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***Original Research Article*** ***Open Access***

# ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM SALAD SAMPLES

**ABDULLAH[1*], AMIN FU[2,1], HASSAN N[1], KHAN FA[3], MOHAMMAD Z[1], DILAWAR S[1], BAKHTIAR M[1], UMER M[1], MEHMOOD K[4], BASHIR K[1*]**

*[1]Department of Health and Biological Sciences, Abasyn University, Peshawar, 25000, Khyber Pakhtunkhwa Pakistan*
*[2]The Department of Neurosurgery, Center for Stem Cell and Regenerative Medicine, UT Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center at Houston (UTHealth), Houston, TX 77030, USA*
*[3]Department of Allied Health Sciences, Iqra National University Peshawar, 25000, Pakistan*
*[4]Agricultural Research Station Serai Naurang (Bannu), 28440 Bannu, Pakistan*
**Correspondence Author Email Address: awanabdullah860@gmail.com; kashif.bashir@abasyn.edu.pk*

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**Abstract** *Salad vegetables are consumed without heat treatment therefore the possibility of food-borne illness is more. Salad can get contaminated with pathogenic microorganisms during growing in the field, harvesting, handling, processing, and distribution. Staphylococcus aureus is one of the most adaptable human pathogens. It can cause a range of illnesses from minor skin infections to life-threatening diseases. The present study was conducted for the isolation of Staphylococcus aureus from salad vegetables from different restaurants in Peshawar. For this purpose, a total of 30 salad samples were collected from different local restaurants located in Peshawar. The samples were diluted in peptone water and inoculated on a Mannitol Salt Agar medium. The S. aureus was confirmed by biochemical tests. 17 samples out of 30 were confirmed positive for S. aureus. The positive samples were then checked for antibiotic susceptibility with antibiotics CLR, FOX, NV, LZD, and VA by inoculating on an agar plate. In positive samples, 47.05% to resistant to NV, 35.29% to LZD, 29.41%) to CLR, 23.52 % to VA, and 17.64% MRSA were detected. A high level of sensitivity of 64.70% was shown by FOX and LZD. Out of 30 samples, 29.98% (average) resistance was noted against 5 antibiotics which indicated a low level of hygienic condition applied during salad making, handling, and processing. Our study concluded that S. aureus was present in salad vegetables of different restaurants in Peshawar. MRSA was also detected in RTE salad during the present study. Further molecular research is needed for the identification of MRSA and VRSA genes.*

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**Keywords:** *Salad vegetables; bacterial contamination; S. aureus; susceptibility pattern*

## Introduction

*Staphylococcus aureus* is included in the group of Gram-positive bacteria which is a non-spore-forming, facultative anaerobic bacteria that belongs to the *Staphylococcus* genus. The genus *Staphylococci* is subdivided into 32 species and subspecies. *S. aureus* is widespread on the skin and mucosa of humans and other animals and also in the environment. *S. aureus* produces enterotoxins which is the cause of food-borne disease (SFD) caused by the consumption of contaminated food by *S. aureus* and their enterotoxins. *S. aureus* is considered to be the common reason for reported food-borne illnesses (Kadariya *et al.,* 2014). Consumption of vegetable salad (tomatoes, onions, cucumber, carrots, cabbage, lettuce, and green chili) is an increasing trend in Pakistan. Almost they are sold in every market. Salad vegetables enhance a healthy life but harbor a different range of pathogenic microbial agents (Shah *et al*., 2015). The salad items show a widespread disparity in total microbial count ranging from 1.6 x $10^6$ to 2.9 x $10^8$ cfu/g at 37 °C associated with the salad. *S. aureus* is found in the majority of salad samples (Itohan., *et al* 2011). Salad vegetables can be contaminated with pathogenic microbes during their growth in fields, and orchards, during harvesting, handling, processing, and dissemination (Tambekar *et al.,* 2006).

