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RESEARCH ARTICLE

Molecular Prevalence, Genetic Diversity and *In silico* Structural Analysis of MRSA Isolated from Eggshell in Commercial Layer Farms

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ABSTRACT

Eggs remain a vital component of nutrition and the economy for many counties, while the routine non-therapeutic use of antimicrobials and high-density animal farming practices may contribute to the emergence and dissemination of Methicillin-Resistant *Staphylococcus aureus* (MRSA). The purpose of the current research was to evaluate the prevalence of *S. aureus* and MRSA isolated from the eggshell surface, evolutionary comparison of locally examined isolates with the isolates originating from other nations. Overall, 384 eggshell samples were procured from different layer farms of the districts Lahore and Kasur and allowed to enrichment broth for proper growth. After this, these samples were further grown on selective media and genotypically analyzed by PCR for the *mecA* gene. According to this study's results, the prevalence of *S. aureus* on a phenotypic and genotypic basis was 35.67% and 17.96%. Among the PCR confirmed *S. aureus* isolates, 68.11% isolates exhibited resistance against Methicillin disc and were considered phenotypically MRSA resistant, while 33.33% isolates were confirmed on PCR to have the *mecA* gene and depicted MRSA positive on a genetic basis. The phylogenetic analysis of MRSA revealed that local isolates exhibited divergence with each other but showed resemblance with the isolates of the neighboring boundaries, including India, Iran, Egypt, and Poland, indicating the transboundary transmission of the pathogen. Furthermore, computational analysis revealed evolutionary similarities of nucleotide and amino acid sequences of local and reference isolates and stability of PBP2a. Also, it also gives the idea of a 3D model of the *mecA* protein and transmission patterns of MRSA across different hosts and regions.

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INTRODUCTION

Poultry is a vibrant and important sector of Pakistan agriculture, contributing 1.3 % to the national GDP. Poultry refers to a category of domesticated birds that are kept for various purposes, such as the production of animal products like meat, eggs, manure, and fibers (Hedman *et al.*, 2020). Commercial poultry contributes 19.170 billion eggs and 2.028 million tons of meat (Hussain *et al.*, 2024). As a consequence, the poultry industry recorded a growth rate of 20–30% per year during the early 1970s, which stabilized to 10–15% annually in the 1980s (Hussain *et al.*, 2015). Table eggs are widely utilized as food and regarded as the utmost affordable, nutrient-dense, and protein-rich source that can contribute to a balanced diet. Even

though the egg's contents are shielded by a semi-permeable membrane and a hard shell, they could still be tainted with microorganisms and could potentially spread infectious agents to people.

Numerous bacterial species of medical significance have previously been identified in table eggs. More significant is the existence of bacteria showing resistance to antimicrobial agents in this dietary source (Elgendi and Amin, 2019). Eggshells contain various pathogens, including Staphylococcus aureus (*S. aureus*), *E. coli*, *Salmonella* spp., *Streptococcus spp*., *Listeria monocytogenes* and *Bacillus spp*. (Pondit *et al.*, 2018). *Staphylococcus* is considered a typical component of chicken flora, found in the respiratory and intestinal systems, as well as on the skin and feathers (Casey *et al.,* 2007). Numerous conditions, such as necrotising pneumonia, otitis media, fasciitis, superficial skin and soft tissue infections, and urinary tract infections, are caused by the bacterial species *S. aureus* (Tamarapu *et al.*, 2001; Harris and Foster, 2002). Furthermore, *S. aureus* is a major contributor to foodborne illness because of its capacity to generate heat-stable Enterotoxin (Argudín and Mendoza, 2010).

Methicillin-resistant *S. aureus* (MRSA) is a significant bacterium that differs genetically from other strains of Staphylococci. MRSA developed resistance via different mechanisms, including horizontal gene transfer and natural selection (Gurusamy *et al.*, 2013). Methicillin-Resistant *Staphylococcus aureus* (MRSA) exhibits resistance to a broad range of β-lactam antimicrobial agents, including penicillin and cephalosporins. This resistance is attributed to the expression of an altered penicillin-binding protein, PBP2a. PBP2a has a markedly reduced affinity for β-lactam antibiotics, rendering them ineffective. The *mecA* gene, found on the chromosomal DNA of MRSA, is responsible for encoding the resistance-associated protein PBP2a. It is carried within the staphylococcal cassette chromosome *mec* (*SCCmec*), a transposable element capable of horizontal transfer, facilitating the spread of resistance genes among *S. aureus* strains.

