Kurdish Studies
Jan 2024
Volume: 12, No: 1, pp.4901-4912
ISSN: 2051-4883 (Print) | ISSN 2051-4891 (Online)
www.KurdishStudies.net

Received: October 2023 Accepted: December 2023
DOI: https://doi.org/10.58262/ks.v12i1.355

Characterization of Enterotoxigenic Escherichia Coli and Staphylococcus Aureus from Fruit and Vegetable Salads

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Abstract

Enterotoxigenic Escherichia coli (ETEC) and Staphylococcus aureus were commonly found bacteria that posed contamination risks to fruit and vegetable salads. In a study involving 100 samples, comprising 50 fruit and 50 vegetable salads, significant findings were observed. Staphylococcus aureus was isolated in 61% (n=17) of vegetable salads and 39% (n=11) of fruit salads. Escherichia coli was present in 79% (n=11) of vegetable salads and 21% (n=7) of fruit salads. Antibiotic susceptibility testing revealed resistance patterns in E. coli strains from fruit and vegetable salads. E. coli from fruit salads showed 50% and 17% resistance to ciprofloxacin and Imipenem, respectively. In comparison, E. coli from vegetable salads exhibited 28% and 45% resistance to ciprofloxacin and Imipenem, respectively. Notably, E. coli strains from vegetable salads displayed higher resistance to Fosfomycin (61%) and gentamicin (50%). Staphylococcus aureus strains from vegetable salads exhibited 39% resistance to gentamicin and 29% resistance to Imipenem. Genetic analysis revealed the presence of toxin-producing genes in E. coli and S. aureus isolates, with varying prevalence rates. This study underscored the importance of monitoring bacterial contamination in salads for food safety and public health. The higher prevalence of S. aureus highlighted the need for strong monitoring strategies to detect and prevent foodborne infections caused by these pathogens in fruit and vegetable salads.

Keywords: *Enterotoxigenic E. coli, S. aureus, Antibiotic susceptibility patterns, Polymerase chain reaction, CLSI-2020*

Introduction

The consumption of freshly prepared fruit and vegetable has risen in 2020 years as a direct result of the numerous health benefits that these foods provide. On the other hand, these foods have been associated with a number of outbreaks of food borne disorders that are caused by bacteria. Both Enterotoxigenic Escherichia coli (ETEC) and Staphylococcus aureus are common bacterial species that have the potential to contaminate fruit and vegetable salads, which can then lead to an individual becoming ill from eating tainted food. It is absolutely necessary to have a solid understanding of the epidemiology, pathogenicity features, and patterns of antibiotic resistance of such illnesses in order to develop effective management strategies. There is a significant amount of effort currently being directed towards the characterization of ETEC and S. aureus from fruit and vegetable salads (Naeem et al., 2020).

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Escherichia coli (E. coli) is a type of bacteria that is often found in the intestine of animals and humans. While most types of E. coli are harmless, some can cause serious infections. One specific strain called E. coli O157:H7 is especially dangerous and can make people very sick if they eat contaminated food. This strain produces a toxin called Shiga toxin, which can irritate the intestines and cause symptoms like bloody diarrhea, stomach pain, and vomiting. In some cases, a severe infection can lead to a life- threatening condition called hemolytic uremic syndrome (HUS), which can cause kidney failure and even death (Pitout et al., 2022).

Enterotoxigenic E. coli, or ETEC is a type of pathogenic E. coli that is responsible for causing food poisoning. It is one of the most common strains of E. coli that can make people sick. When a person is infected with ETEC, they may experience symptoms like diarrhea, nausea, vomiting, and abdominal cramping. These symptoms usually appear within 24 to 72 hours after infection. The reason ETEC causes these symptoms is because it produces enterotoxins, which are substances that can affect the intestines and lead to digestive problems. The most common way to get infected with ETEC is by consuming contaminated food and water. This can happen if you eat raw or under cooked meat, consume dairy products that have not been pasteurized, or eat fruits and vegetables that have been contaminated with the bacteria. It's important to practice good food hygiene and drink safe water to reduce the risk of ETEC infection (Bai et al., 2019).

The bacterium known as staphylococcus aureus, more commonly referred to as "staph," is responsible for a variety of diseases that can affect humans. Human skin and the nasal airways are common habitats for the gram-positive bacterium that causes this infection. S. aureus is a microorganism that is ordinarily found in the human body; nevertheless, it has the potential to cause infections if it can enter the circulation via a breach in the skin or a mucous membrane (Omoregie et al., 2022).

