

**STUDY ON THE PRESENCE OF
ANTIBIOTIC-RESISTANT BACTERIA
IN THE RAW VEGETABLES OF
MIRZAPUR DISTRICT**

काशी हिन्दू
विश्वविद्यालय



BANARAS HINDU
UNIVERSITY

Thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
In
Plant Biotechnology

Submitted by
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Dear Sir,

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By

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ABBREVIATIONS

°C	Degree Celsius	LB	Lysogeny Broth
µl	Microlitre	lbs	Pounds
<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>	MAR	Multiple Antibiotic Resistance
ARB	Antibiotic-Resistant Bacteria	MDR	Multi-drug-resistant
ARGs	Antibiotic-Resistant Genes	MEG	Mobile Gene Element
ATCC	American Type Culture Collection	mg	Milligram
<i>B. cepacia</i>	<i>Burkholderia cepacia</i>	min	Minutes
bp	Base pair	ml	Millilitre
C	Control	MLST	Multilocus sequence typing
CFU	Colony-forming Unit	MPN	Most Probable Number
CLSI	Clinical and Laboratory Standards Institute	MR	Methyl red
CO ₂	Carbon dioxide	MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
Conc.	Concentration	MSA	Mannitol Salt Agar
<i>E. coli</i>	<i>Escherichia coli</i>	MSSA	Methicillin-susceptible <i>Staphylococcus aureus</i>
EDTA	Ethylenediaminetetraacetic acid	NAP	National Action Plan
EHEC	Enterohemorrhagic <i>E. coli</i>	NCR	National Capital Region
EMB	Eosin Methylene Blue	OD	Optical Density
EPEC	Enteropathogenic <i>E. coli</i>	<i>P. luteola</i>	<i>Pseudomonas luteola</i>
ESBL	Extended Spectrum β-lactamases	PCR	Polymerase Chain Reaction
ESBL-E	Extended Spectrum β-lactamases <i>Escherichia coli</i>	pH	Potential of Hydrogen
<i>et al.</i>	<i>Et alia</i> (and others)	psi	Pound per Square inch

EtBr	Ethidium Bromide	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>etc</i>	<i>Et cetera</i> (and the rest)	<i>S. maltophilia</i>	<i>Stenotrophomonas maltophilia</i>
FASSI	Food Safety and Standards Authority of India	spp	Species
g	Gram	STEC	Shiga toxin-producing <i>E. coli</i>
G	Gauge	TBE	Tris-borate-EDTA
GDP	Gross Domestic Product	TSI	Tri Sugar Iron
HCl	Hydrochloric acid	UTI	Urinary Tract Infection
<i>i.e.</i>	<i>Id Est</i> (that is)	U.V.	Ultraviolet
IMViC	Indole, Methyl red, Voges Proskauer and Citrate	<i>viz</i>	<i>Videlicet</i> (namely)
ISO	International Organisation of Standardization	VP	Voges Proskauer
L	Litre	XLD	Xylose Lysine Deoxycholate

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INTRODUCTION

The emergence of antimicrobial resistance (AMR) is a serious threat to global health. AMR happens when viruses, bacteria, fungi and parasites change over time and no longer respond to medications making infections harder to treat and increasing the risk of disease spread, serious illness and death. As a result of drug resistance, antibiotics and other antimicrobial medicines become ineffective and infections become increasingly difficult or impossible to treat (World Health Organisation, 2021).

Microbes, like all living beings, are constantly developing in order to survive. AMR existed even before the first antibiotic was discovered. 30,000-year-old DNA recovered from Beringian permafrost sediments had Antibiotic-Resistant Genes (ARGs) encoding resistance to beta-lactams, tetracyclines and glycopeptides, thousands of years before they were used clinically. (D’Costa *et al.*, 2011).

Among all the antimicrobial-resistant organisms, antimicrobial-resistant bacteria are more significant than other AMR organisms. According to the estimate conducted in 2019 about 1.2 million people and potentially millions more died due to antibiotic-resistant bacterial infections (Murray *et al.*, 2022). AMR is accelerating due to selective pressure applied by antibiotic use, through genetic mutations, or the acquisition of genetic material through plasmid transfer from a resistant bacterium (Lipsitch *et al.*, 2002).

AMR has been highlighted as a global health problem with significant health, political and economic consequences (O’Neill and J., 2014). The advent of AMR has put modern medicine’s progress in jeopardy. Annual deaths due to AMR are anticipated to rise to 10 million worldwide by 2050, it alone is killing more people than road traffic accidents and cancer combined. AMR is a leading cause of death around the world, especially in developing countries (de Kraker *et al.*, 2016). Recent research indicates that antimicrobial resistance would elevate the rate of poverty and impact low-income countries compared to the rest of the world (World Bank, 2017). Studies show that annual global GDP could decrease by approximately 1% and there would be a 5–7% loss in developing countries by 2050. This percentage ultimately translates into \$100-210 trillion. By 2020, multidrug-resistant *tuberculosis* alone might cost the world \$16.7 trillion. (Dadgoster *et al.*, 2019) Several countries are dealing with the introduction of bacteria that are entirely resistant

to antibiotics and these are developing country-specific antimicrobial resistance action plans based on the WHO's global action plan (World Health Organisation, 2019).

India has been introduced as the “AMR Capital of the world” (Gandra *et al.*, 2017). It has one of the greatest burdens of drug-resistant infections in the world, including the highest burden of multidrug-resistant tuberculosis (Government of India, 2017) and frighteningly high resistance among Gram-negative and Gram-positive bacteria such as *Klebsiella pneumoniae* and *Staphylococcus aureus* respectively whereas pathogens such *Salmonella typhi*, *Shigella*, *Pseudomonas* and *Acinetobacter* have been found to have high AMR in regional investigations (Kakkar *et al.*, 2017), even to newer antimicrobials such as carbapenems and faropenem since their introduction in 2010 (Gandra *et al.*, 2016). Every year, it is estimated that more than 50,000 babies die from sepsis caused by bacteria resistant to first-line antibiotics (Laxminarayan *et al.*, 2013). While precise population burden estimates are unavailable, neonates and the elderly are assumed to be the most vulnerable. By the year 2050, India is expected to have two million deaths owing to AMR (Gelband *et al.*, 2015). India spends only 4.7% of its GDP on health, with the government contributing barely one-fourth (1.15%) of it, making the task huge (Bajpai and V., 2018). The typical cost of treating a resistant bacterial illness, according to one study, is more than a year's pay for a rural worker (Chandy *et al.*, 2014). In India, there are no well-documented estimations of AMR's economic impact. The high frequency of malaria, tuberculosis and HIV in India and the lack of laws on the use of antibiotics in humans and the production of food-producing animals suggest that AMR could have a significant impact on the Indian economy (Dixit *et al.*, 2019). India has also framed its National Action Plan (NAP) for AMR (Government of India, 2017).

