



# Phenotypic and genotypic antimicrobial resistance in *Staphylococcus* spp. isolated from raw milk of cows, goats, and ewes †

[Resistencia antimicrobiana fenotípica y genotípica de *Staphylococcus* spp. aislados de leche cruda de vacas, cabras y borregas]

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## SUMMARY

**Background:** Mastitis is a major disease in dairy production, causing economic losses and increasing concern due to antimicrobial-resistant bacteria. The presence of resistant *Staphylococcus* spp. in raw milk from clinically healthy animals may represent a potential public health risk and a challenge for control strategies under a One Health framework. **Objective:** To evaluate antimicrobial resistance patterns and resistance genes in *Staphylococcus* spp. isolated from raw milk of clinically healthy cows, goats, and ewes from three dairy farms included in this study. **Methodology:** Seventy-five isolates (25 per species) were analyzed using disk diffusion antibiograms and PCR detection of *blaZ*, *mecA*, *ermB*, and *tetK* genes. Phenotypic–genotypic agreement and interspecies differences were statistically evaluated. **Results:** High phenotypic resistance to  $\beta$ -lactams was observed, especially in cows (ampicillin 96%, penicillin 92%) and ewes (penicillin 60%). Oxacillin resistance was higher in goats and ewes (52%) than in cows (8%). Resistance to erythromycin and tetracycline was moderate to low. Gene detection showed high prevalence of *mecA* (84–100%) and *blaZ* (80% in cows and goats; 20% in ewes), with significant interspecies differences in *blaZ* and *tetK* ( $p < 0.001$ ). Overall phenotype–genotype agreement was low, although *blaZ* showed the highest diagnostic value. Frequent codetection of resistance genes was observed among isolates, indicating the coexistence of multiple resistance determinants within the same bacterial populations. **Implications:** The low phenotype–genotype concordance highlights limitations of relying on a single diagnostic approach and suggests the need for integrated surveillance combining phenotypic and molecular tools. **Conclusion:** Antimicrobial-resistant *Staphylococcus* spp. are widely present in raw milk from clinically healthy ruminants, with significant interspecies differences. The present study revealed marked differences in antimicrobial resistance profiles and resistance gene distribution among *Staphylococcus* spp. isolated from cows, goats, and ewes. The low phenotype–genotype concordance observed for several antibiotic–gene pairs highlights the complexity of antimicrobial resistance and supports the complementary use of phenotypic and molecular approaches for surveillance and characterization of resistant isolates.

**Key words:** Antimicrobial resistance; *Staphylococcus* spp.; Mastitis; PCR; Raw milk.

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## RESUMEN

**Antecedentes:** La mastitis es una enfermedad importante en la producción lechera que genera pérdidas económicas y creciente preocupación por bacterias resistentes a los antimicrobianos. La presencia de *Staphylococcus* spp. resistentes en leche cruda de animales clínicamente sanos puede representar un riesgo potencial para la salud pública y un desafío para su control bajo el enfoque One Health. **Objetivo:** Evaluar los patrones de resistencia antimicrobiana y los genes de resistencia en *Staphylococcus* spp. aislados de leche cruda de vacas, cabras y ovejas clínicamente sanas procedentes de las tres granjas lecheras incluidas en este estudio. **Metodología:** Se analizaron 75 aislamientos (25 por especie) mediante antibiograma por difusión en disco y detección por PCR de los genes *blaZ*, *mecA*, *ermB* y *tetK*. Se evaluó la concordancia fenotipo–genotipo y las diferencias entre especies. **Resultados:** Se observó alta resistencia fenotípica a  $\beta$ -lactámicos, especialmente en vacas (ampicilina 96%, penicilina 92%) y ovejas (penicilina 60%). La resistencia a oxacilina fue mayor en cabras y ovejas (52%) que en vacas (8%). La resistencia a eritromicina y tetraciclina fue de moderada a baja. La detección génica mostró alta prevalencia de *mecA* (84–100%) y *blaZ* (80% en vacas y cabras; 20% en ovejas), con diferencias interespecíficas significativas en *blaZ* y *tetK* ( $p < 0.001$ ). La concordancia fenotipo–genotipo fue baja en general, aunque *blaZ* mostró el mayor valor diagnóstico. Se observó una codetección frecuente de genes de resistencia entre los aislamientos, lo que indica la coexistencia de múltiples determinantes de resistencia dentro de las mismas poblaciones bacterianas. **Implicaciones:** La baja concordancia fenotipo–genotipo evidencia limitaciones al utilizar un solo enfoque diagnóstico y resalta la necesidad de vigilancia integrada que combine herramientas fenotípicas y moleculares. **Conclusión:** *Staphylococcus* spp. resistentes a antimicrobianos están ampliamente presentes en leche cruda de rumiantes clínicamente sanos, con diferencias significativas entre especies. El presente estudio reveló marcadas diferencias en los perfiles de resistencia antimicrobiana y en la distribución de genes de resistencia entre aislamientos de *Staphylococcus* spp. procedentes de vacas, cabras y borregas. La baja concordancia fenotipo-genotipo observada para varios pares antibiótico-gen destaca la complejidad de la resistencia antimicrobiana y respalda el uso complementario de enfoques fenotípicos y moleculares para la vigilancia y caracterización de aislamientos resistentes.

**Palabras clave:** Resistencia antimicrobiana; *Staphylococcus* spp.; mastitis; PCR; leche cruda.

## INTRODUCTION

Mastitis is one of the most costly and complex diseases affecting the dairy industry due to the wide range of etiological agents, variability in immune response, and limited effectiveness of control measures (Stanek *et al.*, 2024). Coagulase-negative Staphylococci (CoNS), also called non-*aureus* staphylococci (NAS), have emerged as relevant agents of intramammary infection (IMI), especially in subclinical cases (Frey *et al.*, 2013; Nobrega *et al.*, 2018). These species, which include *S. chromogenes* and *S. simulans*, are part of the normal microbiota but can act as reservoirs of antimicrobial resistance genes (Becker *et al.*, 2014; Argemi *et al.*, 2019). Their persistence is enhanced by biofilm formation and survival on the skin and in the environment. Resistance to  $\beta$ -lactams is mediated by the *blaZ* gene, which encodes a class A  $\beta$ -lactamase, and the *mecA* gene, responsible for methicillin resistance via PBP2a production and often carried on the SCCmec element (Maree *et al.*, 2022; Lade and Kim, 2023; Yang *et al.*, 2023). Resistance to tetracyclines involves *tetM* and *tetK*, encoding ribosomal protection and efflux pumps, respectively (Strommenger *et al.*, 2003). Macrolide resistance is frequently linked to *erm* genes (*ermA*, *ermB*, *ermC*), which methylate 23S rRNA and confer the MLSB resistance phenotype (Talebi *et al.*, 2019; Chenouf *et al.*, 2021). The indiscriminate use of antibiotics—especially  $\beta$ -lactams, macrolides, and tetracyclines—for treating clinical mastitis, persistent infections, and during dry-off therapy programs has promoted the selection of multidrug-resistant strains. The use of  $\beta$ -