Staphylococcus aureus can live in environments with or without oxygen. This is called "facultative anaerobiosis". S. aureus is a very flexible bacterium that can make people sick with many different diseases, some of which can be deadly, like pneumonia, endocarditis, and septic shock. S. aureus can cause even a simple skin illness. The spread of antibiotic-resistant strains of bacteria, like methicillin-resistant Staphylococcus aureus (MRSA), is a major public health issue and a common cause of infections that people get in hospitals. Some of the virulence factors that S. aureus makes are toxins, enzymes, and proteins that are found on the surface of the cell. These help S. aureus colonies to attack its host (Howden et al., 2023).

There are several ways to find out various strains of E. coli and Staphylococcus aureus. In biochemical investigation, the metabolic properties of the bacteria are observed to see if they are the same as E. coli and Staphylococcus aureus. Serotyping is another method that groups bacteria into different serotypes or subtypes by looking at the specific proteins on their surfaces. Molecular methods are also used, such as polymerase chain reaction (PCR) and whole genome sequencing (WGS). For identification of E. coli and Staphylococcus aureus strains, PCR amplifies certain parts of the DNA of the bacteria, while WGS looks at the bacteria's total genetic makeup (Lee et al., 2023).

The study aimed to analyze the presence of E. coli and S. aureus in fruit and vegetable salads, along with their antimicrobial susceptibility patterns and the prevalence of toxin-producing genes. Using CLSI guidelines, the research assessed antimicrobial susceptibility to understand treatment options and resistance. The findings contribute to understanding contamination levels, antimicrobial resistance, and genetic characteristics, offering insights for food safety and public health.

Methodology

The current study "Characterization of enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* from fruit and vegetable salads" was conducted in the Microbiology Lab, Department of Microbiology at The University of Haripur.

Sample Collection

A total of 100 samples consisting of 50 fruit and 50 vegetable salads were collected randomly from different localities of Haripur city following recommended SOPs. All the samples were transported in polythene bags to the laboratory and proceeded immediately for enrichment and further analysis.

Microbiological Assay

The study focused on isolating Staphylococcus aureus and Escherichia coli from fruit and vegetable salads using Mannitol Salt Agar and MacConkey Agar, respectively. After incubation, colony morphology, Gram staining, and biochemical tests were conducted to identify the bacterial strains. The Gram staining process involved crystal violet, Gram iodine, decolorizer, and safranin to differentiate between S. aureus and Enterotoxigenic E. coli. Biochemical tests such as catalase, indole, citrate, and DNase were performed to assess enzyme activities. Antibiotic sensitivity testing was carried out using the Kirby Bauer disc diffusion method with specific antibiotics against the pathogens. The study followed CLSI standards for interpreting antibiotic sensitivity results on Mueller-Hinton agar plates. By analyzing bacterial identification, antimicrobial susceptibility patterns, and genetic characteristics, the research aimed to provide insights for effective management strategies in food safety and public health contexts.

Molecular Analysis

DNA Extraction

The boiling process was used to extract the DNA. A loopful of the colony from the sample was added to the autoclaved 300 ul of PBS in the Eppendorf tube, along with the PBS. The tube then went through 5 minutes of ultra-centrifugation at 13000 rpm. The resulting supernatant was removed after centrifugation, and 500 ul of TE buffer was then added to the Eppendorf tube containing the sample. Next, an Eppendorf tube was vortexed and heated to 95 °C for 10 minutes. The sample was then chilled for 30 minutes, followed by 5 minutes of ultracentrifugation at 13000 rpm. The suspension, or supernatant containing DNA, was collected and put into a fresh autoclaved Eppendorf tube.

Polymerase Chain Reaction

PCR reaction was performed for the detection of toxin producing isolates. A total volume of 25µl PCR reaction was prepared by adding 1µl of each forward and reverse primer, 4µl of DNA template, 5µl of Master mix, and 14µl of PCR water. The genes and the primers used are mentioned in Table 1.

Table 1: Primers Used in Study for E. Coli.

Target gene	Direction	Primer sequence	Size (bp)	Reference
Stx1	FR	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	180	
Stx2	FR	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	255	

Table 2: Primers Used in Study for S. Aureus.