An estimated 196.27 million tons of vegetables were produced in India in the year 2020-21. (Ministry of Agriculture & Farmers Welfare, India, 2022). In the farm-to-table cycle, vegetables can be contaminated in the pre-harvest, harvesting and post-harvest stages. Treatment of soil with manure, irrigation with contaminated sewage water, cultivation in areas with an abundant presence of pathogenic microorganisms and so on are sources of contamination of vegetables during harvesting (Mathur *et al.*, 2014). Post-harvest activities that lead to contamination of vegetables include the use of pond and river water to wash vegetable produce, handling of vegetables by workers infected with and carriers of the pathogen(s) and storage in contaminated places (Mritunjay *et al.*, 2015). When these products are consumed raw, microbes can infect humans and cause outbreaks of food poisoning and other enteric diseases (Denis *et al.*,

2016). About 20% of these antimicrobial-resistant infections are attributed to agricultural antibiotic usage rather than clinical treatment (Spellberg *et al.*, 2013)

Escherichia coli is a common cause of bacteremia. Bile and urinary tract infections (UTI), sterile fluids in close proximity to the gastrointestinal system, are common sources of *E. coli*. A small number of serotypes cause disease and virulence factors (e.g., antiphagocytic capsules, adhesions, invasins, lipopolysaccharide and toxins) are well described (Melzer *et al.*, 2007). ESBL (Extended Spectrum-lactamases)-producing Enterobacteriaceae have a substantial danger potential. ESBLs are β -lactamases with a broad activity spectrum that are produced when non-ESBL β -lactamases are mutated. They belong to many types of β -lactamases (such as TEM, SHV and CTX-M), which have a low homology of around 25% at times. (Paterson and Bonomo, 2005). These enzymes are mostly plasmid-coded, making them easier to spread horizontally. They hydrolyze β -lactam antibiotics, causing penicillin, cephalosporin and aztreonam resistance. They've been linked to nosocomial epidemics since the 1980s and they've been on the rise in recent years.

Staphylococcus aureus is a major human pathogen that causes a variety of nosocomial and community-acquired infections, ranging from minor skin and soft tissue infections to wound infections and bacteremia (Fang *et al.*, 2003). Methicillin-resistant *S. aureus* (MRSA) is a kind of *S. aureus* bacteria that has developed resistance to methicillin as well as other antibiotics such as all other β -lactams, aminoglycosides and macrolides (Choi *et al.*, 2003) The methicillin resistance gene (*mecA*) encodes a methicillin-resistant penicillin-binding protein that is not present in susceptible strains and is believed to have been acquired from a distantly related species. Staph food poisoning is a gastrointestinal ailment caused by toxins generated by the bacteria *Staphylococcus aureus* (*S. aureus*). *S. aureus* is found on the skin and in the nose of about 25% of people and animals (Centers for Disease Control and Prevention, 2018).

The present study entitled "Study on the presence of antibiotic-resistant bacteria in the raw vegetables of Mirzapur district" has been carried out with the following objectives:

1. To study the prevalence of *Escherichia coli* and *Staphylococcus aureus* bacteria in the fresh vegetable bought from the retail markets present in the various tehsils of Mirzapur district.
2. To study the prevalence of antibiotic-resistant bacteria (ARB) from the above isolates.
3. To validate the ARB found by detecting ARGs through the PCR method.

REVIEW OF LITERATURE

Vegetable markets or retail markets are the places that offer fresh vegetables or fruits with great variety and they can be brought at an affordable price at these places. But due to the rise of Anti-Microbial Resistance (AMR), they are becoming reservoirs for Antibiotic-Resistant Bacteria (ARB) and point of contact for foodborne diseases in humans. Vegetables generally get contaminated with ARB during post-harvest activities. Many studies have been conducted to monitor AMR across the various retail markets in the whole world in this context literature including recent ones are described as under -

Rouamba et al. (2022) investigated the amount of contamination in irrigation water and lettuce, as well as the antibiotic resistance profile of isolated bacteria. Eighty lettuce samples and ten irrigation waters were gathered from market gardening locations in Boulmiougou, Bissigin, the National School of Public Health, and Tanghin, Burkina Faso. The research on thermotolerant coliforms, *E. coli*, and *Salmonella* was carried out according to ISO guidelines. The resistance profile of isolated *E. coli* and *Salmonella* bacteria was examined using the Kirby-Bauer method with a panel of twenty different antibiotics. The thermotolerant coliforms were found in 68.75% (55) of the eighty lettuce samples, with an average load of 8.75×10^5 CFU g⁻¹. *E. coli* was found in 57.5% of cases (46) and *Salmonella* was found in 11.25% of cases (9) with eight strains of *Salmonella enterica* spp. and one strain of *Salmonella enterica arizona*. Thermotolerant coliforms were found in all irrigation fluids, with an average concentration of 3.11104×10^4 CFU/100mL. *E. coli* was found in 40% (4/10) of irrigation waters, while *Salmonella enterica* spp. was found in 20% (2/10). Ampicillin 28.26%, amoxicillin + clavulanic acid 26.09%, and tetracycline 19.57% antibiotic resistance was discovered in *E. coli* strains.

Roshan et al. (2022) conducted the study to detect the distribution of virulence and enterotoxin genes among MRSA isolates from bovine mastitis. Only 56 of 500 milk samples had Staphylococcus isolates, and only 126 of them were resistant to cefoxitin (75%) and ceftazidime (75%) as well as amoxicillin (71.4%), cefodaxime (67.8%), cefepime (66.1%), oxacillin (64.3%), norfloxacin (60.7%), and gentamicin (60.7%). (58.9%). Among the staphylococci isolates, only 42 were recognized as MRSA strains. MRSA had virulence genes such as *mecA* (100%), *coa* (100%), and *nucA* (100%). Other virulence variables like *hlg* (80.9%),

34/42), pvl (47.6%, 20/42), and spa (92.8%, 39/42) were also mentioned. Out of 42 examined isolates, 11 were found to be negative (26%) for any enterotoxin gene, whereas 7 (16.6%), 6 (14.3%), 18 (42.8%), 1 (2.3%), 26 (61.9%), 27 (64.2%), and 3 (7.1%) were found to be positive for sea, seb, sec, sed, seg, sei, and seq enterotoxin, respectively.

Richter et al. (2021) in South Africa, conducted microbiological safety surveillance on 545 vegetable samples (spinach, tomato, lettuce, cucumber, and green beans) obtained from stores, street dealers, trolley vendors, and farmers' markets. The prevalence of *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* was determined after counting *E. coli*, coliforms, and Enterobacteriaceae. Antibiotic resistance was determined phenotypically and genotypically in *E. coli* isolates (diarrheagenic virulence genes). With a few exceptions, counts of Coliforms, *E. coli*, and Enterobacteriaceae were not significantly different between formal and informal marketplaces. 90% to 98% tomatoes, 70% to 94% spinach, 82% cucumbers, 93% lettuce, and 80% green bean samples were appropriate (100 CFU/g) *E. coli* counts when compared to international norms. *E. coli* was found in 14.86% (n = 81) of the 545 vegetable samples tested, mostly in leafy green vegetables. The virulence genes (lt, st, bfpA, eagg, eaeA, stx1, stx2, and ipaH) were not found in the 67 *E. coli* isolates studied, although 40.30% were multidrug-resistant. Resistance to aminoglycosides (neomycin, 73.13%; gentamycin, 10%), penicillins (ampicillin, 38.81%; amoxicillin, 41.79%; augmentin, 10%), sulfonamides (cotrimoxazole, 22.39%), tetracycline (19.4%), chloramphenicol (11.94%), cephalosporins (cefepime, 34.33%),

