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Hospital-Associated Synanthropic Insects as Carriers of Methicillin-Resistant *Staphylococcus aureus*: Evidence from Lahore, Pakistan

Taskeen Zahra[1], Hafiza Amina Rafiq[1], Marvah Qiass[1], Mehwish[1] and Saba Riaz[1*]

[1]Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan

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***Corresponding Author:**
Saba Riaz
Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan
saba.mmg@pu.edu.pk

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ABSTRACT

The issue of antimicrobial resistance (AMR) is significant in the world, and one of the most important types of pathogens that transmit both nosocomial and community infections is methicillin-resistant *Staphylococcus aureus* (MRSA). **Objectives:** To determine the existence of antibiotic-resistant Gram-positive bacteria in hospital-related insects, to describe the resistance mechanisms and biofilm-forming capabilities of the bacteria. **Methods:** Two hundred houseflies were taken from one hospital, which was a tertiary care hospital in Lahore. The isolates of *S. aureus* were determined by mannitol fermentation, Gram stain, and standard biochemical assays. The Kirby-Bauer disk diffusion method of performing antimicrobial susceptibility testing was conducted as per CLSI (2023) guidelines. A microtiter plate assay was used to determine biofilm formation. PCR detection of the *mecA* and *mecC* genes was done by extracting their genomic DNA. The agar well diffusion assay was employed to determine the activity of FGE as a single entity and in conjunction with the 3-lactam antibiotics. **Results:** Sixteen *S. aureus* isolates (8% of the flies) were recovered. All isolates were methicillin-resistant and carried the *mecA* gene, while the *mecC* gene was absent. Resistance was universal to oxacillin (100%) and nitrofurantoin (100%), and was high against erythromycin (87.5%) and rifampicin (75%). Most isolates (62.5%) were strong biofilm producers. **Conclusions:** Hospital-associated houseflies can serve as reservoirs and potential vectors for multidrug-resistant, biofilm-forming MRSA. The synergy observed between FGE and β-lactam antibiotics highlights the potential of plant-derived adjuncts in addressing resistant infections.

INTRODUCTION

The problem of antimicrobial resistance (AMR) is among the most acute global public-health issues of the 21st century. Recent global analyses estimate bacterial AMR was associated with millions of deaths worldwide in 2019. The overall burden continues to rise, with projections warning of a steep increase in AMR-attributable mortality and societal costs unless decisive action is taken [1-3]. Among resistant pathogens, MRSA remains a leading cause of difficult-to-treat infections in both hospital and community settings. MRSA is associated with severe outcomes, including bloodstream infections, pneumonia, surgical-site infections, and increased length of hospital stay and mortality, and it continues to rank highly in global pathogen–drug burden studies. The principal genetic determinant of methicillin resistance is the *mecA* gene, which encodes an altered penicillin-binding protein (PBP2a) with low affinity for β-lactams; a divergent homologue, mecC, has also been reported but remains less common in many regions [4-6]. The epidemiology of MRSA in Pakistan and the surrounding region demonstrates notable diversity in phenotypic resistance and the distribution of resistance genes. Molecular surveillance from Pakistan and nearby settings has reported variable frequencies of *mecA* and *mecC* among MRSA isolates, highlighting the local circulation of classic and, in some reports, *mecC*-bearing strains, underlining the need for

