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Original Article

Biofilm forming multidrug resistant *Staphylococcus aureus* of dairy origin: molecular and evolutionary perspectives

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Abstract

Background: Biofilm production by *Staphylococcus aureus* is a prevailing cause of multidrug resistance. The evolutionary mechanisms of adaption with host and pathogenicity are poorly understood. **Aims:** The present study aimed to investigate the biofilm-forming potential, associated multidrug resistance, and the evolutionary analysis of *S. aureus* isolated from bovine subclinical mastitis. **Methods:** 122 *S. aureus* isolates were subjected to Congo red agar method (CRA), microtitre plate method (MTP), and PCR to check the biofilm-forming potential. The Kirby-Bauer disk diffusion method was used to evaluate the antibiotic resistance pattern. The *icaA* gene of isolates was subjected to molecular and evolutionary analysis using different bioinformatics tools. **Results:** The results showed that 63.93% of *S. aureus* isolates carried the *icaA* gene and the detection rate of CRA was higher (36.07%) compared to the MTP test (24.59%). A total of 78.21% and 56.41% of biofilm-positive isolates were methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), respectively. All *S. aureus* isolates (100%) showed multidrug resistance. The molecular analysis showed an evolutionary link between isolates and revealed a strong codon bias, three different recombination events, and positive selection in some residues of the semi-conserved segments of the *icaA* gene. **Conclusion:** The study concluded that biofilm-positive isolates have a high tendency to exhibit methicillin, vancomycin, and multidrug resistance. The findings suggest that mutation and selection are the most likely causes of codon bias in the *icaA* gene sequences. The variations led by recombination events and positive selection are suggestive of bacterial strategy to combat antimicrobial effects and to escape the host's immune surveillance.

Key words: Antimicrobial resistance, Biofilm, Evolutionary analysis, Mastitis, MRSA

Introduction

Staphylococcus aureus, belonging to the low G+C content group of the Firmicutes division of bacteria, is responsible for persistent udder infections in bovines leading to tremendous financial losses in terms of decreased milk yield and treatment cost. The pathogen is zoonotic and shows adverse effects on veterinary as well as public health (Chen *et al*., 2020). Biofilm formation is a potential virulence factor of *S. aureus* that is mainly facilitated by the intercellular adhesion genes (*icaA* and *icaD* genes) and is considered one of the most effective defense mechanisms of the pathogen (Ahmed *et al*., 2022).

The undiscriminating and undue usage of antibiotics (beta-lactams, fluoroquinolones, lincosamides, macrolides, and streptogramins) in veterinary as well as human medicine have raised the emerging issue of multidrug-resistant pathogens like methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (Yang *et al*., 2017). Moreover, biofilm production by *S. aureus* not only hinders the antimicrobial penetration but also leads to the horizontal transfer of antibiotic resistance genes within the biofilm (Shin *et al*., 2021) resulting in the reduced antimicrobial susceptibility of the pathogen (Thiran *et al*., 2018). Biofilm aids the bacteria to resist antimicrobials 10-1000 times more than planktonic cells (Begum *et al*., 2007). Biofilm-associated evasion from the host immune system (Fabres-Klein *et al*., 2015) and antimicrobial resistance helps bacterial survival in the udder and thus contributes to the prolonged sustainment of pathogen at farms (Melchior *et al*., 2006).

Molecular tools like sequencing and phylogenetic analysis, with certain limitations, have been reported to evaluate genetic diversity and to trace the evolutionary relationship between various *Staphylococcus* isolates (Abboud *et al*., 2021; Pizauro *et al*., 2021). Genetic diversity is primarily driven by two prime evolutionary processes, recombination and point mutation (Pond *et*

al., 2006). The alternative synonymous codons usage differs among species and even among genes from a single genome. The codon usage pattern in a gene imitates a complex balance among biases engendered by selection, mutation, and random genetic drift (Sharp *et al*., 2005). The codon usage variations among genes of a bacterial genome occur due to high variation in genomic G+C content, variation in mutation biases between the lagging and leading strands of replication, evidence of natural selection on codon usage in many species, and the evidence of extensive horizontal gene transfer among bacteria (Sharp *et al*., 2005). For evolutionary studies, the rapid identification of recombination and non-recombinant fragments in multiple sequence alignments is necessary which can be done using genetic algorithm recombination detection (GARD), one of the accurate programmatic approaches used (Pond *et al*., 2006).

Despite the emergence of multidrug-resistant *S. aureus*, the mechanisms of adaption with the host are poorly understood. The comparative sequence analysis of closely resembling bacteria from different origins, based on phylogenetic analysis, can provide information to understand the mechanisms of adaption with host and pathogenicity.

The current study is planned to investigate the prevalence as well as the antibiogram profiling of biofilm-producing *S. aureus* isolated from bovine subclinical mastitis. Moreover, the phylogenetic analysis of the biofilm-associated *icaA* gene of *S. aureus* was conducted to reveal the genetic diversity among various isolates while the codon usage bias, recombination signals assessment, and haplotype diversity were accessed to trace the evolutionary aspects of the pathogen involved in the adaption with host.

Materials and Methods

Sampling strategy

From January to December 2021, 384 milk samples were collected from 192 cattle and buffaloes located in 14 dairy farms in the vicinity of district Faisalabad, Pakistan (Fig. 1). The milk samples were collected aseptically using the guidelines of the National Mastitis Council, USA (Hogan *et al*., 1999), screened for subclinical mastitis by California mastitis test (Javed *et al*., 2023), and dispatched to the laboratory for the isolation of *S. aureus* as per standard microbiological procedures given by the National Mastitis Council.

Isolation and identification of *S. aureus*

The subclinical mastitis samples were processed for the isolation of *S. aureus* as per standard microbiological procedures given by the National Mastitis Council. The milk samples were initially swabbed on 7% sheep blood agar and were subjected to aerobic incubation at 37°C for 24-48 h. After incubation, the swabbed samples were examined for bacterial growth, colony characteristics, and hemolytic pattern. The presumptive *S. aureus* isolates were further confirmed by Mannitol fermentation, tube coagulase test (Javed *et al*., 2021), and presence of *nuc* and *coa* gene using the primers and conditions according to Louie *et al*. (2000) and Abdul *et al*. (2017), respectively.

