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Genome sequencing and comparative genomic analysis of bovine mastitis-associated non-aureus staphylococci and mammaliicocci (NASM) strains from India

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We describe the whole-genome sequencing and comparative genomic analysis of 22 mastitis-associated NASM strains isolated from India. The mean genome size of the strains was 2.55 Mbp, with an average GC content of 32.2%. We identified 14 different sequence types (STs) among the 22 NASM strains. Of these, ST1 and ST6 of *S. chromogenes* were exclusively associated with bovine mastitis. Genome-wide SNP-based minimum spanning tree revealed the intricate phylogenetic relationships among NASM strains from India, categorizing them into five major clades. Interestingly, mastitis-associated strains formed separate subclades in all the NASM species studied, indicating distinct host-specific co-evolution. The study identified 32 antimicrobial resistance (AMR) genes and 53 virulence-associated genes, providing insights into the genetic factors that could contribute to the pathogenicity of NASM species. Some virulence and AMR genes were found in the predicted genomic islands, suggesting possible horizontal transfer events.

Keywords Bovine mastitis, Non-aureus Staphylococci and Mammaliicocci, MLST, Resistome, Virulome

Bovine mastitis is an endemic disease affecting dairy cattle worldwide. It causes inflammation of the mammary gland, resulting in economic losses due to decreased milk production, veterinary care costs, and culling of infected animals¹. Staphylococci are the most common infectious causative agents of bovine mastitis, and were historically divided into two groups. One group includes *S. aureus*, which is considered more pathogenic, and is a coagulase-positive staphylococcus (CoPS). The other group that is presumed to be less pathogenic, includes other staphylococcal species referred to as the coagulase-negative staphylococci (CoNS) or non-aureus staphylococci (NAS). Some coagulase-positive and coagulase-variable mastitis pathogens (e.g., *S. pseudintermedius*) are also included in the CoNS category². Recently, five Staphylococcus species, i.e., *S. sciuri*, *S. fleurettii*, *S. lentus*, *S. stepanovicii*, and *S. vitulinus*, were reclassified into the new genus, namely, *Mammaliicoccus*³. Together, these organisms, referred to as non-aureus staphylococci and mammaliicocci (NASM), are the most prevalent (ranging from 9.1 to 16.6% of milk samples) agents isolated from intramammary infection (IMI) of dairy cows and the leading cause of subclinical mastitis. Recent studies have identified *S. chromogenes*, *S. epidermidis*, *S. xylosus*, *S. vitulinus*, *S. simulans*, and *Mammaliicoccus sciuri* as the leading causes of IMI in cattle. Among them, *S. chromogenes*, *S. xylosus*, and *S. haemolyticus* are more commonly found in milk samples^{4,5}.

Although NASM has become a leading group of pathogens, knowledge of their virulence and antimicrobial resistance mechanisms is still limited. Biofilm formation has been identified as a crucial virulence factor for NASM, particularly in persistent IMI⁶, and could potentially account for their heightened antibiotic resistance.

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The relatively increased capacity of NASM to form biofilm, as compared to other staphylococci, poses challenges in predicting antibiotic efficacy and the likelihood of persistence and recurrence of IMI. It is therefore crucial to understand the factors governing NASM resistance, persistent infections, and recurrent episodes, emphasizing the need for comprehensive research to enhance our knowledge of these intricate dynamics in the context of mastitis⁷.

NASM encompasses a diverse group of species, each with varying pathogenic potentials. Therefore, it is essential to understand the role of individual species on udder health and milk production^{2,8}. Various molecular subtyping techniques, including pulsed-field gel electrophoresis (PFGE)⁹, random amplification of polymorphic DNA (RAPD) analysis¹⁰, multi-locus sequence typing (MLST), and multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA)¹¹, have been used to investigate the molecular epidemiology of NASM. The whole genome sequence (WGS) analysis is expected to provide the best discriminatory power and better insights into the molecular epidemiology and the genetic determinants responsible for the pathogenicity of NASM. Comparative genomic analysis can identify phylogenetic relationships among various species of NASM and distinctions within and between species.

This study focused on the comprehensive genomic analysis of 22 NASM strains associated with bovine mastitis isolated in India. These strains were fully sequenced, followed by a comparative analysis of their genetic diversity, virulence factors, antimicrobial resistance (AMR) genes, and sequence types. The study also included a perceptive phylogeny analysis of the previously documented NASM genomes from India, which helped understand the genetic differences between the NASM strains associated with or not associated with bovine mastitis.

Methodology

Whole genome sequencing and annotation

Whole genomes of 22 strains of bovine mastitis-associated NASM were sequenced. These strains were collected between 2009 and 2019 from cows (*Bos taurus*) with mastitis, which included 18 strains from sub-clinical and four from clinical mastitis cases in India. The strains were isolated from three states; 18 from Karnataka, and two each from Gujarat and Meghalaya. The strains had been curated at the National Institute of Animal Biotechnology in Hyderabad and the Department of Microbiology at Karnataka Veterinary, Animal & Fisheries Sciences University in Bengaluru. For isolation, milk samples collected from clinical or subclinical mastitis had been subjected to an overnight enrichment in nutrient broth, followed by streaking on selective solid media for *E. coli*, staphylococci and streptococci. Staphylococci had been further subjected to catalase, coagulase and thermonuclease tests, followed by PCR for *nuc* and/or *tuf* genes, which mark the isolates as *S. aureus*. Those that did not belong to *S. aureus* species had been further subjected to biochemical testing, PCR and sequencing for the 16S rRNA gene, and in some cases, development of new PCRs for species identification^{12–14}.

The DNeasy blood and tissue kit (Qiagen) was used to isolate genomic DNA from each strain, following the manufacturer's instructions. The resulting RNA-free DNA was then employed for library preparation and sequenced using the Illumina HiSeq platform at Macrogen, Seoul, South Korea. The process of assembly and analysis of the genome sequences involved multiple steps. First, the Trimmomatic tool was used to eliminate adapters and discard low-quality reads¹⁵. The following settings were applied: ILLUMINACLIP: TruSeq3-PE: fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36. These parameters ensured quality trimming of the raw reads, removing adapter sequences, low-quality bases, and maintaining read length integrity. Adaptor-trimmed high-quality reads were assembled using SPAdes v 3.11.1¹⁶. The quality of the read assessed in checkM analysis¹⁷. Finally, the assembled genome sequences underwent annotation using the PROKKA¹⁸. Species identification was done using the rMLST (ribosomal multilocus sequence typing) tool available at the PubMLST webserver¹⁹.

Multilocus sequence typing

Multilocus sequence typing (MLST) using respective species-specific housekeeping genes was performed using the PubMLST web server (<https://pubmlst.org/>)²⁰. Allelic profiles were compared to the PubMLST database, and sequence types (STs) were identified.

Average nucleotide identity (ANI) estimation

The genome sequences were analyzed to determine their Average Nucleotide Identity (ANI) values using Jspecies (<https://jspecies.ribohost.com>), which included MUMmer ANIm, ANItetra, and BLAST + ANI²¹. *S. gallinarum* STP534, *S. xylosus* SMQ-121, *S. epidermidis* ATCC 14,990, *S. haemolyticus* ATCC 29,970, *S. hominis* FDAARGOS_575, *M. lentinus* Colony453, *M. sciuri* NCTC12103, *S. pseudintermedius* FDAARGOS_930, and *S. chromogenes* 17 A were used as reference genomes for the respective species of NASM. Each genome was assigned a species name based on an ANI cutoff of > 95%²². A heatmap was then generated using the Jspecies data on the Clustviz webserver²³.