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molecular monitoring alongside routine susceptibility testing [7]. Environmental reservoirs and mechanical vectors have been increasingly recognized as contributors to the persistence and dissemination of antibiotic-resistant bacteria. Synanthropic insects, especially houseflies (*Musca domestica*), frequent both clinical and waste environments and can carry viable bacteria on their exoskeletons and in their digestive tracts. Experimental and field studies demonstrate that flies can acquire, transport, and deposit pathogenic bacteria, including MRSA and other multidrug-resistant organisms, making them potential, though often overlooked, vectors in hospital and peri-hospital environments. These findings support the inclusion of vector surveillance and environmental controls in AMR mitigation strategies [8]. Compounding the problem, many MRSA strains form biofilms (surface-attached, matrix-embedded communities) that markedly reduce antibiotic penetration, protect bacteria from host defenses, and facilitate persistent colonization of surfaces and medical devices. Biofilm production, therefore, contributes to treatment failure and environmental persistence, further motivating studies that pair phenotypic (biofilm) and genotypic (resistance gene) characterization of isolates from clinical and environmental sources [9]. Given the dwindling antibiotic pipeline and the high burden of resistant infections, adjunctive and alternative antimicrobial approaches are under active investigation. Natural products, particularly sulphur-containing compounds from *Allium sativum* (garlic) such as allicin, have demonstrated in vitro antibacterial activity against MRSA and other pathogens. A study conducted by Murugaiyan *et al*. highlighted that FGE or purified organosulfur compounds can act synergistically with β-lactam antibiotics, enhancing inhibition zones or lowering minimum inhibitory concentrations, suggesting a possible role as adjuvants to restore or augment antibiotic efficacy. Nonetheless, results vary by strain and method, and further targeted evaluation is required [10, 11]. Against this background, investigation of antibiotic-resistant Gram-positive bacteria isolated from synanthropic insects in hospital environments can illuminate overlooked transmission routes and local resistance gene distributions, while also providing a platform to screen potential adjunctive agents such as FGE. This study characterized Gram-positive isolates recovered from houseflies collected in a tertiary-care hospital in Lahore, performed phenotypic antibiotic susceptibility testing and biofilm assays, and conducted molecular screening for *mecA* and *mecC*. This study also evaluated fresh garlic extract's in vitro synergistic activity with representative β-lactams against methicillin-resistant isolates.

Although MRSA is well recognized as a major nosocomial pathogen, limited data exist regarding the role of synanthropic insects as environmental reservoirs of resistant strains in Pakistani hospital settings. Most local surveillance studies focus primarily on clinical isolates, while potential mechanical vectors such as houseflies remain underexplored. Furthermore, molecular characterization of resistance determinants (mecA/mecC) and biofilm-forming capacity in insect-derived isolates has rarely been investigated in Lahore. In addition, the potential synergistic activity of plant-derived agents such as fresh garlic extract against locally circulating MRSA strains has not been systematically evaluated. Addressing these gaps is essential to better understand environmental transmission pathways and identify complementary antimicrobial strategies. This study aims to clarify the role of synanthropic insects as reservoirs of MRSA in a hospital setting, to describe the local distribution of methicillin resistance determinants, and to explore low-cost adjunctive strategies that might inform future infection-control and therapeutic approaches.

METHODS

The current cross-sectional study was carried out between June and August 2024 at a Tertiary-Care Hospital in Lahore. Synanthropic insects (houseflies, *Musca domestica*) were targeted due to their frequent presence in hospital wards, waste disposal areas, and peri-hospital surroundings. Written informed consent was taken. Sampling was designed to capture flies from diverse ecological niches within the hospital to represent patient-care and waste-handling environments. All laboratory analyses were carried out using standardized microbiological and molecular techniques at the Institute of Microbiology and Molecular Genetics (IMMG), University of the Punjab, Lahore. A total of 200 adult flies were collected using sterile entomological traps and clean sweep nets, ensuring minimal contamination. To minimize sampling bias, traps were placed according to a predetermined sampling schedule that randomized the specific collection points within the major zones (wards, waste bins, and outdoor peri-hospital sites) across different days. While a formal sample size calculation was not performed a priori, a target of 200 flies was set to provide a sufficient sample for prevalence estimation. Based on a conservative expected MRSA prevalence of 10% (from pilot data and regional studies), this sample size provides a margin of error of approximately ±4% at a 95% confidence level, which was deemed adequate for this exploratory survey. Furthermore, this sample size is consistent with similar entomological studies of AMR in the region. Traps were placed at predetermined points (wards, waste bins, and outdoor peri-hospital sites) and checked daily. The flies were caught and put straight into sterile containers and taken to the laboratory within 2 hours, and

