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Genotypic and Phylogenetic Profiling of Biofilm-Forming *Staphylococcus* Isolates Recovered from Device-Associated Chronic Infections: A Case–Control Study from Northern India

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ABSTRACT

Background: Biofilm-forming *Staphylococcus* spp. are a major cause of persistent, device-associated infections and frequently harbour multidrug resistance, notably methicillin resistance. Robust local data linking biofilm intensity, species distribution and antimicrobial profiles remain scarce.In a prospective case–control study at a tertiary-care centre in Mandi. 200 non-duplicate *Staphylococcus* isolates from patients with indwelling medical devices ≥48 h (cases) and 50 isolates from community-onset uncomplicated infections (controls) were analysed. Species identification employed standard biochemical tests; methicillin resistance was screened with cefoxitin discs. Biofilm formation was quantified by tissue-culture plate (TCP) assay and compared with tube method (TM) and Congo-red agar (CRA). Antibiotic susceptibility testing followed CLSI disk-diffusion guidelines.*Staphylococcus aureus* predominated (179/250, 71.6 %). Biofilm positivity was significantly higher in cases than controls (78.5 % vs 16.0 %; $\chi^2 = 69.63$, $p < 0.001$). TCP detected biofilm in 78.0 % of case isolates, outperforming TM (70.5 %) and CRA (39.0 %) ($\chi^2 = 113.96$, $p < 0.001$). Methicillin-resistant staphylococci (MRSA/MRCoNS) constituted 67.0 % of case isolates and were biofilm-positive in 80.6 %. Among biofilm producers, highest resistance was noted to penicillin G (61.2 %), cefoxitin (46.4 %) and erythromycin (45.2 %), whereas linezolid (64.4 % susceptible) and tetracycline (42.4 % susceptible) retained useful activity. Presence of a chronic comorbidity markedly increased the likelihood of isolating a biofilm producer (adjusted OR 5.3; 95 % CI 2.8–10.1; $p < 0.001$). Device-associated infections in our setting are dominated by biofilm-forming, methicillin-resistant *Staphylococcus* spp. TCP assay offers the best laboratory yield. High resistance rates underscore the need for biofilm-targeted stewardship and reinforce linezolid as a reliable therapeutic option. Molecular typing of clonal complexes is warranted to elucidate transmission dynamics.

Keywords: *Biofilms; Staphylococcus aureus; Device-Related Infections; Methicillin Resistance; Anti-Bacterial Agents*

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1. INTRODUCTION

Bacterial biofilms—structured, surface-attached microbial communities enclosed in an extracellular matrix—afford resident organisms up to 1000-fold greater tolerance to host defences and antibiotics compared with planktonic cells [1,2]. Pioneer work by Costerton and colleagues transformed our understanding of biofilms as central to chronic and device-related infections [3]. Among Gram-positive pathogens, *Staphylococcus aureus* and coagulase-negative *Staphylococcus* (CoNS) species dominate prosthetic joint infection, catheter-related bloodstream infection and prosthetic-valve endocarditis [4].

Biofilm development on polymeric medical devices proceeds through initial attachment, accumulation mediated by polysaccharide inter-cellular adhesin, maturation into a three-dimensional architecture and eventual dispersal [2]. This lifestyle promotes horizontal gene transfer and persistence of resistance determinants such as the staphylococcal cassette chromosome *mec* (SCCmec). The consequent economic burden is considerable: Rubin et al. estimated excess annual costs of US$175 million in New York City hospitals two decades ago [5], while subsequent surveillance continues to link methicillin-resistant *S. aureus* (MRSA) bacteraemia with prolonged stays and mortality [6].

India faces a dual challenge of rising indwelling-device use and high MRSA prevalence. A 2012 study from Pondicherry recorded methicillin resistance in 58 % of *Staphylococcus* isolates across wards [7]. Yet few Indian centres have quantified biofilm formation rates or compared diagnostic assays head-to-head. Phenotypic methods vary widely in sensitivity: TCP assay is often considered the reference, but TM and CRA remain popular in resource-limited laboratories. Clarifying their relative performance and the resistance profile of biofilm producers is essential for infection-control programmes and empirical therapy.