Phylogeny analysis using genome-wide SNPs

We used kSNP v.3 to predict genome-wide SNPs, identifying SNPs without requiring genome alignment²⁴. The k-mer value of 17 was optimal for NASM strains by the KChooser tool of the kSNP v3. Genome-wide SNPs were identified and collected in a data matrix known as the 95% majority SNP matrix. Based on the genome-wide SNPs, a phylogenetic tree was constructed for 22 NASM strains using MEGA 11²⁵. Used with the standard method, maximum likelihood with the general reversible substitution model. Additionally, a genome-wide SNP-based phylogenetic tree was constructed for 176 genomes of NASM strains reported from India, which includes 22 strains reported in this study, using Grapetree²⁶.

Identification of antimicrobial resistance (AMR) genes and virulence factors

The resistance gene identifier (RGI) in the comprehensive antibiotic resistance database (CARD) was used to identify the AMR genes using default parameters²⁷. The VFAnalyzer in the virulence factor database (VFDB) was used to identify the genes associated with virulence factors (VF)²⁸. We conducted our analysis in strict mode, which inherently applies a threshold thresholds of $\geq 30\%$ identity and $\geq 80\%$ coverage.

Identification of prophages and genomic islands

The presence of prophages in the genome was determined by PHASTER (PHAge Search Tool—Enhanced Release) (<https://phaster.ca/>)²⁹. Based on the scores, prophages were classified into three groups, i.e., intact, questionable, and incomplete, with the corresponding scores > 90 , $70\text{--}90$, and < 70 , respectively. Genomic islands (GIs) in each genome were predicted using Island Viewer 4 (<http://www.pathogenomics.sfu.ca/islandviewer/>)³⁰.

Genome availability

NASM genome sequence used in this study has been deposited in NCBI with the following accession numbers: GCA_018986335.1, GCA_018996905.1, GCA_019334185.1, GCA_018997025.1, GCA_018997125.1, GCA_018967705.1, GCA_019149065.1, GCA_019149165.1, GCA_019149045.1, GCA_019193065.1, GCA_019148995.1, GCA_019334235.1, GCA_019429675.1, GCA_019149085.1, GCA_019149255.1, GCA_019149245.1, GCA_019149205.1, GCA_019334165.1, GCA_019100515.1, GCA_019165085.1, GCA_018967945.1, GCA_018968045.1.

Results

General features of genome sequences of bovine mastitis-associated NASM strains

The genomes of 22 strains of NASM associated with bovine mastitis were sequenced using the Illumina platform. These strains belong to nine different species of *Staphylococcus* and *Mammaliicoccus* genera (Table 1). For each genome, a minimum of 100X mean sequence coverage was obtained. The draft genome assemblies were checked by the checkM tool, and the genomic features such as contigs, N50, total genome length, and GC content are shown in Table S1. Adapter removal, and the processed high-quality reads were used for *de novo* assembly. The draft genomes contained 24 to 150 contigs with a mean genome size of 2.55 Mbp and an average GC content of 32.2%. The rMLST method uses ribosome protein subunit (*rps*) gene sequences for precise taxonomic identification. Of the 22 NASM strains, 21 were identified by exact matches with 56 *rps* genes. *S. chromogenes* strain K17 was identified by exact matches with 55 *rps* genes. The summary of genome sequences is given in Table 2.

Identification of sequence types based on MLST

MLST schemes are available for five species of NASM, which were used in this study and can be accessed at the pubMLST server. The sequence types (STs) were determined based on the respective species-specific housekeeping genes (Table S2). *M. sciuri* strains were divided into ST114 ($n=2$) and ST115 ($n=1$). Similarly, *S. chromogenes* were represented by ST6 ($n=2$) and ST1 ($n=2$). Three different STs were identified among *S. epidermidis*: ST1158 ($n=2$), ST1157 ($n=1$), and ST924 ($n=1$). ST42 ($n=2$) was identified in *S. haemolyticus*; two other strains representing potential new STs could not be typed. One strain of *S. hominis* belonged to ST79.

Average nucleotide identity analysis

The ANI analysis examined the interrelationship among NASM, where species-specific clustering of strains was observed, as shown in (Fig. 1) and the pairwise average nucleotide identity given in (Table S3). The ANI threshold of 96% was used to establish species delimitation within species-specific boundaries. For instance, *S. chromogenes* strain K17 was identical to the strains K23, K26, and K29, while *S. epidermidis* strain K16.1 matched the strains K4.3, K3.2, and K60. Similarly, *M. sciuri* strain K117.2 shared identity with strains K14 and K9, and *S. xylosus* strain K19 was similar to the strains K46 and SMG. *M. latus* was closer to *M. sciuri* strains and formed a genus-specific cluster. *S. gallinarum* strain clustered together with *S. xylosus* strains. Likewise, *S. pseudintermedius* strain clustered with *S. chromogenes*, while *S. haemolyticus* clustered with *S. hominis*.

S. No.	Name of the species	Number of strains
1	<i>Staphylococcus chromogenes</i>	4
2	<i>Staphylococcus epidermidis</i>	4
3	<i>Staphylococcus gallinarum</i>	1
4	<i>Staphylococcus haemolyticus</i>	4
5	<i>Staphylococcus hominis</i>	1
6	<i>Staphylococcus pseudintermedius</i>	1
7	<i>Staphylococcus xylosus</i>	3
8	<i>Mammaliicoccus latus</i>	1
9	<i>Mammaliicoccus sciuri</i>	3

Table 1. NASM strains used in this study.

S.no.	Strain	Genome size (bp)	No. of Contigs	Sequence Type (ST)	CDS	rRNA	tRNA	Pseudogenes	Accession No.
1	<i>M. latus</i> K169	2,848,578	54	-	2783	3	29	13	GCA_018967945.1
2	<i>M. sciuri</i> K117.2	2,702,737	18	ST114	2672	2	14	15	GCA_018967705.1
3	<i>M. sciuri</i> K14	2,739,519	46	ST115	2723	2	14	18	GCA_019149065.1
4	<i>M. sciuri</i> K91	2,715,464	150	ST114	2735	6	28	26	GCA_019334185.1
5	<i>S. chromogenes</i> K17	2,321,223	26	ST1	2225	2	23	31	GCA_019149165.1
6	<i>S. chromogenes</i> K23	2,349,003	47	ST6	2247	5	42	43	GCA_019334235.1
7	<i>S. chromogenes</i> K26	2,340,405	55	ST1	2249	6	45	44	GCA_019429675.1
8	<i>S. chromogenes</i> K29	2,329,474	31	ST6	2231	2	13	35	GCA_019149085.1
9	<i>S. epidermidis</i> K16.1	2,407,306	76	ST1157	2200	2	9	58	GCA_019149045.1
10	<i>S. epidermidis</i> K3.2	2,455,169	142	ST1158	2281	5	48	60	GCA_019100515.1
11	<i>S. epidermidis</i> K4.3	2,449,159	83	ST924	2222	5	37	70	GCA_019165085.1
12	<i>S. epidermidis</i> K60	2,460,483	67	ST1158	2239	7	44	71	GCA_019149245.1
13	<i>S. gallinarium</i> B13	2,935,302	55	-	2735	4	40	28	GCA_018997025.1
14	<i>S. haemolyticus</i> A11	2,485,414	24	-	2336	4	42	78	GCA_018986335.1
15	<i>S. haemolyticus</i> A3.2	2,497,919	24	-	2355	4	29	80	GCA_018996905.1
16	<i>S. haemolyticus</i> K16.2	2,459,833	108	ST42	2318	5	33	77	GCA_019193065.1
17	<i>S. haemolyticus</i> K47	2,435,548	84	ST42	2300	2	19	64	GCA_019149205.1
18	<i>S. hominis</i> K24	2,162,928	27	ST79	2055	2	48	34	GCA_019149255.1
19	<i>S. pseudintermedius</i> B32	2,517,961	40	-	2329	4	35	42	GCA_018997125.1
20	<i>S. xylosus</i> K19	2,781,766	48	-	2575	2	16	20	GCA_019148995.1
21	<i>S. xylosus</i> K46	2,801,517	88	-	2588	5	46	40	GCA_019334165.1
22	<i>S. xylosus</i> SMG24	2,802,341	29	-	2585	4	39	34	GCA_018968045.1

Table 2. Summary of NASM genome sequences.