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handled under aseptic conditions. The flies were immobilized on ice, surface-sterilized by dipping them in 70% ethanol, 30s and washing them twice in sterile phosphate-buffered saline (PBS, pH 7.4), followed by homogenizing them in 1 mL sterile PBS using a glass tissue homogenizer. Short-term centrifugation of the homogenates for 5 min at 3000 rpm was performed to remove debris, followed by the inoculation of aliquots into Luria-Bertani (LB) broth with colistin (10 2g/mL) to prevent Gram-negative bacteria. Enrichment cultures were incubated overnight at 37 o C in a shaker (150 rpm). The analysis was done through SPSS version 27.0. Data were summarized with the help of descriptive statistics: the summarization of categorical variables (like the frequency of the resistance and the type of biofilm formation) is expressed in frequencies and percentages. Continuous data (i.e., inhibition zone diameters) are expressed as mean plus standard deviation (SD). In the case of the synergy assays, the mean difference in the inhibition zone of each treatment group (antibiotic alone, FGE alone, and antibiotic/FGE) was compared using ANOVA and then using a post hoc test (Tukey) to compare all multiple results. A p-value that was less than 0.05 was treated as significant. The enriched samples were streaked on Mannitol Salt Agar and incubated at 37°C for 24h. Colonies that developed a typical yellow coloration as a result of mannitol fermentation were picked and sub-cultured in fresh MSA to acquire pure cultures. Gram staining was done to verify Gram-positive cocci in grape-like clusters. Further identification was done by catalase and coagulase tests. In further analysis, Presumptive *S. aureus* isolates were stored in tryptic soy broth (TSB) containing 15% glycerol at -80°C. The Kirby-Bauer disk diffusion procedure conducted on Mueller-Hinton agar (MHA; Oxoid, UK) was used to determine the antibiotic susceptibility according to the guidelines of Clinical and Laboratory Standards Institute. Each isolate was prepared into a standardized bacterial suspension (0.5 McFarland standard, which is about 1 × 108 CFU/mL) and inoculated onto MHA plates as a lawn. Commercial antibiotic agar plates were used, which included oxacillin (1 µg), cefoxitin (30 µg), erythromycin (15 µg), nitrofurantoin (300 µg), rifampicin (5 µg), gemifloxacin (5 µg), cephalexin (30 µg), vancomycin (30 µg), tigecycline (15 µg), and trimethoprim sulfamethox Incubation of the plates was done at 35°C, 18 to 24h. CLSI breakpoints were used to interpret zone diameters and were measured. ATCC 25923 *S. aureus* was taken as a quality control strain to ensure the accuracy of the results. Fresh garlic extract (FGE) was made by peeling and weighing the garlic cloves, followed by sterilization of the surface with the use of 70 percent ethanol, followed by swirling in sterile distilled water, then homogenized with the use of a sterile blender in a ratio of 1 g/mL. The average inhibition zone of oxacillin alone was 12.4 +/ -1.2 mm, and the average inhibition zone, when mixed with FGE, was 20.1 +/ -1.5 mm ($p<0.001$). Likewise, the average zone of the cefoxitin by itself was 14.2 + 1.0 mm, and that of the combination was 21.8 +1.7 mm ($p<0.001$). FGE on its own generated an average inhibition zone of 8.5 + 0.8 mm. The homogenate was filtered using Whatman No.1 filter paper to eliminate debris, after which it was sterilized using a 0.22 µm syringe filter. Extracts were prepared and kept at 4°C and consumed within 48 hours. The agar well diffusion assay was used to test synergy. MHA plates with isolates seeded on 0.5 McFarland suspensions had their wells (6 mm diameter) cut. Treatments were: (i) FGE alone (100 µL per well), (ii) antibiotic disc alone, and (iii) antibiotic disc with 50 µL FGE overlaid. Incubation in plates was done at 37°C for 24h, with the measurements of the inhibition in millimeters. Repeats were done on different days to guarantee that the assays were reproducible. The computations of mean zone diameters and SD were done, and a one-way ANOVA paired with the post hoc test of the Tukey test were used to examine the differences between treatments in SPSS-27. A p-value of below 0.05 was a statistically significant value. An improved activity was considered to be a discernible zone diameter change over the antibiotic disc itself. The formation of biofilms was determined by the use of the microliter plate assay [12], modified. In short, 1:100 cultures of isolates in tryptic soy broth (TSB), with 1% glucose, were left as an overnight culture. Four samples of 200 µL each were inoculated in triplicate in sterile flat-bottom 96-well polystyrene microliter plates. Plates were incubated at 37°C without shaking. Planktonic cells in the wells were removed by washing with 3 times of sterile PBS, then the biofilms were air-dried and stabilized by use of the 0.1% solution of crystal violet, followed by 15 min staining of the biofilms using 200 µL of 33% glacial acetic acid solution. The optical density (OD) at 590 nm was measured using a microplate reader (BioTek, USA). The classification of isolates was based on the OD values of isolates relative to the negative control (cut-off ODc = mean OD of control + 3 x standard deviation): non-biofilm, weak, moderate, and strong producers. The phenol chloroform isoamyl alcohol method, with slight modifications, was used to extract Genomic DNA. In brief, an overnight bacterial culture was centrifuged at 10,000 rpm for 5 min, after which it was suspended in TE buffer before being lysed with lysozyme (20 mg/mL, 37°C, 30 min) and subsequently treated with proteinase K and SDS. The extraction of the DNA was done using phenol, chloroform, and isoamyl alcohol, followed by the precipitation of the DNA using ethanol, 70% ethanol, and finally, the DNA was suspended in nuclease-free water. To validate the assay, *S. aureus* ATCC 43300 (*mecA*-positive) was used as a positive, and a *mecC*-positive control was used to confirm that the primers worked and

