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In vitro antimicrobial and antibiofilm activity of phage cocktail against *Mammaliicoccus sciuri*, a causative agent of bovine mastitis

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Research Article

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Abstract

In this research paper the *in vitro* antimicrobial and antibiofilm activity of phage cocktail against the coagulase negative *Mammaliicoccus sciuri* was investigated. Three *M. sciuri* isolates obtained from clinical bovine mastitis samples were characterized and identified by 16S rRNA gene sequencing. Bacteriophages with lytic activity against *M. sciuri* isolates were isolated from dairy farm effluents. Two typical phages were isolated using standard enrichment and plaque assay techniques, purified by polyethylene glycol precipitation, and morphologically characterized based on shape and size using transmission electron microscopy. This was followed by determination of host range using spot tests and stability to varying temperature, pH and UV treatment. The phage cocktail suppressed bacterial activity within 30 min of exposure. Crystal violet assay showed that the tested phages and their cocktail significantly reduced the biofilm biomass of all three *M. sciuri* strains compared to the untreated control *in vitro* within 24 h with a single dosing. Transmission electron micrography of the purified phage particle revealed an icosahedral head and a rigid contractile tail, characteristic of the class *Caudoviricetes*. The findings open new avenues in phage-based antimicrobial approaches for controlling contagious and teat skin opportunistic bacteria causing bovine mastitis.

Contagious bovine mastitis remains a significant impediment to the growth and sustainability of the dairy industry. Mastitis, the inflammation of mammary glands, is a prevailing ailment among dairy cattle, exerting substantial economic losses through reduced milk yield, compromised milk quality and increased veterinary expenses. Although various bacterial pathogens are associated with mastitis, staphylococci emerge as the most frequently isolated bacteria from infected bovine milk. Amid the plethora of mastitis-causing agents, the role of coagulase-negative staphylococci (CNS) in mastitis epidemiology remains underestimated, even though their implications, especially considering their zoonotic potential, are noteworthy.

Staphylococcus sciuri, initially identified by (Kloos *et al.*, 1976), is a versatile bacterium with a propensity for colonizing diverse environments. This bacterial species is inherently methicillin-resistant and carries various homologs of the methicillin-resistance gene *mecA* within its chromosomal DNA. *S. sciuri* strains have made their presence known in clinical settings, causing infections in both human and animal populations (Stepanovic *et al.*, 2003; Frey *et al.*, 2013). This intriguing combination of ecological adaptability and clinical relevance underscores the importance of further investigating the characteristics and implications of *S. sciuri* in various contexts. Recently, there has been a significant taxonomic revision, leading to the reassignment of several bacterial species, including *Staphylococcus sciuri*, *Staphylococcus fleurettii, Staphylococcus lentus, Staphylococcus stepanovicii, and Staphylococcus vitulinus*, to a novel genus known as *Mammaliicoccus*. Within this newly designated genus, *Mammaliicoccus sciuri* is currently identified as the type species (Madhaiyan *et al.*, 2020).

An alternative method for controlling pathogenic *M. sciuri* infections is the utilization of bacteriophages, viruses that specifically target and destroy these bacteria. This promising approach offers a highly targeted and potentially effective means of combating *M. sciuri* while minimizing the risk of antibiotic resistance development. A temperate phage named ZCSS1 was isolated from raw milk (Makky *et al.*, 2023), with remarkable lytic activity against multidrug-resistant *M. sciuri*. The phage was specific to the bacteria and possessed significant antibiofilm activity.

No studies on lytic phages infecting mastitis causing *M. sciuri* have been reported so far. This study addresses this critical gap in our understanding by investigating the efficacy of a lytic bacteriophage against *M. sciuri* isolates responsible for mastitis.