Genome-wide SNP-based phylogeny analysis of NASM

We identified 192 core SNPs and 953,968 non-core SNPs in 22 NASM strains. Based on the distribution of SNPs, a maximum likelihood phylogenetic tree using MEGA 11 was constructed with 100 bootstrap replicate as also mentioned in the branch (Fig. 2). The NASM strains grouped into five species-specific clades. *S. gallinarum* and *S. xylosus* formed a clade (I) and were divided into species-specific subclades. *S. hominis* and *S. haemolyticus* formed a clade (II) and were divided into species-specific subclades. While *S. epidermidis* formed a separate clade (III). *S. pseudintermedius* and *S. chromogenes* formed a clade (IV), and both species were divided into separate subclades. Similarly, *Mammaliicoccus* formed a separate clade (V), while *M. latus* and *M. sciuri* formed species-specific subclades. In summary, the genome-wide SNP-based phylogeny and ANIB-based phylogeny were similar.

In order to further understand the inter-relationship between our strains and other NASM strains from India, a genome-wide SNP-based phylogeny analysis of 176 NASM strains (Table S4), including 22 strains from this study, was conducted. The NASM strains could be grouped into five species-specific clades (Fig. 3A). Clade A consisted of *S. epidermidis*, which formed a major clade with 71 strains from India, divided into two subclades. Clade B was represented by *S. haemolyticus* and *S. hominis* and divided into separate subclades. Clade C comprised *S. haemolyticus*, *S. gallinarum*, and *S. xylosus*. Strains belonging to the species *S. haemolyticus* were represented in both clades B and C. Clade D was represented by *S. chromogenes* and *S. pseudintermedius*. Clade E was represented by the *Mammaliicoccus* species (*M. latus* and *M. sciuri*).

We examined the minimum spanning tree in relation to the source of isolation - whether it was from a cow or some other host. The results were interesting as all mastitis-associated NASM strains isolated from cows formed separate subclades or clusters in all clades (Fig. 3B). On the other hand, NASM strains isolated from other sources, such as humans, plants, dogs, etc. clustered together. This suggests that bovine mastitis-associated strains might have host-specific divergence and evolution.

Identification of antimicrobial resistance genes among mastitis-associated NASM strains

The 22 NASM strains contained antimicrobial resistance (AMR) genes (AMR genes) against 12 classes of antibiotics (Fig. 4). The 22 strains carried 32 AMR genes, while the analysis of all the Indian isolates revealed 57 AMR genes. The number and classes of AMR genes varied significantly among the strains. Two AMR genes, *sdrM* and *sepA* were found in all 22 strains. Of these, *sdrM* forms an efflux pump that confers resistance to norfloxacin and ethidium bromide, while *sepA* forms a multidrug efflux pump that confers resistance to disinfectants and antiseptics. The *norC*, which confers resistance to fluoroquinolone antibiotics, was present in all strains except for *S. pseudintermedius* strain B32, *M. latus*, and *M. sciuri* species.

The *dfrC*, responsible for trimethoprim resistance, and the multidrug efflux pump coding gene *norA*, which confers resistance to fluoroquinolones as well as several antiseptics and disinfectants, were observed in all strains of *S. epidermidis* but absent in other species. The fusidic acid resistance gene (*fusE*) was detected in all strains of *S. chromogenes* but not in other species. The genes associated with resistance to penam drugs (*mecl* and *mecR1*) were only found in *S. epidermidis* strain K4.3. The methicillin-resistant gene (*mecA*) was present in *S. epidermidis*

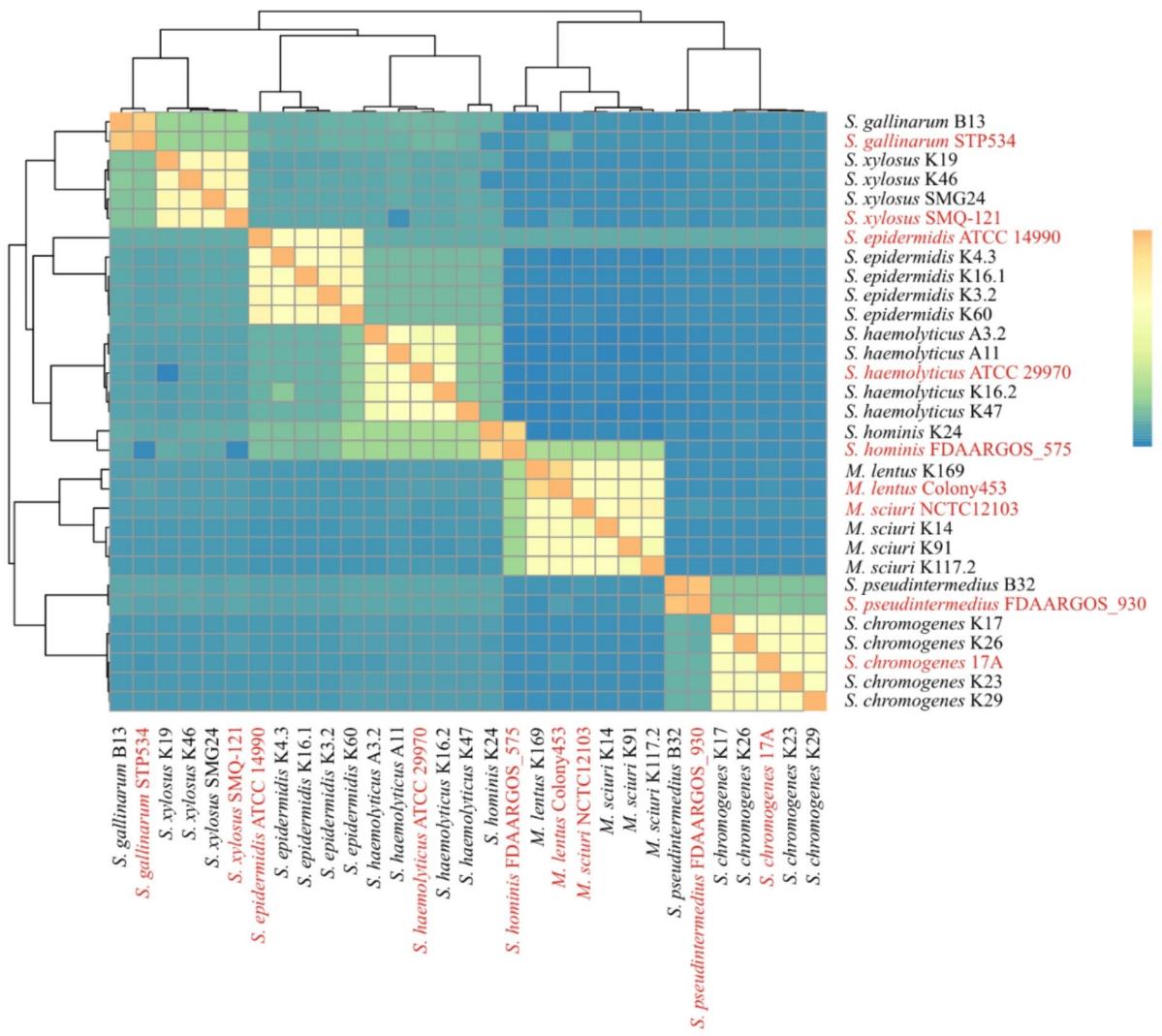


Fig. 1. Heatmap and dendrogram illustrating the phylogenetic relationships based on average nucleotide identity (ANI) values for 22 mastitis-associated NASM isolates. The color scale represents ANI percentage identity, ranging from 75–100%, with warmer colors (orange) indicating higher similarity and cooler colors (blue/green) representing lower similarity. Unit variance scaling was applied to enhance comparability across different strain pairings. Reference genomes, marked in red, provide comparative context.

strain K4.3, *S. haemolyticus* strain K16.2, and *S. haemolyticus* strain K47. The PC1 beta-lactamase (*blaZ*) gene was detected in all strains of *S. haemolyticus*, and *S. epidermidis* strains K3.2 and K4.3.