Zhang et al. (2021) carried out research work that described plasmid-mediated colistin-resistant *E. coli* strains and studied antibiotic resistance and antibiotic resistance genes in *E. coli* isolated from retail ready-to-eat (RTE) foods. A total of 1118 RTE food samples were gathered from marketplaces in 39 Chinese cities, and 126 *E. coli* bacteria were identified, with >95% of them being multidrug-resistant. Tetracycline resistance was found in 95.24% of the isolates, as well as ampicillin resistance (82.54%), trimethoprim-sulfamethoxazole resistance (77.78%), nalidixic acid resistance (74.60%), cephalothin resistance (72.22%), chloramphenicol resistance (66.67%), and streptomycin resistance (53.97%). There were 22 *E. coli* that produced extended-spectrum-lactamase (ESBL) and four *E. coli* that were colistin-resistant. The most frequently discovered resistance genes were TEM, CTX-M, tetA, sul2, strA/strB, aadA, and qnrS. The most common CTX-M kinds were CTX-M-55 and CTX-M-14. The mcr-1 gene was found in all four colistin-resistant *E. coli* isolates. Conjugation and transformation can be used to transfer the mcr-

1 gene to *E. coli* C600. The *mcr-1* genes were discovered in IncX4 and IncHI2 plasmids after whole-genome sequencing.

Giri et al. (2021) assessed the microbiological quality of foods in a typical emerging and growing urban suburb of India where ready-to-eat street foods are rapidly establishing with public health implications and evaluated the potential burden of antibiotic-resistant *E. coli* and *Klebsiella pneumoniae* contaminating ready-to-eat street foods. A total of 100 ready-to-eat food samples were obtained, with *E. coli* accounting for 22.88% and *K. pneumoniae* accounting for 27.12%. ESBL-producing *E. coli* and *K. pneumoniae* were found in 25.42% of the samples, particularly in chutneys, salads, paani puri, and poultry. Antimicrobial resistance was found in cefepime (72.9%), imipenem (55.9%), cefotaxime (52.5%), and meropenem (16.9%) isolate, with 86.44% having a MAR index greater than 0.22. The most common β -lactamase encoding gene was blaTEM (40.68%), followed by blaCTX (32.20%) and blaSHV (32.20%) (10.17%), 20.34% of the isolates, the blaNDM gene was also found.

Gundappa et al. (2021) investigated the percentage of harmful bacteria found in various fruit and vegetable samples found in and around the Kalaburagi market in Karnataka, India. Kalaburagi yielded a total of 940 different samples, with 390 (23.84%) being fruits and 550 (35.27%) being vegetables. The pathogenic bacteria were isolated using a peptone water enrichment culture technique. Traditional microbiological identification approaches were used to identify the bacterial isolates. *E. coli*, *Salmonella*, and *Shigella* were tested for antibiotic resistance using the disc diffusion method. *E. coli* isolates were resistant to Nalidixic acid (86.50%), while Imipenem and Trimethoprim-Sulfamethoxazole had the lowest resistance (19.84%), *Salmonella* isolates were resistant to Ciprofloxacin (86.66%), while Norfloxacin had the lowest resistance (4.76%), and *Shigella* isolates were resistant to Vancomycin (80.35%), while Amoxicillin had the lowest resistance (3.57%). Multiple drug resistance (MDR) was found in *E. coli* (38.88%), *Salmonella* (26.66%), and *Shigella* (10.11%) in that order.

Saksena et al. (2020) The objective of this study was to determine the bacterial contamination and antibiotic resistance in fresh produce sold in Delhi, India. In order to do so, 150 samples of commonly consumed raw fruits and vegetables were obtained from retail and wholesale vendors in Delhi-NCR, India. By growing 25 g of each sample, microbial contamination was investigated. Isolated microorganisms were tested for antimicrobial resistance, and resistance phenotypes were identified. Overall, infections were found in 97.3% of the fruits and vegetables

tested. Coliforms were found in over 75% of the samples, with *E. coli* being found at 17.4%. The majority of vegetables tested positive for coliforms, including ginger (100%), cabbage (80%), cucumber (75%), and tomato (75%) (75%). In eight (5.7%) of the isolates, extended-spectrum beta-lactamases (ESBL) were found, along with carbapenem resistance in four and three Metallo—lactamases producers. Retail shops had a considerably greater rate of *E. coli* isolation than wholesale markets (p value =.04).

Colosi et al. (2020) investigated the prevalence of extended-spectrum β -lactamase (ESBL), AmpC, and carbapenemase-producing Enterobacterales isolates from retailed raw vegetables, as well as if domestic washing is an effective technique of reducing bacterial burden in Romania. Supermarkets (n = 2) and farmer markets (n = 2) were used to obtain seasonal vegetables (n = 165). Following sample processing and isolation, matrix-assisted laser desorption ionization time-of-flight mass spectrometry was used to identify Enterobacterales (MALDI-TOF). The presence of the major ESBL, AmpC, and Carbapenemase genes was determined using a polymerase chain reaction (PCR) multiplex. Isolate resistance profiles. The number of colony-forming units (CFU) of Enterobacteriaceae was compared to different vegetable types. Bacteria that produce β -lactamase were found on 7.9% of vegetables, with 5.5% having the ESBL/AmpC phenotype and 2.4% being Carbapenemase producers. blaSHV (n = 4) was the most often found β -lactamase gene, followed by blaCTX-M and blaTEM (both with n = 3). Antibiotic resistance testing revealed that 46% of isolates were multidrug-resistant, with aminoglycosides (38.5%) being the most common non- β -lactam resistance, followed by first-generation quinolones (38.5%).

Wu et al. (2018) in their study staphylococcal enterotoxin genes, spa-typing, and multi-locus sequence typing were used to characterize *S. aureus* resistant isolates from Chinese retail vegetables. During the period 2011–2016, 24 (5.73%) of 419 retail vegetable samples from 39 cities in China tested positive for *S. aureus*, with a geometric mean of 3.85 MPN/g. *S. aureus* was found in the highest concentration in lettuce (13/84, 15.48%), tomato (7/110, 6.36%), caraway (2/87, 2.30%), and cucumber (2/128, 1.56%), but all other vegetables were clear of the bacteria. The researchers looked at a total of 30 isolates. Most isolates (93.3%) were resistant to ampicillin and penicillin in an antibiotic susceptibility test; however, all isolates were susceptible to linezolid, trimethoprim/sulphamethoxazole 1:19, nitrofurantoin, rifampicin, and teicoplanin. All isolates (30/30, 100%) were resistant or intermediately resistant to at least three antibiotics, with nine isolates (30%) resisting at least ten drugs. Five isolates were cefoxitin-resistant and had mecA

genes, indicating that they were MRSA. The sem gene was found in 86.7% of the isolates, followed by the sec (83.3%), sep (70.0%), seg (56.7%), sel (53.3%), seh (50.0%), seq (50.0%), sej (46.7%), seb (36.7%), sen (36.7%), and ser (33.3%). The seo and seu genes were found in only 6.75% of the isolates. In *S. aureus* isolated from retail vegetables in China, MLST and spa typing revealed a considerable genomic diversity. ST59-t437 was the most common type of MRSA isolate (3/5, 60%), while ST188-t189 was the most common type of MSSA isolate (7/25, 28%).

