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Original article

Exploring the virulence potential of immune evasion cluster genes in methicillin-resistant *Staphylococcus aureus* from cancer patients

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is accountable for a plethora of infections, ranging from minor cutaneous manifestations to grave metastatic conditions. The dissemination of MRSA among cancer patients poses a substantial public health hazard on a global scale. This study explores the association between MRSA and bacteriophage-encoded immune evasion cluster (IEC) genes. This investigation employed a total of 168 pathogenic MRSA collected from 38 cancer and 130 non-cancer patients. A cefoxitin disc diffusion method followed by PCR analysis was used to identify the *mecA* gene. In this study, we employed singleplex and multiplexed PCR techniques to detect specific IEC genes. No association ($p = 0.98$) was observed between the sex and age of patients and MRSA isolates. However, MRSA isolates demonstrated a notable association ($p = 0.01$) with pus samples in non-cancer patients and skin swabs in cancer patients. The resistance profiles of MRSA strains from cancer and non-cancer patients did not show significant differences ($p > 0.05$). Notably, the *sea* gene was found to be more prevalent in MRSA isolates from cancer patients, displaying a significant association ($p = 0.03$). Additionally, this study identified two novel and distinct combinations of IEC types, namely V1 (*sea*, *chp*, *scn*) and V2 (*sea*, *scn*). Cancer patients had higher multidrug resistance and toxin gene abundance than non-cancer patients. The identification of two novel IEC patterns underscores the urgent need to control MRSA dissemination in hospitals and monitor emerging clones.

1. Introduction

Staphylococcus aureus commonly exists asymptomatically in approximately 30 % of individuals, but it can give rise to severe illnesses in both humans and animals. The breach of the body's initial line of defense allows the invasion of these bacteria (Pietrocola et al., 2017). The circulation of multidrug-resistant (MDR) strains of *S. aureus* pose a significant global public health threat. MDR bacteria present substantial clinical challenges, thereby complicating patient treatment and, in certain instances, resulting in therapeutic failure (Nosheen et al., 2017; Qamar et al., 2018). Many clinical and non-clinical strains of Gram-positive and Gram-negative bacteria have become MDR, limiting the availability of effective therapies (Jameel et al., 2014; Nosheen et al., 2017; Ejaz et al., 2021). By 2050, antibiotic-resistant infections will result in approximately 10 million fatalities, surpassing the number of deaths attributed to cancer. Consequently, pursuing alternative therapeutic approaches and the judicious use of antibiotics have become imperative in the present era (Abdalla et al., 2019; Fernández et al., 2020).

S. aureus exhibits a repertoire of virulence factors that play crucial roles in colonization, surface adherence, and biofilm formation. In addition to evading the immune system, S. aureus has a propensity for developing multidrug resistance and exerting toxicity on the host (Shettigar and Murali, 2020). Bacteriophages, which are versatile genetic elements, can be horizontally transferred among

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S. aureus strains and integrated into their genomes. Certain genes within the immune evasion cluster (IEC) of *S. aureus* are encoded by β-hemolysin converting bacteriophages. These genes include staphylokinase (SAK), staphylococcal enterotoxin A (SEA), staphylococcal complement inhibitor (SCIN), and the chemotaxis inhibitory protein of *S. aureus* (CHIPS). Notably, SCIN acts as an inhibitor of the classical, lectin, and alternative complement pathways, impeding opsonophagocytosis, neutrophil-mediated killing of *S. aureus*, neutrophil chemotaxis, and C5a generation (Singh and Phukan, 2019).

There are seven IEC variants, and the proteins SCIN, SAK, SEA, and CHIPS are encoded by *scn*, *sak*, *sea,* and *chips* genes (Nepal et al., 2021). All IEC variants have conserved *scn* along with varied *sak, sea*, and *chip* combinations. These genes encode CHIPS, a human-specific immunological modulator, SEA, and SAK (Rohmer and Wolz, 2021). CHIPS inhibits neutrophil chemotaxis by interacting with both the C5a receptor and the formylated peptide receptor present in neutrophils (Sultan et al., 2018). Neutrophil migration from the bloodstream to the site of infection is a pivotal early event in the nonspecific immune response against invading pathogens, making CHIPS crucial in this process (Hajdamowicz et al., 2019). SEA, a well-known superantigen, functions by down-regulating chemokine receptors on monocytes, while SAK plays a pivotal role in halting human neutrophils' phagocytosis of staphylococci. SAK achieves this by activating bacterial plasminogen, leading to its conversion into plasmin, which aids in the removal of opsonic molecules such as C3b and IgG (Abatángelo et al., 2017). Additionally, SAK also inhibits α-defensins, antimicrobial peptides that possess bactericidal properties (Scudiero et al., 2020). Moreover, the Panton-Valentine leukocidin (PVL), a two-component toxin, leads to the formation of pores in leukocyte cell membranes (Tromp and van Strijp, 2020; Tabassum et al., 2023).