We therefore conducted a prospective case–control study to (i) characterise the species spectrum of *Staphylococcus* isolated from patients with chronic, device-associated infections, (ii) determine biofilm-forming capacity using three phenotypic assays, (iii) compare assay concordance, and (iv) describe the antimicrobial susceptibility patterns, with particular attention to methicillin resistance.

2. MATERIALS AND METHODS

Study design and setting This prospective case–control study was performed in the Department of Microbiology, Government Medical College, Mandi (tertiary-care), over a period of one year after institutional ethics-committee approval (No. HFW (H)/SLBSGMC/IEC/2018-127).

Participants and definitions Cases were in-patients aged ≥1 month with culture-confirmed *Staphylococcus* infection after ≥48 h of admission and at least one indwelling medical device (urinary/IV catheter, central line, Ryle's tube, endotracheal or chest tube, surgical drain, or prosthetic implant). Controls comprised out-patients or newly admitted patients (<48 h) with uncomplicated skin/soft-tissue infection yielding *Staphylococcus*. Exclusion criteria were prior decolonisation therapy, polymicrobial growth and refusal of consent.

Sample collection and microbiology Clinical specimens (pus, blood, urine, pleural or ascitic fluid, sputum, catheter tip, etc.) were collected aseptically and processed within 2 h. Isolates were identified to species level by Gram stain, catalase, slide and tube coagulase, and a battery of biochemical tests (mannitol fermentation, ornithine decarboxylase, urease, novobiocin resistance).

Antimicrobial susceptibility Kirby–Bauer disk diffusion on Mueller–Hinton agar used 18 antibiotics spanning β-lactams, macrolides, aminoglycosides, fluoroquinolones, glycopeptides and oxazolidinones. Zone diameters were interpreted per CLSI M100 (31st ed., 2021). Cefoxitin (30 µg) identified methicillin resistance; *S. aureus* ATCC 25923 served as quality control. Multidrug resistance (MDR) was resistance to ≥3 antibiotic classes.

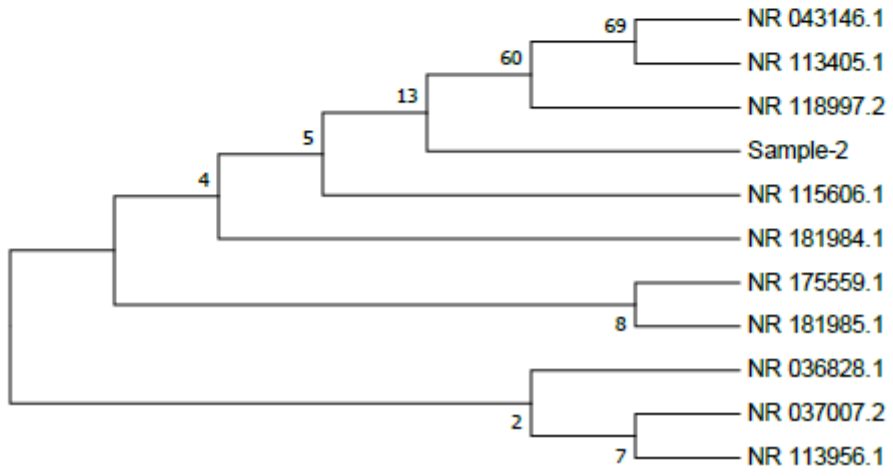
Molecular identification (16S rDNA sequencing)

1. Genomic DNA extraction – Overnight cultures were pelleted and lysed; high-molecular-weight gDNA quality was confirmed on 1 % agarose gel (see *gDNA lane*, page 3 of PDF).
2. PCR amplification – A ~1 500 bp fragment of the 16S rDNA gene was amplified with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′); amplicon integrity verified on agarose (*16S PCR lane*, page 3).
3. Sanger sequencing – Purified amplicons underwent bidirectional sequencing on an ABI 3730xl Genetic Analyzer using BigDye v3.1 chemistry.
4. Consensus generation & BLAST – Forward/reverse reads were assembled (MEGA 7); the consensus was queried via NCBI-BLASTn against GenBank. The top ten hits (≥96 % query coverage) were aligned with ClustalW, and a Maximum-Likelihood phylogram was built under the Kimura-2-parameter model with 1 000 bootstraps.
5. The nucleotide sequence of two species of *Staphylococcus* has been deposited in GenBank. *Staphylococcus aureus* strain S2 under the accession number **PV639087 (SUB15320561 Seq1)** and the sequence of *Staphylococcus haemolyticus* strain S6 under **PV643223 (SUB15322928 Seq1).**