Al-Kharousi et al. (2016) studied whether opportunistic pathogenic bacteria cross geographical barriers by hiding in fruits and vegetables. This study used aerobic plate count (APC) and counts of Enterobacteriaceae, Enterococcus, and *Staphylococcus aureus* to identify some microbial communities of fresh produce from 105 samples of imported fresh fruits and vegetables from various countries around the world, including local samples (Oman). Molecular (PCR) and biochemical procedures were used to identify the bacteria that had been extracted (VITEK 2). 60% of fruits and 91% of vegetables contained Enterobacteriaceae. Enterococcus bacteria were found in 20% of fruits and 42% of vegetables. *E. coli* and *S. aureus* bacteria were found in 22% and 7% of the vegetables, respectively. VITEK 2 and PCR were used to identify 97 bacteria from 21 different species to species level. The most common bacteria were *E. coli*, *Enterobacter cloacae*, *Enterococcus casseliflavu*, and *Klebsiella pneumoniae*.

Karumathil et al. (2016) studied the prevalence of multidrug-resistant (MDR) *Acinetobacter baumannii* on fresh vegetables obtained from Connecticut farmers' markets was assessed in this investigation. Fresh carrots, potatoes, and lettuce samples were sampled and streaked on selective media, such as Leeds *Acinetobacter* and MDR *Acinetobacter* agars. Gram staining, biochemical assays, and PCR were used to identify all morphologically distinct colonies from MDR *Acinetobacter* agar. An antibiotic disc diffusion assay was used to assess the sensitivity of the isolates to 10 antibiotics routinely used in humans, including imipenem, ceftriaxone, cefepime, minocycline, erythromycin, colistin-sulfate, streptomycin, neomycin, doxycycline, and rifampin. Only two samples of potato and one sample of lettuce tested positive for *A. baumannii*. Furthermore, all carrot samples tested negative for the bacterium. *Burkholderia cepacia* (1% potatoes, 5% carrots, and none in lettuce), *Stenotrophomonas maltophilia* (6% potatoes, 2% lettuce, and none in carrots), and *Pseudomonas luteola* (9% potatoes, 3% carrots, and none in lettuce) were among the opportunistic MDR human pathogens recovered from the vegetables. Antibiotic susceptibility testing of the isolates revealed high rates of resistance to the following

antibiotics: In *P. luteola*, ceftriaxone (6 of 6), colistin-sulfate (5 of 6), erythromycin (5 of 6), and streptomycin (4 of 6); In *B. cepacia*; colistin-sulfate (11 of 11) and imipenem (10 of 11); cefepime (7 of 8), ceftriaxone (8 of 8), colistin-sulfate (8 of 8), erythromycin (5 of 8); In *A. baumannii* imipenem (3 of 3), ceftriaxone (3 of 3), erythromycin (3 of 3), and streptomycin (3 of 3) and In *S. maltophilia*, imipenem (4 of 8).

Gundappa et al. (2016) examined the prevalence of harmful bacteria in fruit samples obtained from the supermarkets of Kalaburagi, Karnataka, India. A total of 390 fresh fruit samples were acquired from supermarkets and enriched with peptone water before being plated on various media. Only 93 (23.84%) of the 390 fruit samples tested positive for culture on EMB, XLD, and MacConkeys Agar. Traditional methods were used to identify food pathogens. The prevalence of *E. coli* (55.91%) and *Salmonella* (36.55%) is highest in all fruit samples, while *Shigella* has a relatively low incidence (7.52%)

Shah et al. (2015) evaluated the abundance of Enteropathogenic, Enterotoxigenic, and Shiga toxin-producing pathotypes of *E. coli* found in fresh vegetables and ready-to-eat salads. A total of 260 vegetable and salad mix samples (cucumber, lettuce, spinach, and carrot) were obtained from commercial food markets in Pakistan's southern areas. *E. coli* bacteria were found in about 34% of the vegetable samples. Diarrheagenic *E. coli* pathotypes were found in 32.4% of *E. coli* strains from vegetable samples. Similarly, *E. coli* pathotypes were found in 26.7% of salad samples. From spinach salad samples, 15% (2 Enterotoxigenic *E. coli* and Enteropathogenic *E. coli*) were isolated. From mixed salad type A, 25% (3 Enterotoxigenic *E. coli*, 1 Enteropathogenic *E. coli*, and 1 Shiga toxin-producing *E. coli*) were isolated. From mixed salad type B, 40% (4 Enterotoxigenic *E. coli*, 2 Enteropathogenic *E. coli*, and 2 Shiga toxin-producing *E. coli*) were isolated. Tetracycline resistance was found in 92% of diarrheagenic *E. coli* pathotypes, and Ampicillin resistance was found in 87%.

Kabir et al. (2014) investigated the microbiological quality and antibiotic resistance patterns of pathogenic bacteria isolated from vegetable samples in Dhaka city, Bangladesh. A total of 14 vegetable samples, 7 from local and 7 from super shops, were randomly collected for this study from different locations within the city. By serial dilution and spread plate technique, total heterotrophic bacteria, total coliform, fecal coliform, *Pseudomonas* spp., *Listeria* spp., and *Staphylococcus aureus* were counted in each sample. Enrichment and selective plating procedures were used to assess the presence of *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. Imipenem (10

g), Ceftriaxone (30 g), Sulphamethoxazole (25 g), Ampicillin (10 g), Gentamicin (10 g), Aztreonam (30 g), Cefuroxime (30 g), and Oxacillin (5 g) antibiotic discs were used to investigate the antibiotic sensitivity patterns of the isolated bacteria. *E. coli* was found in larger proportions in the local market veggies (4/7, 57.14%), whereas *Pseudomonas* spp. (5/7, 71.42%) and *Listeria* spp. (5/7, 71.42%) were found in higher proportions in the superstore vegetables (5/7, 71.42%). Pathogenic bacteria isolated from supermarkets exhibited enhanced resistance to antibiotics tested against them (5/8, 62.5%).

Reuland et al. (2014) gathered 119 samples of 15 different types of vegetables from various sources in Amsterdam, the Netherlands, to see if extended-spectrum lactamase (ESBL)-producing Enterobacteriaceae (ESBL-E) are prevalent in retail raw veggies. A microarray was used to characterize ESBL-encoding genes after culture, strain identification, and susceptibility testing. ESBL-E was found in four of the fifteen vegetable varieties. ESBL-E was found in 7 (6%) of the samples. There were three blaCTX-M-15 genes, one blaCTX-M-1 gene, two CTX-M-9 genes, and one SHV ESBL-encoding gene discovered. The ESBL genes were identical to those discovered in human-derived enterobacterial strains.

Sahebnaqah et al. (2013) used the PCR method for the detection of *mecA* and *nuc* genes to estimate the prevalence of MRSA recovered from patients in Tehran, Iran. *S. aureus* isolates were identified using phenotypic methods such as microscopic and colony morphology, as well as catalase and coagulase tests. DNA was taken from all isolates, and PCR was used to determine the presence of the *nuc* and *mecA* genes. The oxacillin disc diffusion test was used to determine MRSA via phenotypic approaches. SPSS software was used to examine the data. 101 isolates possessed the *nuc* gene out of 126 clinical samples identified by phenotypic approach. 78.2% of isolates tested positive for MRSA using an oxacillin disc, however, only 69% tested positive for the *mecA* gene using a PCR technique.