The *mgrA*, which confers resistance to fluoroquinolone antibiotics, was present in *S. haemolyticus* strains A11 and A3.2, and all the strains of *S. epidermidis*. The *salB*, which confers resistance to lincosamide and class A streptogramins, was only found in *M. latus* strain K169. On the other hand the homolog *salC*, was present in all strains of *M. sciuri* whereas *salE* was present in *S. xylosus* strains K19 and K46. The *tetK* gene, which is a tetracycline efflux protein, the *qacJ* gene, which confers resistance to quaternary ammonium compounds, and the SAT-4 gene, which provides resistance to nucleoside antibiotics, were only present in *S. haemolyticus* strains belonging to ST42. The *fosBx1*, which confers resistance to phosphonic acid antibiotics, was found in *S. xylosus* strains K19 and K46, *S. gallinarum* strain B13, *S. epidermidis* strain K16.1, and *M. sciuri* strains and K117.2. The *fexB* coding for a phenol antibiotic efflux pump was only present in *S. xylosus* strain SMG24. The *vanT*, which is a part of codes for a membrane-bound serine racemase which confers resistance to vancomycin, was present in multiple strains, except *S. pseudintermedius*, *S. epidermidis*, *S. hominis*, and *S. gallinarum* species. The *vanW* gene, which is part of the vancomycin operon, was identified exclusively in *S. epidermidis* K60. Additionally, the *vanY* gene, which is also part of the same operon, was present only in *M. latus* K169. Furthermore, *vanY* was found in all strains of *M. sciuri* and in *S. haemolyticus* strains belonging to ST42, as well as in *S. xylosus* SMG24.

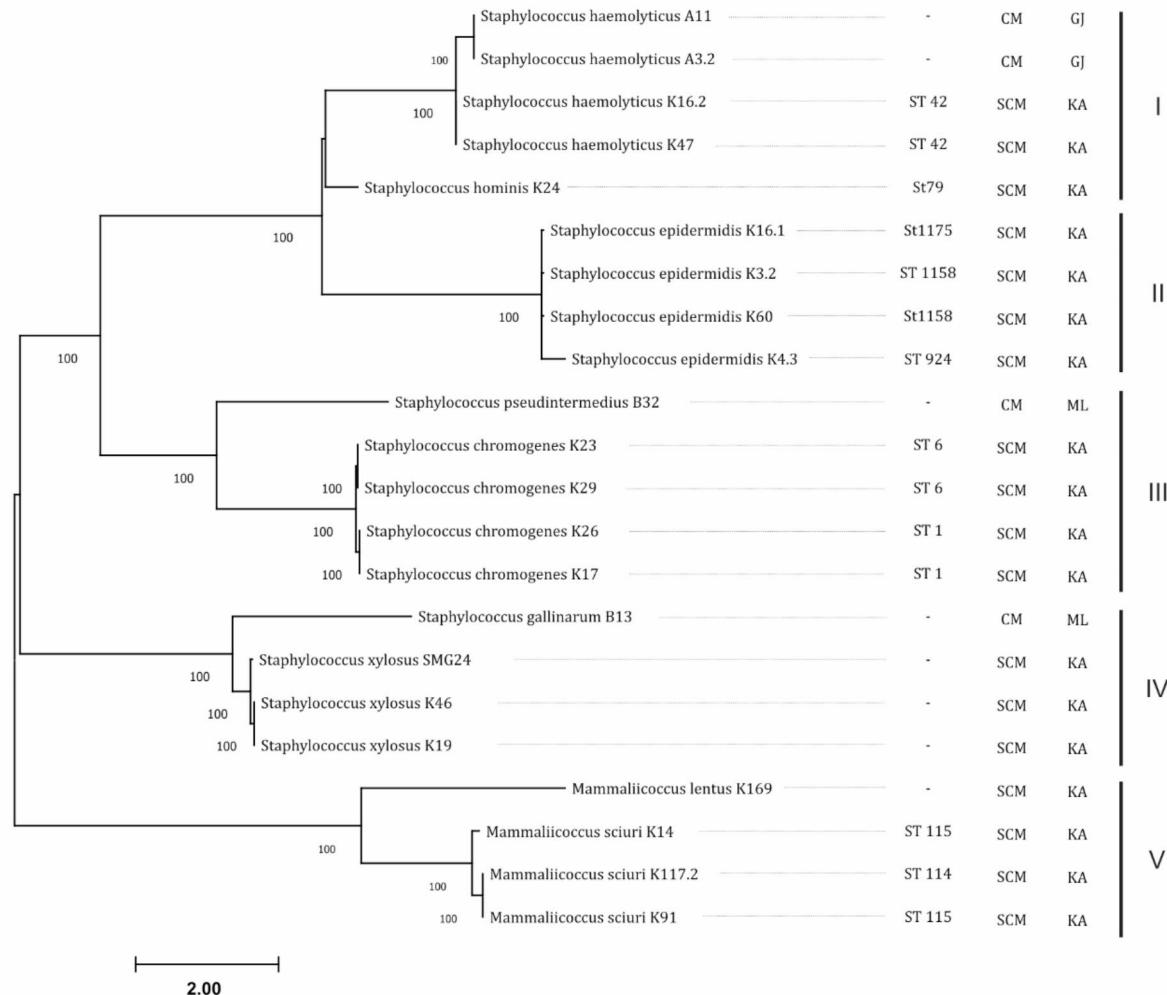


Fig. 2. The genome-wide SNP-based maximum likelihood phylogenetic tree of mastitis-associated NASM strains. The phylogenetic tree was constructed using kSNP v.3, and visualized using Mega 11. The scale bar indicates 2.00 substitutions per nucleotide position and with 100 bootstrap replicates. The strain names, sequence types (STs), clinical (CL) or subclinical (SCL) mastitis, and three different states in India (GJ-Gujarat, KA-Karnataka, and ML-Meghalaya) from where the strains were isolated are indicated, respectively.

When the analysis was extended to all 176 NASM isolates from India, 57 antibiotic-resistant genes were identified in all Indian isolates as against 32 genes for our 22 strains. A complete list of AMR genes from 176 NASM strains is shown in (Table S5). And the AMR hit identity were provided in (Table S6).