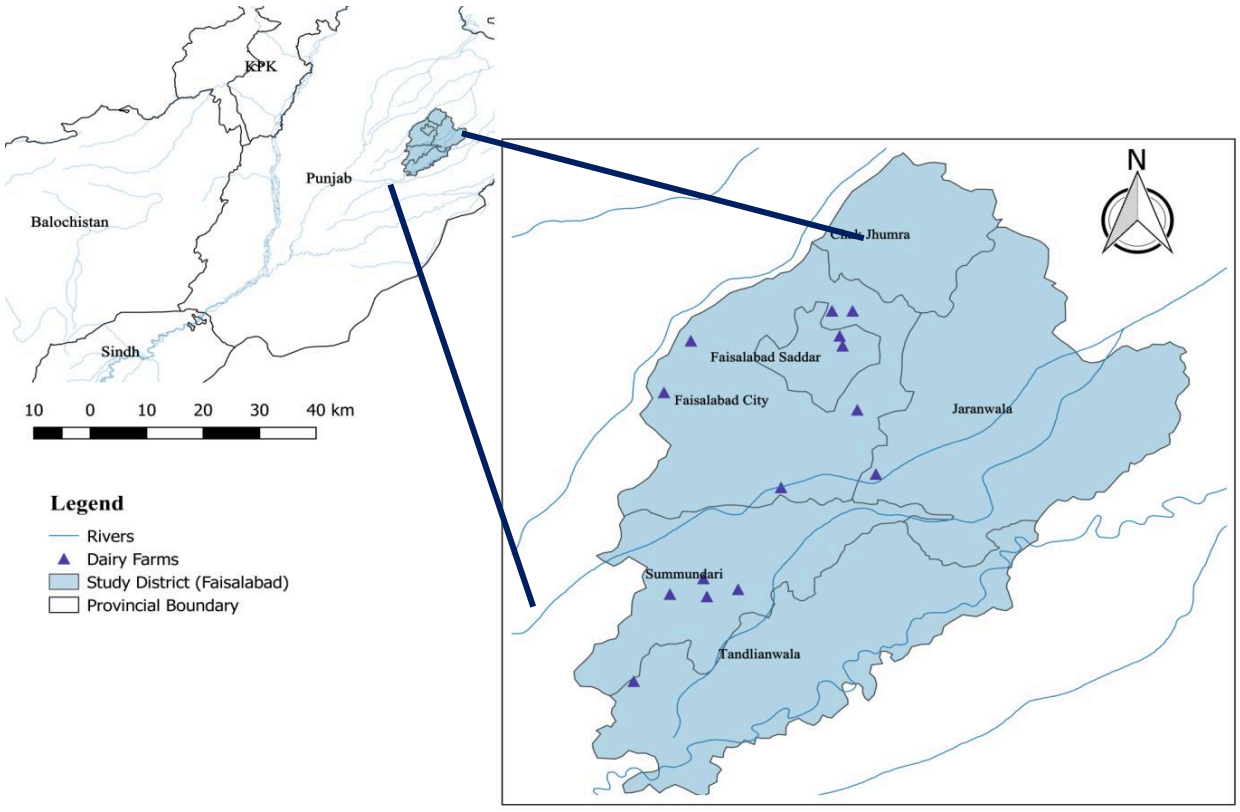

Fig. 1: The map shows the study of dairy farms in district Faisalabad, Pakistan

In vitro biofilm detection methods

The phenotypic and genotypic confirmed *S. aureus* isolates were subjected to biofilm detection methods. The Congo red agar (CRA) method and microtitre plate (MTP) method, both used for phenotypic detection of biofilm, were used. CRA method, being easy to perform, was used for qualitative detection while the MTP method, a more sensitive method, was used for quantitative assessment of biofilm-producing *S. aureus.*

For qualitative detection of biofilm formation, the *S. aureus* isolates were cultured using CRA method as described by Darwish *et al*. (2013). The isolates showing black color and rough edges colonies on CRA were considered biofilm-producers while colonies showing red or dark red color with smooth edges were assumed, non-biofilm producers.

For the microtitre plate (MTP) method, briefly, *S. aureus* colonies were inoculated in tryptic soy broth (TSB), incubated for 37°C overnight, diluted with fresh TSB in 1:100 ratio, followed by the filling of 96 wells of polystyrene plate with 200 µL of diluted broth and incubation for 24 h at 37°C. After incubation, the contents of each well were removed by gentle tapping followed by two times washing with PBS (200 µL), drying, and fixation of attached bacteria by the addition of methanol (200 µL) into wells. The plates were stained with 0.5% crystal violet (160 µL) for 15 min followed by rinsing under tap water and the addition of 95% ethanol (160 µL) to dissolve the stain bound with bacteria. The optical density (OD) of each well was measured at 570 nm using an ELISA reader. Cut-off OD (ODc) was calculated as the three standard deviations above the mean OD of the negative control. Biofilm-producing isolates revealed an OD value greater than ODc while the isolates showing an OD value less than ODc were declared non-biofilm producers (Darwish and Asfour, 2013).

Detection of biofilm-associated *icaA* gene

The DNA from bacterial colonies of confirmed *S. aureus* isolates was extracted using DNA extraction kit (Vivantis Technologies Sdn. Bhd, Malaysia) following the manufacturer's directions. The DNA samples were subjected to a PCR test for the intercellular adhesion (*icaA*) gene that is associated with the biofilm formation of *S. aureus*. A pair of primers (5´-CCT AAC TAA CGA AAG GTA G-3´ and 5´-AAG ATA TAG CGA TAA GTG C-3´) was used in the PCR, leading to a 1315 bp product (Vasudevan *et al*., 2003). The thermal PCR condition was initial denaturation at 92°C for 5 min followed by 30 cycles of denaturation at 92°C, annealing at 56.5°C each for 45 s, and elongation at 72°C for 1 min, and final extension at 72°C for 7 min. The resultant PCR products were subjected to gel electrophoresis (Ghumman *et al*., 2021). A 100 bp DNA ladder (Bioshop® Canada Inc.) was used as a molecular weight marker to visualize the resultant bands on the UV trans-illuminator. The isolates showing bands on 1315 bp were considered biofilm-positive while the isolates showing no bands on the required base pair were considered biofilm-negative isolates.

