persistent infection. Further detailed studies are required for the recent pathogens causing mastitis, including *Providencia hormaechei* and *Burkholderia*.

Conclusion

The findings reveal a diverse range of microorganisms causing bovine mastitis, highlighting both traditional pathogens and emerging opportunistic ones, often in mixed infections. The use of MALDI-TOF-MS proved effective for accurate species identification, especially for rare isolates, and when paired with conventional methods, it enhanced diagnostic accuracy. Antimicrobial susceptibility testing showed significant variability in resistance patterns, reflecting the impact of antibiotic use in dairy herds. Notable resistance genes were identified, indicating the public health risks associated with resistant mastitis pathogens. Additionally, the presence of virulence traits such as biofilm formation suggests a potential for chronic infections. The *par*C gene found in some *M. bovis*

isolates points to emerging fluoroquinolone resistance, underscoring the need for vigilant surveillance. This integrated approach offers insights into the changing epidemiology of bovine mastitis, aiding in the development of better diagnostic and treatment strategies and ensuring animal health and dairy safety.

Conflict of interest

The authors have no conflict of interest to declare.

References

Abd El Tawab, A.A., El-hofy, F.I., Hassan, N.I., El-khayat, M.E., 2019. Prevalence of *Mycoplasma bovis* in bovine clinical mastitis milk in Egypt. Benha Veterinary Medical Journal 36, 57-65.

Abed, A.H., Menshawy, A.M., Zeinhom, M.M., Hossain, D., Khalifa, E., Wareth, G., Awad, M.F., 2021. Subclinical mastitis in selected bovine dairy herds in North Upper Egypt: Assessment of prevalence, causative bacterial pathogens, antimicrobial resistance and virulence-associated genes. Microorganisms 9, 1175.

Ahmed, S.O., Abou-Khadra, S.H., Saad, S.A., Nagati, S.F., 2023. Molecular characterization some bacterial pathogens causing bovine mastitis with special reference to *Mycoplasma bovis*. Slovenian Veterinary Research 60 (25-Suppl), 205-15.

Annavajhala, M.K., Gomez-Simmonds, A., Uhlemann, A.C., 2019. Multidrug-resistant *Enterobacter cloacae* complex emerging as a global, diversifying threat. Frontiers in microbiology 10, 44.

Ayyal Al-Gburi, N.M., 2020. Isolation and molecular identification and antimicrobial susceptibility of Providencia spp. from raw cow's milk in Baghdad, Iraq. Veterinary Medicine International 2020, 8874747.

Babacan, O., 2025. Detection of vancomycin resistance genes with polymerase chain reaction in enterococci isolated from mastitis infection in cows. Veterinarski arhiv 95, 381-391.

Balan, S.S., Nethaji, R., Sankar, S., Jayalakshmi, S., 2012. Production of gelatinase enzyme from Bacillus spp isolated from the sediment sample of Porto Novo Coastal sites. Asian Pacific Journal of Tropical Biomedicine 2, S1811-S1816.

Bürki, S., Frey, J., Pilo, P., 2015. Virulence, persistence and dissemination of *Mycoplasma bovis*. Veterinary microbiology 179, 15-22.

Clark, A.E., Kaleta, E.J., Arora, A., Wolk, D.M., 2013. Matrix-assisted laser desorption ionization–time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clinical Microbiology Reviews 26, 547-603.

Clinical and Laboratory Standards Institute (CLSI), 2020. Performance standards for antimicrobial susceptibility testing (30th edition).

Cobirka M, Tancin V, Slama P., 2020. Epidemiology and classification of mastitis. Animals 10, 2212.

de Souza Ferreira, L., Showemimo, T., Juliano, L.B., Rodriguez, Z., Ruegg, P.L., 2025. Use of MALDI-TOF to identify cryopreserved mastitis pathogens collected from 2003 to 2011 that were originally identified using conventional microbiological methods. JDS Communications 6, 422-426.

Duarte, C.M., Freitas, P.P., Bexiga, R., 2015. Technological advances in bovine mastitis diagnosis: an overview. Journal of Veterinary Diagnostic Investigation 27, 665-672.

Dubey, S.K., Kumar, P., Kumari, N., Rajak, S.K., 2025. Enterobacter hormaechie: An Etiological Agent for Bovine Mastitis Identified through PCR and Sequencing. Uttar Pradesh Journal of Zoology 46, 87-95.

Elbehiry, A. and Abalkhail, A., 2025. Spectral Precision: Recent Advances in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Pathogen Detection and Resistance Profiling. Microorganisms 13, 1473.

Ernø, H. and Stipkovits, L., 1973. Bovine *Mycoplasma*s: Cultural and Biochemical Studies: I. Acta veterinaria scandinavica 14, 436-449.

Freundt, E.A., 1973. Principles of *Mycoplasma* classification. Annals of the New York Academy of Sciences 225, 7-13.