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that a negative result can be trusted. PCR tests were done against the *mecA* and *mecC* genes using primers that were published. The amplifications were conducted in 25 0L reaction mixtures that included 12.5 0L of 2X PCR Master Mix (Thermo Fisher Scientific, USA), 1 0L of each primer (10 0M), 2 0L template DNA (50 ng), and nuclease-free water. My ecA thermocycling conditions were: denaturation (95°C, 5-min); 35 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 45 s); and finally denaturation (72°C, 7-min). In the case of mecC, annealing was done at 58°C. PCR products were packed on ethidium bromide-stained, 1.5% agarose gels, and their position was observed under UV light with the help of a gel documentation system (Bio-Rad, USA). As a molecular size marker, a 100 bp DNA ladder was employed. *MecA* was expected to be 238 bp and mecC 304 bp in size.

RESULTS

From the 200 houseflies collected, 16 isolates (8%) were identified as *Staphylococcus aureus* based on mannitol fermentation on MSA (yellow colonies) and Gram staining, which showed Gram-positive cocci in grape-like clusters. The point prevalence of *S. aureus* carriage was 8% (95% confidence interval (CI): 4.6% to 12.7% These isolates were selected for further phenotypic and molecular characterization. Kirby–Bauer disk diffusion revealed high resistance among the 16 *S. aureus* isolates. All strains (100%) were resistant to oxacillin and nitrofurantoin. Resistance to other agents included erythromycin (87.5%), rifampicin (75%), cephalexin (68.7%), and ciprofloxacin (62.5%). In contrast, only two isolates (12.5%) showed resistance to cefoxitin, while susceptibility to tigecycline and vancomycin was relatively preserved. The mean resistance rate across ten clinically relevant antibiotics was 68.7%. Antibiotic resistance profile of Staphylococcus aureus isolates, showing the percentage of resistance observed against different tested antibiotics (Figure 1).