Antimicrobial susceptibility test

The biofilm-positive *S. aureus* isolates were subjected to the Kirby-Bauer disk diffusion test to detect MRSA and VRSA isolates. The antibiogram profile of bacterial isolates confirmed on different phenotypic and genotypic methods was evaluated as per guidelines of the clinical laboratory standards institute (CLSI, 2019). Various antibiotics from different groups were selected for sensitivity profiling based on their clinical relevance and modes of action. The antibiotic disks used for this procedure include penicillin-G, amoxicillin, cefoxitin, oxacillin, vancomycin, amikacin, gentamycin, ciprofloxacin, levofloxacin, oxytetracycline, trimethoprim/sulphamethoxazole, tylosin, chloramphenicol, fusidic acid, and linezolid. The isolates were declared sensitive or resistant based on the zones of inhibition measured and compared with the guidelines of CLSI (2019).

Evolutionary analysis of biofilm-positive isolates

Sequencing and phylogenetic analysis

The PCR products of positive samples were shipped to a renowned lab for sequencing. The similarity of nucleotide sequences of study isolates, obtained after sequencing, with already reported *icaA*-positive *S. aureus* isolates was checked using the BLAST of NCBI srerver. The BLAST analysis of study isolates revealed a variable similarity with various isolates of other countries. The genetically diverse and representative sequences of study isolates were selected for further phylogenetic analysis. For comparison, the known *icaA* gene sequences of multidrug-resistant isolates from different sample sources, i.e. milk, feces, food, and human samples of various countries, were retrieved from the Genbank database. The Clustal W alignment method of BioEdit software (version 7.5.0.3) was adopted to align and analyze the multiple sequences of study isolates as well as the already reported isolates (Rasheed *et al*., 2023). MEGA X software version 10.1.6 was used for the construction of a phylogenetic tree using the Maximum Likelihood method with bootstrap analysis of 1000 replicates (Tamura *et al*., 2011). The purpose of phylogenetic tree was to compare the evolutionary relationship of local study sequences with similar reported sequences from other countries.

Recombination analysis

To investigate the evidence of recombination, the nucleotide sequences were first subjected to identify haplotypes (*Na*), to estimate the polymorphic sites (*S*), the average number of nucleotide differences (*K*), and nucleotide diversity (π) by using the DnaSP 5.10 software (Librado, 2009). The detection of breakpoints and recombinant signal assessment in nucleotide sequences were done using the online genetic algorithm recombination detection (GARD) tool of the Datamonkey webserver (Pond *et al*., 2006).

Tests for selection

To identify the sites subjected to selection in the *icaA* gene, the DnaSP 5.0 was used to calculate the parameter (ω) for functional alleles by estimating the dN/dS of the *icaA* gene (Librado, 2009). Furthermore, the Nei-Gojobori method was performed by MEGA7 software to calculate the codons (Nei and Gojobori, 1986) with the Jukes and Cantor correction. Standard error estimates were derived from 1000 bootstrap replicates. Z test of positive selection was calculated by MEGA7 (Tamura *et al*., 2011). The median-joining network was used to evaluate the haplotype diversity. The DnaSP software (version 5.10.01) was used to determine the haplotypes, and Network software (version 4.6.1.4) created a median connecting network of 13 haplotypes.

Statistical analysis

The results of PCR and antimicrobial resistance were expressed in relative or absolute frequencies. Multiple antibiotic resistance index (MAR index) was calculated as the number of antibiotics to which the isolate is resistant divided by the number of antibiotics to which the isolate is subjected.

Results

Comparison of different biofilm detection methods

The culturing of the subclinical mastitis samples and the identification of the *nuc* gene confirmed the *S. aureus* in 122 (31.77%) milk samples. The *S. aureus*-associated subclinical mastitis was more prevalent in buffaloes (35.94%) compared to cattle (27.6%). Biofilm detection through both *in vitro* methods i.e. CRA and MTP test revealed a higher prevalence in cattle isolates compared to buffalo isolates. CRA method showed that 44 out of 122 *S. aureus* strains (36.07%) were capable of forming biofilm while the MTP test showed biofilm-forming capability in 30 *S. aureus* strains (24.59%). Twenty-six out of 122 *S. aureus* isolates (21.31%), showed biofilm production both by CRA and MTP test while 74 strains (60.65%) were found non-biofilm producers on both methods (Table 1). There were some discrepancies noted among the results of both methods as the findings revealed that 18 *S. aureus* strains (14.75%) were found biofilm producers on CRA while non-biofilm producers on the MTP test. Similarly, 4 strains (3.28%) positive for biofilm on the MTP test were found biofilm non-producers on the CRA method (Table 1). The findings suggested that the biofilm detection rate of CRA is higher compared to the MTP test.

Molecular detection confirmed the *icaA* gene in 78 *S. aureus* strains (63.93%). The detection rate of the *icaA* gene was higher in the subclinical mastitis of cattle (n=43) compared to buffaloes (n=25). The comparative analysis of the results of the CRA test and MTP test was made with that of PCR, a more sensitive method. The comparison of results between the CRA test and presence of the *icaA* gene showed that among 44 biofilm-positive strains for the CRA test, 40 strains were carrying the *icaA* gene. Among 78 *icaA*-positive isolates, 38 isolates were found non-biofilm producers on the CRA test. A total of 40 isolates were found negative for both the CRA and PCR. The sensitivity and specificity of the CRA test, calculated by Med-Calc statistical software, were 90.91% and 51.28%, respectively (Table 2). Similarly, the comparison of results between the MTP and PCR showed that all except one biofilm-positive strain based on the MTP test were PCR positive. A total of 49 isolates carrying the *icaA* gene were found biofilm-negative on the MTP test while 43 isolates were found negative for biofilm formation on both MTP as well as PCR methods. The sensitivity and specificity of the MTP test were found to be 96.67% and 46.74%, respectively (Table 2).