lactams, macrolides, and tetracyclines for the treatment of mastitis may contribute to the selection of resistant bacterial populations. In Mexico, antimicrobial resistance in livestock has been identified as an emerging challenge for animal and public health, emphasizing the need for continued surveillance and responsible antimicrobial use (García-Alarcón *et al.*, 2025). Several international studies have documented the presence of resistance genes in isolates from bovine milk. For example, Chajęcka-Wierzchowska *et al.* (2015) identified CoNS strains harboring *mecA*, *tetK*, and *ermC*, while Jiménez-Velásquez *et al.* (2020) reported multidrug-resistant isolates in Colombia. Additionally, Fidelis *et al.* (2024) demonstrated that regional genetic variability can influence local therapeutic efficacy. In Mexico, information on resistance genes in CoNS is still limited, particularly in isolates from goat and ewe milk. Some studies suggest that *S. aureus* strains isolated from ovine milk do not carry the *mecA* gene, or that methicillin-resistant strains may have a human origin, raising questions about interspecies transmission (Lade and Kim, 2023). In contrast, methicillin-resistant CoNS strains have been documented in goats, emphasizing the need for further research in the country (Shi *et al.*, 2025). Therefore, integrating phenotypic and genotypic approaches may provide complementary information for the characterization of antimicrobial resistance in *Staphylococcus* spp. associated with dairy production systems. However, the relationship between resistance phenotypes and the distribution of resistance genes may vary among bacterial species and host animals, and remains insufficiently characterized in dairy

ruminants in Mexico. This study aimed to evaluate antimicrobial resistance patterns and resistance genes in CoNS and *Staphylococcus aureus* isolated from raw milk samples of clinically healthy cows, goats, and ewes from the three dairy farms included in this study.

## MATERIALS AND METHODS

### Sampling sites and animal inclusion criteria

The study was conducted in three dairy production units located in different states of north-central Mexico, where a total of 300 raw milk samples were collected, equally distributed among cows, goats, and ewes. Samples were obtained from Holstein cows in Torreón, Coahuila; Toggenburg goats in Tequisquiapan, Querétaro; and East Friesian ewes in Jilotepec de Molina Henríquez, State of Mexico. A convenience sampling strategy was used. Farms were selected based on accessibility, willingness to participate, and availability of lactating animals at the time of sampling. All animals had an average of three parturitions and a body condition score of 3, indicating optimal nutritional and health status at the time of sampling. Animals were sampled during routine milking regardless of clinical signs of mastitis or indirect tests such as the California Mastitis Test (CMT). This methodological strategy was chosen to obtain an unbiased representation of udder health status, including clinically healthy animals and those with possible subclinical infections not visually detectable. The absence of clinical selection criteria enabled an objective evaluation of the presence of pathogens and antimicrobial resistance genes, independently of apparent clinical status. In each production unit, 100 raw milk samples (one per gland) were collected under strict hygienic conditions, resulting in a total of 300 milk samples (100 per animal species). All samples were subjected to bacteriological culture. The study focused on *Staphylococcus* spp. isolates because of their recognized importance in mastitis epidemiology and antimicrobial resistance surveillance. To allow balanced statistical comparisons among animal species, a total of 75 *Staphylococcus* spp. isolates were selected for phenotypic and genotypic analyses, including 25 isolates from cows, 25 from goats, and 25 from ewes. Therefore, the study should be interpreted as an observational analysis of *Staphylococcus* spp. isolates obtained from these three farms and should not be considered representative of dairy production systems in north-central Mexico as a whole. For sample collection, teats were disinfected with swabs soaked in 70% alcohol, the first three streams of milk were discarded, and approximately 30 mL were collected in sterile, pre-labeled polypropylene tubes. Samples were handled aseptically, frozen at  $-20^{\circ}\text{C}$ , and transported to the Teaching and Research Laboratory of the Department of Medicine and Zootechnics of Ruminants, FMVZ-

UNAM, where bacterial culture, isolation, and microbiological identification were performed. This study was reviewed by the Institutional Committee for the Care and Use of Animals (CICUA) of the Faculty of Veterinary Medicine and Animal Science, UNAM. As the procedures involved only routine animal management practices (i.e., milking), ethical approval was not required.

### Bacteriological analysis

Milk samples were gradually thawed and then homogenized by vortexing for 30 seconds to release bacteria trapped in fat globules. For primary isolation, 30  $\mu\text{L}$  of milk were inoculated onto blood agar plates using a quadrant streaking technique to obtain well-separated colonies. Plates were incubated at  $37^{\circ}\text{C}$  for 24 hours under aerobic conditions. In cases where no bacterial growth was observed, incubation was extended for an additional 48 hours before being considered negative. Developed colonies were evaluated based on macroscopic morphology, including shape, color, hemolysis, and relative colony count. Representative colonies were used to prepare fixed smears stained with Gram stain for microscopic observation and guidance in bacterial identification. Initial identification of the genus *Staphylococcus* spp. was based on cell morphology, clustering, and staining characteristics under the microscope, as well as catalase and coagulase tests. Isolates classified as CoNS were identified to the species level using the commercial API® Staph system (BioMérieux Laboratories), following the manufacturer's instructions. Antimicrobial susceptibility of *Staphylococcus* spp. isolates was assessed using the agar disk diffusion method described by Kirby-Bauer, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (2023). A bacterial inoculum was prepared from each isolate and adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately  $1.5 \times 10^8$  CFU/mL). The inoculum was uniformly spread using a sterile swab over the surface of Mueller-Hinton agar plates to ensure confluent bacterial growth. Antibiotic disks containing penicillin, ampicillin, oxacillin ( $\beta$ -lactams), erythromycin (macrolide), and tetracycline were placed on the plates. The antimicrobial panel was selected to represent the resistance mechanisms targeted by the genes evaluated in this study. Penicillin and ampicillin were included to assess  $\beta$ -lactam resistance associated with *blaZ*, oxacillin was selected as the phenotypic marker of methicillin resistance because it is an accepted surrogate marker according to the Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. Erythromycin was selected to evaluate macrolide resistance related to *ermB*, and tetracycline was included because of its association with *tetK*-mediated resistance. Thus, the antimicrobial panel was designed to facilitate phenotype-genotype