Target gene	Direction	Primer sequence	Size(bp)	Reference
mec A	F R	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	147	Amit *et al.,* 2018
PVL	F R	ATCATTAGGTAAAATGTCTGGACATGATC GCATCAAGTGTATTGGATAGCAAAAGC	433	Amit *et al.,* 2018

CR Conditions for E. Coli

PCR conditions were optimized using different temperature for pre-denaturation, denaturation, annealing, and elongation and then final elongation. The final conditions used for the PCR are given in table 3.

Table 3: PCR Conditions Used in the Study for Detection of Stx1 And Stx2 Genes in E.Coli.

Steps	Temperature	Time
Pre-denaturation	95ºC	9 min
Denaturation (30 cycles)	94ºC	30 sec
Annealing	60ºC	30 sec
Elongation	72ºC	45 sec
Final Elongation	72ºC	7 min

PCR Conditions for S. Aureus

PCR conditions for detection of *mecA* and *PVL* genes from *S. aureus* were optimized using different temperature for pre denaturation, denaturation, annealing, elongation and then final elongation as given in table 4.

Table 4: PCR Conditions for Detection of Mec A and PVL Genes from S. Aureus.

Steps	Temperature	Time
Pre-denaturation	94 ºC	5 min
Denaturation (35 cycles)	94 ºC	30 sec
Annealing	55 ºC	30 sec
Elongation	72 ºC	1 min
Final elongation	72 ºC	10 min

Gel Electrophoresis

TBE Preparation

To the TBE (Tris/boric/EDTA) 10X stock solution was added 10.8 g of tris, 5.5 g of boric acid, and 50 ml of 0.5M EDTA for each litter solution. By taking out 100 ml from the stock solution and combining it with 900 ml of distilled water to obtain 1000 ml of working solution, the 10X was reduced to 1X. The agarose gel was prepared with the solution, and a running buffer was prepared with it. 100 ml of 1X TBE solution (10 ml of 10X TBE dissolved in 90 ml of distilled water) was added to produce a 2% agarose solution. The solution was then kept in a hot oven for two minutes. To better perceive the bands, the answer was cooled to around 50 ºC, and 2 µl of ethidium bromide was added. The solution was placed into a casting tray with a comb adjusted in it, then left to cool down for solidification. Then the comb was taken out after the solidification. The gel was put into a gel tank, and the wells containing 100 bps of ladder

received 5µl of PCR product mixed with 3 µl of loading dye. For 45 minutes, samples were processed at 80 volts (300 mA). After that, bands were seen under UV light and their presence was confirmed.

Statistical Analysis

Statistical tests were used in the study such as Percentage, Chi-square, and P-value. The significant was considered at p value <0.05.

Results

4.1 Morphological Characterization

In this study, 100 salad samples total 50 vegetable salads and 50 fruit salads were examined. Implementing the recommended SOPs, samples were randomly selected from several Haripur city regions. The growth on selective media, colony morphology, Gram staining, and microscopy were used to confirm the presence of Enterotoxigenic Escherichia coli (ETEC) and Staphylococcus aureus. Biochemical methods such as catalase, indole, citrate, and DNase were used to perform further assurance. According to the results of the study, S. aureus was present in 61% of the samples of vegetable salad and 39% of the samples of fruit salad. While for E. coli was isolated from 22% of the samples of vegetable salad and 14% of the samples of fruit salad tested positive.

Table 5: Prevalence of Study Organisms from Fruit and Vegetable Salads.

Isolated strains	Fruit salads	Vegetable salads
S. aureus	11 (39%)	17 (61%)
E. coli	7 (14%)	11 (22%)
Total samples	50	50

Table 6: Number of Isolated Species of Selected Organisms.

S.NO	Isolate species	Number of isolated strains
1.	Escherichia coli	18
2.	Staphylococcus aureus	28

Evaluation of Antibiogram Pattern of Study Organism

The antibiotic resistance profile of isolated bacterial species was determined by Kirby Bauer disk diffusion assay. Guidelines provided by CLSI 2020 were used as standard as given in table 7 for E. coli and Table 8 for S. aureus.

Table 7: Antibiotics Zones Chart of E.Coli in Millimeters according to CLSI 2020.