Approximately 250 diverse food-borne diseases have been identified. The term food poisoning can be defined as any kind of illness or disease when contaminated food is eaten. Gastroenteritis is mainly caused by *Staphylococcus aureus* due to eating food in which enterotoxigenic *staphylococci* have grown and produced toxins that are stable at high temperatures (100°C for 1 h) (Bhatia and Zahoor 2007). *staphylococcal* food poisoning is mainly caused by both Coagulase-positive (CPS) and coagulase-negative (CNS) *staphylococci*. CPS and

CNS have received increasing consideration because of their active part in disseminating antibiotic resistance markers (Osman *et al.,* 2015). The resistant strains of *S. aureus* include community-associated MRSA (CA-MRSA), livestock-associated (LA-MRSA), Hospital associated MRSA (HA-MRSA) can originate in foods that are intended for human feeding. The sources of contamination of food especially animal and plant-origin foods may be livestock along with humans that are involved in the food handling process (Sergelidis *et al.,* 2017). In 1960 Methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified as a nosocomial pathogenic bacteria and it is related to severe community-acquired and nosocomial diseases mostly in immune-compromised patients (Vanegas López *et al.,* 2012). Researchers recommend that selective media based on cefoxitin are superior to those based on oxacillin for the detection of MRSA (Smyth *et al.,* 2005). The presence of *S. aureus* might indicate insufficient sterility during salad elaboration however the enterotoxigenicity of the strains highlights the risk of consumer's intoxication (Estrada *et al*., 2013).

Methicillin-resistant *S. aureus* (MRSA) is the causative agent of infections and is mostly being treated by Vancomycin. However, the isolation of the first vancomycin-resistant MRSA was done in 1996. Various vancomycin-resistant *S aureus* (VRSA) strains in different countries like USA, France, Korea, South Africa, and Brazil have confirmed that the emergence of vancomycin resistance in *S. aureus* is a global issue (Hiramatsu *et al.,* 2001). Nowadays different antibiotics are being used against *S. aureus* including vancomycin, penicillin G, linezolid, oxacillin, imipenem, and some other antibiotics. The *S. aureus* is becoming resistant to some of these antibiotics (Aydin *et al.,* 2011). As per the above statements the current study was designed to assess the following aims and objectives.

## Materials AND Methods

### Sample collection

A total of 30 samples of RTE salad were collected from different restaurants located on Ring Road, Charsadda Road, Kohat Road, University Road, and Dalazak Road. These samples were collected in an antiseptic zip-lock bag and transported to the laboratory for analysis. The samples were immediately processed for further analysis.

### Sample processing

### Media preparation

Two types of media including Peptone Water and Mannitol Salt Agar (MSA) were prepared as follows.

### Peptone Water

15-gram powder was taken and dissolved in 1 liter of distilled water and Peptone Water was organized. It was shaken well to dissolve completely and disinfected by autoclaving at 121 °C for 15 minutes. The medium was incubated at 37 °C for 24 hrs for sterility check. The sample was added to a medium and incubated at 37 °C for 12 - 18 hrs.

### Mannitol Salt Agar (MSA)

MSA medium was prepared by dissolving 108 g of powder in 1 liter of distilled water. The media was sterilized by autoclaving at 121 °C for 15 minutes. The media was then cooled to 60 °C, shaken, and poured in antiseptic petri plates aseptically. Plates were incubated at 37 °C for 24 hrs for sterility check. The plates were inoculated with specimens and incubated at 37 °C for 24 hrs.

### Sample inoculation

Each sample in 25 g of size was added to 250 ml of peptone water, grinded, homogenized carefully for 4 min, and incubated for 18 h. This overnight culture was then serially diluted up to $10^{-1}$ - $10^{-4}$. A 100 µl suspension from $10^{-2,}$ $10^{-3,}$ and $10^{-4}$ diluent was taken and spread on a mannitol salt agar plate. The plates inoculated with test organisms were incubated for 24 to 48 hrs at 37 °C. Bacterial colonies were observed.

### Bacterial identification

### Gram Staining

A clean glass slide was added with a drop of distilled water. A bacterial colony was taken and mixed with water dropped on a slide. The slide was heat-fixed by passing over the flame 3-4 times. The smear was stained with crystal violet for a minute and then washed with water. Iodine liquid was used to flood the slide for 1-2 minutes and washed with water. 95% ethanol was used to decolorize the slide for a few seconds and then washed with water. Safranin was added for 1-2 minutes and washed with water. The slide was air-dried and observed in the microscope.

### Catalase Test

A microscopic slide was placed on a petri plate. 1 drop of 3 % hydrogen peroxide was placed on a microscopic slide. A small inoculum was taken with a sterile wire loop and introduced into hydrogen peroxide ($H_2O_2$), and the bubble formation was observed.

### Coagulase Test

A microscopic slide was placed on a petri plate. The serum containing coagulase was placed on a microscopic slide. A small amount of inoculum was taken with a sterile wire loop and mixed with serum on a slide. The slide was rocked gently and a macro clump was observed.