Identification of virulence factors among mastitis-associated NASM strains

We identified 53 virulence factors among the 22 NASM strains associated with bovine mastitis (Fig. 5). Notably, no single virulence gene was present in all the studied strains, and each strain exhibited a unique combination of virulence factors. Adherence-related Genes, such as *icaA*, *icaB*, and *icaC* (intercellular adhesins), were common in *S. xylosus* and *M. sciuri* strains. Notably, the *spa* gene, encoding *Staphylococcus* protein A, was exclusive to the *S. pseudintermedius* strain, highlighting strain-specific adherence mechanisms. Enzyme-coding genes, lipase (*lip*), and thermonuclease (*nuc*) genes were present in all staphylococcal species but absent in *Mammaliicoccus*. Immune evasion genes and capsule protein genes (*capB*, *capC*) were found in most strains but absent in *M. sciuri*, *M. latus*, and *S. pseudintermedius*. The adenosine synthase (*adsA*) gene was exclusively found in *S. pseudintermedius* and *S. chromogenes*. Toxin-related genes were strain-specific, with leukotoxin genes (*lukD* and *lukE*) found exclusively in the *S. pseudintermedius* strain, and cytolsin (*cylR2*) detected in *S. xylosus*, *S. hominis*, *S. haemolyticus*, and *S. gallinarum* strains. Only *S. epidermidis* K4.3 harbored genes encoding the Type VII secretion system (T7SS), *esaA*, *esaB*, *esaG*, *esaA*, *esaB*, *esaC*, and *esaX*, which may contribute to its unique virulence potential. The *vctC* gene, responsible for iron uptake, was found in all *S. xylosus* strains, while the

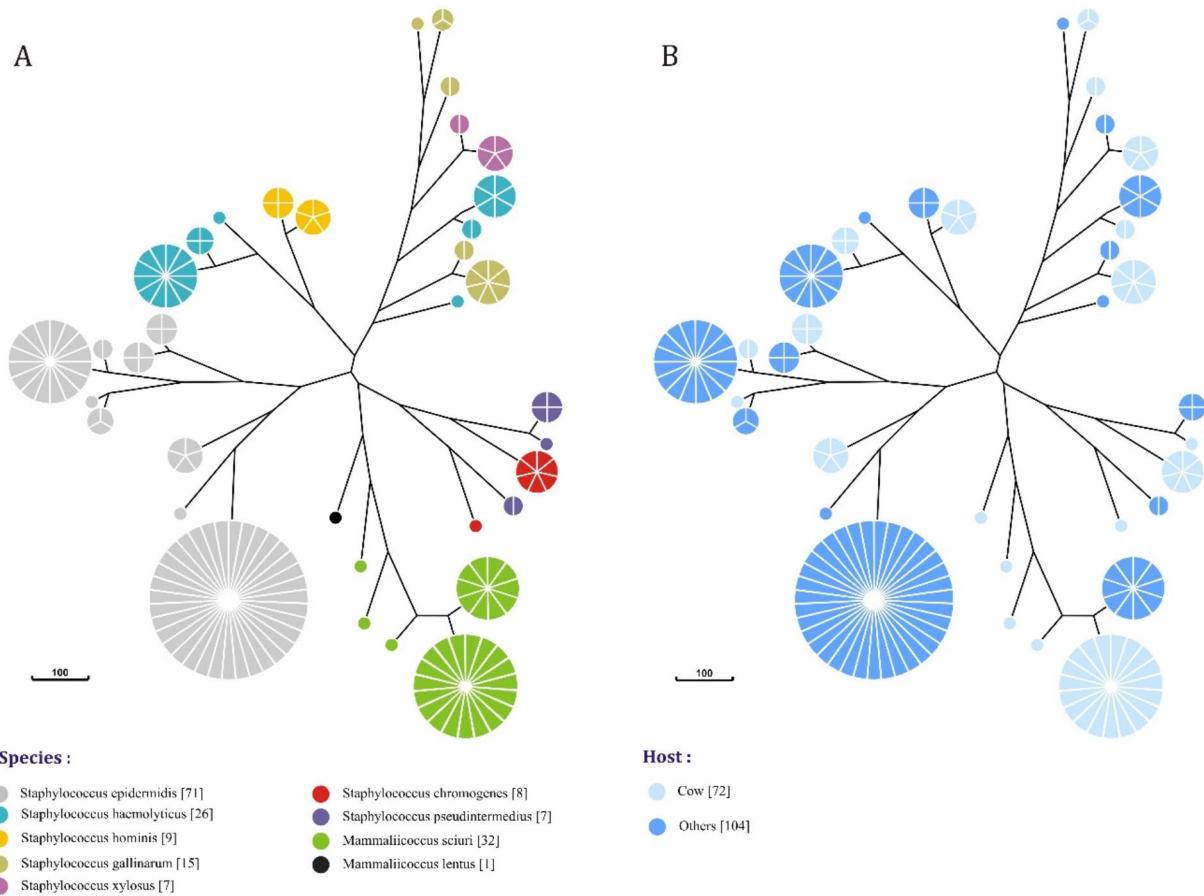


Fig. 3. Minimum spanning tree (MST) of 176 NASM genomes reported from India. SNPs were predicted using kSNP v.3 and the tree was generated using GrapeTree. A – color codes denote the species names; B – color codes denote the isolation source, cow / other hosts.

copper uptake gene (*ctpV*) was identified only in *S. epidermidis*. Genes associated with intracellular survival, such as *ndk* (phagosome arrest), were prevalent across *Mammaliicoccus* strains.

Prophage sequence-associated with NASM

We identified nine intact prophage sequences among our NASM strains. All the prophage sequences contain all the genes required for a phage life cycle, as shown in (Figure S1). Two prophage sequences, *Staphylococcus* phage vB Saus IMEP5 and uncultured caudovirales phage clone 10 S.1, were found in *S. gallinarum*. *Staphylococcus* phage vB SepS BE21 and *Staphylococcus* phage S-CoN Ph24 were found in *S. epidermidis* strains K60 and K16, respectively. *Staphylococcus* phage SAP3 was found in *S. chromogenes* strains K29 and K23. Similarly, *S. haemolyticus* strains A3.2 and A11 harbored *Staphylococcus* phage IME-SA4. *S. xylosus* contained sequences of the *Staphylococcus* phage StB20. The presence of prophage sequences is suggestive of their potential role in the pathogenicity of NASM, however further comprehensive investigation is required.

Genomic islands of NASM

The results of the analysis of genomic islands in the 22 NASM strains are summarized in Table 3, and a representative image of the predicted genomic island is shown in (Figure S2). The number of GIs in each genome varied from 3 to 9, coding for 85 to 250 genes. These GIs mainly consisted of hypothetical protein-coding genes, transposases, and transcriptional regulators. Some known virulent factors and AMR genes were identified in a few genomic islands. For example, the *fusB* gene, which encodes metallothiol transferase, leading to fosfomycin resistance, was found in *M. latus*. The *cadC* gene, responsible for cadmium resistance, transcriptional regulatory protein *Yyc*, which regulates the two-component system *WalR/WalK* regulatory protein and *fusB* were found in *S. sciuri* strains. The *lip* gene, coding for the virulence-related enzyme lipase, and *yyc* were found in *S. chromogenes* strains. *S. epidermidis* strains carried the *ebh* gene, coding for extracellular matrix-binding protein related to adherence, *blaZ*, coding for b-lactamases responsible for penicillin resistance, *msr* gene, which is an ABC-F subfamily ribosomal protection protein conferring resistance to erythromycin and streptogramin B class of macrolides, *qac* gene, which is a multidrug efflux pump resistant to fluoroquinolone antibiotics, *ssaA*, which is staphylococcal secretory antigen, *cadC*, and *yyc*.