comparisons rather than to provide a comprehensive assessment of all antimicrobial classes used in dairy production systems. Plates were incubated at 37 °C for 24 hours. After incubation, inhibition zones were measured in millimeters using a ruler. Interpretation of results followed CLSI breakpoint values, classifying each isolate as susceptible, intermediate, or resistant to each antibiotic.

### Molecular identification of bacteria

DNA extraction from bacterial isolates was performed using the Quick-DNA Miniprep Plus Kit (Zymo Research, USA) according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry (Velab, Mexico) and standardized to 100 ng/μL. Working dilutions were subsequently prepared at 10 ng/μL and used as template DNA for PCR reactions. Molecular identification of *Staphylococcus* spp. was performed using primers described by Mason *et al.* (2001), targeting the 16S ribosomal RNA gene and generating a 791 bp amplicon. In addition, four primer pairs were used to detect antimicrobial resistance genes. The primers for *blaZ* and *ermB* were designed specifically for this

study by retrieving target gene sequences from the NCBI database, evaluating their specificity using BLAST, and designing oligonucleotides with DNAMAN software version 10.0.2 (Table 1). Primer design considered amplicon size, melting temperature compatibility, and sequence conservation within the target genes to minimize potential cross-reactivity with non-target organisms. Each PCR reaction consisted of 12.5 μL of DreamTaq Green PCR Master Mix, 1 μL of each primer (forward and reverse, 10 μM), 1.3 μL of DNA at 10 ng/μL, and nuclease-free water to a final volume of 25 μL. Amplifications were performed in a Prime Thermal Cycler 5PRIMEG/02 using the conditions described in Table 2. A clinical oxacillin-resistant *S. aureus* isolate was included as a positive control, whereas nuclease-free water was used as a negative control in each reaction to monitor contamination. Amplification products were considered valid only when the expected fragment size was observed in the positive control and no amplification occurred in the negative control. PCR products were analyzed by electrophoresis in 2% agarose gels prepared with 1X TAE buffer, stained with GelRed® (Millipore), and visualized under a transilluminator (Accuris 58-E3100).

**Table 1. Primers used for molecular identification of *Staphylococcus* spp. and detection of antimicrobial resistance genes in isolates recovered from raw milk of clinically healthy cows, goats, and ewes from three dairy farms in north-central Mexico.**

| Gene        | Primer sequences                                                                   | Size (bp) | Resistance group | Reference                              |
|-------------|------------------------------------------------------------------------------------|-----------|------------------|----------------------------------------|
| 16S rRNA    | 5'-CCTATAAGACTGGGATAACTTCGGG-3'<br>5'-CTTTGAGTTTCAACCTTGCGGTCG-3'                  | 791       | 16S rRNA gene    | Mason <i>et al.</i> (2001)             |
| <i>mecA</i> | 5'-ACT GCT ATC CAC CCT CAA AC -3'<br>5' CTG GTG AAG TTG TAA TCT GG -3'             | 163       | β-lactams        | Jiménez-Velásquez <i>et al.</i> (2020) |
| <i>blaZ</i> | 5'- CAA AGA TGA TAT AGT TGC TTA TTC TCC -3'<br>5'- TGC TTG ACC ACT TTT ATC AGC -3' | 182       | β-lactams        | Own Design                             |
| <i>tetK</i> | 5'- TCG ATA GGA ACA GCA GTA-3'<br>5'- CAG CAG ATC CTA CTC CTT-3'                   | 169       | Tetracyclines    | Jiménez-Velásquez <i>et al.</i> (2020) |
| <i>ermB</i> | 5'- CTA TCT GAT TGT TGA AGA AGG ATT-3'<br>5'- GTT TAC TCT TGG TTT AGG ATG AAA-3'   | 156       | Macrolides       | Own Design                             |

**Table 2. PCR thermocycling conditions used for amplification of the 16S rRNA gene and antimicrobial resistance genes (*mecA*, *blaZ*, *tetK*, and *ermB*) in *Staphylococcus* spp. isolates recovered from raw milk of clinically healthy cows, goats, and ewes.**

| Gene        | Initial denaturation (1 cycle) | Denaturation | Annealing    | Extension    | Number of cycles | Final extension (1 cycle) |
|-------------|--------------------------------|--------------|--------------|--------------|------------------|---------------------------|
| 16S rRNA    | 95 °C / 5 m                    | 95 °C / 60 s | 65 °C / 60 s | 72 °C / 60 s | 30               | 72 °C / 5 m               |
| <i>mecA</i> | 95 °C / 5 m                    | 95 °C / 30 s | 55 °C / 30 s | 72 °C / 60 s | 40               | 72 °C / 5 m               |
| <i>blaZ</i> | 95 °C / 5 m                    | 95 °C / 30 s | 63 °C / 30 s | 72 °C / 60 s | 40               | 72 °C / 5 m               |
| <i>tetK</i> | 95 °C / 5 m                    | 95 °C / 30 s | 58 °C / 30 s | 72 °C / 60 s | 40               | 72 °C / 5 m               |
| <i>ermB</i> | 95 °C / 5 m                    | 95 °C / 30 s | 56 °C / 30 s | 72 °C / 60 s | 40               | 72 °C / 5 m               |