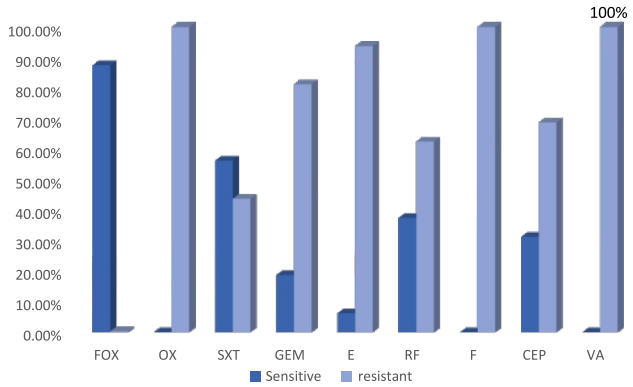

Figure 1: Antibiotic Resistance Profile of *Staphylococcus Aureus* Isolates Against Different Tested Antibiotics

FGE enhanced the activity of β-lactam antibiotics against MRSA isolates. Zones of inhibition produced by oxacillin or cefoxitin discs increased significantly when combined with FGE compared to antibiotics alone. FGE alone also produced measurable antibacterial activity, but the effect was markedly potentiated in combination. It is important to note that these findings are preliminary and represent a preclinical exploration of FGE's potential synergistic effects. Further in vivo validation and mechanistic investigations are required before any therapeutic implications can be drawn. The individual use of the garlic extract and antibiotics (OX and CTX) on the MRSA exhibited intermediate zones of inhibition, which means that the two have their antimicrobial effect independently (Figure 2).

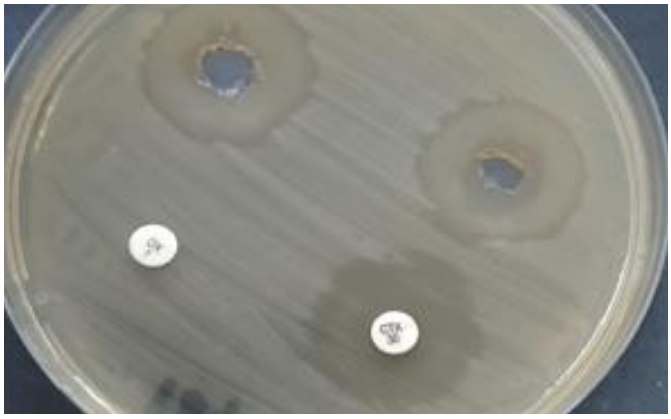

Figure 2: The Separate Application of Garlic Extract and Antibiotics Against MRSA

Conversely, there was a significantly improved zone of inhibition in the combination of garage extract using OX and CTX than when they were administered separately. This means that it has a synergistic effect that enhances the antibacterial effect against MRSA (Figure 3).

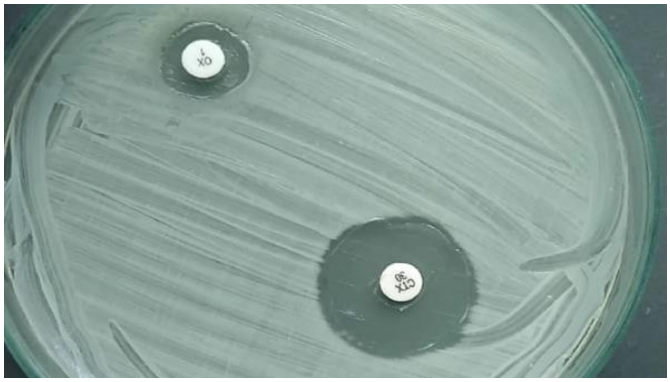

Figure 3: Concomitant Effect of Garlic Extract with OX and CTX

Quantitative biofilm assays revealed that the majority of isolates exhibited substantial biofilm-forming ability. Specifically, 10 of 16 isolates (62.5%) were categorized as strong biofilm producers, 3 isolates (18.7%) as moderate, and 2 isolates (12.5%) as weak producers, while only a single isolate (6.3%) showed no measurable biofilm formation. The predominance of strong biofilm producers suggests that most of these MRSA strains possess a considerable capacity for persistence and survival on abiotic surfaces, which may facilitate prolonged environmental

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contamination and contribute to transmission in healthcare settings(Figure 4).