Materials and methods

Isolation of CNS from infected milk

Twenty-one milk samples from cases of bovine mastitis were aseptically collected in sterile bottles and transported to the laboratory at 4°C. The milk samples were streaked onto BHI (brain heart infusion, Himedia, Mumbai) agar and incubated aerobically at 37°C for 24 h. The bacterial isolates were sub-cultured to obtain pure colonies. Gram-positive cocci, that were catalase positive and oxidase negative were identified as *Staphylococci.* Coagulase activity was determined by tube coagulase test using rabbit coagulase plasma (Himedia, Mumbai). The coagulase activity was confirmed by amplification of the *Coa* gene using a primer pairs *coaf* 5′-ATA GAG CTG ATG GTA CAG G-3′ and *coar* 5′-GCT TCC GAT TGT TCG ATG C-3′ (Aslantaş *et al*., 2007).

Molecular characterization of CNS

The bacterial DNA of all three isolates was extracted using a Genelute™ bacterial genomic DNA kit (Sigma-Aldrich). The partial genome sequence of the amplicon derived from PCR targeting the 16S rRNA gene using universal primer pairs 27F (5′ AGAG TTTGATCCTGGCTCAG3′) and 1492R (5′ GGTTACCTTGTT ACGACTT 3′) (Weisburg *et al*., 1991) helped identify the species. The sequences were purified and sequenced at Gene Spec Pvt. Ltd., Kochi. The phylogenetic tree of the three isolates was constructed by the neighbor-joining method using MEGAX software.

Antibiotic sensitivity testing (AST)

Sensitivity testing of the three identified *M. sciuri* strains was performed by the disk diffusion method (Bauer *et al*., 1966). The test was performed as per CLSI (2023) guidelines using Mueller-Hinton agar and antibiotic disks manufactured by Himedia, Mumbai, India. Antibiotic sensitivity of the isolates were determined against azithromycin (15 µg), amoxycillin-clavulanate (30 µg), ampicillin (10 µg), gentamicin (10 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), tetracycline (30 µg), co-trimoxazole (25 µg), cefoperazone (75 µg), amikacin (30 µg), doxycycline hydrochloride (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), linezolid (30 µg) and levofloxacin (5 µg) disks. The results were interpreted based on the antimicrobial susceptibility interpretation chart.

Evaluation of biofilm formation

Congo red agar prepared by adding sucrose (50 g/l) and Congo red indicator (8 g/l) aided in detecting biofilm production. Congo red agar plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production (Freeman *et al*., 1989).

Isolation of bacteriophages against M. sciuri

To isolate bacteriophages effective against *M. sciuri* strains, sewage samples from dairy farms with reported cases of bovine mastitis were collected. The phages were isolated using multiple host enrichment techniques (Vaiyapuri *et al*., 2021). Briefly, the samples were centrifuged at $2200 \times g$ to collect the supernatant. Simultaneously, in 200 ml of nutrient broth containing 10 mM $CaCl_2$, activated cultures of *M. sciuri* were inoculated at a rate of one per cent. The supernatant was then aseptically transferred and incubated overnight in a rotary shaker at 100 rpm. Following incubation, the mixture was centrifuged at 8000 *g* for 20 min at 4°C. The supernatant was filtered through a 0.45 µm pore size mixed cellulose esters membrane syringe filter (Merck Millipore Ltd., Cork, Ireland) and stored at 4°C for further analysis (Feyereisen *et al*., 2019).

The presence of phage was evaluated using the spot test and further confirmed using a double agar overlay method (Sambrook *et al*., 2001). The presence of phages resulted in the development of plaques in the plate. The phages were quantified using the formula PFU/ml = number of plaques/D × V, where PFU/ml is the number of phages in plaque-forming units/ml, D is the dilution factor, and V is the volume of supernatant solution. Phages were purified by polyethylene glycol (PEG) precipitation (Sambrook and Russell, 2006) by treating 20 ml of each filtered phage supernatant with 10% (w/v) PEG8000 (Merck, Germany) overnight at 4°C to allow the phages to precipitate. The next day, after pelleting the samples by centrifugation at $11\,000 \times g$ for 10 min at 4°C, the supernatant was decanted without disturbing the pellet. The pellet was resuspended in 5 ml of SM Buffer (Himedia), added to an equal volume of chloroform, and vortexed for 30 s to remove PEG and cell debris. The phage solution was then centrifuged at $3000 \times g$ for 15 m at 4°C. The phage-rich aqueous phase was stored at 4°C.