Numerous risk factors contribute to acquiring methicillin-resistant *S. aureus* (MRSA), encompassing suboptimal antibiotic usage, prolonged hospitalization, age, low host immunity, intravascular devices, artificial breathing, and many other disabilities (Lozano et al., 2020). Cancer patients are particularly prone to multiple predisposing factors for infection. Apart from the underlying malignancy, treatments such as bone marrow transplants, radiation therapies, stem cell transplants, and surgical interventions can significantly impair or suppress the immune system. Consequently, understanding the pathogenic genotype, virulence factors, and antibiotic resistance profiles exhibited by MRSA strains holds great potential to benefit cancer patients undergoing treatment (Li et al., 2021).

The current investigation aims to elucidate the potential involvement of IEC genes in MRSA infections observed explicitly in cancer patients. By exploring this aspect, a more profound comprehension of MRSA's virulence mechanisms and their pathological consequences within the context of cancer patients can be achieved. Additionally, establishing the connection between these virulence factors will provide crucial insights for the development of innovative anti-virulence strategies aimed at effectively controlling drug-resistant MRSA infections.

2. Materials and methods

2.1. Ethical approval and patient specimens

A total of 168 pathogenic MRSA isolates obtained from the Shaukat Khanum Memorial Cancer Hospital and Research Center (SKMCH&RC) Lahore, Pakistan, were utilized in this investigation. Ethical approval for the project was granted by the SKMCH&RC's Study Center Ethics Committee, and all participants provided informed consent to partake in the study, and the data was kept confidential. Clinical specimens, encompassing sputum, abscesses, aspirates, tissue samples, ear fluid, high vaginal swabs, throat swabs, pleural fluid, wound swabs, and skin lesions, were collected from cancer patients between October 2020 and February 2021. Of the obtained isolates, 130 were derived from individuals without cancer, while 38 isolates were specifically obtained from cancer patients.

Cultivation of the collected specimens was carried out on mannitol salt agar, chocolate agar, and blood agar plates, incubated at a temperature of 37 °C. The identification of *S. aureus* was achieved by assessing colony characteristics, mannitol fermentation, and coagulase and catalase assays performed on the clinical samples. The cefoxitin disc method was employed to detect MRSA, followed by PCR analysis to identify the *mecA* gene based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021). In addition to the microbial data, demographic information pertaining to each patient was collected, including sex, age, sites exhibiting positive cultures, and the patient's cancer history.

2.2. Antibiotic susceptibility testing (AST)

We performed AST employing the disc diffusion technique (CLSI, 2021). A panel of twelve antimicrobial agents (Oxoid, Basingstoke, United Kingdom) was used to determine the antimicrobial resistance (AMR) profiles. We used the antibiotic discs of chloramphenicol (30 μg), clindamycin (2 μg), linezolid (30 μg), vancomycin (30 μg), gentamycin (10 μg), fusidic acid (10 μg), erythromycin (15 μg), oxacillin (1 μg), penicillin (10 units), ciprofloxacin (5 μg), co-trimoxazole (1.25 μg) and tetracycline (30 μg). A reference strain of *S. aureus*, ATCC 25923, was included as a control in the experiments.

2.3. Genomic DNA extraction and gel electrophoresis

Following manufacturer guidelines, bacterial genomic DNA was extracted using a GenJet DNA extraction kit (Thermo Fisher Scientific, Waltham, United States). Microcentrifuge tubes containing 1.5 ml of sterile DNA were stored at −20 °C after collection. NanoDrop instrument (Thermo Fisher Scientific, Waltham, United States) was used to assess the quality and yield of isolated DNA.