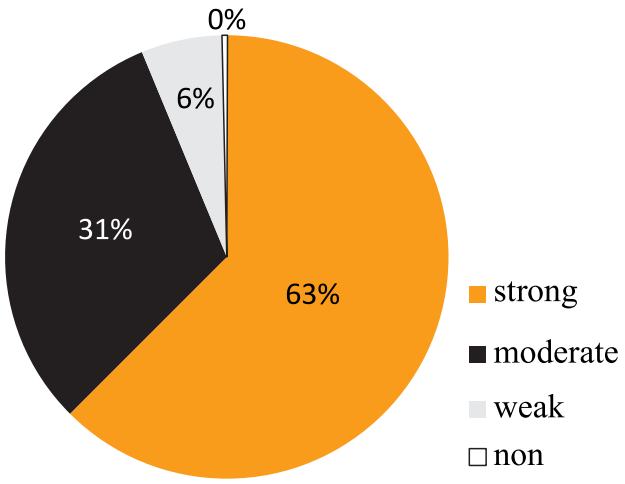

Figure 4: The Percentage Distribution of *Staphylococcus Aureus* Isolates Categorized as Biofilm Producers

PCR analysis confirmed the presence of the *mecA* gene in all 16 *S. aureus* isolates, supporting their phenotypic resistance to methicillin. Amplification consistently produced the expected 238 bp product, which was clearly visualized by gel electrophoresis under UV illumination. In contrast, none of the isolates yielded amplicons with *mecC*-specific primers, indicating the absence of this resistance determinant in the tested population. The universal detection of *mecA* highlights it as the dominant genetic mechanism of methicillin resistance among these isolates, while the lack of *mecC* is consistent with its relatively rare occurrence in South Asia compared to European settings. Summary of *Staphylococcus aureus* isolates showing their source location, antibiotic resistance profiles, biofilm strength, and *mecA* gene detection(Table 1).

Table 1: Key CSC Markers and Their Expression in Various Cancer Types

Isolate ID	Source Location	Ox	Fox	Ery	Rif	Cpx	Cep	Nit	Van	Tig	TMP-SMX	Biofilm Strength	mecA
SA01	Ward	R	S	R	R	R	R	R	S	S	R	Strong	+
SA02	Ward	R	R	R	S	R	S	R	S	S	R	Strong	+
SA03	Waste Area	R	S	R	R	R	R	R	S	S	R	Moderate	+
SA04	Ward	R	S	R	R	S	S	R	S	S	R	Strong	+
SA05	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA06	Ward	R	S	R	R	R	S	R	S	S	S	Moderate	+
SA07	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA08	Ward	R	S	R	S	S	S	R	S	S	R	Weak	+
SA09	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA10	Ward	R	S	R	R	R	S	R	S	S	R	Strong	+
SA11	Waste Area	R	S	R	R	R	S	R	S	S	R	Moderate	+
SA12	Ward	R	S	R	S	R	R	R	S	S	R	Weak	+
SA13	Ward	R	S	R	R	R	R	R	S	S	R	Strong	+
SA14	Waste Area	R	S	R	R	R	R	R	S	S	R	Strong	+
SA15	Ward	R	S	R	R	R	R	R	S	S	R	Strong	+
Sa16	Waste Area	R	S	R	S	S	S	R	S	S	S	Non-Biofilm	+

Ox = Oxacillin, Fox = Cefoxitin, Ery = Erythromycin, Rif = Rifampicin, Cpx = Ciprofloxacin, Cep = Cephalexin, Nit = Nitrofurantoin, Van = Vancomycin, Tig = Tigecycline, TMP-SMX = Trimethoprim–sulfamethoxazole; R = Resistant, S = Susceptible, + = Present.