Determination of host range

Host rage of the phages against different coagulase-negative staphylococci and other pathogens was determined by spot-testing (Oliveira *et al*., 2017) against the bacterial isolates stated in online Supplementary Table S2. To 10 ml of top agar (0.7% agarose) supplemented with one drop of 10 mM calcium chloride, 100 µl of bacterial overnight culture was added and mixed well. This solution was then poured onto a Petri dish containing nutrient agar (used as the bottom agar) and gently swirled to ensure even distribution. On the top agar, ten microliter of a purified phage solution was spotted (10^8 PFU/ml). Plates were solidified and incubated inverted at 37°C for 18 h. A clear zone at the applied site indicated a positive reaction and a negative if no clearance appeared.

Morphological characterization of phages

Transmission electron microscopy (TEM) aided in describing the morphological features of the phages. On a carbon-coated TEM grid, 100 µl of PEG-concentrated phages with a titer of 10 (PFU)/ml were placed and allowed to settle. After blotting off the excess sample, 2% phosphotungstic acid (pH 7) was used to negative-stain the phage particles. HT7700 transmission electron microscope operated at Sophisticated Analytical Instrument Facility (SAIF) at ICAR-IIHR located at Hesaraghatta, Bengaluru, was employed for the purpose.

Physical characterization of phages: thermal stability

The phage suspension was incubated in a water bath maintained at 20, 30, 40, 50, 60, 70 and 80°C for 4 h followed by cooling and determining the phage activity by spot test (Shaaban *et al*., 2020).

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Physical characterization of phages: tolerance to pH

Purified phage suspension was dispensed in sterile TSB broth adjusted to pH values ranging from 2 to 10 adjusted using 0.1 N HCl and 0.1 N NaOH. After incubating the mixture for 10 m, the residual phage activity was determined by the spot test (Shaaban, *et al.*, 2020).

Physical characterization of phages: stability to UV radiation

The purified phage suspension was exposed to a UV lamp with a wavelength of 210 nm for 6 h at 15 cm from the lamp, followed by a spot test to detect the phage infectivity at intervals of 1 h (Shaaban, *et al.*, 2020).

Antibiofilm activity of phage

Initially, biofilms were grown on microtiter plates by adding 180 µl of diluted fresh suspension of *M. sciuri* strains in a 96-well microtiter plate and incubating at 37°C for 48 h. After incubation, the biofilm was washed twice with phosphate-buffered saline to remove planktonic bacteria. Phage ϕ-*M. sciuri* D at a MOI of one (final concentration of 10^7 PFU/ml), Phage ϕ-*M. sciuri* A at a MOI of 10 (final concentration of 10^9 PFU/ml) and a mixture of ϕ-*M. sciuri* D and ϕ-*M. sciuri* A (phage mixture) was added to the wells with preformed biofilm. One well with biofilm without phage treatment was the positive control, while a well with sterile TS broth was the negative control. After incubation at 37°C for 18 h, wells were decanted, washed with phosphate buffered saline, stained with 0.1% crystal violet for 15 min and rinsed with distilled water. After air-drying the plates, adding 30% glacial acetic acid eluted the stain. The absorbance was measured at 600 nm using a microplate reader (Thermoscientific) to quantify the biofilm biomass (Liu *et al.*, 2022).

In vitro *bacteriolytic activity*

The isolate *M. sciuri* ADMS3 was the most pathogenic among the three isolates based on its AST and biofilm production. For determining the bacteriolytic activity, phages (ϕ-*M. sciuri* D, ϕ-*M. sciuri* A and PM- Phage mixture) were co-cultured with *M. sciuri* ADMS3 (OD = 0.5) for 4 h in TSB cultures (1.71×10^6) at MOIs of 1 and 100 and incubated at 37°C at 180 rpm. In addition, sterile TSB formed the negative control. *M. sciuri* ADMS3 in TSB was the positive control. The absorbance (OD600) of the culture broth was measured every 30 min after the onset of incubation. A decrease in absorbance compared to positive control at each measurement time point indicated the bacteriolytic activity (Liu, *et al.*, 2022).