2.4. Polymerase chain reaction

PCR amplification of multiple target genes was conducted using a PE 9600 thermocycler (PerkinElmer Inc., Waltham, United States) with a 15 μL reaction volume. Within each 15 μL PCR reaction mixture, the components included 0.5 μL (10 pM) of each oligodeoxynucleotide primer, 2 μL of bacterial genomic DNA, 7.5 μL of 2 × Master Mix green, and 2.5 μL of nuclease-free water. The PCR technique was employed to detect the presence of specific genes, namely *mecA*, *agr*, *pvl*, *scn*, *sea*, *sak*, *sep*, and *chip*. For the amplification of *mecA*, *pvl*, and *agr* genes, singleplex PCR was utilized (Fig. 1), while *scn*, *chp*, and *sea* genes were multiplexed, and *sea* and *sak* genes were amplified in separate multiplex reactions (Fig. 2). A comprehensive list of primer sequences and their corresponding amplification conditions is provided in Table 1 (van Wamel et al., 2006). Following amplification, a 1.5 % agarose gel electrophoresis employing 0.5 × TBE buffer was performed to visualize the PCR products. Ready-to-use DNA ladders of 1 kb and 250 bp were used as size markers (Thermo Fisher Scientific, Waltham, United States).

2.5. Phage typing

Phage typing was employed to classify the IEC genes, resulting in the assignment of types A to H, following the PCR outcomes.

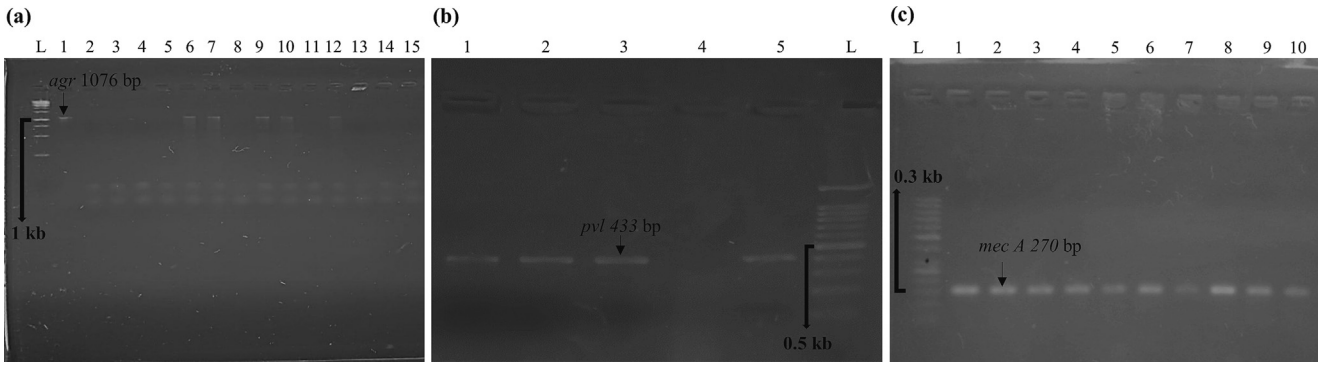

Fig. 1. Gel electrophoresis results showcasing the amplified *agr*, *pvl* and *mecA* genes. In panel (a), the amplification of *agr* genes from 15 samples is displayed, with the position of the gene estimated using a 1 kb ladder as a reference. Moving to panel (b), the PCR amplification of *pvl* genes is depicted, and the approximate size of the amplified product is indicated using a 100 bp ladder. Finally, panel (c) illustrates the amplification of *mecA* genes. On the left-hand side, a DNA ladder of 100 bp is provided as a size marker, while samples 1 to 10 exhibit the presence of *mecA* genes.