## Statistical analysis

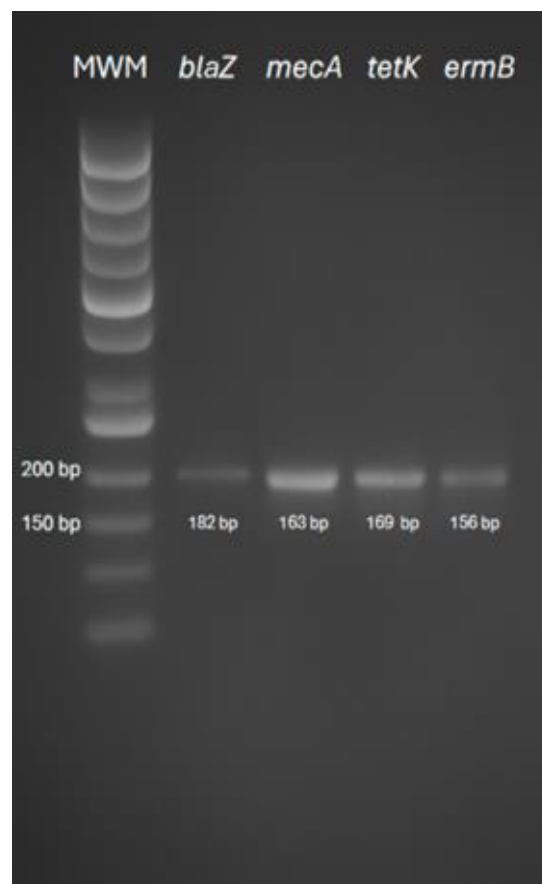
All statistical analyses were performed using Python 3.10. Associations between categorical variables were evaluated using Chi-square tests when test assumptions were met, whereas Fisher's exact test was applied in contingency tables with expected cell frequencies lower than five, particularly in comparisons involving infrequent *Staphylococcus* species or low-frequency resistance categories. This approach ensured the validity of statistical inference when sample sizes were small. Phenotype–genotype agreement was assessed using Cohen's kappa coefficient. Diagnostic performance measures, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), were calculated from contingency tables. Binary logistic regression models were fitted separately for each antibiotic–gene pair to evaluate factors associated with phenotypic antimicrobial resistance. In each model, phenotypic resistance (resistant/susceptible) was used as the dependent variable, whereas animal species (cow, goat, or ewe) and the presence or absence of the corresponding resistance gene were included as explanatory variables. Animal species was treated as a categorical predictor, with ewe isolates serving as the reference category. Statistical analyses were conducted using the SciPy, Scikit-learn, and Statsmodels libraries, and statistical significance was established at  $p < 0.05$ .

## RESULTS

A total of 75 *Staphylococcus* spp. isolates were obtained, equally distributed among the three sampled animal species: 25 from cows, 25 from goats, and 25 from ewes. All isolates were identified to the species level using the API® Staph system (BioMérieux Laboratories), which revealed 12 different *Staphylococcus* species. Each isolate underwent antimicrobial susceptibility testing (antibiogram) and endpoint PCR for phenotypic and genotypic resistance detection, respectively (Figure 1). The most frequently identified species was *S. aureus* (25 isolates, 34.25%), followed by *S. xylosus* (15 isolates, 19.1%), *S. simulans* (13 isolates, 17.81%), *S. chromogenes* (6 isolates, 8.22%), and *S. lentus* (4 isolates, 5.48%). Less frequent species included *S. epidermidis* (3 isolates, 4.11%), *S. cohnii*, *S. caprae*, and *S. capitis* (2 isolates each, 2.74%), as well as *S. warneri*, *S. sciuri*, and *S. hominis* (1 isolate each, 1.37%) (Table 3).

In cow isolates, the highest phenotypic resistance was observed to ampicillin (96%) and penicillin (92%), followed by erythromycin (16%), oxacillin (8%), and tetracycline (4%). Genotypic analysis revealed the presence of *mecA* in 84%, *blaZ* in 80%, *ermB* in 72%, and *tetK* in 32% of isolates. Among goat isolates, the highest resistance was observed for oxacillin (52%),

followed by ampicillin (48%), penicillin (44%), erythromycin (40%), and tetracycline (24%). The *mecA* gene was detected in 84% of isolates, whereas *blaZ* and *ermB* were each detected in 80% and 72% of isolates, respectively. The *tetK* gene was present in 32% of goat isolates. A particularly notable finding was the detection of *mecA* in 100% of ewe isolates, representing the highest prevalence observed among the three host species. In ewe isolates, resistance was most frequent to penicillin (60%), followed by oxacillin (52%), ampicillin (48%), erythromycin (44%), and tetracycline (28%). In contrast, *blaZ* was detected in only 20% of ewe isolates, whereas *tetK* showed a high prevalence (96%) and *ermB* was detected in only 4%. The distribution of phenotypic resistance and resistance gene frequencies among animal species is shown in Figure 2.



**Figure 1.** PCR for the *blaZ*, *mecA*, *tetK*, and *ermB* genes. PCR amplification of antibiotic resistance genes in *Staphylococcus* spp. The amplified products correspond to the genes *blaZ* (182 bp), *mecA* (163 bp), *tetK* (169 bp), and *ermB* (156 bp). MWM: 50 bp molecular weight marker. Bands were visualized by electrophoresis on a 2% agarose gel stained with GelRed® (Millipore SCT123).