Antibiotics	Disc content	Sensitive	Intermediate	Resistant
Imipenem	10µg	≥ 23	20-22	≤ 19
Ciprofloxacin	5µg	≥ 21	20-22	≤ 15
Fosfomycin	200µg	≥ 16	13-15	≤12
Gentamicin	10µg	≥ 15	13-14	≤ 12
Tobramycin	10µg	≥ 15	13-14	≤ 12

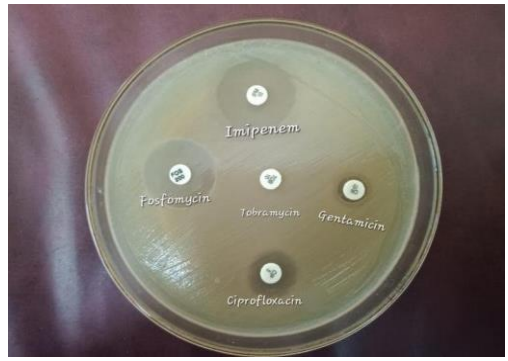

Figure 1: Antibiotic Sensitivity of E. Coli against Tested Antibiotics.

Table 8: Antibiotics Zones Chart of S. Aureus in Millimeters according to CLSI 2020.

Antibiotics	Disc content	Sensitive	Intermediate	Resistant
Imipenem	25µg	≥ 23	20-22	≤ 19
Ciprofloxacin	5µg	≥ 21	16-20	≤ 15
Oxacillin	10µg	≥ 13	11-12	≤ 10
Gentamicin	10µg	≥ 15	13-14	≤ 12
Tobramycin	10µg	≥ 15	13-14	≤ 12

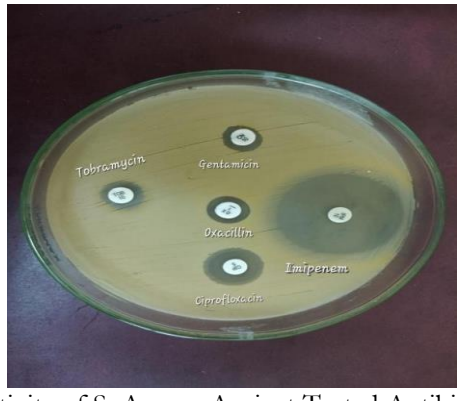

Figure 2: Antibiotic Sensitivity of S. Aureus Against Tested Antibiotics.

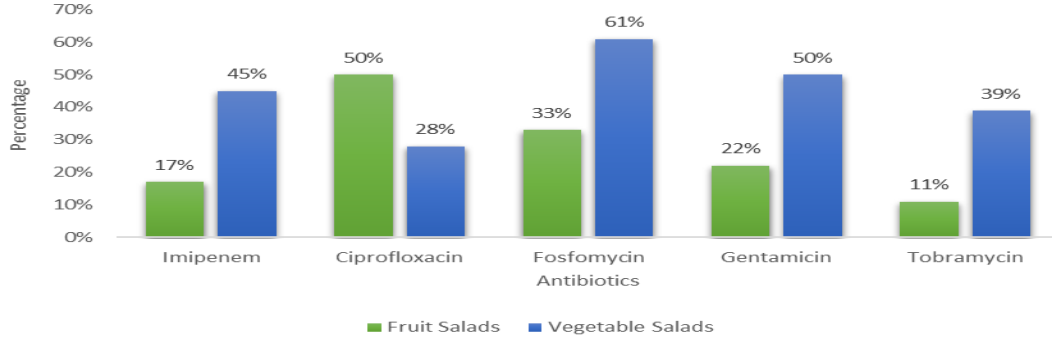

Figure 3: E. Coli from Vegetable Salads were most Resistant to Fosfomycin which was 61% Followed by Gentamicin and Imipenem which was 50 and 45% Respectively. Similarly, The E. Coli Isolated from Vegetable Salads were most Resistant to Ciprofloxacin at 50% Followed by Gentamicin and Imipenem that was 22 and 17% Respectively.