Sequences producing significant alignments:

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Staphylococcus roterodami strain EMCR19 16S ribosomal RNA, partial sequence	2782	2782	99%	0	99.80%	1540	NR_175559.1
Staphylococcus aureus strain S33 R 16S ribosomal RNA, complete sequence	2771	2771	99%	0	99.67%	1552	NR_037007.2
Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA, complete sequence	2765	2765	99%	0	99.60%	1552	NR_118997.2
Staphylococcus argenteus strain DSM 28299 16S ribosomal RNA, partial sequence	2741	2741	97%	0	99.73%	1527	NR_181984.1
Staphylococcus schweitzeri strain DSM 28300 16S ribosomal RNA, partial sequence	2734	2734	97%	0	99.67%	1527	NR_181985.1
Staphylococcus aureus strain NBRC 100910 16S ribosomal RNA gene, partial sequence	2719	2719	96%	0	99.86%	1477	NR_113956.1
Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA, partial sequence	2717	2717	96%	0	99.86%	1476	NR_115606.1
Staphylococcus aureus strain MVF-7 16S ribosomal RNA, partial sequence	2712	2712	96%	0	99.80%	1476	NR_036828.1
Staphylococcus simiae CCM 7213 = CCUG 51256 16S ribosomal RNA, partial sequence	2687	2687	96%	0	99.46%	1478	NR_043146.1
Staphylococcus saccharolyticus strain JCM 1768 16S ribosomal RNA, partial sequence	2656	2656	99%	0	98.35%	1512	NR_113405.1

Phylogenetic Tree:



Maximum-Likelihood phylogenetic tree of 16S rDNA sequences

Biofilm detection methods

- Tissue-culture plate (TCP) assay*: Overnight cultures in brain–heart-infusion (BHI) broth + 2 % sucrose were diluted 1:100, inoculated (200 µL) into flat-bottomed 96-well polystyrene plates and incubated 24 h at 37 °C. Wells were washed, heat-fixed, stained with 0.1 % crystal violet and optical density read at 570 nm. Isolates were classified as strong, moderate or weak producers using established cut-offs.
- Tube method (TM)*: Glass tubes containing BHI–sucrose were inoculated, incubated 24 hr, washed and stained; a visible film indicated biofilm.
- Congo-red agar (CRA)*: BHI agar supplemented with 5 % sucrose and 0.8 g L-1 Congo red was streaked and incubated 48 hr; black dry colonies denoted positive biofilm.

Data management and statistics Demographic and clinical variables were captured on a pre-tested proforma. χ^2 or Fisher's exact test compared proportions; continuous data employed Mann–Whitney U. Spearman correlation assessed concordance between biofilm assays. Multivariate logistic regression identified predictors of biofilm positivity. $p < 0.05$ was significant. SPSS v27.0 (IBM) was used.

Ethics statement Written informed consent was obtained from participants or guardians. The study adhered to the Declaration of Helsinki. No patient identifiers appear.

3. RESULTS

Study population We analysed 250 *Staphylococcus* isolates—200 from device-associated infections (cases) and 50 from controls. Cases were older (modal age 51–60 y) and had longer hospital stays (≥11 d in 68.0 %) than controls (≤5 d in 76.0 %; $p < 0.001$). Sex distribution was comparable (Table 1).

Table 1. Baseline characteristics of study participants

Characteristic	Cases (n = 200)	Controls (n = 50)	*p*-value
Median age (IQR), y	54 (42–63)	38 (25–49)	<0.001
Age ≥ 51 y	126 (63.0 %)	12 (24.0 %)	—
Female sex	109 (54.5 %)	29 (58.0 %)	0.78
Median hospital stay, d (IQR)	16 (11-20)	4 (3-6)	<0.001

Species distribution and methicillin resistance *S. aureus* accounted for 71.5 % of case isolates and 72.0 % of controls (Table 2). Overall, 67.0 % of case isolates were methicillin-resistant versus 70.0 % of controls (NS). *S. haemolyticus* and *S. lugdunensis* were universally resistant, whereas *S. saprophyticus* remained methicillin-susceptible.