**Table 3. Distribution of *Staphylococcus* spp. isolates by species and animal source (cows, goats, and ewes).**

| Species               | Cows | Goats | Ewes | Total     |
|-----------------------|------|-------|------|-----------|
| <i>S. aureus</i>      | 21   | 2     | 2    | 25        |
| <i>S. xylosus</i>     | 1    | 3     | 11   | 15        |
| <i>S. simulans</i>    | 1    | 8     | 4    | 13        |
| <i>S. chromogenes</i> | 2    | 3     | 1    | 6         |
| <i>S. lentus</i>      | 0    | 0     | 4    | 4         |
| <i>S. epidermidis</i> | 0    | 1     | 2    | 3         |
| <i>S. cohnii</i>      | 0    | 2     | 0    | 2         |
| <i>S. caprae</i>      | 0    | 2     | 0    | 2         |
| <i>S. capitis</i>     | 0    | 2     | 0    | 2         |
| <i>S. hominis</i>     | 0    | 0     | 1    | 1         |
| <i>S. warneri</i>     | 0    | 1     | 0    | 1         |
| <i>S. sciuri</i>      | 0    | 1     | 0    | 1         |
|                       |      |       |      | <b>75</b> |

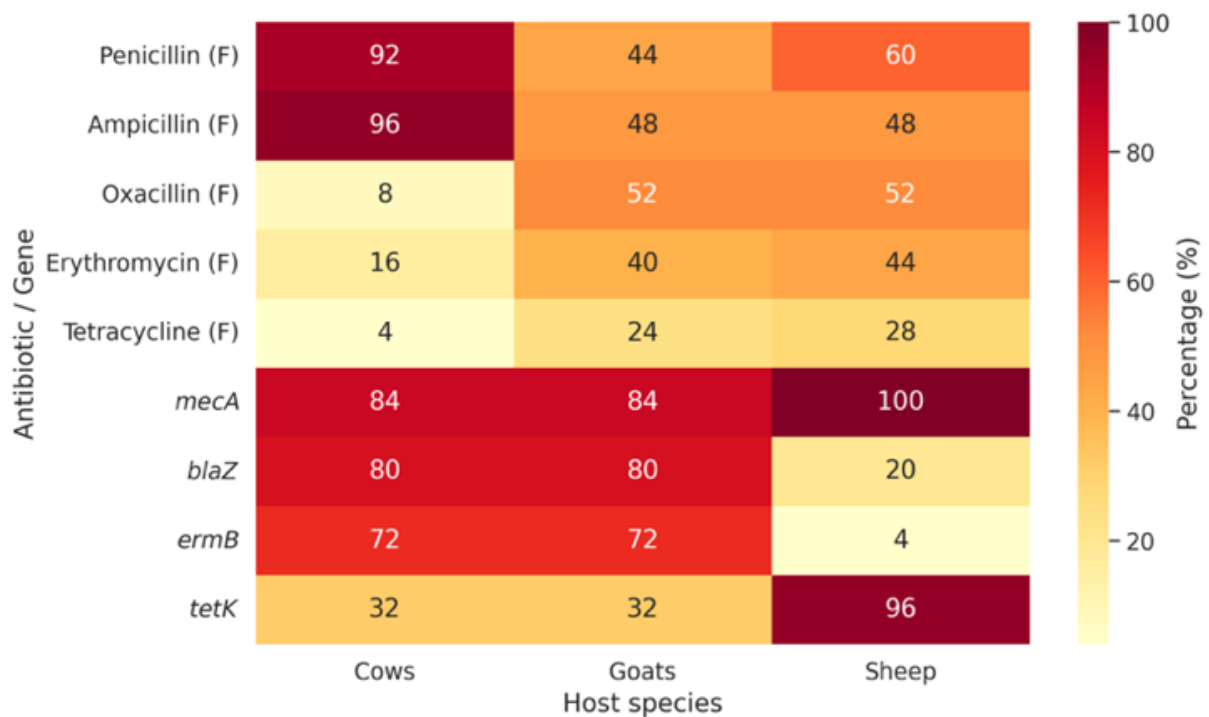
## Phenotypic resistance

### Intraspecies comparison of bacterial species

Chi-square tests showed no significant differences in resistance patterns among bacterial species isolated from cows ( $p > 0.05$ ), indicating a homogeneous phenotypic resistance profile. In goats, a significant difference was found for ampicillin resistance ( $p < 0.05$ ), but no associations were detected for the remaining antibiotics ( $p > 0.05$ ). In ewes, significant differences were observed for penicillin, oxacillin, and tetracycline resistance ( $p < 0.05$ ), suggesting an association between resistance profile and bacterial species.

### Interspecies comparison for a given *Staphylococcus* species

Resistance patterns were compared between animal species for isolates of the same *Staphylococcus* species. For *S. aureus*, statistically significant differences were observed for oxacillin and tetracycline ( $p < 0.05$ ), but not for penicillin, ampicillin, or erythromycin ( $p > 0.05$ ). Similar non-significant results ( $p > 0.05$ ) were found for *S. chromogenes*, *S. xylosus*, *S. simulans*, and *S. epidermidis*.



**Figure 2. Percentage distribution of phenotypic antimicrobial resistance and resistance genes in *Staphylococcus* spp. isolated from cows, goats, and ewes.** Heatmap showing the percentages of phenotypic resistance (F) to five antibiotics and detection of resistance genes (*mecA*, *blaZ*, *ermB*, and *tetK*) in *Staphylococcus* spp. isolates from raw milk of cows, goats, and ewes. Values represent the percentage of resistant isolates (phenotype) or gene-positive isolates (genotype) for each animal species. A high prevalence of *mecA* and *blaZ* was observed in bovines, as well as *tetK* in ovines.

### Phenotypic resistance variability by animal species

Significant differences ( $p < 0.05$ ) were found among cows, goats, and ewes for three of the five antibiotics evaluated, confirming that animal species influences the expression of phenotypic resistance.

### Association between phenotypic resistance and resistance genes

2×2 contingency tables were created for each antibiotic–gene pair (penicillin–*blaZ*, ampicillin–*blaZ*, oxacillin–*mecA*, erythromycin–*ermB*, tetracycline–*tetK*). Fisher's exact test ( $p < 0.05$ ) showed a significant association only for *blaZ* and ampicillin resistance. No significant associations were found for *blaZ* and penicillin, *mecA*, *ermB*, or *tetK* ( $p > 0.05$ ), suggesting that gene presence alone does not necessarily result in a resistant phenotype. A Chi-square test was also applied to compare the proportion of gene-positive isolates among animal species. Highly significant differences were found for *blaZ* and *tetK* ( $p < 0.001$ ), while *mecA* and *ermB* showed more homogeneous distributions ( $p > 0.05$ ).