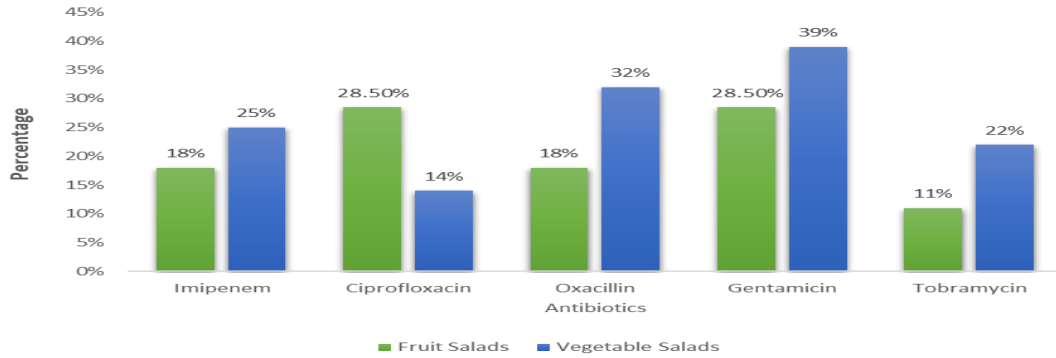

Figure 4: S. Aureus Isolated from Fruit Salads were most Resistant 28% to Fosfomycin and Gentamicin Followed by Oxacillin and Imipenem to whom they were Equally 18% Resistant. S. Aureus Isolated from Vegetable Salads were most Resistant to Gentamicin 39% Followed by Oxacillin and Ciprofloxacin that is 32 and 28% Respectively.

4.2 PCR Amplification of Enterotoxigenic Genes

By using specific primers (mec A, PVL for S. aureus and Stx1, Stx2 for E. coli) 100 bp DNA ladder were used to observe the PCR product. mec A product size 147 bp were observed in 13(13.4%) samples and PVL with product size 433 observed in 9(8.5%) samples, while Stx1 product size 180bp were observed in 6(5.5) samples and Stx2 with product size 255 observed in 3(3.4%) samples.

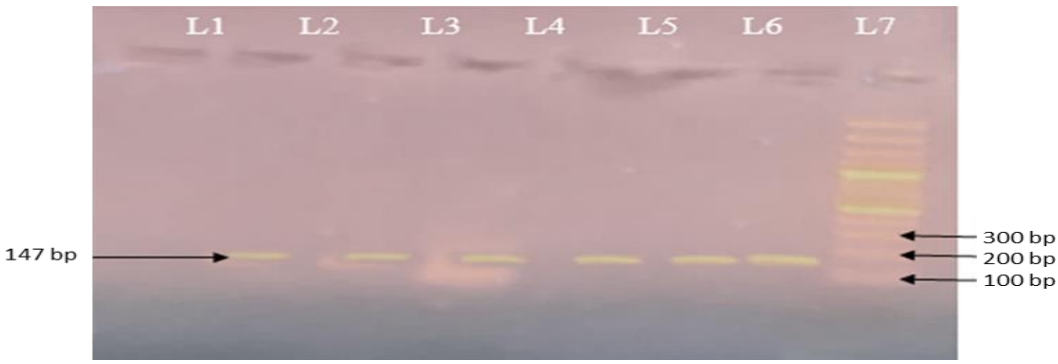

Figure 5: Agarose Gel Electrophoresis of Meca Gene Through PCR. L7 Shows the 100bp Ladder, L1 to L6 Shows the 147bp Bands.

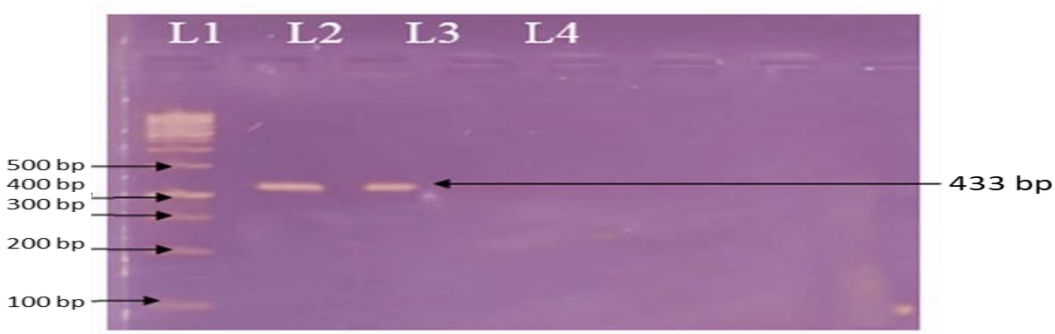

Figure 6: Agarose Gel Electrophoresis of PVL Gene Through PCR. L1 Shows the 100bp Ladder, L2 to L3 Shows the 433bp Bands.