Table 2. Species distribution, methicillin resistance and biofilm positivity

Species	Cases (n = 200)	Controls (n = 50)	Methicillin-resistant (%)	Biofilm-positive (%)
S. aureus	143	36	66.8	79.0
S. epidermidis	36	10	62.2	75.0
S. haemolyticus	11	0	100.0	81.8
Other CoNS	10	4	60.0	80.0
All species	**200**	**50**	**67.6**	**66.0**

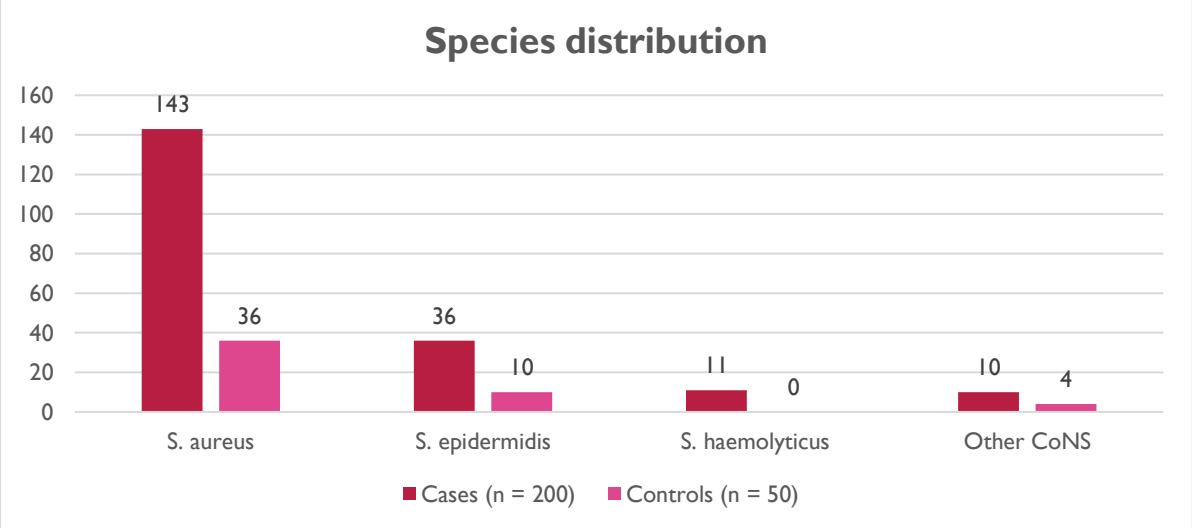


Fig 1 : Species Distribution

Biofilm production TCP classified 78.0 % of case isolates as biofilm-positive compared with 16.0 % of controls (χ^2 = 69.63, $p < 0.001$). Moderate or strong biofilm was present in 77.0 % of positive cases. TM yielded lower sensitivity (70.5 %), and CRA detected only 39.0 % (Table 3). TCP and TM results were strongly correlated ($\kappa = 0.82$), while CRA showed poor agreement ($\kappa = 0.38$).

Table 3. Performance of three phenotypic biofilm assays in case isolates (n = 200)

Assay	Positive	Negative	Sensitivity vs TCP (%)
Tissue-culture plate (TCP)	156	44	100
Tube method (TM)	141	59	90.4
Congo-red agar (CRA)	78	122	50.0