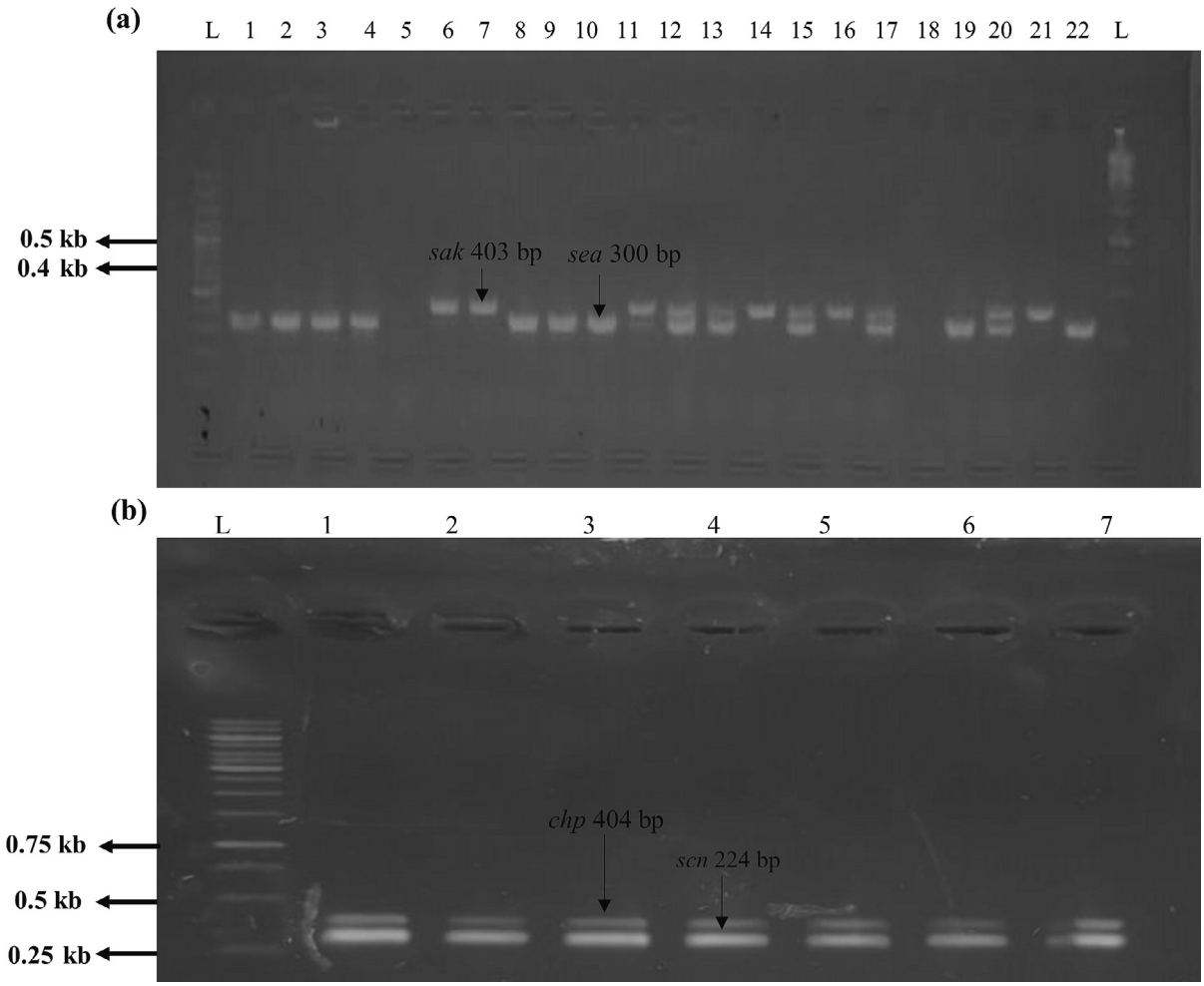

Fig. 2. Gel electrophoresis results of the IEC types obtained from MRSA isolates using multiplex PCR. To estimate the size of the amplified genes, a 100 bp ladder was utilized during the multiplex PCR process. Panel (a) visually represents the detection of the *sac* and *sea* genes through multiplex gel electrophoresis. Moving to panel (b), the gel clearly demonstrates the detection of the *chp* and *scn* genes within the amplified products obtained from multiplex PCR.

Type A isolates were characterized by the presence of *scn*, *sak*, *sea*, and *chp* genes. Type B isolates contain *scn*, *sak*, and *chp* genes. Type C isolates exhibit *scn* and *chp* genes. Type D isolates possess *scn*, *sak*, and *sea* genes. The presence of *scn* and *sak* genes defines type E isolates. Type F isolates harbor *sep*, *sak*, *chp*, and *scn* genes. Type G isolates contain *scn*, *sak*, and *sep* genes. Lastly, type H isolates solely display the *scn* gene (Farzi et al., 2021).

2.6. Statistical analysis

The data obtained from the study was analyzed using IBM's SPSS v. 26.0 (SPSS Inc., Chicago, United States). Graphical representations of the data were generated using GraphPad Prism v. 9.3.1 (GraphPad Software Inc., San Diego, United States). A chi-square test was employed to assess the presence of statistical significance

Table 1
PCR primers and thermal profiles for all the genes reported in this study.