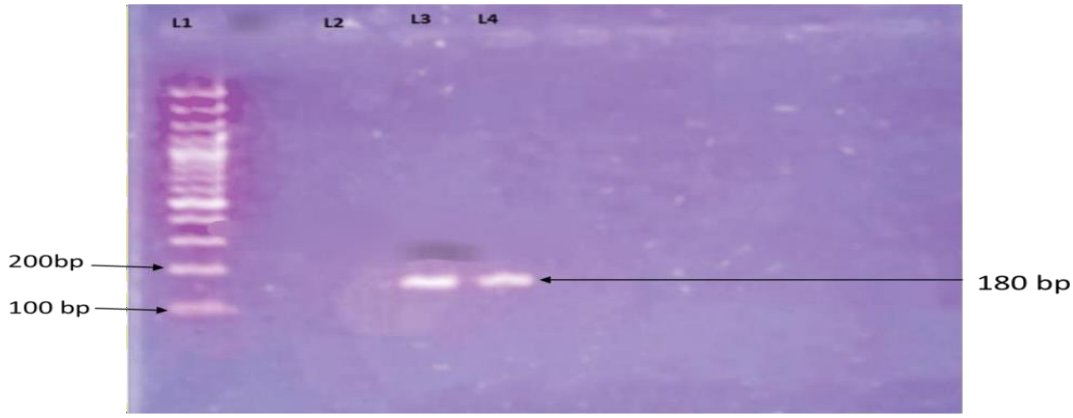

Figure 7: Agarose Gel Electrophoresis of Stx1 Gene Through PCR. L1 Shows the 100bp Ladder, L3and L4 Shows the 180bp Bands.

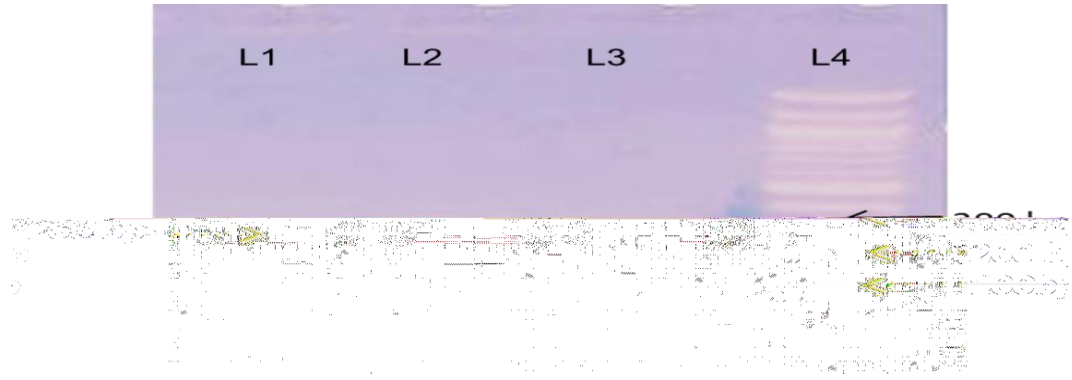

Figure 8: Agarose Gel Electrophoresis of Stx2 Gene Through PCR. L4 Shows the 100bp Ladder, L1 To L3 Shows the 255bp Bands.

Table 9: Prevalence of Enterotoxigenic Genes Isolated from Fruit Salad & Vegetable Salads.

Total Isolates	**Detected Gene (%)**		
E. coli	Stx1	Stx2	Stx1+Stx2
18(36%)	5 (27.7%)	2 (11.1%)	1 (5.5%)
S. aureus	mec A	PVL	mec A+PVL
28(56%)	11(39.2%)	7(25%)	2(7.1%)

Table 10: Comparative Analysis of Strains Isolated and Gene Detected by Chi-Square.

Strains	**Fruit salads**	**Vegetable salads**	**mecA/PVL**	**Stx1/Stx2**	**Chi-square value**	**P-value**
E. coli	7(21%)	11(79%)	13(46.3%)	6(33.2%)	4.1832	.04082*
S. aureus	11(39%)	17(61%)	9(32.1%)	3(16.6%)		

Discussion

Isolated E. coli strains isolated from vegetable salads were mostly resistant towards Ciprofloxacin but its resistance was comparatively lower in isolates of fruit salads. Similarly, S. aureus strains isolated from vegetable salads were more resistant to selected antibiotics as

compared to isolates of fruit salads. The difference in resistance to these isolates based on their sources can be due to the separate handling and storage conditions of both types of salads. A study conducted by Smith et al. (2018) also reveals variations in the distribution of sensitivity, intermediate, and resistance categories for the antibiotics. Some antibiotics show notable differences, such as ciprofloxacin and Fosfomycin, while others have relatively similar patterns, such as Imipenem and gentamicin. These variations highlight the dynamic nature of antibiotic susceptibility patterns and the importance of regularly monitoring and updating antibiotic resistance data for effective treatment decision-making.