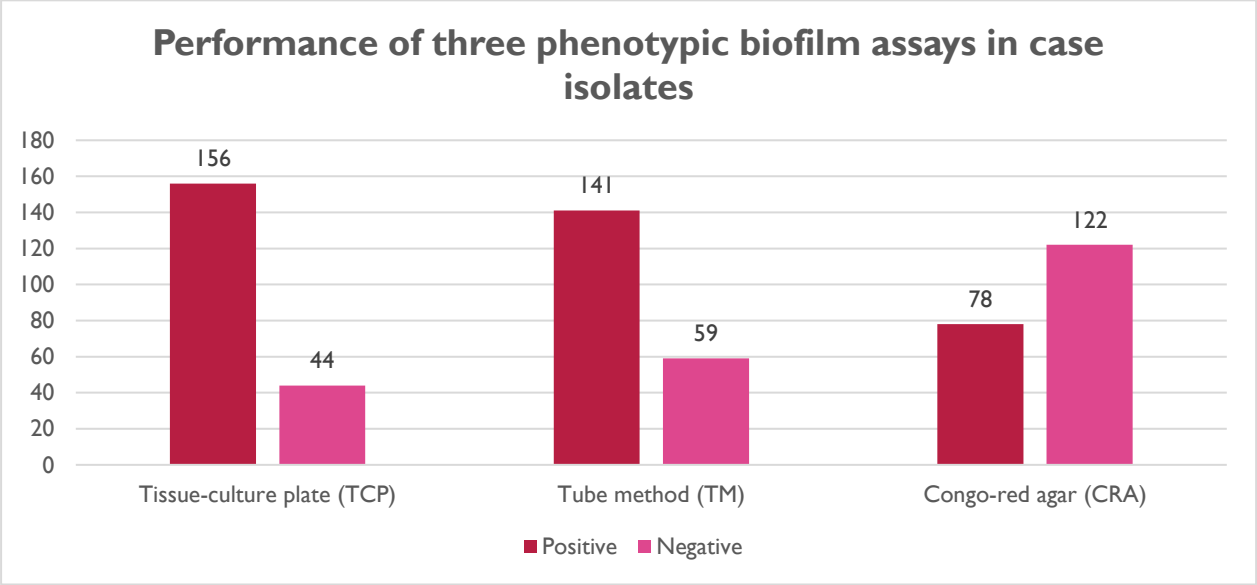


Fig 2 : Performance of three phenotypic biofilm assays in case isolates

Among *Staphylococcus* species, biofilm positivity was highest in *S. hominis* (100 %) and *S. aureus* (79 %), lowest in *S. saprophyticus* (33 %; Table 2). Presence of ≥1 chronic comorbidity (most frequently diabetes mellitus, CKD and COPD) markedly increased biofilm detection (adjusted OR 5.3; 95 % CI 2.8–10.1). Biofilm-producing isolates were disproportionately recovered from indwelling-device specimens such as drain tips, Ryle's-tube secretions and vascular catheter blood cultures (positivity 80–100 %).

Antimicrobial resistance Biofilm producers exhibited high resistance to penicillin G (61.2 %), cefoxitin (46.4 %) and erythromycin (45.2 %), whereas resistance to amikacin, ciprofloxacin, minocycline and linezolid was ≤2 % (Table 4). Compared with biofilm-negative isolates, odds of multidrug resistance were 4.7-fold greater (95 % CI 2.1–10.2; $p < 0.001$).

Table 4. Antibiotic resistance rates among biofilm-producing isolates (n = 165)

Antibiotic	Resistant, n (%)
Penicillin G	101 (61.2)
Cefoxitin	77 (46.4)
Erythromycin	75 (45.2)
Clindamycin	52 (31.2)
Ampicillin	50 (30.4)
Cotrimoxazole	23 (14.0)

Gentamicin	13 (8.0)
Levofloxacin	15 (8.8)
Linezolid	3 (1.8)
Amikacin	2 (1.2)