Detection of methicillin, vancomycin, and multidrug resistance

The antimicrobial susceptibility test showed that all biofilm-producing *S. aureus* exhibited diverse antibiotic resistance profiles to 15 different antibiotics. The antibiotic resistance increases with the density of biofilm

Table 1: Biofilm forming capability in *S. aureus* isolates using CRA and MTP tests

Method	Results	CRA		MTP	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)
CRA	Positive	44 (36.07)	0 (0.00)	26 (21.31)	18 (14.75)
	Negative	0 (0.00)	78 (63.93)	04 (03.28)	74 (60.65)
MTP	Positive	26 (21.31)	12 (09.84)	30 (24.59)	0 (0.00)
	Negative	18 (14.75)	66 (54.10)	0 (0.00)	92 (75.41)

Table 2: Comparison of results between *icaA* gene expression and *in vitro* biofilm detection methods

Method	Results	PCR (*icaA*)			Sensitivity (%)	Specificity (%)
		Positive	Negative	Total		
CRA	Positive	40	4	44	90.91	51.28
	Negative	38	40	78		
	Total	78	44	122		
MTP	Positive	29	01	30	96.67	46.74
	Negative	49	43	92		
	Total	78	44	122		

Table 3: Relative and absolute frequency of phenotypical antimicrobial resistance for biofilm-positive *S. aureus* isolates in bovine mastitis

Antibiotic class	Antibiotic	Biofilm detection methods		
		CRA (n=44)	MTP (n=30)	*icaA* gene (n=78)
β-Lactams	Cefoxitin (30 µg)	39 (88.64)	24 (80.00)	61 (78.21)
	Oxacillin (1 µg)	34 (77.27)	21 (70.00)	63 (80.77)
	Amoxicillin (10 µg)	36 (81.82)	22 (73.33)	64 (82.05)
Aminoglycosides	Gentamicin (10 µg)	27 (61.36)	20 (66.67)	65 (83.33)
	Amikacin (30 µg)	11 (25.00)	09 (30.00)	25 (32.05)
Quinolones	Ciprofloxacin (5 µg)	31 (70.45)	23 (76.67)	58 (74.36)
	Levofloxacin (5 µg)	19 (43.18)	14 (46.67)	30 (38.46)
	Moxifloxacin (5 µg)	12 (27.27)	07 (23.33)	17 (21.79)
Glycopeptides	Vancomycin (30 µg)	31 (70.45)	18 (60.00)	44 (56.41)
Tetracyclines	Oxytetracycline (30 µg)	37 (84.09)	26 (86.67)	60 (76.92)
Macrolides	Tylosin (30 µg)	09 (20.45)	05 (16.67)	21 (26.92)
Chloramphenicol	Chloramphenicol (10 µg)	06 (13.64)	06 (20.00)	18 (23.08)
Sulfonamides	Trimethoprim + Sulfamethoxazole (1.25 µg + 23.75 µg)	37 (84.09)	26 (86.67)	63 (80.77)
Fusidanes	Fusidic acid (10 µg)	04 (09.09)	03 (10.00)	11 (14.10)
Oxazolidinones	Linezolid (30 µg)	13 (29.55)	07 (23.33)	19 (24.36)

CRA: Congo red agar, and MTP: Microtitre plate test

Table 4: Multidrug resistance profiles of biofilm producing *S. aureus* isolates to the tested antibiotics

Sr. No.	Antibiotic resistant pattern	No. of antibiotic classes	MAR index
1	FOX, OX, AMX	1	0.2
2	FOX, OX, CN	2	0.2
3	FOX, AMX, CN	2	0.2
4	AMX, TMP, LEV	3	0.2
5	AMX, CN, CIP	3	0.2
6	AK, LZ, FOX	3	0.2
7	AMX, CHL, CN	3	0.2
8	VAN, AMX, CN, TMP	4	0.27
9	VAN, T, AMX, LEV	4	0.27
10	FOX, CIP, TMP, TY	4	0.27
11	VAN, T, FOX, OX, CN	4	0.33
12	VAN, AMX, FOX, TMP, CN	4	0.33
13	AK, T, MOX, TY, FOX, AMX	5	0.4
14	LZ, AMX, FOX, OX, TMP, CN	4	0.4

MAR index: Multiple antibiotic resistance index, AMX: Amoxicillin, VAN: Vancomycin, CN: Gentamicin, T: Oxytetracycline, TY: Tylosin, TMP: Trimethoprim+sulfamethoxazole, FOX: Cefoxitin, Ox: Oxacillin, AK: Amikacin, CIP: Ciprofloxacin, LEV: Levofloxacin, MOX: Moxifloxacin, and CHL: Chloramphenicol

formed by isolates. The results showed that out of 78 *icaA* positive *S. aureus* isolates, 61 (78.21%) isolates were MRSA while 44 (56.42%) isolates were VRSA. The isolates showed high antibiotic resistance to trimethoprim/sulfamethoxazole, oxytetracycline, amoxicillin, and gentamycin in a decreasing manner while biofilm-positive MRSA isolates were sensitive to fusidic acid, chloramphenicol, tylosin, and moxifloxacin (Table 3).

The multidrug resistance (MDR) of biofilm-positive isolates tested against fifteen antimicrobials showed an overall 100% MDR. All of the isolates were found resistant to more than two antimicrobials. The multiple antibiotic resistance index (MAR) for isolates was calculated as the number of antibiotics resistant to an isolate divided by the total number of antibiotics tested for susceptibility. MAR index depicts the extent of microbial exposure to antibiotics used within the community. Overall, the *S. aureus* isolates showed resistance against 3 to 6 antibiotics out of 15 antibiotics tested, generating a MAR index of 0.2-0.4. Similarly, the study isolates were found resistant from one antibiotic group to 5 different antibiotic groups (Table 4).

Evolutionary analysis

Phylogenetic analysis

The phylogenetic analysis of the *icaA* gene of local isolates showed close nucleotide sequence similarity with each other and with already reported isolates from different sample sources and countries. All except one cattle isolate (Milk Catt-10 Pakistan), showed a resemblance with other isolates. Similarly, the buffalo isolates (Milk Buff-02 Pakistan and Milk Buff-93 Pakistan buffalo) showed a high resemblance with each other but were found genetically different from the other isolate (Milk Buff-03 Pakistan) (Fig. 2).