Primer	Sequence (5′-3′)	PCR conditions	Product size
scn-F	TGAGGCACAAGCTAGCACAAGCT	1 min 94 °C, 30 sec 94 °C, 30 sec 50 °C, 1 min 72 °C, 5 min 72 °C (30 cycles)	224 bp
scn-R	TGAAGTTGATATTTTGCTTCTGACATTTTC		
sak-F	TGAGGTAAGTGCATCAAGTTCA		403 bp
sak-R	CCTTTGTAATTAAGTTGAATCCAGG		
chip-F	TTTACTTTTGAACCGTTTCCTAC		404 bp
chip-R	TGCATATTCATTAGTTTTTCCAGG		
sea-F	GGTTATCAATGTGCGGGTGG		322 bp
sea-R	CAAATAAATCGTAATTAACCGAAGGTTC		
sep-F	GACCTTGGTTCAAAAGACACC		275 bp
sep-R	TGTCTTGACTGAAGGTCTAGC		
mecA- F	TCCAATTACAACTTCACCAGG	5 min 94 °C, 1 min 94 °C, 30 sec 54 °C, 2 min 72 °C, 10 min 72 °C (30 cycles)	270 bp
mecA-R	CCACTTCATATCTTGTAACG		
pvl-F	ATCATTAGGTAAAATGTCTGGACATGATCCA	10 min 94 °C, 45 sec 94 °C, 45 sec 55 °C, 1:15 min 72 °C (35 cycles)	433 bp
pvl-R	GCATCAAGTGTATTGGATAGC AAAAGC		
agr-F	TATGGTCCTGCAGCAACTCTAA	4 min 94 °C, 1 min 94 °C, 2 min 60 °C, 3 min 74 °C, 3 min 74 °C (30 cycles)	1076 bp
agr-R	CTTGCGCATTTCGTTG		

between antibiotic resistance and the occurrence of IEC genes in cancer patients. This analysis defined a p-value below 0.05 as a significant association or correlation.

3. Results

3.1. Clinical features of S. aureus isolates

The pathogens were obtained from a total of 93 (55 %) male and 75 (45 %) female individuals. Interestingly, a higher prevalence of MRSA was observed in the age of 21–––60 years, compared to the younger and older age groups, which typically exhibit less active immune systems. However, no associations were found between the incidence of MRSA and either the sex or age of the patients ($p > 0.05$).

The majority of MRSA isolates in cancer patients were obtained from skin swabs (24; 56 %), followed by tissues (7; 16 %) and pus samples (7; 16 %). In non-cancer patients, most MRSA isolates were detected in pus samples (76; 61 %) and skin swabs (22; 18 %). We observed an association between MRSA isolates and pus samples in non-cancer patients ($p = 0.01$) and between MRSA isolates and skin swabs in cancer patients ($p = 0.01$), as shown in Table 2.

3.2. AMR of S. aureus isolates

MRSA isolated from cancer patients displayed high resistance rates to penicillin (100 %), erythromycin (82 %), ciprofloxacin (76 %), and fusidic acid (58 %). Similarly, MRSA isolates from non-cancer patients exhibited substantial resistance levels. Penicillin resistance was observed in the highest proportion of isolates (99 %), followed by erythromycin (81 %), ciprofloxacin (79 %), and fusidic acid (54 %). Nevertheless, no significant associations were identified regarding the antibiotic resistance patterns between MRSA infections in cancer and non-cancer patients. Notably, no resistance was observed against linezolid, vancomycin, and chloramphenicol among the isolates. Furthermore, no statistically significant disparities were detected in the resistance profiles of MRSA isolates (Fig. 3).

Table 2
Prevalence and occurrence of MRSA isolates in cancer and non-cancer patients.