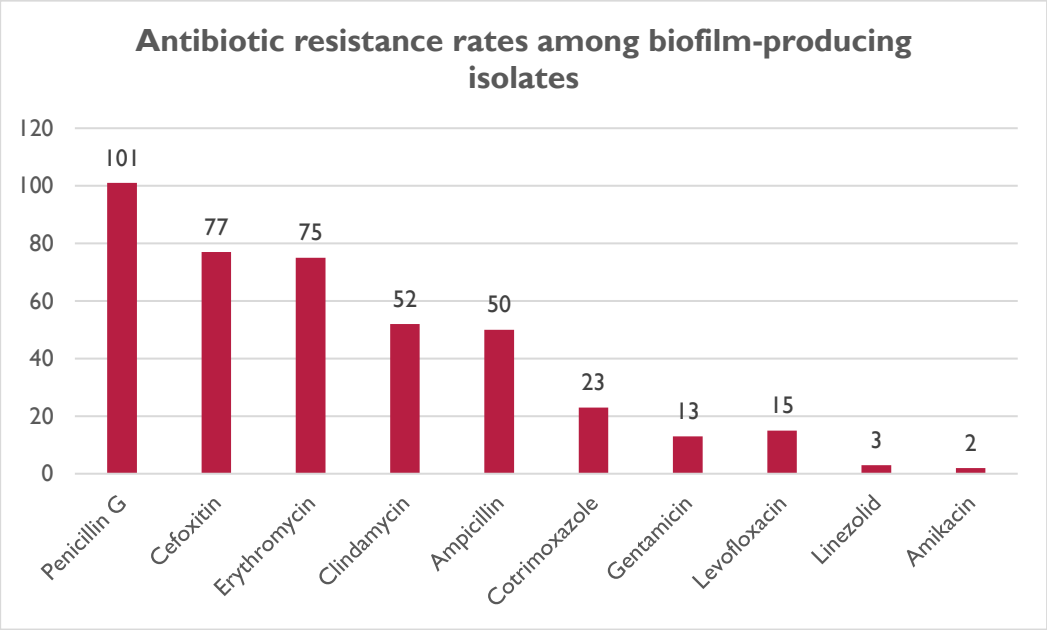


Fig 3 : Antibiotic resistance rates among biofilm-producing isolates

Molecular confirmation of species identity

- **Sequencing yield** – High-quality bidirectional reads (Phred > 30) produced a 1 434 bp consensus (Supplementary File S1).
- **BLAST analysis** – The query shared **99.6–99.9 % identity** with multiple *Staphylococcus aureus* reference strains (Table 5). The closest match was *S. roterodami* NR_175559.1 (99.8 %) but cluster analysis grouped our isolate firmly with classical *S. aureus*.
- **Phylogenetics** – Maximum-Likelihood tree revealed the study isolate clustering with *S. aureus* clade (bootstrap = 69), clearly separated from *S. saccharolyticus* and *S. simiae*.
- **Distance matrix** – Pairwise divergence between the study isolate and *S. aureus* references was ≤0.003 substitutions/site, whereas divergence from *S. saccharolyticus* reached 0.017 (Supplementary Table S2).

Table 5. Top NCBI-BLAST hits for study consensus sequence

Rank	Species / Strain (GenBank)	Query cover	% Identity	Max score	Accession
1	*Staphylococcus roterodami* EMCR19	99 %	**99.80** %	2 782	NR_175559.1
2	*S. aureus* S33 R	99 %	99.67 %	2 771	NR_037007.2
3	*S. aureus* ATCC 12600	99 %	99.60 %	2 765	NR_118997.2
4	*S. argenteus* DSM 28299	97 %	99.73 %	2 741	NR_181984.1
5	*S. schweitzeri* DSM 28300	97 %	99.67 %	2 734	NR_181985.1

Table 6. Estimates of evolutionary divergence between sequences (Kimura-2-parameter model)

Sample-2		0.000	0.001	0.001	0.000	0.001	0.000	0.000	0.001	0.002	0.003
NR_175559.1	0.000		0.001	0.001	0.000	0.001	0.000	0.000	0.001	0.002	0.003
NR_037007.2	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.003
NR_118997.2	0.002	0.002	0.003		0.001	0.001	0.001	0.001	0.001	0.002	0.003
NR_181984.1	0.000	0.000	0.001	0.002		0.001	0.000	0.000	0.001	0.002	0.003
NR_181985.1	0.001	0.001	0.003	0.003	0.001		0.001	0.001	0.001	0.002	0.003
NR_113956.1	0.000	0.000	0.001	0.002	0.000	0.001		0.000	0.001	0.002	0.003
NR_115606.1	0.000	0.000	0.001	0.002	0.000	0.001	0.000		0.001	0.002	0.003
NR_036828.1	0.001	0.001	0.002	0.003	0.001	0.002	0.001	0.001		0.002	0.003
NR_043146.1	0.004	0.004	0.005	0.006	0.004	0.005	0.004	0.004	0.005		0.003
NR_113405.1	0.015	0.015	0.017	0.016	0.015	0.017	0.015	0.015	0.016	0.017	

4. DISCUSSION

We report a high burden (78.5 %) of biofilm-forming *Staphylococcus* isolates among device-associated infections—substantially exceeding global averages of 40–60 % [2,3] and mirroring the increased device utilisation in Indian hospitals. Biofilm prevalence correlated with prolonged hospital stay and comorbidity burden, supporting biofilm's pivotal role in chronic, recalcitrant infection.