### Comparison of resistance and gene distribution among animal species

To compare the frequency of phenotypic resistance and the distribution of resistance genes among cows, goats, and ewes, Chi-square tests of homogeneity were applied to evaluate proportional differences between groups. In cases involving 2×2 tables or low expected frequencies, Fisher's exact test was used to ensure the statistical validity of the results. Significant differences ( $p < 0.05$ ) were found among cows, goats, and ewes for resistance to penicillin, ampicillin, and oxacillin. No significant differences were observed for erythromycin or tetracycline ( $p > 0.05$ ), indicating that phenotypic resistance varies by host species. Significant interspecies differences ( $p < 0.001$ ) were observed for the distribution of *blaZ* and *tetK*, indicating marked variability between host species. In contrast, *mecA* and *ermB* showed more uniform distributions ( $p > 0.05$ ), despite their high overall prevalence. A Venn diagram analysis was conducted to identify frequent combinations of resistance genes. Figure 3a shows co-detection of *mecA*, *blaZ*, and *tetK*: 15 isolates harbored all three genes; the most common pair was *mecA* + *blaZ* (22 isolates), followed by *mecA* + *tetK* (21). Four isolates had *blaZ* + *tetK*, but no *mecA*, while nine had only *mecA*, and four had only *blaZ*. Notably, no isolates carried *tetK* alone. Figure 3b shows the co-occurrence of *mecA*, *blaZ*, and *ermB*: 22

isolates had all three; *mecA* + *blaZ* was the most frequent pair (15), followed by *mecA* + *ermB* (9), and *blaZ* + *ermB* (6). Additionally, 21 isolates carried only *mecA*, and 2 carried only *blaZ*. As with *tetK*, *ermB* was not detected alone, suggesting it is usually associated with other resistance genes.

### Phenotype–Genotype concordance measures

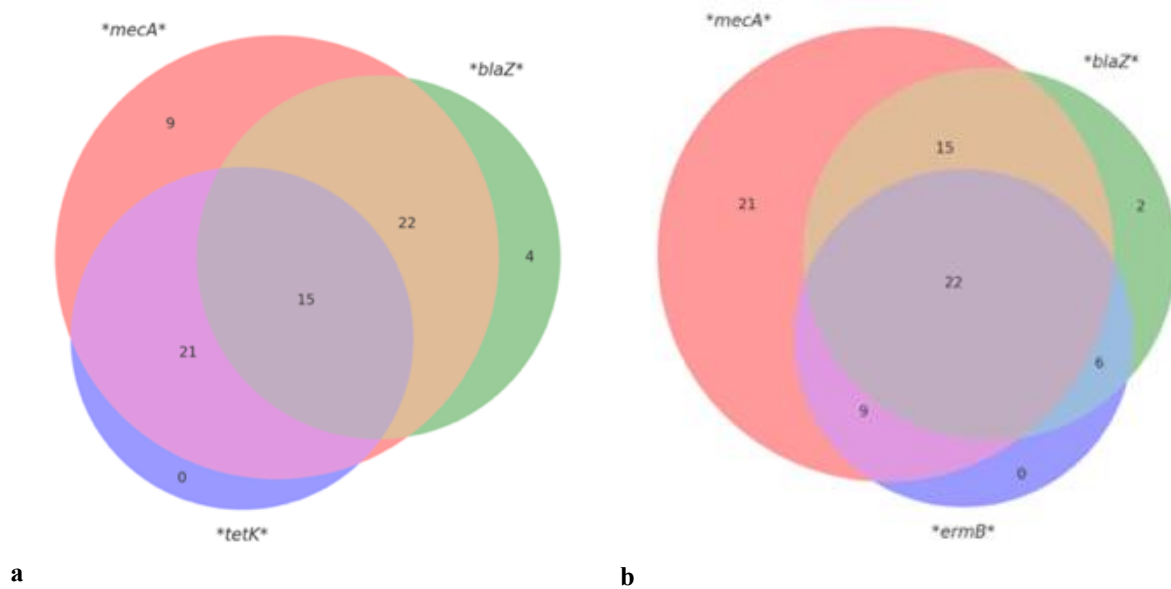
Cohen's kappa coefficient and diagnostic performance measures were calculated for each antibiotic–gene pair. Overall, phenotype–genotype concordance was low, with the highest agreement observed for *blaZ* and ampicillin. Complete values for sensitivity, specificity, positive predictive value, negative predictive value, and Cohen's kappa coefficient are presented in Table 4.

### Logistic regression analysis

Binary logistic regression was performed to assess whether phenotypic resistance could be predicted from the presence of corresponding resistance genes and animal species. None of the genes (*blaZ*, *mecA*, *ermB*, *tetK*) showed a statistically significant association with phenotypic resistance ( $p > 0.05$ ). In contrast, animal species did show a significant association in some cases ( $p < 0.05$ ): cow-derived isolates were more likely to be resistant to ampicillin, oxacillin, and tetracycline compared to ewe-derived isolates. These results suggest that host species may influence phenotypic resistance more strongly than gene presence alone.

### Diagnostic performance of PCR for resistance detection

Diagnostic parameters were calculated to evaluate the utility of PCR as a complementary tool to antibiograms. Among the five antibiotic–gene pairs, *mecA* had the highest sensitivity (93%) for predicting oxacillin resistance but low specificity (13%), indicating a high rate of false positives. *blaZ* showed sensitivities of 65% and 69% for predicting resistance to penicillin and ampicillin, with PPVs of 71% and 73%, respectively. In contrast, *ermB* had low sensitivity (40%) and a PPV of only 27%, limiting its diagnostic utility. *tetK* had moderate sensitivity (57%) and relatively high NPV (83%), suggesting it may be useful for ruling out tetracycline resistance. Overall, the findings highlight low phenotype–genotype concordance and emphasize the importance of integrating phenotypic and molecular methods for more accurate surveillance (Table 4).



**Figure 3.** a) Co-occurrence of the resistance genes *mecA*, *blaZ*, and *tetK*. Co-occurrence of the resistance genes *mecA*, *blaZ*, and *tetK* in *Staphylococcus* spp. isolates from raw milk. Fifteen isolates carried all three genes simultaneously, while the most frequent combination was *mecA* + *blaZ* (22 isolates), followed by *mecA* + *tetK* (21 isolates). No isolate carried the *tetK* gene alone, suggesting its frequent co-detection with other genetic resistance determinants. b) Co-occurrence of the resistance genes *mecA*, *blaZ*, and *ermB*. Co-occurrence of the resistance genes *mecA*, *blaZ*, and *ermB* in *Staphylococcus* spp. isolates from raw milk. Twenty-two isolates carried all three genes simultaneously. The most frequent partial combinations were *mecA* + *blaZ* (15 isolates), *mecA* + *ermB* (9 isolates), and *blaZ* + *ermB* (6 isolates). No isolates carried the *ermB* gene alone, suggesting that its presence is typically associated with other genetic resistance determinants.