### Blood agar Test

To prepare Blood agar media, 40 g of powder was dissolved in 1 liter of distilled water. The media was autoclaved for sterilization at 121 °C for 15 minutes. The media was cooled to 50 °C, and fresh antiseptic sheep blood was added and mixed gently. The 15-20 ml prepared media was poured into a sterile Petri plate. The specimens were spread on prepared blood Agar plates. The incubation was done at 37 °C for 24 hours. Lysis of RBC (hemolysis) was observed after incubation.

### Antibiotic resistance

Antimicrobial activities of isolated strains were determined by Kirby-Bauer method (Biemer and James 1973).

**Nutrient broth preparation & inoculation**

The nutrient broth was prepared by mixing 8 g powder in 1 liter of distilled water. The medium was autoclaved at 121 °C for 15 minutes. The inoculum of overnight grown culture was added to the prepared nutrient broth and incubated for 24 hrs. Nutrient Agar was prepared by dissolving 28 g powder in 1 liter of distilled water. The media was autoclaved for sterilization at 121 °C for 15 minutes. After sterilization, the media was cooled to 60 °C and poured into petri plates. The sterility was checked by incubating the plates at 37 °C for 24 hrs. Microbial inoculum was taken from incubated nutrient broth and spread on prepared nutrient agar plates with the help of cotton swabs to prepare bacterial lawn. The selected antibiotic discs were located on nutrient agar plates containing bacterial lawn followed by incubation of inoculated plates at 37 °C for 24 hrs.

**Results**

In the present study, 30 samples of salads were taken from various restaurants situated in Peshawar city. Salad samples were examined for the presence of *S. aureus*. Results revealed that about (56.66 %) of all salad samples showed bacterial growth on the MSA medium. A high frequency of bacterial isolates was obtained from a local restaurant located on Charsada Road (75 %), Ring Road, and Dalazak Road (50 %), followed by Kohat Road (40 %) and University Road (66.66 %).

**Table 1: Samples collected from various locations with their percentage**

| S.NO | Location | Total samples | Positive sample | Percentage (%) |
|---|---|---|---|---|
| **1** | Charsadda Road | 8 | 6 | 75 |
| **2** | Ring road | 8 | 4 | 50 |
| **3** | Dalazak Road | 6 | 3 | 50 |
| **4** | Kohat Road | 5 | 2 | 40 |
| **5** | University Road | 3 | 2 | 66.66 |

Bacterial isolates were then processed for identification. Catalase, coagulase, and blood agar hemolysis were performed to check the sample's confirmation. 17 bacterial isolates appeared purple under the microscope which showed gram-positive cells. The bubble formation of bacterial isolates showed that they were catalase-positive. These isolates were further checked by coagulase test by clotting each isolate in blood serum indicating that are coagulase positive. These isolates showed growth on the Blood Agar medium with hemolysis. During the current study, different antibiotics were checked against the isolated *S.aureus*. The bacterial strains were evaluated as resistant, intermediate, and sensitive by measuring the zones of inhibition around antibiotic disks. A few *S. aureus* isolates were resistant to various antibiotics.

**Table 2: (a) Antibiotics against different isolates with their zone of inhibition in mm**

| Isolates No | Zone of inhibition of following antibiotics(mm) | | | | |
|---|---|---|---|---|---|
| | NV(30 µg) | LZD(30 µg) | FOX(30µg) | CLR(15µg) | VA(30 µg) |
| 1 | 21 | 30 | 29 | 28 | 20 |
| 2 | 22 | 30 | 30 | 30 | 19 |
| 3 | 7 | 30 | 26 | 16 | 25 |
| 4 | 30 | 30 | 27 | 30 | 9 |
| 5 | 14 | 25 | 12 | 17 | 13 |
| 6 | 7 | 8 | 10 | 12 | 12 |
| 7 | 13 | 19 | 26 | 9 | 13 |
| 8 | 13 | 10 | 12 | 8 | 16 |
| 9 | 14 | 25 | 20 | 23 | 12 |

**Note:** R= Resistant, I=Intermediate, S= Sensitive

CLSI standard for NV = (R= ≤12 I = 12-16 S = ≥16)

CLSI standard for LZD = (R = ≤20, I = 20-21, S = ≥21)

CLSI standard for FOX = (R = ≤21, I = 21-22, S = ≥22)

CLSI standard for CLR = (R = ≤13, I = 14-17, S = ≥18)

CLSI standard for VA = (R = ≤12, I =12, S = ≥12)