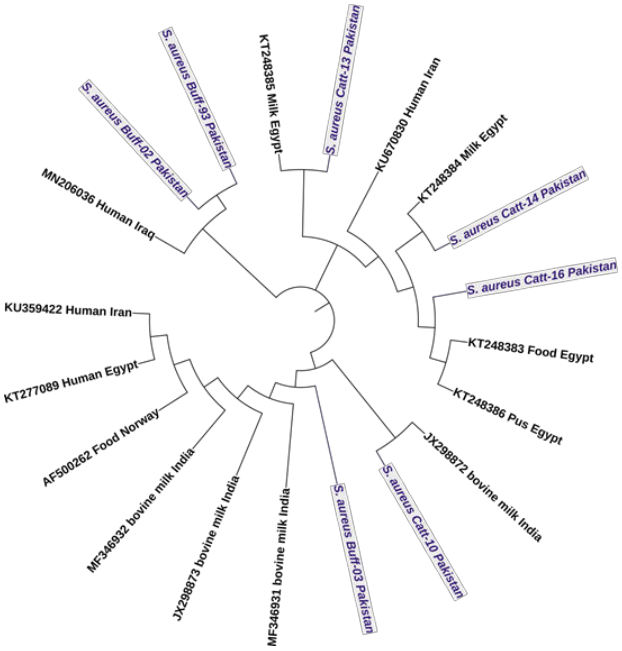

Fig. 2: The dendrogram represents the *icaA* gene diversity in *S. aureus* study isolates compared to other sequences obtained from the Gene bank. The blue color denotes the study isolates

Comparing the study isolates with already reported ones showed a close resemblance with some isolates from Egypt, India, and Iraq. Among cattle reference isolates, the two isolates revealed identity with the *S. aureus* isolated from milk samples of Egypt (accession No.: KT 248384; KT248385). One study isolate showed a high resemblance with the Indian isolate from milk origin (accession No.: JX298872). From this study isolates, one cattle and two buffalo isolates were found genetically diverse and showed a lesser resemblance with different isolates from Egypt and Iraq, respectively (Fig. 2).

Conserved region analysis

DnaSP was used to perform the analysis of the conserved DNA region module in this study. The analysis of the conserved DNA region module revealed that the sequence with a length of 1149 contained three conserved segments, referred to as segment 1 ranging from 123-297 with a conservation score of 0.433, and a homozygosity value of 0.920 at 0.0015 P-value, segment 2 ranging 654-736 with a conservation score of 0.422, and a homozygosity value of 0.914 at 0.0322 P-value, and the conserved segment 3 ranging 644-752 with a conservation score of 0.427 and a homozygosity value of 0.915 at 0.0209 P-value. The conservation of amino acid sequences in conserved regions varied from 92.2% to 98.6% of the amino acid sequences (Fig. 3). When individual segments were examined for selection, it was shown that residues in conserved segments had experienced purifying selection. Still, certain residues in semi-conserved segments were under positive selection. Each of the codons for these residues contained nucleotides that were highly variable within their respective semi-conserved sequences, making them the most variable codons. One of the segments described here was a novel segment that does not overlap with antigenic determinants previously found. Positive selection evidence in several semi-conserved segment residues showed that their variation is important in strategy to combat antimicrobial effects and to evade host immune surveillance.

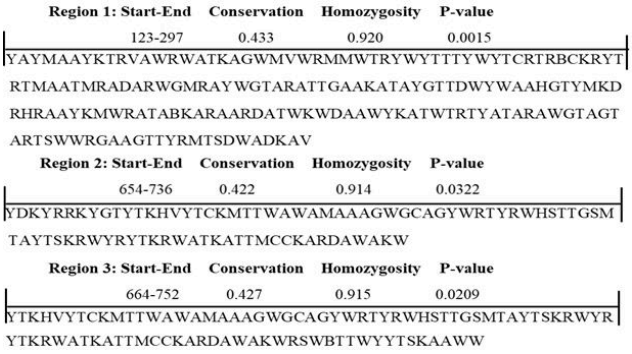

Fig. 3: The detection of three highly conserved DNA fragments by performing conserved region analysis within DnaSP

Codon usage bias analysis

Codon use bias analysis of open reading frames (ORFs) of the *icaA* gene showed a strong codon bias with a higher effective number of codons (ENC) value (48.435). The codon bias index (CBI) exhibited a negative connection pattern with ENC. ORFs in the *icaA* gene had a larger codon use bias, according to both ENC (48.435) and CBI (0.329). Furthermore, G+C concentration was low in all studied sequences of the *icaA* gene, according to GC3 and GCC data. The average relative synonymous codon use (RSCU) value was calculated to discover the best codons to employ in the *icaA* gene (Table 5). Except for AUG (M), GUA (V), GAA (E)/GAG (E) in the *icaA* gene, and GGC (G)/GGG (G) in the *icaA* gene, there was no codon use bias (Table 5). Furthermore, the bulk of optimum codons with RSCU values closes to 1 end in U or A, implying that the *icaA* codon use was skewed towards synonymous codons.

Recombination analysis

For the *icaA* gene, a recombination study was performed to find potential evolutionary links between genes. The research revealed three recombination events. Each recombination sequence, including the major and minor parents, came from the *icaA* gene, which was obtained from a biofilm-producing *S. aureus* strain. GARD analysis found evidence of recombination breakpoints. GARD examined 5120 models at a rate of 30.30 models per second. The alignment contained 759 potential breakpoints, translating into a search space of 72874879 models with up to 3 breakpoints, of which 0.01% was explored by the genetic algorithm (Fig. 4). The Akaike Information Criterion (AICc) score was compared for the best fitting GARD model that allowed for different topologies between segments (9148.1), assumed the same tree for all the partitions inferred by GARD, and allowed different branch lengths between