This study was designed to find the prevalence of *S. aureus* and MRSA isolated from commercial layer eggshells. Furthermore, phylogenetic and *in silico* studies were performed to assess the evolutionary relationship and inter species transmission of local MRSA isolates with isolates from other countries.

MATERIALS AND METHODS

Sampling Area and population: Sampling was carried out on commercial layer farms in the Punjab's districts, including Lahore and Kasur, Pakistan (Fig. 1). A total of 384 fresh chicken eggs were collected from different layer stocks by employing a convenient sampling approach. The egg shells and adhering material over collected eggs were swabbed with sterile cotton swabs and these samples were brought to the Medicine Research Lab (MRL) Department of Veterinary Medicine, University of Veterinary and Animal Sciences, Lahore, Pakistan, for further analysis by maintaining the cold chain.

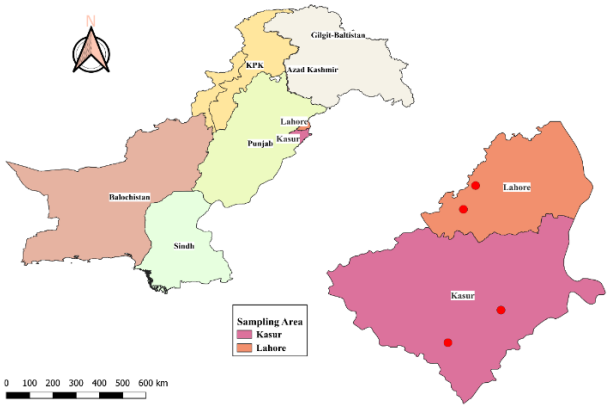


Fig. 1: Study map of the sampling area

Isolation and phenotypic characterization of *S. aureus:* The samples underwent enrichment culturing containing nutrient broth in the laboratory and were maintained at 37 °C for 24 hours to get robust bacterial proliferation. After enrichment, egg samples were subjected to 5% blood agar followed by *S. aureus* selective media, Mannitol Salt agar (MSA). Microbiological verification of *S. aureus* was done based on slightly raised and golden yellow colonies of *S. aureus* on MSA (Muzammil *et al.*, 2022). Furthermore, confirmation was done based on Gram staining protocol, catalase, and coagulase tests.

DNA extraction and confirmation of *nuc* gene: For genetic confirmation, DNA was harvested from phenotypically verified *S. aureus* samples using a DNA purification kit. Subsequently, these isolates were genotypically validated by amplifying the *nuc* gene having product size of 270 bp using specific primers (F: GCGATTGATGGTGATACGGTT and R: AGCCAAGCCTTGACGAACTAAAGC) and PCR conditions comprised 3 minutes of an initial denaturation at 94°C, followed by 1.5 minutes of final denaturation at 94°C, 2 minutes of annealing at temperature of 55°C, and extension at 72°C for 3 minutes as per guidelines of Mkize *et al.* (2017).

Phenotypic and genotypic confirmation of MRSA: For phenotypic identification of the *mecA* gene, confirmed *S. aureus* isolates were streaked on Muller-Hinton agar (MHA) using a Kirby-Bauer disc diffusion method. Cefoxitin discs having a concentration of 30µg were placed in the MHA plates and allowed for incubation at 37 °C for 24 hours. After this, inhibition zones were analysed via

Vernier caliper and contrasted with the Clinical and Laboratory Standards Institute (CLSI, 2019). Methicillin-resistant *S. aureus* (MRSA) isolates showed inhibition zones of ≤21 mm with the cefoxitin disc, while methicillin-sensitive *S. aureus* (MSSA) isolates displayed zones greater than 22 mm.

MRSA was genotypically confirmed through PCR by detecting the occurrence of the *mecA* gene in the purified DNA samples. Primers used for *mecA* amplification were (F-TGGCATTCGTGT CACAATCG and R- R-CTGGAACTTGTTGAGCAGAG) as used by (Rasheed *et al.*, 2025) with a product size of 310bp. The PCR protocol was included 5 minutes of initial denaturation at 94 °C, followed by 34 cycles of final denaturation at 94 °C for 1 minute, 1 minute of annealing at 54 °C, and 1 minute of extension at 72 °C while 10 minutes of final extension at 72 °C.