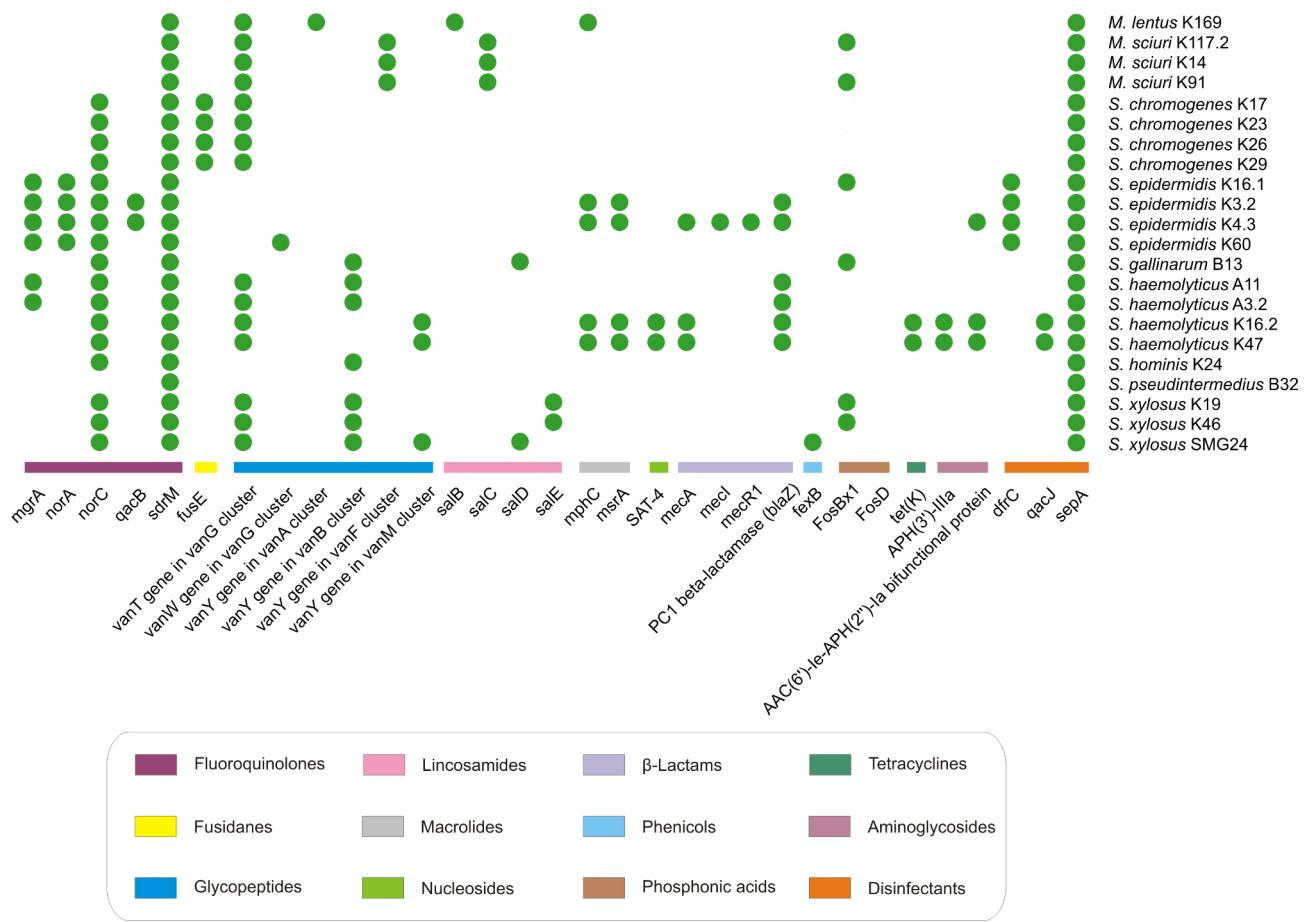
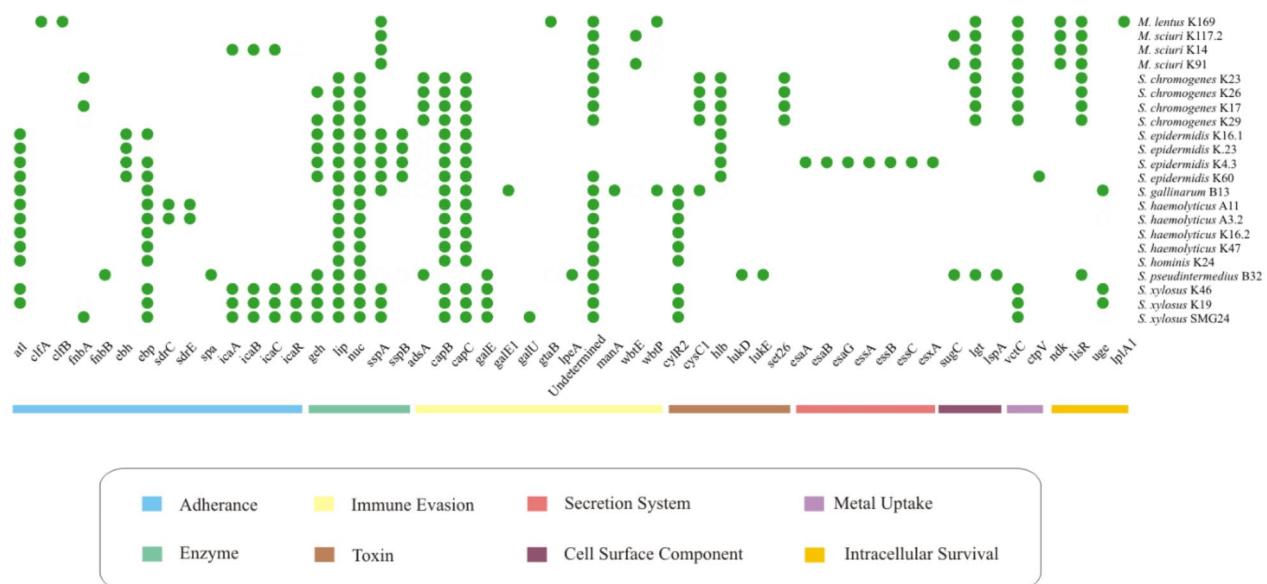


Fig. 4. The resistome of 22 mastitis-associated NASM isolates, hierarchically clustered based on the presence (green dots) or absence of 32 antimicrobial resistance (AMR) genes.