Table 5: Sites/Codons with alignment gaps were excluded in single DNA sequences

UUU-F 16 (1.23)	UCU-S 2 (0.86)	UAU-Y 12 (1.60)	UGU-C 4 (1.60)
UUC-F 10 (0.77)	UCC-S 2 (0.86)	UAC-Y 3 (0.40)	UGC-C 1 (0.40)
UUA-L 18 (2.25)	UCA-S 3 (1.29)	UAA-* 4 (1.00)	UGA-* 4 (1.00)
UUG-L 11 (1.38)	UCG-S 2 (0.86)	UAG-* 4 (1.00)	UGG-W 7 (1.00)
CUU-L 5 (0.63)	CCU-P 2 (4.00)	CAU-H 5 (1.43)	CGU-R 3 (1.00)
CUC-L 3 (0.38)	CCC-P 0 (0.00)	CAC-H 2 (0.57)	CGC-R 0 (0.00)
CUA-L 9 (1.13)	CCA-P 0 (0.00)	CAA-Q 6 (1.09)	CGA-R 3 (1.00)
CUG-L 2 (0.25)	CCG-P 0 (0.00)	CAG-Q 5 (0.91)	CGG-R 1 (0.33)
AUU-I 18 (1.42)	ACU-T 4 (0.94)	AAU-N 9 (1.06)	AGU-S 2 (0.86)
AUC-I 6 (0.47)	ACC-T 2 (0.47)	AAC-N 8 (0.94)	AGC-S 3 (1.29)
AUA-I 14 (1.11)	ACA-T 8 (1.88)	AAA-K 11 (1.22)	AGA-R 8 (2.67)
AUG-M 12 (1.00)	ACG-T 3 (0.71)	AAG-K 7 (0.78)	AGG-R 3 (1.00)
GUU-V 6 (1.14)	GCU-A 5 (1.18)	GAU-D 7 (1.27)	GGU-G 3 (1.09)
GUC-V 7 (1.33)	GCC-A 2 (0.47)	GAC-D 4 (0.73)	GGC-G 4 (1.45)
GUA-V 5 (0.95)	GCA-A 9 (2.12)	GAA-E 11 (1.83)	GGA-G 2 (0.73)
GUG-V 3 (0.57)	GCG-A 1 (0.24)	GAG-E 1 (0.17)	GGG-G 2 (0.73)

* Indicates stop codon

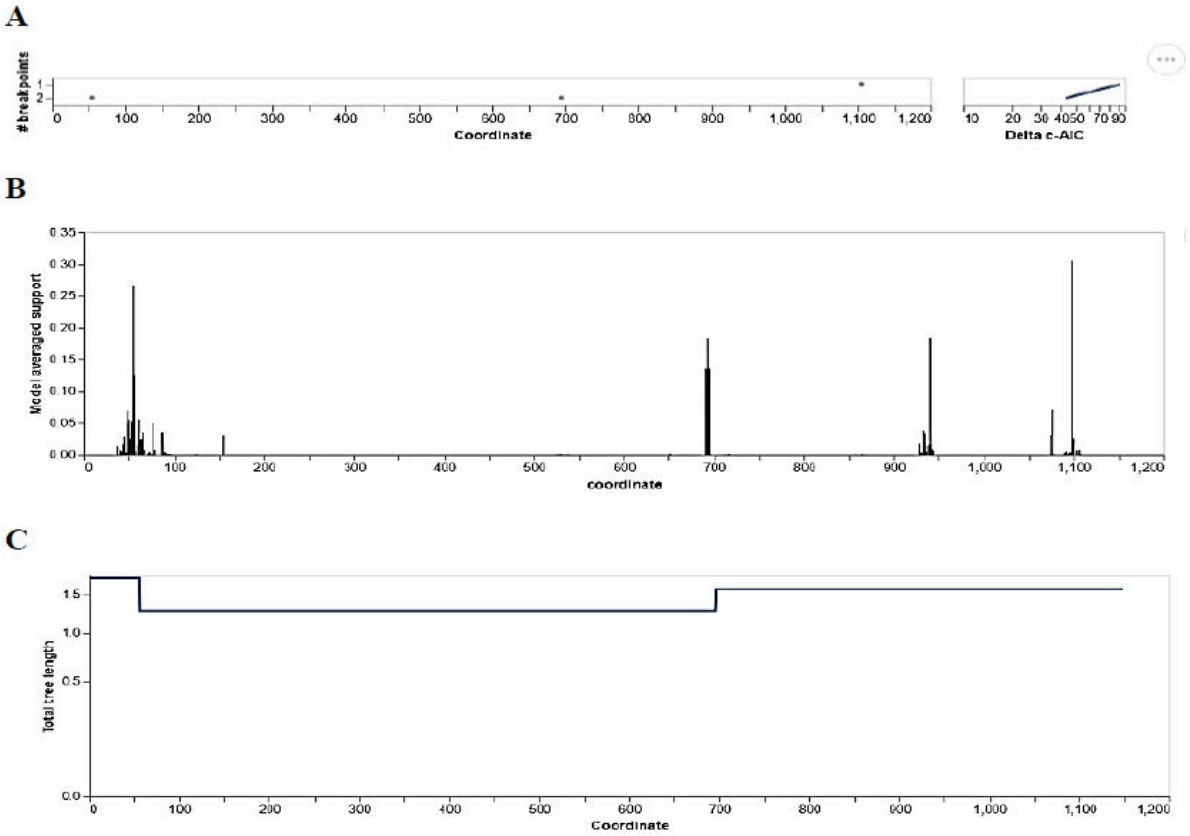

Fig. 4: Performance of GARD to detect recombination events in biofilm-associated *icaA* gene of *S. aureus* isolated from bovine mastitis. (**A**) Left: The best placement of breakpoints inferred by the algorithm for each number of breakpoints considered. Right: The improvement in the c-AIC score between successive breakpoint numbers (log scale), (**B**) Model-averaged support for breakpoint placement, and (**C**) Total tree length by partition

partitions (9357.6). The model suggested at least one of the breakpoints reflecting a true topological incongruence as the multiple tree model can be preferred over the single tree model by an evidence ratio of 100 or greater.

Median-joining network and selection analysis

A network plot for 13 coding haplotypes of the *icaA* gene was created to demonstrate the phylogeny of *icaA* gene, and the groupings matched to taxa in the phylogenetic tree. The median-joining network (Fig. 5) revealed that the phylogenetic association of *icaA* gene coding haplotypes (H-1) with the other 12 haplotypes is more closely connected to the buffalo haplotype (H2), which was combined using the same median vector displaying the mutation sites. Several haplotypes were

found to be shared by multiple species, such as the H2, H3, H8, and H9 haplotypes, which are shared with each other's, which could be due to ancestral polymorphism conservation (Fig. 5). The ratios of non-synonymous (dN) and synonymous (dS) sites in *icaA* gene of *S. aureus* were used to calculate the selection pressure. Several codon sites including 136, 212, 220, 229, 236, 277, 310, 313, 367, 376, 379, 394, 398, 421, 454, 460, 517, 520, 529, 535, 541, 553, 574, 580, 583, 598, 646, 649, 667, 709, 718, 736, 748, 760, and 766 have been identified under selection pressure with a dN/dS ratio of greater than 1, whereas the other sites went under negative selection with a dN/dS ratio of less than 1.