**Table 4.** Diagnostic performance and phenotype–genotype concordance of antimicrobial resistance genes in *Staphylococcus* spp. isolates from raw milk of cows, goats, and ewes. (PPV: positive predictive value; NPV: negative predictive value;  $\kappa$ : Cohen's kappa coefficient).

| Antibiotic   | Gene        | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Cohen's $\kappa$ |
|--------------|-------------|-----------------|-----------------|---------|---------|------------------|
| Penicillin   | <i>blaZ</i> | 65.0            | 50.0            | 71.0    | 43.0    | 0.15             |
| Ampicillin   | <i>blaZ</i> | 69.0            | 56.0            | 73.0    | 50.0    | 0.24             |
| Oxacillin    | <i>mecA</i> | 93.0            | 13.0            | 39.0    | 75.0    | 0.04             |
| Erythromycin | <i>ermB</i> | 40.0            | 46.0            | 27.0    | 61.0    | -0.12            |
| Tetracycline | <i>tetK</i> | 57.0            | 48.0            | 20.0    | 83.0    | 0.03             |

## DISCUSSION

This study revealed a high prevalence of resistance to  $\beta$ -lactam antibiotics, particularly penicillin, ampicillin, and oxacillin, in *Staphylococcus* spp. isolated from raw milk. Cows showed the highest phenotypic resistance to ampicillin (96%) and penicillin (92%), followed by ewes (60% penicillin) and goats (48–44%). Oxacillin resistance was notably elevated in goats and ewes (52%) but remained low in cows (8%). These findings align with previous studies in Korea and Colombia (Jiménez-Velásquez *et al.*, 2020; Kang *et al.*, 2024) and confirm the clinical relevance of *mecA* and *blaZ* in dairy staphylococci (Frey *et al.*, 2013; Chenouf *et al.*, 2021). A total of 75 isolates representing 12 different *Staphylococcus* species were identified, with *S. aureus* being the most prevalent (34.25%), followed by *S.*

*xylosus*, *S. simulans*, and others. This bacterial diversity may explain part of the heterogeneity observed in resistance profiles among and within animal species. At the genetic level, *blaZ* and *mecA* were detected in 80–84% of isolates from cows and goats, and *mecA* reached 100% prevalence in ewes. Additionally, *tetK* was highly prevalent in ewes (96%), while *ermB* appeared mainly in cows and goats (72%). Despite these frequencies, phenotypic resistance to tetracycline and erythromycin remained low to moderate, indicating discordance between genotype and phenotype. For instance, *ermB* showed a negative kappa value ( $\kappa = -0.12$ ), and *tetK* showed low concordance (sensitivity 57%, PPV 20%). These inconsistencies may result from gene silencing, variable expression, or alternative resistance mechanisms (Yang *et al.*, 2023; Kang *et al.*, 2024).

One of the most notable findings of the present study was the high prevalence of *mecA* (84–100%) among *Staphylococcus* spp. isolates, particularly considering that all samples were obtained from clinically healthy animals. Although the frequency of *mecA* varies considerably among studies, CoNS have been widely recognized as important reservoirs of methicillin resistance determinants, including *mecA* (Becker et al., 2014; Argemi et al., 2019). Differences in bacterial population structure, host species, sampling strategies, and antimicrobial exposure may contribute to the variability reported in the literature. In the present study, the predominance of CoNS among several host groups may have contributed to the high overall prevalence of *mecA*. Nevertheless, the marked discordance observed between *mecA* detection and phenotypic resistance to oxacillin highlights the complexity of methicillin resistance in *Staphylococcus* spp. The presence of *mecA* does not necessarily result in a resistant phenotype, as gene expression may be influenced by regulatory mechanisms, heterogeneous expression among bacterial subpopulations (heteroresistance), or the presence of resistance genes that remain unexpressed under routine laboratory conditions (Chenouf et al., 2021). Furthermore, although oxacillin remains an accepted phenotypic marker according to CLSI guidelines, cefoxitin has been reported to provide improved phenotypic detection of *mecA*-mediated resistance in some *Staphylococcus* species, which may have contributed, at least in part, to the low phenotype–genotype agreement observed in the present study. Because antimicrobial use and management practices were not directly evaluated, the possible contribution of  $\beta$ -lactam selective pressure should be regarded as a hypothesis supported by previous studies rather than as a conclusion derived from the present data. Collectively, these findings reinforce the complementary value of phenotypic susceptibility testing and molecular detection for the surveillance and characterization of methicillin-resistant *Staphylococcus* spp. Species-specific differences were also evident. Cows displayed the highest  $\beta$ -lactam resistance. Although differences in management practices, antimicrobial exposure, and production systems could contribute to this pattern, these factors were not directly evaluated in the present study (Pascu et al., 2022). In contrast, goats and ewes—commonly raised under extensive systems—showed higher oxacillin resistance and greater prevalence of *tetK* and *ermB*. Physiological differences among host species, together with potential differences in management and antimicrobial exposure, may contribute to the observed variations; however, these factors were not specifically investigated in this study (França et al., 2012; Balemi et al., 2021). Interestingly, significant intraspecies variability was found, particularly in ewes, where *Staphylococcus* species showed different resistance profiles to penicillin, oxacillin, and tetracycline ( $p <$