Friman, M.J., Eklund, M.H., Pitkälä, A.H., Rajala-Schultz, P.J., Rantala, M.H.J., 2019. Description of two *Serratia marcescens* associated mastitis outbreaks in Finnish dairy farms and a review of literature. Acta Veterinaria Scandinavica 61, 54.

Gaur, V., Varia, O., Patel, R., Patel, P., Kumari, P., Patel, D., 2025. Advances in Molecular Diagnostics of Red Complex Bacteria: The Role of PCR in Periodontal Pathogen Detection and Clinical Implications. Scholars Academic Journal of Biosciences 5, 536-541.

Gautier-Bouchardon, A.V., Ferré, S., Le Grand, D., Paoli, A., Gay, E., Poumarat, F., 2014. Overall decrease in the susceptibility of *Mycoplasma bovis* to antimicrobials over the past 30 years in France. PloS one 9, e87672.

Gelgie, A.E., Desai, S.E., Gelalcha, B.D., Kerro Dego, O., 2024. *Mycoplasma bovis* mastitis in dairy cattle. Frontiers in Veterinary Science 11, 1322267.

Guaita, A., Paterlini, F., Posante, A., Boldini, M., Rolfi, C., Daminelli, P., 2025. Ten-Year Monitoring of Bovine Mastitis-Causing Bacteria in Northern Italy and Evaluation of Antimicrobial Resistance in Raw Milk. Microorganisms 14, 46.

Hashem, Y.A., Abdelrahman, K.A., Aziz, R.K., 2021. Phenotype–genotype correlations and distribution of key virulence factors in *Enterococcus faecalis* isolated from patients with urinary tract infections. Infection and Drug Resistance 1713-1723.

Hogan, J.S., Gonzalez, R.N., Harmon, R.J., Nickerson, S.C., Oliver, S.P., Pankey, J.W., Smith, K.L., 1999. Laboratory handbook on bovine mastitis. National Mastitis Council, Madison, WI 78, 485-488.

Khalil, D., Becker, C.A., Tardy, F., 2017. Monitoring the decrease in susceptibility to ribosomal RNAs targeting antimicrobials and its molecular basis in clinical *Mycoplasma bovis* isolates over time. Microbial Drug Resistance 23, 799-811.

Kiplimo, M., Njagi, L., Mbindyo, C., Muguro, A., Muinde, P., Ngotho, M., 2025. Prevalence, characterization, and antibiotic susceptibility profiles of bovine mastitic bacteria in Nyeri County, Kenya. German Journal of Microbiology 5, 21-29.

Li, R., Wang J, Sun, X., Liu, L., Wang, J., Yuan, W., 2021. Direct and Rapid Detection of *Mycoplasma bovis* in Bovine Milk Samples by Recombinase Polymerase Amplification Assays. Frontiers in Cellular and Infection Microbiology 11, 639083.

Liakopoulos, A., Mevius, D., Ceccarelli, D., 2016. A review of SHV extended-spectrum β-lactamases: neglected yet ubiquitous. Frontiers in Microbiology 7, 1374.

Liu, J., Liang, Z., Zhongla, M., Wang, H., Sun, X., Zheng, J., Ding, X., Yang, F., 2024. Prevalence and molecular characteristics of enterococci isolated from clinical bovine mastitis cases in Ningxia. Infection and Drug Resistance 2121-2129.

Massella, E., Russo, S., Filippi, A., Garbarino, C.A., Ricchi, M., Bassi, P., Toschi, E., Torreggiani, C., Pupillo, G., Rugna, G., Carta, V., 2025. Does Bovine Raw Milk Represent a Potential Risk for Vancomycin-Resistant Enterococci (VRE) Transmission to Humans?. Antibiotics 14, 814.

Maunsell, F., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D., 2011. *Mycoplasma bovis* infections in cattle. Journal of Veterinary Internal Medicine 25, 772-783.

Moreira, G., Pinho, L., Mesquita, J.R., Silva, E., 2025. *Serratia marcescens* Isolates from Bovine Mastitic Milk: Antimicrobial Resistance and Virulence Features. Antibiotics 14, 892.

Murphy, M.P. and Caraher, E., 2015. Residence in biofilms allows *Burkholderia cepacia* complex (Bcc) bacteria to evade the antimicrobial activities of neutrophil-like dHL60 cells. Pathogens and Disease 73, ftv069.

Nasrollahian, S., Graham, J.P., Halaji, M., 2024. A review of the mechanisms that confer antibiotic resistance in pathotypes of *E. coli*. Frontiers in Cellular and Infection Microbiology 14, 1387497.

Nicholas, R., Ayling, R., McAuliffe, L., 2008. *Mycoplasma* diseases of ruminants. CABI.

O'Toole, G.A., 2011. Microtiter dish biofilm formation assay. Journal of visualized experiments: JoVE, 2437.

Paramasivam, R., Gopal, D.R., Dhandapani, R., Subbarayalu, R., Elangovan, M.P., Prabhu, B., Veerappan, V., Nandheeswaran, A., Paramasivam, S., Muthupandian, S., 2023. Is AMR in dairy products a threat to human health? An updated review on the origin, prevention, treatment, and economic impacts of subclinical mastitis. Infection and Drug Resistance 16, 155-178.