Isolation of bacteriophage-insensitive mutants

Phage-resistant mutants were isolated to investigate the potential causes of reduced ϕ *M. sciuri* A activity (Habusha *et al.*, 2019). Activated culture of *M. sciuri* ADMS3 (OD = 0.5) mixed with ϕ *M. sciuri* A at a MOI of 100 was incubated at 37°C for 10 m for phage adsorption and infection. Subsequently, this mixture was added to 4 ml of 0.4% nutrient agar, gently mixed, and poured into a Petri plate containing bottom agar. Incubation was carried out at 37°C for 24–48 h until bacteriophage-insensitive mutants (BIMs) became visible. A single BIM was selected and stored in 50% glycerol in TSB at −80°C. BIMs were later tested for phage sensitivity by spot test (Liu, *et al.*, 2022).

Genomic data availability

The nucleotide sequences obtained were analyzed and compared with reference sequences available with the GenBank by NCBI-BLAST and submitted to https://www.ncbi.nlm.nih.gov/ with accession numbers (OQ919146, OQ834909, OQ919246).

Statistical analysis

All the data obtained in the experiment were analyzed statistically using SPSS software package (SPSS 22). The categorical variables were summarized using frequency and percentage. A phylogenetic tree was constructed using the neighbor joining method in MEGAX Software (version 10.2.6).

Results

Characterization of CNS

Among the twenty-one isolates obtained from culturing the milk samples, three were Gram-positive, catalase-positive, oxidase-positive and coagulase-negative. The coa gene was absent on PCR testing (Fig. 1a). Amplification of the 16S *rRNA* gene produced 1465 bp amplicons (Fig. 1b). The NCBI BLAST analysis of the three isolates, ADMS1(OQ919146), ADMS3(OQ834909) and ADMS5(OQ919246) showed similarity 95.29 and 99.93% respectively to *Staphylococcus sciuri (Syn. Mammaliicoccus sciuri)*. The sequences were deposited at NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with accession No. https://www.ncbi.nlm.nih.gov/nuccore//. The phylogenetic tree constructed using MEGAX software (Fig. 1c) depicts the evident diversity between the isolates. Based on AST, the isolate ADMS3 was resistant to amikacin, amoxicillin-clavulanate, ampicillin, and tetracycline. The isolate ADMS1 showed resistance to amikacin, ampicillin, and tetracycline, while ADMS5 was resistant to amoxicillin-clavulanate, ampicillin, and cefotaxime. (online Supplementary Table S1). All three isolates showed positive biofilm formation, as evidenced by the production of black colonies with a dry crystalline consistency in Congo red agar (online Supplementary Fig. S1).

Characterization of phages

Two phages, namely ϕ-*M. sciuri* D and ϕ-*M. sciuri* A were isolated from the pooled effluent samples. The phage showed lytic activity against all three *M. sciuri* isolates. Analysis revealed that the phage ϕ-*M. sciuri* D had an icosahedral head 163.37 nm long and 193.15 nm wide. The tail was long and contractile (267.14 nm length and 66.95 nm width) with short fiber, classified as a member of the Myoviridae family of the order Caudovirales. And ϕ-*M. sciuri* A had an icosahedral head of 89.0 nm (length), 76.3 nm (width), a long contractile tail with 122.5 nm (length), and 23.3 nm (width) with long fiber (online Supplementary Fig. S2). So, it belongs to the family Siphoviridiae and the order Caudovirales. All-tailed bacterial and archaeal viruses with icosahedral capsids and double-stranded DNA are currently assigned to the class *Caudoviricetes* (Turner *et al.*, 2023).