Schwaiger et al. (2011) studied to what extent fresh produce from Germany plays a role as a carrier and reservoir of antibiotic-resistant bacteria. 1001 vegetables (fruit, root, bulbous vegetables, salads, and cereals) were collected and bacteriologically evaluated from 13 farms and 11 stores. The microdilution method was used to assess phenotypic resistance of *Enterobacter cloacae* (n=172), *Enterobacter gergoviae* (n=92), *Pantoea agglomerans* (n=96), *Pseudomonas aeruginosa* (n=295), *Pseudomonas putida* (n=106), and *Enterococcus faecalis* (n=100) against up *P. agglomerans* and *E. gergoviae* showed the strongest resistance to β -lactams when it came to

cefaclor (41% and 29%). Doxycycline (23%), erythromycin (21%), and rifampicin (65%) resistance rates were also found in *E. faecalis*, spectinomycin (28%), and mezlocillin (12%) resistance rates in *E. cloacae*, and streptomycin (19%) resistance in *P. putida*. Aminoglycosides amikacin, apramicin, gentamicin, neomycin, netilmicin, and tobramycin showed comparatively low resistance rates in *P. aeruginosa* (4%); 11% were resistant to streptomycin. There were no enterococci that were resistant to glycopeptides.

Nipa et al. (2011) carried out the experiment with the purpose to look at the microbiological quality of raw salad greens as well as their potential as a source of antibiotic-resistant bacteria. Tomato, cucumber, carrot, green chili, lemon, coriander leaf, peppermint, and beetroot were collected from two open markets and two supermarkets in Chittagong, Bangladesh. Coliform and faecal Coliform were substantially infected in all of the vegetables (> 1100 CFU/100ml). Tomatoes had a microbial count of 9.0104 CFU/ml to 3.8105 CFU/g, cucumbers had 5.5104 CFU/g to 1.9106 CFU/g, carrots had 1.2104 to 2.6106 CFU/g, green chili had 1.0104 to 4.0105 CFU/g, lemon had 1.5105 to 1.2106 CFU/g, coriander leaf had 5.87105 to 1.8106 CFU/g, peppermint had 2.2×10^5 to 7.7×10^5 CFU/g and Beetroot had 5.0×10^3 to 5.4×10^5 CFU/g. The majority of the vegetables were found to be free of yeast and mold. There were 266 bacterial isolates from ten genera and three fungi, *Rhizopus*, *Penicillium*, and *Aspergillus*. The most common bacteria were *Enterobacter* spp. (21.80%), *Pseudomonas* spp. (19.17%), *Vibrio* spp. (16.92%), *Lactobacillus* spp. (15.04%), *Staphylococcus* spp. (10.15%), *Klebsiella* spp. (9.04%), *E. coli* (4.89%), *Citrobacter* spp. (2.26%), *Ser* (0.37%). Antibiotic susceptibility was assessed on 1/5th of selected isolates from the Karnafully market. In 98.06% of isolates, multiple drug resistance was found, with resistance to two to seven antibiotics.

Guan et al. (2005) used *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Shigella* and tested for their ability to survive or thrive in pesticide solutions used in the horticultural industry (Ambush 240EC, Benlate T-N-G, Botran, Bravo 50075WP, Captan 80WDG, Parasol, and Vendex 50W). Individual cultures were injected at 4 log CFU/ml with insecticides diluted to the lowest recommended spray doses with sterile saline in the lab. Bacterial survival in the samples and the saline control were counted using agar surface plating or hydrophobic grid membrane filtration after a 96 hours incubation at 21°C. The harmful germs were somewhat inhibited by the majority of the formulations examined. In Bravo 500, all injected bacteria survived or thrived. *Salmonella* spp. had the best chance of surviving in pesticide

solutions, while *Listeria* spp. had the worst chance. *Salmonella* survival changed depending on the kind of formulation when the incubation temperature or pesticide concentration was increased. Both organisms were recovered from the leaves and fruit skins of sprayed tomato plants following the prescribed 1-day-to-harvest interval when a bacterial cocktail including *S. enteritidis* and *E. coli* O157:H7 was introduced to Bravo 500 at a concentration of 6 log CFU/ml. When sprayed in saline (at least 26 and 56 days, respectively), *E. coli* and *Salmonella* lived longer on tomato leaves than when sprayed in Bravo 500 (>45 hours and <15 days, respectively). Pathogen growth in other pesticide formulations was not observed in the laboratory, despite the fact that *S. serovars*, *S. typhimurium*, and *S. heidelberg* grew in the fungicide Bravo and *S. enteritidis* grew in the insecticide Vendex within 96 hrs at 21°C. Higher temperatures (to 30°C) or doubling pesticide dosages had no effect on *S. heidelberg* survival or had a detrimental effect.

Schroeder et al. (2003) investigated the presence of Resistant *E. coli* in retail raw meat markets. For this, they collected Four 432 generic *E. coli* isolates from the ground and whole retail beef, chicken, pork, and turkey obtained from Greater Washington, DC, USA during the years 1998 to 2000. Tetracycline resistance was found in 59% of the isolates, as well as sulfamethoxazole resistance in 45%, streptomycin resistance in 44%, cephalothin resistance in 38%, and ampicillin resistance in 38% (35%). Resistance to gentamicin (12%), nalidixic acid (8%), chloramphenicol (6%), ceftiofur (4%), and ceftriaxone (4%) was also found, but to a lesser level (1%). 16% of the isolates tested positive for one antibiotic, 23% for two, 23% for three, 12% for four, 7% for five, 3% for six, 2% for seven, and 2% for eight. Three *E. coli* isolates were found to have Shiga toxin genes (stx2) after PCR testing; all were zero non-typeable and came from ground beef samples purchased on the same day at the same supermarket. Each of the antimicrobials tested was effective against one of the Shiga toxin-producing *E. coli* (STEC) isolates.

Biochemical tests:

Garg and A. (2022) conducted this study on all staphylococcal isolates at the Shri-Aurobindo Medical College & P.G. Institute in Indore, India's microbiology lab. Each material (apart from blood) underwent gram staining, and results were recorded. Each specimen was grown overnight at 37°C in an aerobic environment on Blood agar and MacConkey's agar. By using phenotyping techniques such as the Gram stain, catalase test, slide, and tube coagulase test, growth on mannitol-salt agar, VP test, Phosphates test, and bacitracin susceptibility test, the

staphylococcal isolate was identified. According to CLSI recommendations, antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method. Chromogenic agar's sensitivity, specificity, and positive predictive value for MRSA detection were 83.7%, 80.2%, and 71.9%, respectively. 89 of the 100 cefoxitin-resistant bacteria tested positive for *mecA*, while only 11 tested negative for *mecA*.

Alizadeh et al. (2021) collected 235 urine samples randomly collected from patients referred to different laboratories in Amol. Each sample was first individually cultivated on EMB agar before being examined utilizing biochemical tests such as IMViC, TSI, and urease. Antibiogram testing was done to determine the imipenem sensitivity in samples that tested positive for *E. coli*. Polymerase Chain Reaction was used to detect the presence of the *papEF*, *sfaD*, and *fimH* genes following DNA extraction from samples resistant to imipenem using the boiling method (PCR). SPSS version 22 was used to examine the study's findings. Man-Whitney and Chi-square tests with a significance level of $P < 0.05$ were used in the statistical analyses. 75 (31/9%) of the 235 urine samples were positive for *E. coli* germs. In addition, 47 samples (62/7%) contained isolates of *E. coli* that were resistant to imipenem. Following the PCR reaction, it was discovered that 28 isolates (59/6%) were positive for the *fimH* gene, 8 isolates (17%) were positive for the *papEF* gene, and 19 isolates (40/42%) were positive for the *sfaD* gene. The findings indicated that among the strains examined, the *fimH* gene was the one that was most frequently found in *E. coli* that had been isolated from urinary tract infections in the city of Amol. This outcome was not statistically significant ($P < 0.05$). Additionally, there was no statistically significant correlation ($P < 0.05$) found between the variables in the analysis of the findings of antibiotic resistance and the presence of fimbrial virulence genes.