**Table 2: (b) Antibiotics against different isolates with their zone of inhibition in mm**

| Isolates No | Zone of inhibition of following antibiotics(mm) |
|---|---|

| | NV(30 µg) | LZD(30 µg) | FOX(30µg) | CLR(15µg) | VA(30 µg) |
|---|---|---|---|---|---|
| 10 | 14 | 7 | 25 | 20 | 16 |
| 11 | 14 | 18 | 21 | 19 | 13 |
| 12 | 4 | 13 | 24 | 9 | 14 |
| 13 | 9 | 22 | 24 | 22 | 5 |
| 14 | 4 | 25 | 24 | 15 | 12 |
| 15 | 11 | 23 | 21 | 22 | 7 |
| 16 | 9 | 25 | 27 | 10 | 10 |
| 17 | 7 | 23 | 26 | 16 | 13 |

**Note:** R= Resistant, I=Intermediate, S= Sensitive
CLSI standard for NV = (R= ≤12 I = 12-16 S = ≥16)
CLSI standard for LZD = (R = ≤20, I = 20-21, S = ≥21)
CLSI standard for FOX = (R = ≤21, I = 21-22, S = ≥22)
CLSI standard for CLR = (R = ≤13, I = 14-17, S = ≥18)
CLSI standard for VA = (R = ≤12, I =12, S = ≥12)

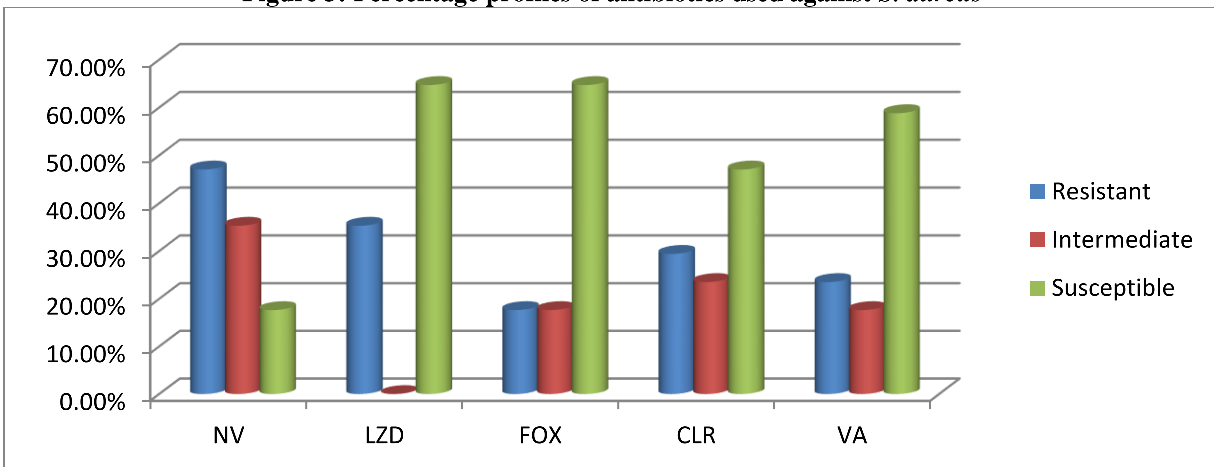
Out of 17 bacterial isolates, NV showed a zone of inhibition against *S. aureus* in the range of 4-30 mm while LZD showed a zone of inhibition in the range of 7-30 mm. FOX showed a zone of inhibition 10-30 mm against bacterial isolates. The zone of inhibition of CLR was noted in the range of 8-30mm while VA showed a zone of inhibition in the range of 5-25 mm. Table 3 showed resistance, intermediate, and sensitive levels of isolates against 5 mentioned antibiotics. A total of 29.98% (average) of *S. aureus* isolates were resistant to one to five antibiotics. The antibiotic Novobiocin was noted with higher resistance (47.05%) followed by Linezolid (LZD) (35.29 %). Clarithromycin (CLR) (29.41%), Vancomycin (23.52%), and Cefoxitin (FOX) (17.64 %) as given in Table 3. *S. aureus* strains which were resistant to FOX (17.64 %) were considered MRSA.