Characteristics	Cancer patients (n = 38)	Non-cancer patients (n = 130)	p-value
	n (%)	n (%)	
Sex			
Male	21 (60 %)	72 (54 %)	0.98
Female	17 (40 %)	58 (46 %)	
Age groups			
0–20	2 (5 %)	20 (15 %)	0.29
21–40	18 (47 %)	65 (50 %)	
41–60	7 (18 %)	16 (12 %)	
61–80	11 (29 %)	29 (22 %)	
Sources of MRSA isolates			
Sputum	2 (5 %)	7 (6 %)	0.77
Pus	7 (16 %)	76 (61 %)	0.01
Skin swab	24 (56 %)	22 (18 %)	0.01
Tissues	7 (16 %)	6 (5 %)	0.23
Others	3 (7 %)	14 (11 %)	0.86

3.3. Genotypic characterization

Genomic DNA extraction was performed on a total of 168 distinct clinical strains, ensuring non-repetitiveness, to investigate the distribution frequencies of IEC types in both types of patients. To confirm the identification of MRSA isolates, PCR analysis targeting the *mecA* gene was conducted. The *mecA* gene was successfully detected in all 168 samples, further confirming their MRSA classification. Moreover, virulence factors, including *pvl* and *agr* genes, were also screened among the MRSA strains.

3.4. Occurrence of IEC gene

All MRSA strains were subjected to testing for the presence of various IEC genes, and it was found that all strains exhibited positive results for the *scn* gene. The distribution patterns of *pvl*, *chp*, *sak*, and *agr* genes were remarkably similar among MRSA isolates obtained from both cancerous and non-cancerous patients. However, a significant association ($p = 0.03$) was observed between the *sea* gene and MRSA isolates from cancer patients, with the *sea* gene found in 76 % of cases (Table 3).

3.5. Phage typing

The study presented eight distinct forms of IEC genetic variants (A-E, H, V1, and V2) carried by β-hemolysin converting bacteriophages (Fig. 4). Analysis of clinical isolates in this investigation revealed a predominant presence of type A (21 %, 36/168) and type B (15 %, 26/168). It is noteworthy, within this context, that two novel IEC combinations were identified in our study, specifically *sea*, *chp*, *scn* and *sea*, *scn*, which were referred to as V1 (8 %, 13/168) and V2 (23 %, 39/168), respectively. A substantial proportion of cancer patients exhibited the presence of both aforementioned novel combinations (Fig. 5).

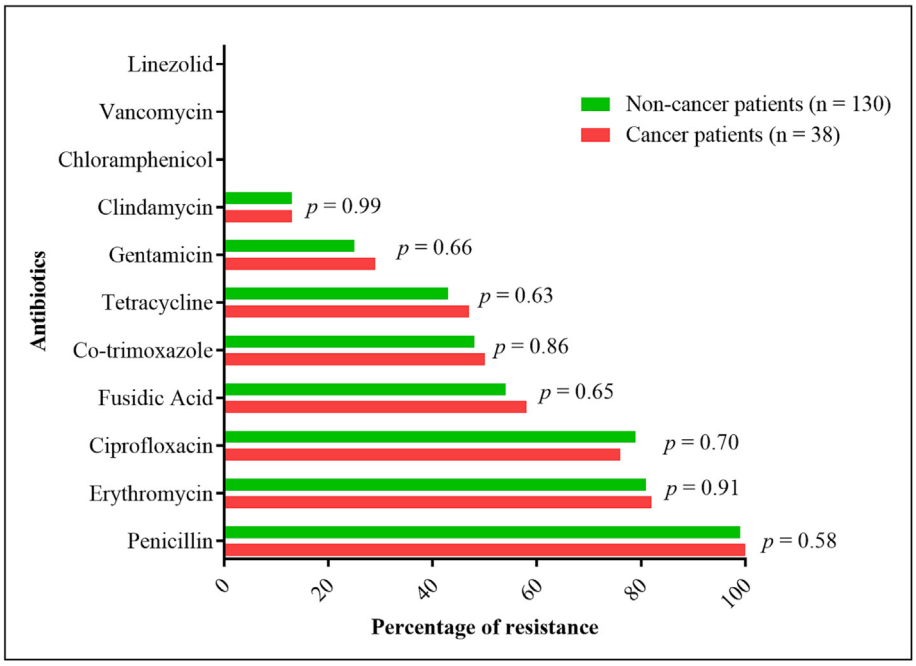

Fig. 3. Antibiotic resistance profile of MRSA among cancer and non-cancer patients.

Table 3
Prevalence and occurrence of IEC genes in MRSA isolated from cancer and non-cancer patients.