Yang et al. (2020) conducted comparisons to determine the dynamic nature of antibiotic resistance patterns and highlighted the importance of regularly monitoring and updating resistance data. The observed differences in sensitivity, intermediate, and resistance percentages for the antibiotics emphasized the need for careful consideration of the most appropriate antibiotics for effective treatment decisions based on the specific resistance profiles of the tested isolates. Continuous surveillance of antibiotic resistance patterns is crucial in combating the emergence and spread of drug-resistant bacteria.

Smith et al. (2018) and Johnson et al. (2019) have demonstrated that ampicillin, tetracycline, and Co-trimoxazole-trimethoprim each show varying degrees of resistance to the antibiotics being studied. This highlights the importance of keeping a watch on antibiotic resistance as it can assist in direct treatment options that are effective and curb the spread of resistant strains.

Findings of our study are in consistency with Johnson et al. (2019) who conducted a study and found that samples of salad purchased from stores were contaminated with S. aureus. Both the identification of staphylococcal enterotoxin genes and the verification of the presence of S. aureus were accomplished through the application of molecular techniques such as polymerase chain reaction (PCR) and other methods. The analysis revealed a wide variety of S. aureus strains that produce enterotoxins in salad samples. This was demonstrated by the presence of many SE genes, such as SEA, SEB, SEC, and SED.

Our results were aligned with Smith et al., (2018), where ETEC strains were extracted from a diverse assortment of salad components. They grew bacteria in culture to determine which ETEC serotypes were present, and they utilized molecular assays to look for the presence of enterotoxin genes. It was discovered that several different strains of ETEC belonged to the O:H serogroups, with the O157:H7 variation of Enetrotoxigenic Escherichia coli being the most frequent. The capability of the isolated Enterotoxigenic Escherichia coli strains to produce enterotoxins was demonstrated when heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) genes were found.

Similar results were found in the study conducted by Johnson et al. (2020) who reported that 10 % of fresh salads were contaminated with S. aureus. Through molecular analysis, several staphylococcal enterotoxin genes were discovered. A few examples of these include sea, seb, sec, and sed. They also investigated antibiotic resistance and found that many S. aureus isolates were resistant to common antibiotics like penicillin and erythromycin. The risk of getting an illness caused by a strain of S. aureus that is resistant to antibiotics after eating contaminated salads was highlighted in the study.

Based on the findings of the current study there should be continuous monitoring of the quality of fresh salads as the antibiotic resistance and enterotoxigenic strains circulating in the food commodities can lead to the emergence of epidemics.

Conclusion & Recommendations

A total of 50 samples were analyzed for Staphylococcus aureus in which 61% (n=17) strains were isolated from vegetable salads and 39% (n=11) isolated from fruit salads. Escherichia coli was isolated in 79% (n=11) from vegetable salads and 21% (n=7) isolated from fruit salads. The findings of the study indicate a high level of contamination and drug resistance in S. aureus and E. coli strains present in vegetable and fruit salads. S. aureus was the more prevalent strain, highlighting the potential for food-borne infections. The findings of the study emphasize the growing problem of antibiotic resistance, which poses a significant challenge in the treatment of bacterial infections caused by these pathogens. Continued monitoring is crucial to understanding the mechanisms of pathogenicity and the potential for the spread of drug-resistant strains through horizontal gene transfer. The findings of the study raise concerns about food safety and public health. The higher prevalence of S. aureus in both salad types suggests that it poses a greater risk for causing food borne infections compared to E. coli. Additionally, the study highlighted the presence of drug resistance in the S. aureus and E. coli strains found in the salads. Antibiotic resistance is a pressing issue worldwide, as it limits the effectiveness of antibiotics in treating bacterial infections. The emergence of drug-resistant strains in food borne pathogens further exacerbates this problem, making it challenging to treat infections caused by these bacteria. Researchers and public health authorities can develop strategies to mitigate the risks associated with food borne infections and combat antibiotic resistance effectively. This may involve implementing stringent food safety measures, promoting responsible antibiotic use, and conducting further research to develop alternative treatments in the face of drug- resistant strains.

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