Determination of host range

A spot test and a subsequent plaque assay were performed to determine the host range of the two phages against coagulase-negative isolates and other pathogenic strains (online Supplementary Table S2). Online Supplementary Figure S3 shows the

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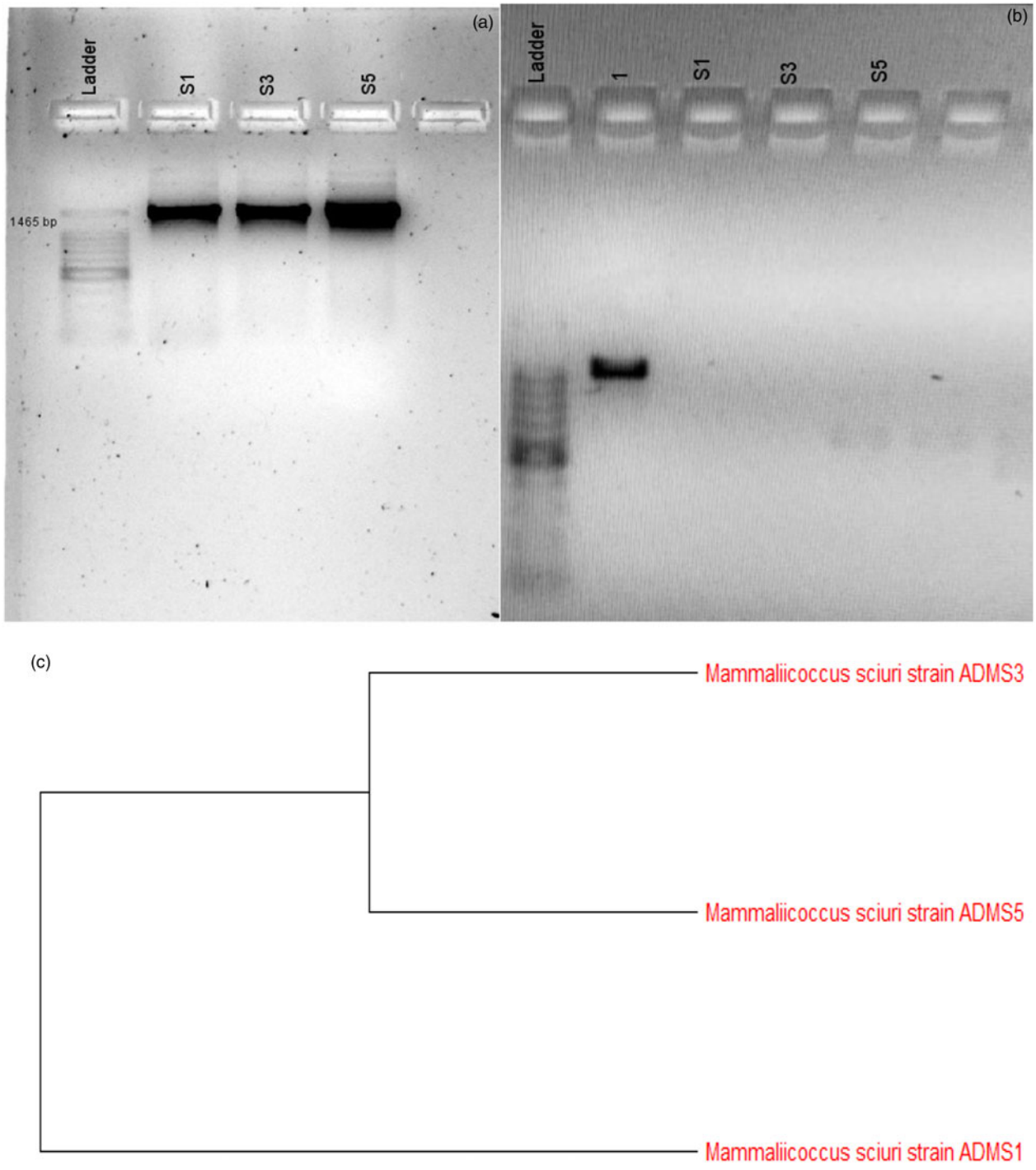

Figure 1. PCR: (a) 16 S rRNA gene targeting products amplified by 27F/149R primers. (b) Amplification of *coa* gene of *Staphylococci* 1-positive control. S1-ADMS1, S3-ADMS3, S5-ADMS5 (c) Phylogenetic tree, which depicts the diversity between the isolates.

interpretation of spot test results. From this, it is very evident that ϕ-*M. sciuri* A could infect isolates ADMS 1, 3, 5, 16 and *S aureus* MTCC 96 and ϕ-*M. sciuri* D could infect only ADMS 1, 3, 5, 16.