Genes	Cancer patients (n = 38)	Non-cancer patients (n = 130)	*p*-value
	n (%)	n (%)	
scn	38 (100 %)	130 (100 %)	0.58
sea	29 (76 %)	75 (58 %)	0.03
pvl	23 (61 %)	90 (69 %)	0.31
chp	20 (53 %)	73 (56 %)	0.7
sak	19 (50 %)	65 (50 %)	0.99
agr	3 (8 %)	6 (5 %)	0.43
sep	0 (0 %)	0 (0 %)	–

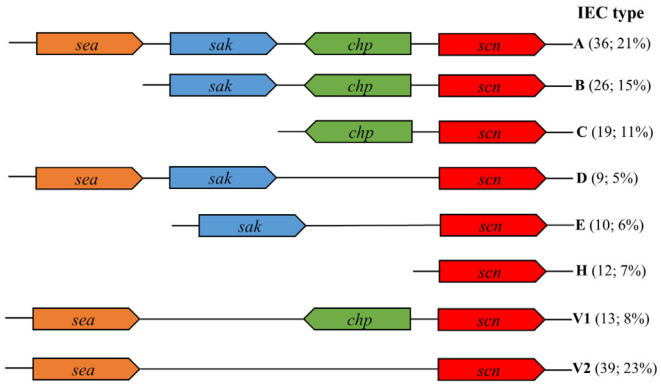

Fig. 4. Diagrammatic representation illustrates the various IEC types observed in cancer and non-cancer patients.

4. Discussion

It has been extensively documented that MRSA infections among cancer patients have severe clinical consequences and a high mortality rate (Li et al., 2021). Therefore, it is paramount to elucidate the morphological, genotypic, and pathogenic characteristics of MRSA isolates to develop effective therapeutic strategies against MRSA infections in cancer patients, especially considering the limited information available on MRSA features among hospitalized cancer patients in Pakistan.

The MRSA infections observed in cancer patients were predominantly healthcare-associated, whereas community-acquired MRSA infections were identified among the outpatients. Previous studies have reported varying prevalence rates of MRSA in different countries. In France, the prevalence was reported as 44.4 % (Lanoix et al., 2011), while in Japan it was 77.8 % (Miyake et al., 2007). Libya reported a prevalence of 35.5 % (Zorgani et al., 2012), Egypt reported 70 % (Khamees et al., 2015), and Iran reported 28 % (Eslami Nejad et al., 2010). In contrast, our study shows a cumulative prevalence of MRSA in cancer patients at 23 %.

S. aureus is a leading cause of human infections and is attributed to the rise of drug resistance in community-acquired infections. The role of immune evasion molecules is critical in unraveling the *S. aureus* virulence to develop novel therapeutic strategies. We report the *pvl* gene in 61 % of cancer and 69 % of non-cancer patients. The cancer patients infected with MRSA isolates presented 40 % *pvl* gene in cancer patients in the USA (Baharvand et al., 2022). A variable occurrence of *pvl* gene has been reported in China (45.2 %), Iran (26.3 %), Jordan (100 %), and Germany (6.2 %) among non-cancer patients (Gao et al., 2019; Abbasi et al., 2021). The primary role of *pvl* is to induce tissue necrosis by specifically targeting and damaging neutrophils. In cancer patients, *pvl*-encoded MRSA infections are mostly associated with skin and soft tissue infections (SSTIs). It is still debated whether *pvl* is associated with more severe infections or poorer outcomes. Despite *pvl*-positive MRSA infections being common in SSTIs, *pvl* was not always linked to worse clinical outcomes for cancer patients with additional risk factors (Campo et al., 2011).

The MRSA isolates demonstrated resistance to multiple classes of antibiotics commonly used for treating MRSA infections (Chai et al., 2022). The risk ratio associated with MRSA isolated from cancer patients revealed a resistance pattern to erythromycin, ciprofloxacin, fusidic acid, co-trimoxazole, tetracycline, gentamicin, and clindamycin. Notably, all MRSA isolates resisted at least two distinct antibiotic classes, indicating the substantial selective pres-