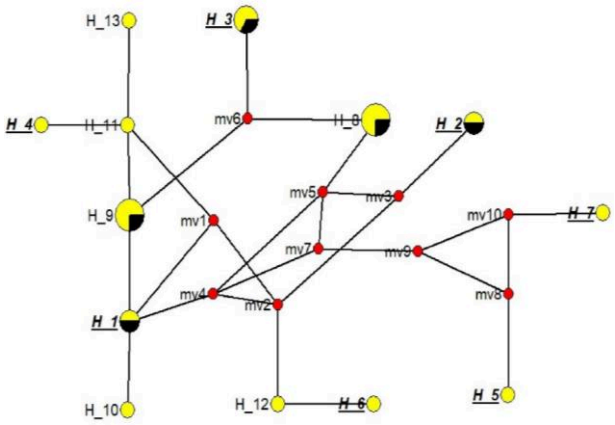

Fig. 5: Neighbor-Joining network of haplotypes found in *icaA* gene of *S. aureus* species

Discussion

Assessment of biofilm detection methods

Biofilm is a main factor involved in the pathogenesis of mastitis that helps the bacteria to survive in a wide range of hostile environments. Biofilm formation is accountable for the high incidence and ineffective treatment of *S. aureus* mastitis (He *et al*., 2014). The combination of CRA and MTP tests has been adopted in many research studies to detect staphylococcal biofilms (Vasudevan *et al*., 2003). In this study, the results of comparing these methods for *in vitro* biofilm detection revealed that the CRA method showed a high detection rate compared to the MTP test. This discrepancy found in the detection rates of the two methods supported the findings of previous studies (Vasudevan *et al*., 2003; He *et al*., 2014). Both methods, despite the relatively large differences in biofilm detection rates, still have a positive predictive value (Seo *et al*., 2008). However, contrary to our findings, some studies have reported a lower biofilm detection rate of CRA than the MTP test (Wiśniewska *et al*., 2008; Melo *et al*., 2013) which could be due to incongruity in the medium category, dilution, incubation period, wavelength measurement, and determination criteria. Although the CRA method is less time consuming, easy to perform, and recommended by many researchers (Jain and Agarwal, 2009; Kouidhi *et al*., 2010), the MTP test has high sensitivity and specificity. In this study, the MTP test showed higher sensitivity (96.67%) compared to the CRA method (90.91%) which was in agreement with the findings of Melo *et al*. (2013). Despite the differences in detection rates, both two assays could be selected as appropriate tools for biofilm detection.

Antimicrobial resistance pattern associated with biofilm-producing *S. aureus*

Biofilm-producing pathogens exhibit high resistance to various antimicrobials making the therapeutic strategies ineffective (Hassan *et al*., 2011). The timely detection and screening of biofilm-positive isolates followed by antimicrobial sensitivity tests are important for the selection of suitable antimicrobial agents (Neopane *et al*., 2018). The current study showed that 78.21% of biofilm-positive isolates were MRSA which supports the findings reported by Islam *et al*. (2019). The high resistance to various beta-lactam antibiotics like cefoxitin, oxacillin, and amoxicillin reported in this study might be due to the production of beta-lactamase enzyme or acquisition of *mecA* gene leading to the formation of altered penicillin-binding protein (PBP-2a) (Haran *et al*., 2012; Ba *et al*., 2014). Contrary to the findings reported by Neopane (1997), a high prevalence of vancomycin resistance (56.41%) has been reported in this study. VRSA is an emerging and prevailing issue, threatening animal health that might be due to acquired resistance as happened in the case of methicillin (Marques *et al*., 2017). The high resistance to antibiotics like oxytetracycline, amoxicillin, and gentamycin found in this study supports the findings of studies that reported a similar resistance pattern in staphylococcal isolates (Aqib *et al*., 2018; Neopane *et al*., 2018). Isolates of the current study showed higher resistance to gentamicin in comparison to that of previous studies (Abdolahi and Khodavandi, 2019). In this study, moxifloxacin, tylosin, Linezolid, and fusidic acid were found sensitive antibiotics which were in agreement with the findings of Altaf *et al*. (2019). The unavailability of quality antimicrobials, self-medication, the usage of antibiotics without prior consultation with authorized personnel, and the lack of laboratory facilities for antimicrobial susceptibility tests are responsible factors for increasing antimicrobial resistance in *S. aureus* isolates (Ansari *et al*., 2014).

Molecular and phylogenetic analysis of local isolates

As the phenotypic characters may develop from different genetic determinants, it is pertinent to investigate biofilm formation at the genetic level (He *et al*., 2014). The emergence of antimicrobial resistance mechanisms and the spread of the relative pathogens can be anticipated by a proper understanding of evolutionary events responsible for the development of antimicrobial resistance (Miragaia, 2018). Many types of research aimed to characterize the molecular diversity in *S. aureus* (Da Silva Soares *et al*., 2021; Hu *et al*., 2021). In this study, the *icaA* gene was confirmed in 63.93% of isolates which is in agreement with the findings of various

studies conducted on milk samples that reported the 56.25%-86.60% prevalence of *icaA* gene in *S. aureus* isolates (Melchior *et al*., 2009; Melo *et al*., 2013; He *et al*., 2014; Aslantaş and Demir, 2016; Khoramrooz *et al*., 2016). The phylogenetic analysis revealed the similarity of the study isolates with isolates of India, Iraq, and Egypt which might be due to the movement of animals across borders, trade of animals, import and export of dairy products, and traveling of humans across the countries, which become important factors responsible for the transmission of pathogens (Fèvre *et al*., 2006).