S.No.	NASM strains	Number of Islands	Number of genes in GIs	No. of genes coding for hypothetical Proteins	Virulence & and antimicrobial resistance genes in genomic islands
1	<i>M. lentinus</i> K169	3	90	69	<i>fosB</i> 2
2	<i>M. sciuri</i> K117.2	6	148	79	<i>fosB</i> , <i>cadC</i> , <i>yyc</i> (<i>walR/K</i>)
3	<i>M. sciuri</i> K14	5	100	58	
4	<i>M. sciuri</i> K91	5	120	68	<i>fosB</i> , <i>cadC</i> , <i>yyc</i> (<i>walR/K</i>)
5	<i>S. chromogenes</i> K17	4	115	88	<i>yyc</i> (<i>walR/K</i>)
6	<i>S. chromogenes</i> K23	3	85	73	
7	<i>S. chromogenes</i> K26	5	132	86	<i>yyc</i> (<i>walR/K</i>)
8	<i>S. chromogenes</i> K29	3	97	81	<i>lip</i>
9	<i>S. epidermidis</i> K16.1	5	88	68	<i>cadC</i> , <i>yyc</i> (<i>walR/K</i>)
10	<i>S. epidermidis</i> K3.2	4	109	56	<i>ebh</i> , <i>blaZ</i> , <i>msr</i> , <i>qacA</i> , <i>qacR</i> , <i>yyc</i> (<i>walR/K</i>)
11	<i>S. epidermidis</i> K4.3	7	150	87	<i>blaZ</i> , <i>msr</i> , <i>ssaA</i> , <i>yyc</i> (<i>walR/K</i>)
12	<i>S. epidermidis</i> K60	8	139	95	<i>cadC</i> , <i>yyc</i> (<i>walR/K</i>)
13	<i>S. gallinarium</i> B13	9	163	103	
14	<i>S. haemolyticus</i> A11	5	131	73	<i>blaZ</i> , <i>cadC</i> , <i>farB</i> , <i>yyc</i> (<i>walR/K</i>)
15	<i>S. haemolyticus</i> A3.2	5	132	74	<i>blaZ</i> , <i>cadC</i> , <i>farB</i> , <i>arsR</i> , <i>yyc</i> (<i>walR/K</i>)
16	<i>S. haemolyticus</i> K16.2	7	114	48	<i>blaZ</i> , <i>msr</i> , <i>qacC</i> , <i>cadC</i> , <i>farB</i> , <i>yyc</i> (<i>walR/K</i>)
17	<i>S. haemolyticus</i> K47	7	118	53	<i>blaZ</i> , <i>msr</i> , <i>cadC</i> , <i>yyc</i> (<i>walR/K</i>)
18	<i>S. hominis</i> K24	6	186	115	<i>ebh</i> , <i>yyc</i> (<i>walR/K</i>)
19	<i>S. pseudintermedius</i> B32	7	250	163	<i>spa</i> , <i>lip</i> , <i>msr</i> , <i>ssaA</i> , <i>yyc</i> (<i>walR/K</i>)
20	<i>S. xylosus</i> K19	5	164	83	<i>lip</i> , <i>yyc</i> (<i>walR/K</i>)
21	<i>S. xylosus</i> K46	4	137	70	<i>yyc</i> (<i>walR/K</i>)
22	<i>S. xylosus</i> SMG24	7	193	116	<i>cadC</i>

Table 3. Salient features of predicted genomic islands of NASM strains.

S. haemolyticus strains carried *farB*, which is the cytoplasmic transporter protein that is part of the *farAB* efflux pump that confers resistance to fatty acids, *arsR*, coding for arsenic resistance, and *blaZ*, *cadC*, *yyc*, *msr*, and *qac* genes. *S. hominis* strains contained *ebh* and *yyc* genes. The *spa*, *lip*, *msr*, *ssaA*, and *yyc* genes were found in *S. pseudintermedius* strains. *S. xylosus* strains carried *lip*, *yyc*, and *cadC*. All *S. haemolyticus* strains consisted of similar islands. *S. haemolyticus* strains K16 and K47 had similar islands, GI-1, 2, and 3. *S. haemolyticus* strains A11 and A3 consisted of GI-2 in common. *S. epidermidis* strains K60 and K16 possessed GI-3 in common. In GI-1 of *S. chromogenes* strain K29 was similar to the GI-2 of *S. chromogenes* strain K23. The GI-1 was found in common in *M. sciuri* strains K117 and K91. A complete list of genomic islands from the 22 NASM strains is shown in (Table S7).

Discussion

Mastitis significantly threatens the dairy industry, causing substantial revenue losses globally. Other than *S. aureus*, the non-*aureus* *Staphylococcus* and *Mammaliicoccus* (NASM) are largely responsible for subclinical and clinical mastitis. The disease progression involves a dynamic process shaped by multiple factors, such as the host's genetics, host as well as bacterial resistance mechanisms, host immune response, geographical influences, virulence factors (VFs) and the genetic variability of the bacterium. A comprehensive genome analysis of mastitis-associated NASM strains from diverse geographical regions could enhance our understanding of these factors, aiding in assessing pathogenic potential and infection risk, disease manifestation, and transmission dynamics.

We conducted a comparative genomic study on 22 strains of NASM that caused bovine mastitis in three states of India. We sequenced their whole-genomes and identified STs specific to each species, along with the distribution of AMR genes and virulence factors. In previous studies, an ANI threshold of < 96% was suggested for identifying NASM at the species level³¹. By comparing the nucleotide sequences of all the 22 strains, we found that members of the same species had ANI values consistently above 96%, indicating significant genomic similarity. On the other hand, members of different species had less than 96% ANI, indicating a clear genomic distinction between different NASM species. Our study supports the 96% ANI threshold value for species differentiation. This threshold helps understand and classify microbial diversity within this group, making it a valuable criterion for delineating species boundaries. The genome-wide SNP-based phylogeny analysis supported the ANI-based analysis, and a similar phylogenetic classification was observed.

The minimum spanning tree (MST) based on SNPs can help us understand the complex relationships among closely related strains. It can reveal patterns of co-evolution, host specialization, and potential transmission pathways. We noticed that strains associated with bovine mastitis clustered together as separate subclades in all the NASM genomes. Previous studies have reported that *S. chromogenes* ST-1 and ST-6 strains are associated with bovine mastitis^{32,33}. Our observation also documents that *S. chromogenes* ST-1 and ST-6 are associated with bovine mastitis. Additionally, we found that *S. epidermidis* genotypes ST111 and ST59 are associated with bovine

mastitis, which is consistent with another earlier report³⁴. Similar sub-clusters unique to particular hosts have been observed in other pathogens³⁵. In several European countries, *S. aureus* CC8 strains have been linked with bovine mastitis. Recently, we reported that *S. aureus* CC97 strains isolated from India were also associated with mastitis³⁶. Further research is needed to understand the relevance of subclade-specific SNPs and their possible association with bovine mastitis to better understand their pathogenicity.

The increase in drug-resistant strains makes it challenging to treat bovine mastitis with antimicrobial intervention. In our study, we found 32 AMR genes in the 22 NASM genomes. Only one of the 22 strains had *mecA* and other *mec*-related genes. Previous studies have documented the presence of *mecA*-positive *S. epidermidis* strains in bovine milk samples and the clonal dissemination of multi-drug-resistant *S. epidermidis* strains carrying *mecA* within herds³⁷. The emergence of methicillin-resistant *S. epidermidis* (MRSE) in cattle highlights the need for increased attention, with some researchers suggesting that animals infected with methicillin-resistant NASM should be culled³⁸. The *S. epidermidis* strain K4.3 investigated in this study exhibited resistance to methicillin, classifying it as MRSE. However, the other 21 NASM strains analyzed were methicillin-sensitive staphylococcus (MSS) and did not contain *mecA*, *mecR1*, or *mec1* genes in their genomes.

The *blaZ* gene was discovered in 6 out of 22 NASM strains. This gene is responsible for the production of penicillinase, which is the primary mechanism of penicillin resistance in NASM³⁹. Multi-drug resistant (MDR) efflux pumps were dispersed throughout the strains and were not associated with specific species or STs. The regulation of MDR efflux pumps is a complex process, requiring multiple regulators to express these elements^{40,41}. Therefore, the presence of these efflux pumps may not necessarily translate to an AMR phenotype.

The *vanG* operon confers resistance to vancomycin by altering bacterial cell wall precursors, thus reducing the binding affinity of the antibiotic. Within this operon, the *vanT* gene encodes a serine racemase enzyme that converts L-serine to D-serine. D-serine can then substitute for D-alanine in the peptidoglycan structure, effectively lowering vancomycin's binding ability, as the antibiotic specifically targets the D-Ala-D-Ala sequence in the cell wall. Additionally, *vanW* is thought to have a regulatory role, potentially aiding in stabilizing or enhancing the resistance mechanism, though its precise function remains to be fully understood. Together, *vanT* and *vanW*, along with other genes within the *vanG* operon, work collectively to produce a robust defense against vancomycin, particularly in species like *Clostridioides difficile* and *Enterococcus faecalis*, where the operon biological processes are still being studied⁴².