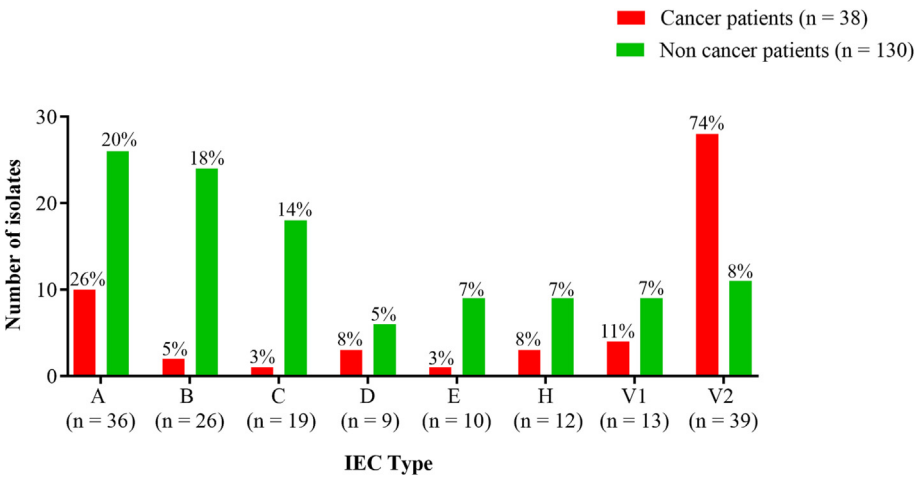

Fig. 5. A comprehensive summary of the virulence genes and IEC types observed in clinically associated MRSA strains.

sure exerted by antibiotic usage on this group. Linezolid, vancomycin, and chloramphenicol were identified as the preferred therapeutic agents for MRSA infections in both cancer and non-cancer patients. The effectiveness of vancomycin in managing MRSA cases among cancer patients has been well-documented in existing literature (Hadied et al., 2015). The variations in antibiotic resistance profiles between isolates from cancer and non-cancer patients may be attributed to differences in patient pathologies and treatment approaches (Nanayakkara et al., 2021). Cancer patients often experience more extended hospital stays compared to non-cancer patients, leading to a higher likelihood of antibiotic prescription as part of routine care. This suggests that cancer patients could potentially act as reservoirs for bacterial resistance. The emergence of resistance to multiple antibacterials concurrently poses challenges in treating MRSA cases (Khan et al., 2018).

It is widely recognized that different strains of *S. aureus* produce distinct staphylococcal enterotoxins and a toxin associated with toxic shock syndrome (Roetzer et al., 2022). However, the virulence factors and IEC types among MRSA isolates from cancer and non-cancer patients in the Pakistani population remain largely unexplored. This study is the first investigation of the toxin genotypes of MRSA in Pakistan, as there are no published studies on these genes in Pakistani MRSA isolates. Additionally, we report significant variations of phage types compared to preceding reports.

Interestingly, our study revealed a higher occurrence of the Type A IEC variant in both cancer (26 %) and non-cancer patients (20 %), which contrasts with a previous study that predominantly observed Type B isolates (Ariyarad et al., 2019). Other studies from the Netherlands reported the highest prevalence of IEC type B variants among isolates (van Wamel et al., 2006; Verkaik et al., 2011), as type B is the predominant variant in human infectious isolates. Notably, a greater prevalence of Type E IEC variants has been documented in MRSA strains from Malaysia (Chai et al., 2022). Our study identified 6 % Type E and 15 % Type B IEC variants among all isolates. Our investigation unveiled two unreported combinations of IEC patterns: *sea*, *chp*, *scn* and *sea*, *scn,* which were named as V1 and V2 variants, respectively. In cancer patients, V2 IEC accounted for 74 % of the cases, which is the highest rate among all IEC variants. These findings imply that IEC types might vary among isolates from different geographical regions. It is, therefore, imperative to control the transmission of MRSA within medical settings. In addition, hospitals should implement robust monitoring and surveillance plans that include IEC typing methods to identify patients' safety and therapeutic measures quickly. The study acknowledges several limitations. Firstly, the uneven sample size may restrict the generalizability of the findings. However, to mitigate this limitation, random sampling was employed. Additionally, the small sample size prevented further subset analysis, such as examining the influence of age and gender. Future studies with a larger sample size and the ability to conduct subset analysis are recommended to enhance the comprehensiveness and robustness of the findings.

5. Conclusion

This study concludes that despite sharing certain virulence factors, the MRSA strains exhibit different characteristics in cancer and non-cancer patients. The cancer patients presented considerably more toxin genes and higher MDR frequencies. Additionally, this study discovered two previously unreported IEC patterns *sea*, *chp*, *scn,* and *sea*, *scn,* that have been categorized as V1 and V2, respectively. Notably, type V2, type A, and type B were the three most frequent IEC patterns found in our study. These findings indicate that MRSA has the potential to jeopardize patients with cancer.

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Declaration of Competing Interest

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

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