Dash et al. (2012) collected UTI urine samples from hospitalized patients, and isolated strains were characterized using biochemical tests such as Gram staining, indole, oxidase, catalase, methyl red, Voges Proskauer, citrate utilization, hemolysis, motility, hydrolysis of gelatin and urea, and fermentation and utilization of glucose, lactose, and sucrose. It was discovered what traditional and conventional antibiotics isolates were sensitive to. It was shown from this investigation that 56.75% of the isolates were gram-negative. Indole positive was detected in 95.23% of the isolates from Gram-negative bacteria. Catalase, methyl red, nitrate reduction, motility, hemolysis, carbohydrate fermentation on broth medium, and carbohydrate utilization on Triple sugar iron agar were all present in 100% of the indole-positive isolates. Oxidase, Voges-

Proskauer, urease, citrate, and gelatinase tests all came back negative. Additionally, it was found that all indole-positive isolates produced positivity on diverse mediums, including MacConkey agar and EMB agar. According to antibiotic sensitivity testing, all characterized strains were completely drug-resistant. According to the study's conclusions, 20 strains of multidrug-resistant uropathogenic *E. coli* were discovered.

Liu *et al.* (2021) The biotic and abiotic factors that influence the variance of ARGs in composting, as well as their putative processes, are the focus of this review. It includes dynamic variations of biotic factors such as bacterial community, mobile genetic elements (MGEs), and ARG existence forms, which are direct driving factors of ARG fate during composting, as well as abiotic indicators such as pH, moisture content, antibiotics, and heavy metals, which interfere with ARG richness indirectly by influencing the succession of bacterial community and abundance of MGEs. The effect of temperature on ARGs is dependent on whether the ARGs are intracellular or extracellular, according to the researchers.

Skandalis *et al.* (2021) their review paper is focused on the emergence of AMR in the environment and approached this topic through one health concept. It focuses on the mechanisms of ARG transmission and the hotspots of antibiotic contamination resulting in the subsequent emergence of ARGs. It follows the transmission of ARGs from farm to plant and animal food products and provides examples of the impact of ARG flow in clinical settings. Understudied and emerging antibiotic resistance selection determinants, such as heavy metal and biocide contamination, are also discussed in it.

Taneja *et al.* (2019) their findings incorporate all the spheres of the One Health concept from the Indian perspective, they reported that India has one of the highest rates of resistance to antimicrobial agents used in both humans, food animals, and the environment especially the water bodies, along with ARG presence. They also told the specific socio-economic and cultural factors which prevent the containment of AMR in India. According to them, injudicious use of antimicrobials and inadequate treatment of waste waters are important drivers of AMR in India.

Chen *et al.* (2019), in this review they highlight the main findings on the composition and function of the plant microbiome and underline the potential of plant microbiomes in the dissemination of antibiotic resistance via food consumption or direct contact.

Thanner *et al.* (2016) explored the importance of antimicrobial resistance (AMR) in terms of animal and human health in this review study, as well as current understanding and information

gaps in the emergence and transmission of AMR in livestock and plants. They provided the data needed to construct risk assessments for AMR in agriculture as well as dangers to animals and people through the food chain.

Ventola and Lee (2015) in this article AMR was introduced along with its history, causes and mechanism. They also listed different types of ARB which are currently prevalent.

Detection of ARGs by PCR method

Bajpai et al. (2017) determined the prevalence of ESBL (blaTEM, blaCTX-M, and blaSHV) genes among the members of Enterobacteriaceae. This study was carried out in 2015 in the Department of Microbiology and Molecular Medicine of a Teaching Tertiary Care Hospital. There was a total of 526 urine samples examined. Seventy-eight isolates were subjected to a polymerase chain reaction for the detection of ESBL they found ESBL genes 18 (45%) of phenotypically confirmed ESBL producers and 20 (52.5%) of phenotypically confirmed non-ESBL producers. The gene that predominated was blaTEM (48.7%), followed by blaCTX-M (7.6%) and blaSHV (5.1%).

Skoková et al. (2013) looked at how *E. coli* was detected and characterized in raw vegetables and sprouted seeds sold in the Czech Republic. A total of 24 (26.4%) of the 91 samples tested positive for *E. coli*. The disc diffusion method and the E-test were used to determine antibiotic resistance. The genes encoding virulence - eaeA, hly, stx1, and stx2 - as well as genes encoding resistance to tetracycline - tet(A), tet(B), tet(C), and tet(G) and to β -lactams - blaTEM, blaSHV, and blaCTX - were detected by polymerase chain. Two isolates had the blaTEM gene, three had the tet(B) gene, and one had the tet(A) gene. The hly, stx1, and stx2 genes were not detected, while the eaeA gene was found in three (11.1%) of the imported vegetable isolates.

Extraction of Genomic DNA

Omar et al. (2014) investigated and compared genomic DNA extraction from Gram-negative and Gram-positive bacteria utilizing a one-hour microwave approach with enzymatic and boiling procedures. When the extraction process incorporated microwave pre-heating, high DNA concentration and purity were reported for both MRSA and ESBL strains (80.1 and 91.1 g/ml; OD 260/280, 1.82, and 1.70, respectively). PCR detection of mecA and CTX-M validated the purity of the DNA.

Queipo-Ortuño *et al.* (2008) This study's objective was to examine the impact of boiling as a DNA preparation technique on the effectiveness of real-time PCR's amplification process when used to diagnose human brucellosis from serum samples. Ten brucellosis patients' serum samples were examined using a SYBR green I LightCycler-based real-time PCR and DNA extracted by boiling. It was not possible to avoid the presence of inhibitors like immunoglobulin G (IgG), which were extracted along with the template DNA, in DNA created by boiling lysis of the bacteria isolated from serum. Serum was precipitated to separate and concentrate the IgG before being examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting to determine and confirm the presence of IgG. The amplification process was entirely prevented when serum volumes greater than 0.6 ml were used. IgG had an inhibitory impact in serum samples, but it was independent of concentration and could be removed by dilution by a factor of 1/10 and 1/20 in water.

MATERIALS AND METHODS

Present investigation entitled “Study on the presence of antibiotic-resistant bacteria in the raw vegetables of Mirzapur district” was carried out at Rajiv Gandhi South Campus, Barkachha, Institute of Agricultural Sciences, Banaras Hindu University and details are described under the following heads:

3.1 Experimental location

The present research work has been conducted in Rajiv Gandhi South Campus of Banaras Hindu University, Barkachha, Mirzapur which is situated at 25.0329533 latitudes and 82.6028692 longitudes. The climate of R.G.S.C. is predominately dry, the winter season is short from December to February but summer is long from March to November. It has an average rainfall of 1059 mm, of which 90% is received from the South-west monsoon.