0.05). In contrast, no such differences were observed in cows, and only ampicillin resistance differed significantly among goat isolates. These results suggest a more heterogeneous distribution of resistance within certain host species. When comparing resistance in the same bacterial species across different animals, *S. aureus* showed significant interspecies differences in resistance to oxacillin and tetracycline, while other species like *S. chromogenes* and *S. simulans* did not. This reinforces the influence of host species on resistance patterns beyond the bacterial identity. The co-detection of resistance genes was frequent. Venn diagrams revealed that *mecA* + *blaZ* was the most common combination, followed by *mecA* + *tetK* and triple gene profiles including *ermB*. Notably, neither *ermB* nor *tetK* appeared alone, suggesting gene clustering and possible co-transfer via mobile genetic elements. For example, *ermB* and *tetK* were always detected in combination with other genes, and 21 isolates carried only *mecA*, while 4 carried only *blaZ*. These patterns may reflect genetic hitchhiking and the co-mobilization of resistance determinants. This raises concerns for horizontal gene transfer and persistence of multidrug resistance even in the absence of clinical disease (Shi et al., 2025; Michael et al., 2023). Low agreement between molecular and phenotypic methods was a consistent theme. *mecA* had high sensitivity for oxacillin resistance (93%) but very low specificity (13%), indicating high false-positive rates. *blaZ* was the most reliable predictor (71% sensitivity and PPV for penicillin), whereas *ermB* and *tetK* showed poor diagnostic performance. These findings support the use of PCR as a complementary—not substitutive—tool, especially for rapid screening (Rusenova et al., 2022). Statistical analysis confirmed that only the *blaZ*–ampicillin pair showed a significant association ( $p < 0.05$ ), while all other gene–antibiotic combinations (including *mecA*, *ermB*, and *tetK*) lacked statistical significance. This underlines the importance of interpreting molecular results within their phenotypic context. Analyzing gene distribution by species, the high frequency of *tetK* among ewe isolates suggests possible differences in the selective pressures acting on *Staphylococcus* populations in this host species. However, information regarding antimicrobial usage was not available, preventing direct assessment of this hypothesis. In contrast, *blaZ* and *mecA* were widespread in cattle. Similar resistance patterns have been reported in dairy herds where dry cow therapy and intramammary  $\beta$ -lactam administration are commonly practiced (Pascu et al., 2022). However, these management practices were not evaluated in the present study. The presence of these genes in non-resistant isolates suggests subclinical reservoirs and environmental persistence facilitated by biofilm formation and suboptimal hygiene practices (Viñes et al., 2022; Regecová et al., 2021). Logistic regression analysis further supported these findings: no resistance gene showed a statistically significant association with

phenotypic resistance ( $p > 0.05$ ), while animal species did. For example, cow-derived isolates were more likely to be resistant to ampicillin, oxacillin, and tetracycline than those from ewes, suggesting that host-related factors may have stronger predictive value than gene presence alone. The public health implications are significant. The identification of *Staphylococcus* spp. harboring *mecA*, *blaZ*, and other resistance genes in raw milk samples, even from healthy animals, underscores the risk of gene dissemination via food products. In rural areas where unpasteurized milk is consumed, gene transfer to human pathogens such as *S. aureus* or *E. coli* is a real concern (Taher *et al.*, 2020; Ning *et al.*, 2023). The circulation of antimicrobial resistance genes in food-producing animals highlights the importance of continued surveillance and antimicrobial stewardship efforts within dairy production systems. From an epidemiological standpoint, combining phenotypic and genotypic methods is essential for robust surveillance. This study contributes valuable baseline data for antimicrobial resistance mapping and highlights the need for stratified monitoring by species and region (Rusenova *et al.*, 2022; Soares *et al.*, 2019). While PCR showed limited specificity, its high negative predictive value for genes like *mecA* and *tetK* could assist in identifying susceptible strains and optimizing diagnostic resources. The findings advocate for a One Health strategy, integrating veterinary, human, and environmental surveillance. Resistance genes circulating in dairy herds may ultimately reach the human population through direct contact or food chains, particularly when surveillance and regulatory frameworks are lacking (McEwen and Collignon, 2018; Tkadlec *et al.*, 2023). Strengthening antimicrobial stewardship, enforcing veterinary prescription guidelines, and promoting farmer education are vital next steps. An additional limitation of this study is that several *Staphylococcus* species were represented by a small number of isolates. Consequently, some statistical comparisons involving individual bacterial species may have had limited statistical power, and the corresponding estimates should be interpreted with caution. Future studies including larger numbers of isolates per species would provide more robust statistical comparisons. In conclusion, this study demonstrates high frequencies of antimicrobial resistance in *Staphylococcus* spp. isolated from raw milk collected from the three sampled dairy farms, with variable distributions of resistance genes and low phenotype–genotype concordance. The low agreement observed between phenotypic resistance and resistance gene detection highlights the complementary value of phenotypic and molecular approaches for antimicrobial resistance surveillance. Additionally, although oxacillin is an accepted phenotypic marker according to CLSI guidelines, the use of cefoxitin has been reported to provide improved detection of *mecA*-mediated

resistance in some *Staphylococcus* species, which may have contributed, in part, to the low phenotype–genotype agreement observed in the present study. PCR proved valuable as a complementary diagnostic tool but should not replace conventional antimicrobial susceptibility testing. The combination of regression analysis, gene co-detection patterns, and species-related resistance trends emphasizes the need to consider bacterial, host, and environmental variables jointly. Furthermore, the co-detection of multiple resistance genes and their species-specific distribution underscore the importance of integrated diagnostic strategies, updated regulatory frameworks, and a coordinated One Health approach to antimicrobial resistance monitoring and control.

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**Conflict of interest.** The authors declare that they have no conflicts of interest.

**Compliance with ethical standards.** All procedures involving animals were conducted in accordance with institutional guidelines for animal care and use. This study was reviewed by the Institutional Committee for the Care and Use of Animals (CICUA) of the Faculty of Veterinary Medicine and Animal Science, National Autonomous University of Mexico (UNAM). As the procedures involved only routine animal management practices (milk sampling during regular milking), formal ethical approval was not required.

**Data availability.** The data supporting the findings of this study are available from the corresponding author upon reasonable request.

**AI disclosure statement.** The authors declare that no generative artificial intelligence tools were used in the preparation of this manuscript.

**Author Contribution Statement (CRediT).** **R. González-López** – Methodology, Validation, Investigation, Writing – original draft. **S.E. Acevedo-Monroy** – Methodology, Formal analysis, Validation, Writing – review & editing. **L.A. Contreras-Méndez** – Investigation, Project administration, Supervision, Writing – review & editing. **R.A. Ruiz-Romero** – Conceptualization, Methodology, Investigation,

Supervision, Writing – original draft, Funding acquisition.

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