Phylogenetic analysis of MRSA isolates: The visible bands obtained from DNA electrophoresis were excised and purified using a commercial gel purification kit (Thermo Scientific GeneJET) and subsequently submitted to a reputable lab for the purpose of sequencing. Isolates identified as MRSA through both phenotypic and molecular characterization methods were examined by sequencing, and representative sequences from these were chosen for subsequent in silico analysis. For comparative analysis, *mecA* gene sequences previously reported on NCBI from different species in Pakistan and neighboring countries like Iraq, China and India were retrieved. A phylogenetic analysis was performed using the MEGA XI application with the Maximum Likelihood method and 1000 bootstrap iterations to assess the genetic relatedness among local isolates and with reference sequences.

In Silico analysis of MRSA isolated from layer egg: Nucleic acid and amino acid sequence alignments were carried out to identify nucleotide substitutions that could result in changes to the amino acid sequence, potentially affecting the protein's three-dimensional structure and overall function. For alignment, the Clustal Omega tool was used, and for conserved motif analysis MEME suite was used (Sievers and Higgins, 2014; Bailey *et al.*, 2015). To analyze the 3D model of protein, a bioinformatics tool named Swiss model was used. Also, the secondary structure comparison and physiochemical traits of protein were evaluated by SOPMA (Self-Optimized Prediction Method with Alignment**)** and ProtParam.

Statistical analysis: The Thrush field formula was used to calculate the prevalence of *S. aureus* and MRSA (Thrushfield, 2007).

RESULTS

Prevalence of *S. aureus* and MRSA isolated from layer egg samples: Overall, the prevalence of *S. aureus* on a phenotypic and genotypic basis was found to be 35.67% and 17.96% respectively. While on Cefoxitin disc, 68.11% *S. aureus* confirmed isolates exhibited resistance and were declared as MRSA on a phenotypic basis. On PCR basis of *mecA* in isolates, 33.33% of isolates demonstrated resistance and were declared as genotypically confirmed MRSA. Furthermore, higher prevalence of *S. aureus* and MRSA was found in district Lahore layer farms in comparison with district Kasur layer farms, and results also demonstrated that the Nick chick breed was more vulnerable to *S. aureus* and MRSA infection than the Rhode Island Red, as shown in Table 1.

Phylogenetic analysis of MRSA isolates: Phylogenetic characterization of *mecA* isolates (E5 and E12) was performed by making comparison of local sequences with pre-existing published sequences of *mecA* gene on NCBI using MEGA-XI software. Results indicated that our local MRSA isolates revealed divergence from each other and a more convergent association with the neighboring countries. Furthermore, E5 isolate exhibited higher similarity index with isolates of Iran, Thailand, and India having accession numbers (MW052034, CP172401, CP062469), while E12 showed convergent relationship with neighboring countries like India, Egypt, and Poland, with accession numbers MW195499, PV386794, CP170689 respectively (Fig 2).

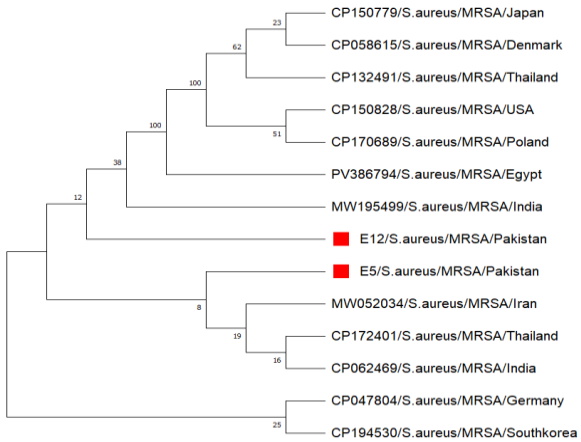


Fig. 2: Phylogenetic analysis of MRSA isolates

Computational analysis of *mecA* gene using bioinformatics tools

Nucleotide sequence alignment and conserved motifs construction: Nucleotide alignment using Clustal Omega software revealed that local sequences exhibited higher similarity with the isolates of Iran, India, Germany, and South Korea, while lower alignment similarity with the isolates of

the USA, Japan, and Denmark as shown in Fig 3. Moreover, MEME suite analysis showed five conserved sequences in all isolates except Egypt. The local and reference isolates showed *p*-value ranging between 2.27e-101 to 4.04e-101 as in Fig 4. The five identified candidate motifs were visualized in blue, yellow, red, green, and arctic blue respectively. Although they shared common binding sites and width, but each motif has a difference in E-value as shown in Table 2.