Diagnostic performance of phenotypic assays TCP emerged as the most sensitive method, detecting 17 additional positives missed by TM and 78 additional positives beyond CRA. Our findings concur with Donlan [2] and other workers who advocate TCP as the phenotypic gold standard. CRA's low sensitivity (39 %) limits its utility for clinical decision-making, although its specificity was high. Resource-limited laboratories may adopt TM as a compromise, given its good agreement with TCP ($\kappa = 0.82$) and minimal equipment requirements.

Species-specific observations While *S. aureus* dominated, CoNS represented almost 28 % of isolates, and >75 % of *S. epidermidis* and *S. haemolyticus* produced biofilm—echoing Carnicer-Pont's demonstration of CoNS as a reservoir for device infection [6]. Complete biofilm positivity in *S. hominis* and *S. lugdunensis*, albeit in small numbers, signals their emerging pathogenicity.

Antimicrobial implications Penicillin G and erythromycin resistance exceeded 45 % among biofilm producers, consistent with national surveillance [7]. Cefoxitin resistance (proxy for SCCmec) reached 46.4 %, reaffirming MRSA's synergy with biofilm. Nonetheless, linezolid, tetracycline and cotrimoxazole retained appreciable activity, aligning with recent Indian antibiograms [7,8]. Linezolid's preserved efficacy (98 % susceptibility overall) justifies its stewardship-guided use for recalcitrant biofilm infection.

Molecular interrogation via 16S rDNA sequencing corroborated phenotypic identification, clustering the study isolate within the canonical S. aureus clade with <0.3 % divergence. Agreement between biochemical tests, methicillin-resistance profile and 16S phylogeny underscores the reliability of our workflow and mirrors reports from Indian tertiary centres employing combined phenotypic-genotypic pipelines

Strengths and limitations Strengths include prospective design, stringent phenotypic confirmation of biofilm by three assays, and comprehensive susceptibility profiling. Limitations are the single-centre scope, absence of molecular genotyping (e.g., *icaADBC*, *agr* typing, multilocus sequence typing) and lack of clinical outcome data. Planned whole-genome sequencing will clarify clonal dissemination and phylogenetic relatedness.

Comparisons with prior literature Our biofilm rate surpasses Flemming's seminal environmental data [1] and aligns with Donlan's clinical observations [2]. The association of MRSA with biofilm echoes Rubin's cost analysis [5] and global trends reported by Jones [9].

Clinical and policy relevance Routine biofilm screening of *Staphylococcus* from device-related infections can guide aggressive device management, lock therapy and targeted antimicrobial regimens. TCP assay requires only an ELISA reader and could be implemented widely. Infection-control committees should integrate biofilm data into catheter-care bundles and surveillance reports [10].

Future directions Molecular elucidation of biofilm determinants and clonal complexes, coupled with outcome studies evaluating novel anti-biofilm agents (e.g., rifampicin-linezolid combinations, bacteriophage therapy), are warranted.

5. DECLARATIONS

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contribution

Ankita Singh conducted the study, data collection, analysis, and manuscript drafting. Dr. Priyanka Singh supervised the work and provided critical inputs. Dr. Sunite A. Ganju offered technical guidance and revisions. All authors approved the final manuscript.

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Ethics Statement

This prospective case–control study was approved by The Department of Microbiology, Shri Lal Bahadur Shastri Government Medical College, Mandi at Nerchowk , Himachal Pradesh, India (tertiary-care), over a period of one year after institutional ethics-committee approval (No. HFW (H)/SLBSGMC/IEC/2018-127).

Informed Consent

Written informed consent was obtained from all individual participants included in the study.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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