Stability of phages

The phage ϕ-*M. sciuri* D was stable between 10 and 50°C, while no lytic activity observed at 60 and 70°C. The phage ϕ-*M. sciuri* D was lytic between pH 2 and 9. The exposure of phages to UV rays did not result in loss of lytic activity for up to 4 h. Phage ϕ *M. sciuri* A was lytic from 10 to 60°C and no lytic activity was observed at 70°C. ϕ *M. The phage sciuri* A was stable at a pH from 2 to 10 and retained the lytic activity post-UV exposure for up to 3 h.

Antibiofilm activity

Crystal violet assay to determine the antibiofilm activity of the phages Φ-*M. sciuri* D and an against *M. sciuri* ADMS1,

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ADMS3, and ADMS5 resulted in the reduction of biofilm following exposure to the phages at 10^7 PFU/ml for 24 h (online Supplementary Fig. S4). From the figure, statistical analysis revealed that the PM alone could significantly ($P < 0.05$) reduce biofilm activity which depends on strains.

In-vitro *bacteriolytic activity*

The *in-vitro* phage inhibition assay of *M. sciuri* ADMS3 with phage ϕ-*M. sciuri* D (MOI = 1), ϕ-*M. sciuri* A (MOI = 10), PM (A + D) demonstrated a reduction in bacterial growth at 60 and 270 min. Interestingly, PM reduced the bacterial growth at 30 min (online Supplementary Fig. S4). Further, co-incubation of *M. sciuri* ADMS3 and ϕ *M. sciuri* A in a double layer plate for more than 24 h resulted in observable colonies of varying sizes. These mutants showed no lysis zone on performing the spot test. These mutants were named S31–S35.

Discussion

Biochemical (Chen *et al.*, 2008) and molecular tests confirmed the identity of three *M. sciuri* isolates obtained from the infected milk samples. Notably, these isolates were resistant to penicillin, aminoglycosides and tetracycline. The prevalence of antimicrobial resistance in CNS is often higher than that of *Staphylococcus aureus*, although little is known regarding the antimicrobial resistance of CNS species (Taponen *et al.*, 2015). *In-vitro* resistance of the isolates to multiple classes of antimicrobials poses a significant challenge for treatment and effective control of contagious bovine mastitis. CNS species are known to mutate faster and be more receptive to the horizontal transfer of the genes coding antibiotic resistance than other mastitis-causing pathogens (Schukken *et al.*, 2009; Otto, 2013). *M. sciuri* is a biofilm-producing organism (Stepanović *et al.*, 2001), as evidenced in the Congo red agar culture. The organism is an opportunistic animal pathogen hypothesized to be a source of genes coding for virulence and antibiotic resistance for other staphylococci. *M. sciuri* strains frequently carry homologs of the *mecA* gene, which codes for a PBP2a-like protein linked to methicillin resistance found on the staphylococcal cassette chromosome *mec*(SCCmec) (Adegoke, 1986; Devriese, 1990; Couto *et al.*, 2003; Nemeghaire *et al.*, 2014), which could be the underlying reason for the beta-lactam resistance profile among the isolates obtained in this study.

The study describes the isolation of two phages, ϕ-*M. sciuri* A and ϕ-*M. sciuri* D, which successfully infected three *M. sciuri* isolates obtained from cases of bovine mastitis. Considering phages for clinical use, it is crucial to establish their lytic nature and to ensure the absence of lysogenic genes that could lead to the incorporation of phage genetic material into the bacterial host (Gill and Hyman, 2010). Morphologically, ϕ-*M. sciuri* D belonged to the family Myoviridae and ϕ-*M. sciuri* A to family Siphoviridiae. Both families belong to the Order Caudovirales. Staphylophages in commercial preparations generally belong to the family Myoviridae (Kornienko *et al.*, 2020). Most CNS phages belong to the Siphoviridiae family (Deghorain and Van Melderen, 2012).