Analysis of conserved DNA regions of local isolates

Bacteria usually exhibit niche-driven genome composition by adopting various strategies to adapt in diverse habitats including reductive evolution to lose genes, acquisition of valuable genes or by horizontal gene transfer, positive section, genetic recombination, etc (Chaudhry *et al*., 2020). One of the evolutionary mechanisms capable of bringing about incompatible interactions between closely related species has been identified as rapid divergence (Wang *et al*., 2019). The current analysis supports the possibility that fast divergence is a factor in the emergence of hybrid mismatches. A gene is considered conserved if having greater than or equal to 80% DNA conservation and genetic distances less than or equal to 3.0%, while considered semi-conserved if having less than 80% DNA conservation but a slightly higher degree of variability, greater than or equal to 4.6% (Pluta *et al*., 2018). According to these criteria, the *icaA* gene comprises three highly conserved segments, one of which is a novel segment that does not overlap with antigenic determinants previously found. The current analysis identified only the conserved segments, showing signs of positive selection, and the semi-conserved residues, showing purifying selection. These low levels of variability reflect a common aspect of *S. aureus* genomes, which is their high degree of conservation between strains.

Codon usage bias analysis of *icaA* gene

Codon usage bias is a long-standing direction in molecular evolution (Ran *et al*., 2014). The codon usage bias analysis in the current study revealed a low G+C content in the *S. aureus* sequences that is supported by previous studies (Suzuki *et al*., 2012). Codon usage bias analysis of open reading frames (ORFs) of the *icaA* gene conducted in this study showed a higher effective number of codons (ENC) value (48.435). The effective number of codons (ENC) used by a gene is usually calculated to estimate synonymous codons bias, independent of the number of codons and amino acid compositions (Sau *et al*., 2005). The values of the effective number of codons vary from 20 (when one codon is used per amino acid) to 61 (when all the codons are used with equal probability). The higher ENC found in the current study suggested a strong codon usage bias in the *icaA* gene. Highly biased genes with higher ENC are usually highly expressed (Sharp and Cowe, 1991). The strong codon bias found in the current study might be associated with various variables like GC content, the extent of gene expression, transcriptional selection, and amino acid conservation (Ran *et al*., 2014). The codon usage variations among genes of a bacterial genome occur due to high variation in genomic G+C content, variation in mutation biases between lagging and leading strands of replication, evidence of natural selection on codon usage in many species, and evidence of extensive horizontal gene transfer among bacteria (Sharp *et al*., 2005).

Assessment of recombination events by GARD analysis

The current study used GARD analysis to check the recombination events in the sequences of the *icaA* gene. The same tool has been used by various previous studies (Pond *et al*., 2006). By examining haplotypes, it is possible to determine the presence of recombinant strains in a population. The current study found evidence of recombination breakpoints and revealed three recombination events. The relationship suggests that *icaA* genes have evolved to have high expression levels. These results were supported by the findings of previous studies that found evidence of recombination in the *S. aureus* genome including homologous recombination, mobile genetic elements, and large-scale chromosomal replacements (Driebe *et al*., 2015). Recombination has the potential to play a substantial role in bacterial survival by lowering the number of harmful mutations that are integrated into a bacteria's genome on an individual basis (Sheppard *et al*., 2018). Evidence of recombination in *S. aureus* has been found in initial studies but most of them suggested that point mutation was mainly responsible for *S. aureus* clonal variants (Driebe *et al*., 2015). This kind of recombination has been shown to avoid mutation accumulation, assist in adapting to new hosts or environmental changes, and overcome host resistance (Haddad Kashani *et al*., 2018). Furthermore, genetic recombination-induced mosaic pattern in bacterial genes is responsible for antibiotic resistance and thus treatment failure (Woegerbauer *et al*., 2015).

Median-joining network and selection analysis

The selection pressure calculation is based on the hypothesis that synonymous substitutions are neutral which is justified as the selection affects much stronger non-synonymous sites compared to the synonymous sites. However, it is a fact that synonymous sites can be subjected to selection driven by either RNA secondary structure or codon usage (Ran *et al*., 2014). In the current study, the median-joining network was made and selection pressure, calculated as the ratios of non-synonymous (dN) and synonymous (dS) sites of a gene (Ran *et al*., 2014), showed a ratio of >1 indicating positive or forward selection for some sites while more sites showed negative or purifying selection. Another study conducted to analyze the selection pressure for the

spa gene of *S. aureus* depicted that no codon showed a low selection pressure value, and thus showed no possibility of positive selection in any part of a repeat of the specified gene (Koreen *et al*., 2004). The negative selection in many codons in the current study was similar to the findings of the study showing significant purifying or negative selection for *spa* gene repeats (Koreen *et al*., 2004). The power of purifying selection differs greatly within genomes of different organisms, between genes of an evolving genome, and between different sites of a gene. Usually, the strength of purifying selection is more in the cases of high effectual population size like bacteria (Koonin *et al*., 2010). Although positive selection has been observed in the current study, the presence of strong purifying selection and less positive selection in the codons of any gene is suggestive of variations in a gene produced intrinsically by the organism rather than accumulating variations associated with outer resources that make them suitable genetic markers for global epidemiological studies (Koreen *et al*., 2004). The findings of our investigation indicate that selection and mutation are the most likely causes of codon bias. It is explained by positive selection theory because codon bias adds to the efficiency and/or accuracy of protein expression and as a result, it is subjected to selective pressure. While this is going on, the mutational explanation proposes that codon bias exists as a result of the non-randomness of mutational patterns. However, despite the fact that the mechanism behind codon bias selection is still up in the air, a substantial connection has been discovered between the GC content and codon usage patterns in this study.

In the current study, we concluded that *S. aureus* is a prevalent cause of bovine subclinical mastitis in study districts. *S. aureus* isolates (63.93%) carried biofilm-associated *icaA* gene, and a high frequency of biofilm-forming potential was shown by isolates on both *in vitro* methods with a higher detection rate with CRA compared to the MTP test. Biofilm-producing *S. aureus* isolates exhibited a high tendency of methicillin, vancomycin, and multidrug resistance on the antimicrobial susceptibility test. As a whole, the current analysis of genome diversity revealed unique evolutionary features of *S. aureus* adaption to non-human environments. The findings suggest that mutation and selection are most likely causes of codon bias in the *icaA* gene sequences. The study proposes that the evidence of positive selection in some residues of the semi-conserved segments suggests that their variation is involved in the bacterial strategy to combat antimicrobial effects and to escape the host's immune surveillance. However, further research is needed to understand the evolutionary mechanisms associated with host adaption and antimicrobial resistance strategies.

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Conflict of interest

The authors declare no financial or non-financial interests to disclose.

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