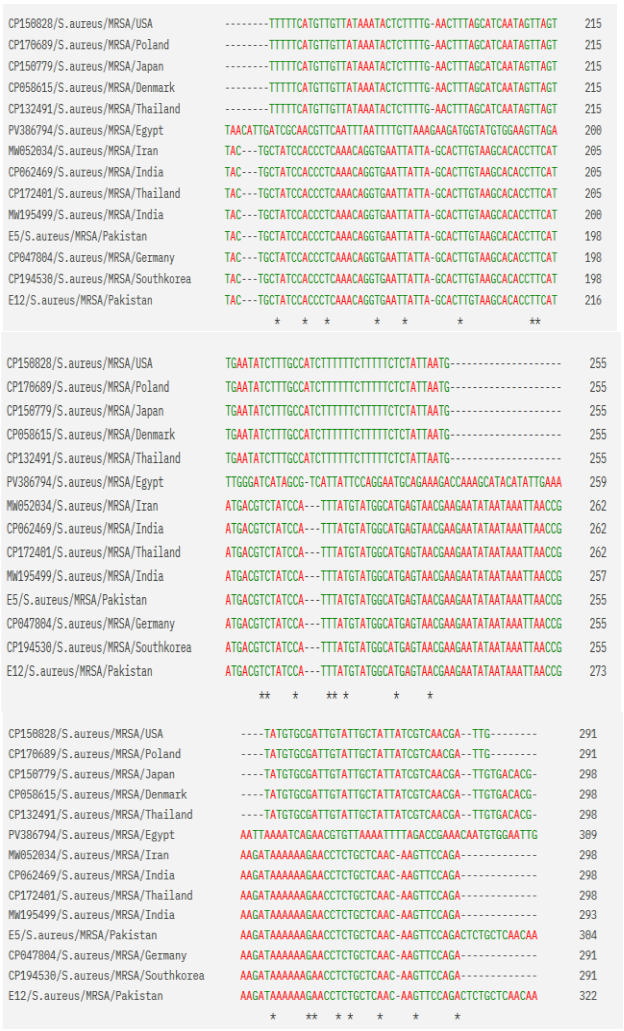


Fig 3. Alignment of *mecA* nucleotide

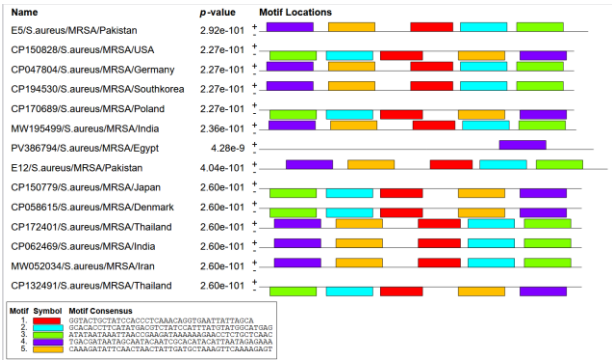


Fig. 4: Conserved motifs of nucleotide sequence

Table 1: Prevalence of *S. aureus* and MRSA

Sampling Area	Breed	No. of samples	Prevalence of *S. aureus*		Prevalence of MRSA	
			Phenotypic (%)	Genotypic (%)	Phenotypic (%)	Genotypic (%)
Lahore	Nick chick	96	40 (41.67)	23 (23.95)	17 (73.91)	9 (39.13)
	Rhode Island Red	96	32 (33.33)	14 (14.58)	9 (64.28)	5 (34.71)
Kasur	Nick chick	96	36 (37.50)	20 (20.83)	14 (70.00)	6 (30.00)
	Rhode Island Red	96	29 (30.20)	12 (12.50)	7 (58.33)	3 (25.00)
Total		384	137(35.67)	69 (17.96)	47(68.11)	23 33.33)

Table 2: Discovered common motifs of *mecA* gene

Discovered candidate motifs	E- value	Binding sites	Width
	3.1e-191	13	39
	2.3e-211	13	43
	4.8e-187	13	43
	2.1e-185	14	43
	4.8e-187	13	43

Amino acid alignment and motifs study: Clustal Omega-based amino acid alignment revealed a high degree of similarity between the local isolates and the reference sequences (Fig. 5), and 3 motifs were observed by using MEME suite. All these motifs were conserved in each isolate except Egypt sequence which showed only one motif (Fig. 6). Motif analysis showed that protein formed by each sequence is the same as PBP2a. All the conserved motifs have different p value binding sites and width as shown in Table 3.