The revised 2022 taxonomy update by the International Committee on Taxonomy of Viruses bacterial virus subcommittee classified it as a member of the new class Caudovirales (Turner, *et al.*, 2023). The phages were stable at extremely diverse conditions such as temperature, pH, and UV exposure, therefore, these phages can resist challenging conditions. When considering phages for clinical trials, such variables should be accounted for. The host range of phages was restricted to *M. sciuri* species, indicating the high specificity and narrow spectrum of both phages. Furthermore, both phages significantly reduced the biomass of 48-h-established *M. sciuri* biofilms *in vitro*. Interestingly, ϕ-*M. sciuri* D exhibited better antibiofilm capabilities compared to ϕ-*M. sciuri* A, as observed from the graphical representation (online Supplementary Fig. S5).

The PM demonstrated efficient biofilm-reducing properties comparable to ϕ-*M. sciuri* D and superior to ϕ-*M. sciuri* A. Phages exerted a significantly high bacteriolytic activity against the ADMS3 strain compared to the other two strains, highlighting the importance of this specific strain in future studies. Evaluation of the *in-vitro* bacteriolytic activity of both phages individually and in combination against ADMS3 indicated that ϕ-*M. sciuri* D and the PM efficiently reduced bacterial growth within 60 and 30 m, respectively. In comparison, ϕ-*M. sciuri* A was less efficient against ADMS3. Assuming BIM (bacteriophage insensitive mutants) could have resulted in lower efficacy of this phage, we attempted and successfully isolated BIM from ADMS3. The isolation of BIM throws light on the bacterial resistance mechanisms against phage attacks. Studies are needed to evaluate the characteristics of these mutants in terms of their activity. This study highlights the potential of phage therapy as an effective and targeted strategy for preventing and controlling bovine mastitis caused by *M. sciuri* strains, particularly in the context of their increasing antimicrobial resistance and biofilm-producing capabilities. Future research should focus on optimizing phage cocktails and understanding bacterial resistance mechanisms to further enhance the efficacy of phage therapy in clinical applications.

Our study aimed to isolate and characterize bacteriophages against mastitis-causing *M. sciuri* strains. The phages were efficient individually and in combination, particularly against biofilms. The findings suggest a potential application of phages to control bovine mastitis caused by teat skin opportunistic pathogens. We also report the occurrence of BIMs warranting further investigation into phage cocktails. The findings underscore the potential of phage therapy as a rapid and effective option for preventing and controlling bovine mastitis.

In conclusion, three coagulase-negative staphylococci (CNS) isolates, ADMS1, ADMS3, and ADMS5, were isolated and characterized from cases of subclinical mastitis in cows. Sequence analysis of the 16S rRNA gene confirmed their identity as *M. sciuri* with 95.29 and 99.93% similarity. These isolates were resistant to multiple antibiotics, including amikacin, amoxicillin-clavulanate, ampicillin, and tetracycline, pointing to the challenges in managing bovine mastitis caused by antimicrobial-resistant CNS species. Additionally, two phages, ϕ-*M. sciuri* D and ϕ-*M. sciuri* A were isolated from the effluents collected from the farms infected with the *M. sciuri* isolates. ϕ-*M. sciuri* D belongs to the Myoviridae family and ϕ-*M. sciuri* A to the Siphoviridiae family. The stability studies revealed that ϕ-*M. sciuri* D was stable between 10 and 50°C, pH 2 to 9, and against UV exposure up to 4 h. ϕ-*M. sciuri* A was stable from 10 to 60°C, pH 2 to 10, and post-UV exposure for up to 3 h. While ϕ-*M. sciuri* A demonstrated a broader host range, infecting isolates ADMS 1,3,5, 16, and S. aureus MTCC 96, ϕ-*M. sciuri* D displayed a narrower host range, infecting only ADMS 1, 3, 5, 16. Increased stability and narrow host specificity suggest the potential for targeted therapeutic applications against *M. sciuri*. Both ϕ-*M. sciuri* D and ϕ-*M. sciuri* A exhibited significant antibiofilm activity against *M. sciuri* strains, indicating their potential efficacy in combating biofilm-producing *M. sciuri* strains. Despite the promising antibacterial activity observed in the phage

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inhibition assays, the emergence of phage-resistant mutants (S31–S35) underscores the need for continued research into phage cocktails to overcome bacterial resistance mechanisms.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029924000384.

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