The *vanA* and *vanB* clusters confer glycopeptide resistance through a slightly different mechanism. They encode a D, D-carboxypeptidase enzyme known as VanY in both clusters, which cleaves the terminal D-alanine residue from peptidoglycan precursors. This cleavage prevents vancomycin from binding effectively by allowing the formation of an alternative structure, such as D-Ala-D-lactate, that the antibiotic binds poorly⁴³. The *vanM* and *vanF* operons similarly utilize *vanY* to promote the formation of D-Ala-D-Lac by removing the terminal D-alanine, thereby reducing vancomycin's effectiveness. The presence of *vanY* across *vanM* and *vanF* suggests a common evolutionary origin, likely from bacteria within the *Bacillaceae* family⁴⁴. Collectively, these operons (*vanA*, *vanB*, *vanG*, *vanM*, and *vanF*) demonstrate a coordinated multi-gene response that modifies peptidoglycan precursors, allowing bacteria to evade vancomycin and continue synthesizing their cell walls despite the antibiotic's action.

We have identified 53 virulence-associated genes among the 22 NASM genomes. Of these, 15 genes are critical for adherence and biofilm development. The *ica* operon, which produce polysaccharide intercellular adhesins (PIA) and is a commonly found genetic component in biofilm formation⁴⁵ stands out as a crucial player. The *ica* gene was present in *S. xylosus* and *M. sciuri* strain K14. The impact of *ica* genes is particularly noticeable within NASM species linked to the human environment⁴⁶. The diversity of studies examining different variants of the *ica* genes poses a challenge in understanding the involvement of this gene in biofilm formation⁴⁷. All of our NASM strains, excluding *Mammaliicoccus* spp., have genes that code for lipases. Cell-wall-associated proteins and enzymes play a significant role in the pathogenesis of staphylococci and are essential targets for drug development⁴⁸. Lipases in *S. aureus*, known as SAL, have been identified in community-associated methicillin-resistant strains^{49,50}.

The *spa* gene, a major antigen of *S. aureus*, was exclusively detected in *S. pseudintermedius*. The *sspA* gene was found in 12 strains, while the *sspB* gene was found exclusively in *S. epidermidis*. In *S. epidermidis*, the *sspA* and *sspB* genes were found to coexist, indicating a potential interplay or cooperative function. The initial player in the staphylococcal proteolytic cascade is aureolysin, a metalloprotease. This enzyme undergoes a rapid process of autocatalytic activation. Subsequently, the activated aureolysin plays a crucial role in activating the *sspA* serine protease, which in turn, serves as a critical activator for the *sspB* cysteine protease^{51,52}. Such cascades are often pivotal in regulating various cellular processes and contribute to the pathogenicity and adaptability of members of the genus *Staphylococcus*. The acquisition of essential nutrients such as iron and copper is vital for the survival and virulence of Staphylococcus species, especially in iron-limited environments like host tissues where metals are tightly bound by proteins such as transferrin and lactoferrin. In non-*aureus* staphylococcal strains, metal uptake genes, including those for siderophores and iron transport systems, play a crucial role in enabling bacterial growth and regulating virulence-associated functions^{53,54}. The identification of genes such as *vctC* for iron uptake and *ctpV* for copper regulation underscores the importance of metal acquisition in the pathogenic potential of NASM strains, particularly in bovine mastitis. The presence of *vctC* in all *S. xylosus* strains highlights the essential nature of iron uptake, while the strain-specific occurrence of *ctpV* in *S. epidermidis* K60 suggests unique adaptations to copper stress.

The type VII secretion system (T7SS) has been recently identified and selectively distributed in various pathogens, including *Mycobacterium tuberculosis* and *S. aureus*. The T7SS plays an essential role in the virulence of human pathogens. In the case of *M. tuberculosis*, the T7SS is very important for bacterial access to the host cytosol. In *S. aureus*, T7SS exports several virulence-associated proteins⁵⁵. The presence of T7SS cluster was reported in *S. lugudensis*, in addition to *S. aureus*⁵⁶. Staphylococci possess genes enabling the production of

capsular polysaccharides that form a protective shield against phagocytosis by the host's immune cells. This capsule enhances virulence and bacterial persistence, highlighting encapsulation as a crucial mechanism for evading immune detection and contributing to pathogenicity. The *capB* and *capC* genes were detected in all NASM species except *Mammaliicoccus* spp. and *S. pseudointermedius*.

Prophages are responsible for the horizontal gene transfer, which in turn contributes to virulence^{57,58}. The prophage *Staphylococcus* StB20 was found in *S. xylosus* strain SMG24. This prophage is reported to exhibit specific proteolytic cleavages in the carboxy-terminal degradation of its tail tape measure proteins (TMP) in *S. aureus*⁵⁹. However, the roles of prophages NASM strains are largely unknown.

Genomic islands (GIs) are horizontally transferred regions carrying specific genes that confer certain traits to bacteria. These traits include metabolic processes, pathogenicity, antibiotic resistance, and symbiosis. GIs help bacteria establish mutually beneficial relationships with eukaryotic hosts. They often carry genetic material related to virulence or adaptive traits and are commonly located near tRNA or transposase genes at one end of the island. Antibiotic-resistant genes found in GIs make them carriers for the spread of antibiotic resistance, enhancing bacterial species' survival when exposed to antibiotics⁶⁰. Coupling virulence or adaptive traits with antibiotic resistance in GIs plays a significant role in shaping bacterial populations' resilience and adaptability to environmental challenges, especially when exposed to antibiotics. In the 22 NASM that we studied, most islands carried the two-component regulatory system, which includes the *walRK* gene. This gene plays a crucial role in regulating the expression of genes associated with cell wall metabolism, influencing autolysis, biofilm formation, and virulence. *WalK* is also involved in sensing the D-Ala-D-Ala moiety of Lipid II, serving as a signal for active cell wall synthesis. The genes *yyCH* and *yyCI* are co-transcribed with *walRK* and modulate its activity. In *S. aureus*, disrupting *yyCH* and *yyCI* genes downregulated the *walRK* regulon⁶¹. The importance of these regulatory components and their roles in governing cell wall-related processes vary across bacterial species. The *fosB*, *blaZ*, and several AMR genes were identified among the GIs, suggesting the possibility of transfer of these traits among the mastitis-associated pathogens.

Conclusion

Whole genome sequencing and phylogeny analysis of bovine mastitis-associated NASM strains isolated from India revealed species-specific and host-specific clustering. The study identified multiple genes responsible for antibiotic resistance and virulence factors. Certain virulence factors were found to be specific to particular species, and some were specific to particular STs. The analysis also found that some virulence and AMR genes were located in genomic islands, which suggests possible horizontal transfer events.

Data availability

NASM genome sequence used in this study has been deposited in NCBI with the following accession numbers: GCA_018986335.1, GCA_018996905.1, GCA_019334185.1, GCA_018997025.1, GCA_018997125.1, GCA_018967705.1, GCA_019149065.1, GCA_019149165.1, GCA_019149045.1, GCA_019193065.1, GCA_019148995.1, GCA_019334235.1, GCA_019429675.1, GCA_019149085.1, GCA_019149255.1, GCA_019149245.1, GCA_019149205.1, GCA_019334165.1, GCA_019100515.1, GCA_019165085.1, GCA_018967945.1, GCA_018968045.1.

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Author contributions

N.R.H., J.R., and S.I. designed the experiments; M.A. and S.C. propagated and characterized the NASM strains from the various States of India; V.R. and R.S. performed WGS and analysis; V.R., S.I., J.R., and N.R.H. wrote the paper. The authors read and approved the final manuscript.

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Declarations

Competing interests

We declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional information

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