DISCUSSION

This study recovered *Staphylococcus aureus* from houseflies collected in a tertiary-care hospital environment in Lahore, Pakistan. This represents the first systematic survey of hospital-associated flies in Lahore for MRSA carriage with both phenotypic and genotypic characterization. All 16 isolates identified as MRSA carried the *mecA* gene, 8% (95% CI: 4.6-12.7%) the *mecA* gene, showed multidrug resistance to commonly used antibiotics, and most were strong biofilm producers. These findings are consistent with regional molecular surveillance showing a dominance of *mecA* among MRSA isolates in Pakistan and variable but generally low prevalence (68.7%) of *mecC* [13, 14]. A recent molecular survey from the region reported *mecA* as the principal determinant in MRSA isolates, with *mecC* present in a minority of strains, reinforcing the prominence of *mecA* in this setting [15, 16]. The recovery of MRSA from synanthropic insects echoes a growing body of evidence that insect pests (particularly houseflies) can act as mechanical carriers and environmental reservoirs of antimicrobial-resistant bacteria in healthcare settings. Multisite surveillance studies and reviews have repeatedly identified flies in hospital and peri-hospital environments as vectors that harbor diverse bacterial species, including

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S. aureus, many carry genes encoding resistance to clinically important antibiotics [17]. Although such studies generally do not prove direct transmission to patients, they highlight an under-recognized environmental route by which resistant organisms can persist and potentially contaminate surfaces, food, or wounds. Therefore, our observation of MRSA in flies aligns with recent international surveillance data that call for including insect control and environmental monitoring within broader AMR containment strategies [18]. The high frequency of strong biofilm producers among our isolates (≈62.5%) is of particular concern because biofilms markedly increase bacterial persistence on abiotic surfaces and medical devices and decrease susceptibility to antibiotics. This high biofilm prevalence among insect-derived MRSA isolates represents a novel observation for this setting. Comparable studies from Pakistan and neighboring regions report similarly high proportions of MRSA isolates with substantial biofilm formation. This supports the idea that biofilm production is widespread among clinically relevant MRSA lineages in this geography. The confluence of multidrug resistance, presence of *mecA*, and robust biofilm formation in isolates found on flies underscores the potential for environmental persistence and the added challenge such strains pose to routine cleaning and disinfection in hospitals [19]. In vitro findings that FGE potentiated the activity of β-lactam antibiotics against MRSA is supported by experimental data describing the antimicrobial and synergistic effects of garlic-derived organosulfur compounds such as allicin. Several laboratory studies and reviews have demonstrated that crude garlic extracts and purified allicin exhibit antibacterial and antibiofilm activity against MRSA and can act synergistically with conventional antibiotics under controlled conditions. However, these findings should be interpreted as preliminary and preclinical, reflecting laboratory-based observations only. While these results are promising and suggest low-cost adjunctive options for resource-limited settings, they remain in vitro and exploratory in nature, and translation to clinical application would require rigorous pharmacological, toxicological, and in vivo efficacy testing [20].

This study was limited by its single-center design and relatively small number of MRSA isolates, which may restrict the generalizability of the findings to other healthcare settings. Whole genome sequencing and detailed molecular typing were not performed, limiting deeper insights into clonal relationships and transmission dynamics. Additionally, the synergistic effects of fresh garlic extract were evaluated only through in vitro assays, without in vivo validation or mechanistic analysis. Future research should include multi-center surveillance, genomic characterization, and longitudinal environmental monitoring to clarify transmission pathways. Further pharmacological and toxicological investigations are also required to determine the clinical feasibility of plant-derived adjunct therapies against multidrug-resistant MRSA.

CONCLUSION

Hospital-associated flies in Lahore can harbor mecA-positive, multidrug-resistant, and biofilm-forming MRSA strains. Finding mecA-positive, multidrug-resistant, biofilm-forming S. aureus on hospital-associated flies emphasizes the need to broaden infection-control measures to include vector surveillance and control in hospital settings, particularly in tropical and resource-constrained environments with abundant synanthropic insects. Integrating environmental surveillance of insects into AMR monitoring programs could provide early indicators of circulating resistance genes in the hospital ecosystem. Additionally, the observed in vitro synergy between FGE and β-lactams suggests a potential role for natural product research in identifying adjunctive agents that could restore or enhance antibiotic activity.

Authors' Contribution

Conceptualization: SR
Methodology: TZ, HAR, MQ
Formal analysis: HAR, M
Writing and Drafting: HAR, MQ, M
Review and Editing: HAR, MQ, M, TZ, SR

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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