3.2 Experimental laboratory

Veterinary Physiology and Veterinary Biochemistry laboratory in the Department of Veterinary Physiology and Biochemistry, Institute of Agricultural Sciences, Rajiv Gandhi South Campus, Banaras Hindu University, Mirzapur was accessed for the present investigation.

3.3 Experimental materials

For this research fresh vegetables had been collected from various vegetable markets in the Mirzapur district. Experimental materials comprise five types of fresh vegetables viz. Cabbage, Carrot, Coriander, Onion, and Radish can be directly consumed as salads without cooking after some processing. For this experiment, only the inner eatable parts were taken to observe the contamination of bacteria.

3.4 Materials and Equipment

3.4.1 Glassware

- a) **100 ml, 250 ml, and 1000 ml Conical Flask (Duran®)**

Used for making culture media.

- b) **100 ml and 500 ml Measuring Cylinder (Duran®)**

Used for measuring the volume of chemicals and water used in the experiment.

- c) **15*150 ml test tubes (Riviera Pvt. Ltd.)**

Used for making broth in which bacterial colonies were incubated and stored.

d) Capped glass bottles (Duran®)

Used for storing distilled water and glycerol stock.

3.4.2 Equipments

a) Forma Steri Cycle i160 CO₂ Incubator (Thermo Fischer Scientific)

Used for providing the ideal environment for the incubation of bacteria.

b) 1300 series Class II Biological Safety Cabinet (Thermo Fischer Scientific)

The whole experiment was conducted in the biological safety cabinet as it provided the aseptic condition which is required during the experiment for it to be contamination free.

c) Electronic Balance type ATX224 (Shimadzu)

The chemicals and culture media powder were weighted on it. It has the least count of 0.1 mg or 0.0001g.

d) Vertical Autoclave NSW-227 (Narang Scientific Works Pvt. Ltd.)

Required for the sterilization of the culture media and other chemicals with the help of steam.

e) Oven Universal NSW-143 (Narang Scientific Works Pvt. Ltd.)

Used for the sterilization of all the glassware used in the experiment.

f) Refrigerator (Samsung - Twin Cooling)

Used for storing chemicals, mother culture and subcultures at -4°C or -20°C as required.

g) Ultra-low Temperature Freezer Forma 900 series (Thermo Fischer Scientific)

Used for Cryopreservation i.e., keeping samples at -20°C for the long-time storage.

h) High-speed cooling centrifuge 5424-R (Eppendorf)

Used for centrifugation during DNA isolation.

i) Agarose gel electrophoresis apparatus (Bio-Rad)

Used for Gel electrophoresis. It includes

- Electrophoresis chamber – electrophoresis occurred in it.
- Power supply unit – used to supply current at a constant potential.
- Casting tray – used for solidifying Agarose gel matrix.
- Comb – used for making wells

j) Nanodrop one^c (Thermo Fischer Scientific)

Used for the quantitative and qualitative analysis of DNA.

k) GelDoc Go imaging system (Bio-Rad)

Used for mixing.

l) Digital Water Bath (Thermostat)

Used for DNA isolation

m) Digital Hotplate stirrer (Bio-rad)

Used for heating culture media before sterilization so that they could mix well.

3.4.3 Plasticware

a) Disposable Aseptic Vented Petri Dish 90mm (Tarsons)

Used for making agar plates on which bacterial colonies were incubated and stored.

b) 2.5 µl ,10µl, 20 µl, 200 µl and 1000 µl Pipettes and tips (Eppendorf)

Used to take small volumes of chemicals and water in µl when required.

c) Cryovials (Tarsons)

Used as containers for the cryopreservation of the samples.

d) 0.2 ml PCR tubes (Tarsons)

In this PCR reaction took place.

e) 0.5 ml and 1.5 ml microcentrifuge tubes (Tarsons)

Used during DNA isolation and Primer dilution.

3.4.4 Culture Media

a) Luria Bertani Broth (HiMedia®)

The Luria Bertani Broth or Lysogeny Broth or LB Broth was used for the general multiplication of bacteria during the preparation of mother culture and subcultures in test tubes.

Preparation of 1 L of LB Broth

- 1) Suspended 25 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Dispensed into test tubes in 10 ml amount.
- 5) Sterilized media by putting them in the vertical autoclave at 15 lbs (121°C) for 15 min.

Table 1: Composition of LB Broth

Ingredients	gm/L
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25°C)	7.5±0.2

b) Luria Bertani Agar (HiMedia®)

The Luria Bertani Agar or LB Agar was used for the general multiplication of bacteria required before biochemical tests. This agar was used because it does not contain any dye that can interfere with the results during biochemical tests.

Preparation of 1 L of LB Agar plates

- 1) Suspended 40 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Sterilized the dissolved media by putting it in the vertical autoclave at 15 lbs (121°) for 15 min.
- 5) Conical flask containing media was kept in a biosafety cabinet in which it was exposed to U.V. radiation for 15 min.
- 6) Poured approx. 25 ml of media in Petri plates
- 7) The plates solidified after 30 min.

Table 2: Composition of LB Agar

Ingredients	gm / L
Tryptone	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
Final pH (at 25°C)	7.5±0.2

c) EMB Agar (HiMedia®)

Eosin methylene blue agar (EMB) is a selective and differential medium used to isolate faecal coliforms/*E. coli*.

Preparation of 1 L of EMB Agar plates

- 1) Suspended 35.96 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Sterilized the dissolved media by putting it in the vertical autoclave at 15 lbs (121°C) for 15 min.
- 5) Conical flask containing media was kept in a biosafety cabinet in which it was exposed to U.V. radiation for 15 min.
- 6) Poured approx. 25 ml of media in Petri plates.
- 7) The plates solidified after 30 min.

Table 3: Composition of EMB Agar

Ingredients	gm / L
Peptone	10.000
Dipotassium hydrogen phosphate	2.000
Lactose	5.000
Saccharose (Sucrose)	5.000
Eosin – Y	0.400
Methylene blue	0.065
Agar	13.500
Final pH (at 25°C)	7.2±0.2

d) Mannitol Salt Agar (HiMedia®)

Mannitol salt agar (MSA) is a selective, differential and indicator medium that is used to isolate and identify *S. aureus*.

Preparation of 1 L of MSA Agar plates

- 1) Suspended 111.02 gm of powder media in 1000 L of distilled water in a conical flask.

- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Sterilized the dissolved media by putting it in the vertical autoclave at 15 lbs (121°C) for 15 min.
- 5) Conical flask containing media was kept in a biosafety cabinet in which it was exposed to U.V. radiation for 15 min.
- 6) Poured approx. 25 ml of media in Petri plates.
- 7) The plates solidified after 30 min.

Table 4: Composition of MSA Agar

Ingredients	gm / L
Proteose peptone	10.000
HM peptone B (beef extract)	1.000
Sodium chloride	75.000
D- Mannitol	10.000
Phenol red	0.025
Agar	15.000
Final pH (at 25°C)	7.4±0.2

e) Tryptophan broth (HiMedia®)

It was used for the biochemical analysis of *E. coli* for the detection of indole production.

Production of 1L of Tryptophan broth

- 1) Suspended 16 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Dispensed into test tubes.
- 5) Sterilized media by putting them in the vertical autoclave at 15 lbs (121°C) for 15 min.