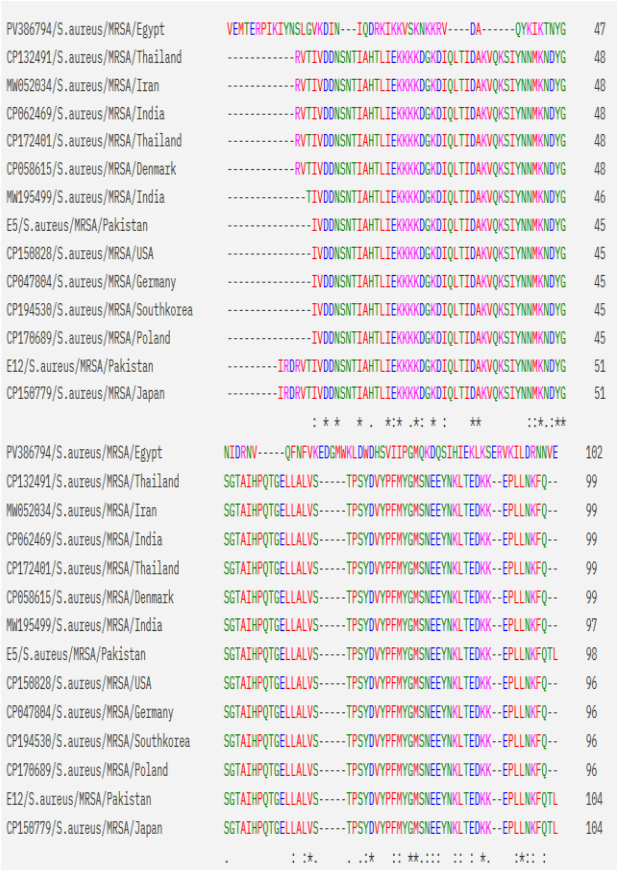
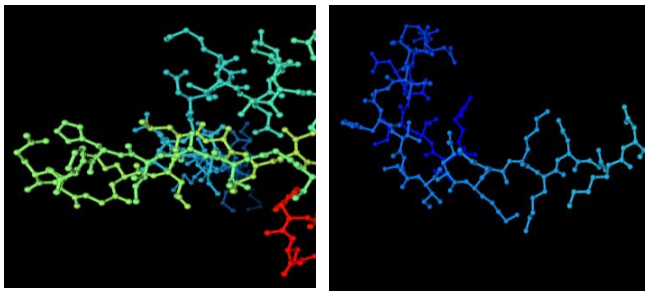


Fig. 5: Alignment of Amino acid sequence

Fig. 6: Conserved motifs of Amino acid sequence

Table 3. Discovered common motifs of PBP2a

Discovered candidate motifs	E- value	Binding sites	Width
	4.8e-498	13	50
	1.5e-232	13	29
	5.3e-111	14	15



(E5) **(E12)**

Fig. 7: 3D view of PBP2a

Table 4: Secondary structure comparison of *mecA* protein by SOPMA

Sample ID	Alpha helix	Extended strand	Beta turn	Random coil
E5	33.00	25.00	10.00	32.00
E12	33.02	25.47	11.32	30.19

3D modeling and Secondary structure comparison of PBP2a: The three-dimensional view revealed that the PBP2a model formed by both isolates is similar in structure to each other, as shown in Fig. 7. Secondary structure comparison revealed that alpha helices and random coil were higher in percentage than extended strand and beta turn, as shown in Table 4. Higher proportion of alpha helices and random coil showed the stability of the protein. Physicochemical traits of the protein formed by both MRSA isolates were given in Table 5.