Potter, R.F., D'Souza, A.W., Dantas, G., 2016. The rapid spread of carbapenem-resistant Enterobacteriaceae. Drug Resistance Updates 29, 30-46.

Pritchard RE, Prassinos AJ, Osborne JD, Raviv Z, Balish MF, 2014. Reduction of hydrogen peroxide accumulation and toxicity by a Catalase from *Mycoplasma* iowae. PLoS One 9, e105188.

Quinn, P.J., Markey, B.K., Carter, M.E., 2002. Veterinary Microbiology and Microbial Disease. John Wiley & Sons

Ramírez-Larrota, J.S., Eckhard, U., 2022. An introduction to bacterial biofilms and their proteases, and their roles in host infection and immune evasion. Biomolecules 12, 306.

Raymond, B.B., 2020. Pathogen evasion strategies. Cellular Microbiology 23, e13300.

Rhodes, K.A. and Schweizer, H.P., 2016. Antibiotic resistance in Burkholderia species. Drug Resistance Updates 28, 82-90.

Roșu, R.D., Morar, A., Tîrziu, E., Herman, V., Ban-Cucerzan, A., Popa, S.A., Pătrînjan, R.T., Pocinoc, A., Ghițan, B.L., Imre, K., 2025. Occurrence and Antimicrobial Susceptibility of Major Bacterial Pathogens Associated with Subclinical Mastitis in Dairy Cows in Western Romania. Microorganisms 14, 26.

Różańska, H., Kubajka, M., Weiner, M., 2021. Occurrence of extended-spectrum beta-lactamase- and *Amp*C-type cephalosporinase-producing *Escherichia coli* in mastitic cow's milk. Medycyna Weterynaryjna 77, 291-294.

Schroeder, M., Brooks, B.D., Brooks, A.E., 2017. The complex relationship between virulence and antibiotic resistance. Genes 8, 39.

Silva, S.M.D., Ramos, B.A., Sá, R.A.D., Silva, M.V.D., Correia, M.T., Oliveira, M.B.D., 2022. Investigation of factors related to biofilm formation in Providencia stuartii. Anais da Academia Brasileira de Ciências 94, e20210765.

Storgårds, E., Haikara, A., Juvonen, R., 2006. Brewing control systems: microbiological analysis. In Brewing. Woodhead Publishing, pp. 391-426.

Subhi, A., Saad, S.A., Osman, K., Hashad, M.E., Deif, H.N., 2023. Prevalence and Antibiogram of *Escherichia coli* isolates recovered from bovine milk. Journal of Applied Veterinary Sciences 8, 82-90.

Tavares, M., Kozak, M., Balola, A., Sá-Correia, I., 2020. *Burkholderia cepacia* complex bacteria: a feared contamination risk in water-based pharmaceutical products. Clinical Microbiology reviews 33, 10-1128.

Tomanić, D., Kladar, N., Kovačević, Z., 2025. Antibiotic Residues in Milk as a Consequence of Mastitis Treatment: Balancing Animal Welfare and One Health Risks. Veterinary Sciences 12, 1159.

Tommasoni, C., Fiore, E., Lisuzzo, A., Gianesella, M., 2023. Mastitis in dairy cattle: on-farm diagnostics and future perspectives. Animals 13, 2538.

Wang, L.J., Yang, X., Qian, S.Y., Liu, Y.C., Yao, K.H., Dong, F., Song, W.Q. 2020. Identification of hemolytic activity and hemolytic genes of Methicillin-resistant *Staphylococcus aureus* isolated from Chinese children. Chinese Medical Journal 133, 88-90.

Yeh, T.K., Lin, H.J., Liu, P.Y., Wang, J.H., Hsueh, P.R., 2022. Antibiotic resistance in *Enterobacter hormaechei*. International journal of antimicrobial agents 60, 106650.

Zaatout, N., 2022. An overview on mastitis-associated *Escherichia coli*: pathogenicity, host immunity and the use of alternative therapies. Microbiological Research 256, 126960.

Zhang, Z., Yao, Y., Yang, J., Jiang, H., Meng, Y., Cao, W., Zhou, F., Wang, K., Yang, Z., Yang, C., Sun, J., 2023. Assessment of adaptive immune responses of dairy cows with Burkholderia contaminans-induced mastitis. Frontiers in Microbiology 14, 1099623.

Zuo, J., Lv, Z., Lian, L., Wu, Z., Fu, S., Zhang, H., Wu, J., Pan, Z., Yu, Y., Chen, W., Jiang, W., Yin, H., Chen, Z., Yi, Y., Han, X., Miao, J., 2025. Difference Analysis on Virulence Genes, Biofilms and Antimicrobial Susceptibility of *Escherichia coli* from Clinical and Subclinical Bovine Mastitis. Veterinary Sciences 12, 132.