**Table 3: Percentage of different antibiotics used against *S. aureus***

| Antibiotics | NV | LZD | FOX | CLR | VA |
|---|---|---|---|---|---|
| **Resistant** | 47.05% | 35.29% | 17.64% | 29.41% | 23.52% |
| **Intermediate** | 35.29% | 0% | 17.64% | 23.52% | 17.64% |
| **Susceptible** | 17.64% | 64.70% | 64.70% | 47.05% | 58.82% |

It is also clear from Table 3 that 64.70% of isolated samples were sensitive to Cefixotin (FOX), 64.70% were susceptible to LZD, and 47.05% was susceptible to CLR, while low susceptibility of Novobiocin was noted against *S. aureus* 17.64 %. Among the tested antibiotics Novobiocin and Clarithromycin showed high intermediate levels for *S. aureus* (35.29%) and (23.52%) respectively while LZD showed a 0% of intermediate level.

**Figure 3: Percentage profiles of antibiotics used against *S. aureus***




**Discussion**

Over the last few years, RTE salad become popular in grocery stores and restaurants all over the world. The salad preparation comprises general handling and the use of uncooked ingredients which are particularly exposed to microbial contaminants that cause food-borne diseases Almualla *et al.,* (2010). *S. aureus* is one of the most important causes of food-born infection (intoxication) contamination by a food worker is the only common cause of *S. aureus* outbreaks Bennett *et al.,* (2018). Methicillin-Resistant *Staphylococcus aureus* (MRSA) a main pathogen in humans, is presently prevalent in various hospitals

around the world and has also emerged in the community. In recent times, Kluytmans *et al.,* (2009) stated that MRSA has been recognized as the main pathogen in food production, animal and people in contact with these animals and food.

Our study comprised 30 RTE salads samples which were investigated for isolation of *S. aureus*. The study revealed that about 17 out of 30 samples were positive for *S. aureus.* Which were biochemically identified by using biochemical tests. Out of 30 samples, 56.66% were positive for *S. aureus*. Our results here are in comparison with Baumgartner *et al.,* (2014) and Vicedo *et al.,* (2006) who also isolated *S. aureus at* 41% and 42.07% by traditional culture method. A total of 29.98% (average) of *S. aureus* species from salad vegetables were resistant to one to five antibiotics. The result of this study is lower as compared to the Nipa *et al.,* (2011) stated that 98.06% of bacterial isolates were resistant to two to seven antibiotics. The antibiotic Novobiocin was noted with higher resistance 47.05% followed by Linezolid (35.29%), Clarithromycin (29.41%), Vancomycin (23.52%), and Cefoxitin (17.64 %) as given in table 3. *S. aureus* which was resistant to FOX (17.64 %) was considered MRSA. *S. aureus* isolates were 17.64% resistant to Cefoxitin which is an almost similar result that was reported by Sina *et al*., (2011) about the methicillin inefficacy against 15.18% of the *S. aureus* bacterial species. Our results are also in agreement with Kim *et al*., (2004) the researchers recorded a 64% methicillin resistance rate, a constitutive macrolide (CLR) resistance phenotype was common and no isolates were resistant to linezolid. It is also clear from Table 3 that 64.70% of isolated samples were sensitive to Cefixotin (FOX) and LZD followed by Vancomycin (58.82%). Low susceptibility of Novobiocin was noted against *S. aureus* (17.64 %). Among the tested antibiotics Novobiocin (NV), and Clarithromycin (CLR) showed a high percentage of intermediate level for *S. aureus* 35.29%*, and* 23.52% respectively. While the Cefoxitin (FOX) and Vancomycin (VA) showed 17.64% of intermediate level. Our results here are in comparison with Akanbi *et al.,* (2017) who also investigated the antibiotic susceptibility of *S. aureus.* This revealed varying susceptibility to cefoxitin (76.7%), vancomycin (50%) and some other antibiotics.

**Conclusion**

Our study concluded that *S. aureus* was present in salad vegetables of different restaurants in Peshawar. MRSA was also detected in RTE salad during the present study. A high level of sensitivity was noted to Cefoxitin while a low level of susceptibility of isolates recorded for Novobiocin. Further molecular research is needed for identification of MRSA and VRSA genes.

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**Declaration**

**Data Availability statement**

All data generated or analyzed during the study have been included in the manuscript.

Consent for publication

Not applicable

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**Conflict of interest**

The authors assure that there were no financial relationships involved that could be perceived as a conflict of interest.

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**Author, Disclosure Statement**

**Ethics approval**

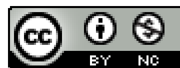
The study permitted by the Ethical Committee of Abasyn University Peshawar.

**AutAuthhor's Contributions**

Abdullah did the experiments and wrote the manuscript. FUA, and HB conceived the study and design, AAK, ZU reviewed the manuscript. All the authors participated in the experimentation & optimizations

**Consent for Publication**

Not applicable



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