Table 5: Structural and compositional characteristics of PBP2a protein

Sample ID	MW	AA	Theoretically pI	TNC	TPC	Half-life (hours)	(II)	(AI)	GRAVY
E5	11341.82	100	5.61	14	12	20	32.05	81.90	-0.695
E12	12082.68	106	6.12	15	14	20	29.29	83.68	-0.698

Note: MW=Molecular weight, AA=Number of Amino Acids, TNC=Total Negatively charged residues, TPC=Total Positively Charged residues, II=Instability index, AI=Aliphatic index

DISCUSSION

A notable hazard to public health is the problem of antibiotic-resistant genes, especially those brought on by resistant forms of *S. aureus*. This issue is particularly noticeable in individuals receiving long-term antibiotic treatment, therefore, infection control becomes more challenging (Chinemeren *et al.*,

2022;Tălăpan *et al.*, 2023). The antibiotic resistance characteristics of *S. aureus* strains discovered in egg samples have raised concerns about the transmission of resistant microbial strains circulating through animals, food, and environmental reservoirs (Ahmad *et al.*, 2021). When *S. aureus* contaminated food is consumed, it can cause *staphylococcal* food poisoning. Eggs are susceptible to contamination and can serve as a reservoir for *Staphylococcus aureus*; notably, the thermostable enterotoxins secreted by this pathogen may persist even after cooking (Romano *et al.*, 2023;Khoothiam *et al.*, 2023). Only a few research have been done on the *S. aureus* prevalence in eggshell and AMR genes (Zhang *et al.*, 2023).

Antibiotics are frequently used to treat production species and in poultry rations, which increases MRSA load in eggs and promotes eggs contamination with resistant pathogen (Abreu *et al.*, 2023). This ongoing investigation found the *S. aureus* prevalence from egg swab samples was observed 35.67% on phenotypic basis while *nuc* gene basis prevalence was recorded 17.96%. This study is in accordance with the findings of (Zhang *et al.*, 2023) while on genotypic basis, current *S. aureus* prevalence was higher than the study (Pondit *et al.*, 2018), which noted 10.45% prevalence. In contrast 80%, 77% and 53% of *S. aureus* prevalence was recorded from egg shell surface (Elgendi and Amin, 2019; Verma *et al.*, 2023; Bose *et al.*, 2025). The high prevalence of *S. aureus* may be due to inadequate cleaning of layer houses, lack of egg collection frequency, high humidity, warm temperatures, and poor ventilation in layer houses create ideal conditions for bacterial growth and persistence on surfaces and in the environment.

The *mecA* gene was detected in 33.33% of *S. aureus* isolates from the layer eggshell surface. A different study performed in Egypt by (Eid *et al.*, 2015) noted 36.36% prevalence of the *mecA* gene, which was in line with the results of current study. Furthermore, many authors demonstrated a higher MRSA prevalence that was 55% and 57% (Rahimi and Karimi, 2015; Bounar *et al.*, 2018). In this study there was a difference between the phenotypic and genotypic positive *mecA*. On phenotypic basis higher prevalence of MRSA was found 68.11% while based on *mecA* gene confirmation on PCR prevalence of MRSA was 33.33%. This discrepancy associated with the hyper production of beta lactamase and lacks the typical genetic mechanism for such resistance, so such type of isolates exhibited resistance in the disc but were found negative for *mecA* on PCR. Additionally, they reported that a different class of strains known as modified *S. aureus* (MODSA) have altered penicillin binding proteins instead of acquiring a new PBP, which is how traditional MRSA works (Eid and Erfan, 2015).

Routine or non-therapeutic use of antibiotics in feed or water promotes the emergence of MRSA strains in poultry farms. The resemblance between the isolated sequences and previously identified sequences from various regions and organisms indicates the probability for cross-species and inter-regional transmission of pathogens. The microbial isolations from this research were comparable to isolates originating from different nations, according to phylogenetic analysis. This might be due to the global dairy trade, cross-border animal trafficking, animal trading, and international travel by people, and classified as important contributors to the spread of infections (Fèvre *et al.*, 2006).

The evolutionary processes linked to rapid divergence were the cause of the differences in nucleotide sequence alignment between standard assemblies and indigenous samples (Ballhausen *et al.*, 2014). The study isolates had high proportions of the alpha helical segment, then irregular coil, elongated strand, and polypeptide turn region, according to secondary structure comparison. The half-life calculates the amount of time needed for fifty percent of the protein to degrade. Proteins also show strong AI, with significant amounts of aliphatic residues supporting enhanced thermo stability (Gasteiger *et al.*, 2005). A minimal value of GRAVY index represents hydrophilicity, which enhances the proteins affinity for different hydrophilic compounds (Alaidarous *et al*., 2020).

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Conflict of Interest: The authors have no competing interests.

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