Table 5: Composition of Tryptophan broth

Ingredients	gm / L
Casein enzymic hydrolysate	10.000
Sodium chloride	5.000
DL-Tryptophan	1.000
Final pH (at 25°C)	7.5±0.2

f) MR-VP broth or Glucose Phosphate Broth (HiMedia®)

Used in the biochemical analysis of *E. coli*. MR-VP Medium or Glucose Phosphate Broth was used for the performance of the Methyl Red and Voges-Proskauer tests in the differentiation of the coli-aerogenes group.

Production of 1L of MR-VP broth

- 1) Suspended 17 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Dispensed into test tubes in 10 ml amount.
- 5) Sterilized media by putting them in the vertical autoclave at 15 lbs (121°C) for 15 min.

Table 6: Composition of MR-VP broth

Ingredients	gm / L
Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2

g) Tri Sugar Iron Agar (HiMedia®)

Used in the biochemical analysis of *E. coli*. Triple Sugar Iron Agar Medium was used for the identification of gram-negative enteric bacilli on the basis of dextrose, lactose and sucrose fermentation, and hydrogen sulfide (H₂S) production.

Preparation of TSI agar slant

- 1) Suspended 64.42 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/ lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Mixed well and distributed into test tubes.
- 5) Sterilize by maintaining at 15 lbs pressure (121°C) for 30 minutes.
- 6) Allowed the medium to set in sloped form with a butt about 2.5cm long.

Table 7: Composition of TSI Agar

Ingredients	gm/ L
Beef extract	3.000
Peptone	20.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Dextrose Monohydrate	1.000
Ferrous sulphate	0.200
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.000

h) Simmons Citrate Agar (HiMedia®)

Used in the biochemical analysis of *E. coli*. It was used for the differentiation of the members of Enterobacteriaceae based on citrate utilization.

Preparation of Simmons citrate agar slant

- 1) Suspended 24.28 gm of powder media in 1000 L of distilled water in a conical flask.
- 2) Constantly shook the conical flask so that no granule/lumps were formed.
- 3) Gently heated the conical flask to dissolve the media completely.
- 4) Mixed well and distributed into test tubes.

- 5) Sterilize by maintaining at 15 lbs pressure (121°C) for 30 minutes.
- 6) Allowed the medium to set in sloped form with a butt about 2.5cm long.

Table 8: Composition of Simmons Citrate Agar

Ingredients	gm/ L
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH (at 25°C)	6.8±0.2

3.4.5 Chemicals

i) Ceftazidime pentahydrate

It is the antibiotic used for the selection of ESBL *E. coli* /ESBL-E.

j) Oxacillin sodium salt monohydrate

It is the antibiotic used for the selection of MRSA.

k) Methyl red solution (HiMedia®)

Methyl Red Indicator was used for the detection of acid production from glucose fermentation.

Table 9: Composition of Methyl red solution

Ingredients	-
Methyl red	0.200 gm
Ethyl alcohol	60.000 ml
Distilled water	40.000 ml

l) Kovacs's Indole Reagent (HiMedia®)

Used in the biochemical analysis of *E. coli*. It was used for the detection of the presence of indole produced by microorganisms due to tryptophan deamination.

Table 10: Composition of Kovacs's Indole reagent

Ingredients	-
p-dimethylamino benzaldehyde	5.000 gm
Amyl alcohol	75.000 ml
Hydrochloric acid concentrated (37%)	25.000 ml

m) Voges-Proskauer Reagent A or Barritt's reagent A (HiMedia®)

Used in the biochemical analysis of *E. coli*. This reagent was used in the Voges-Proskauer test for the detection of acetoin production by bacterial culture.

Table 11: Composition of Voges Proskauer Reagent A

Ingredients	-
α -Naphthol (1-Naphthol)	5.000 gm
Absolute ethanol	100.000 ml

n) Voges-Proskauer Reagent B or Barritt's reagent B (HiMedia®)

Used in the biochemical analysis of *E. coli*. This reagent was used in the Voges-Proskauer test for the detection of acetoin production by bacterial culture.

Table 12: Composition of Voges Proskauer Reagent B

Ingredients	-
Potassium hydroxide	40.000 gm
Distilled water	100.000 ml

o) 10x TB buffer (HiMedia®)

Used during the dilution of primers.

p) Agarose (UltraPure™)

Used for the preparation of the Agarose gel matrix.

q) 10x TBE buffer (HiMedia®)

It was diluted to 1x conc., then used as the running buffer, and in the preparation of Agarose gel matrix in Agarose gel electrophoresis.

r) Ethidium bromide crystals (HiMedia®)

A stock of 10 mg/ml conc. was prepared in by dissolving 10 mg EtBr crystals in distilled water. It was used because upon binding of the molecule to the DNA and illumination with a UV light source, the DNA banding pattern can be visualized.

s) 100 bp Ladder

Used in Gel Electrophoresis. It helped in determining the DNA bands size (number of base pairs) when observed in the transilluminator.

t) 6x Loading dye Bromophenol Blue (BioAtlas)

Used for loading DNA samples in wells of the Agarose gel matrix during gel electrophoresis.

Table 13: Composition of loading dye

Ingredients	-
Tris-HCl (pH 7.5)	10mM
Bromophenol blue	0.03% (w/v)
Xylene cyanol FF	0.03% (w/v)
Glycerol	60% (v/v)
EDTA	60 mM

u) 2x PCR TaqMixture (HiMedia®)

Used during PCR.

v) Glycerol (HiMedia®)

Used in the cryopreservation of bacterial colonies.

w) 70% Ethanol

Used as a surface disinfectant

x) Molecular biology grade water for PCR (HiMedia®)

Used during PCR.

y) Primers (Eurofins Genomics India Pvt. Ltd.)

Used during PCR.

Dilution of primer

Diluted primers to 1:10 ratio in PCR-grade water, for every 1 nMoles, add 10 µl of PCR-grade water

Table 14: Primer's details

Gene		Primer sequence (5'-3')	Product size (bp)	nMol
<i>bla</i> TEM	F	CTTATTCCCTTTTTTGCGGCATTTTGCCTTC	843	31.80
	R	GTCTGACAGTTACCAATGCTTAATCAGTGAG		26.40
<i>bla</i> CTX	F	GCTTTGCGATGTGCAGCACCAGTAAAGT	570	29.90
	R	CACGATCTTTTGGCCAGATCACCGC		34.50
<i>mecA</i>	F	CGCATCAATAGTTAGTTGAATATCTTTGCCATC	232	25.30
	R	GCGGTTATGTTGGTCCCATTA ACTCTG		33.20

3.4.6 Other materials

- **Inoculation loop**

Used to inoculate and streak bacterial colonies in the test tubes and on the agar plates.

- **Absorbent cotton**

Used for covering the mouths of test tubes.

- **Forceps**

Used for taking out Coriander leaves from the sample and also to handle small pieces of other vegetables samples parts used in the experiment.

- **Knife**

Used for peeling and cutting to get the experimental material ready.

- **Spirit lamp**

Used for the sterilization of inoculating loop.

- **Test tube rack**

Used for keeping test tubes during the experiment.

- **Marker pen**

Used for marking/labeling whenever necessary in the experiment.

- **Aluminum foil**

Used for covering the mouth of the conical flask and while measuring the powder media it was used as a container on the weighing machine.

- **Ziplock bag**
Used to keep the fresh samples taken from the retail market until they reached the experimental laboratory.
- **Distilled water**
Used in the preparation of many chemicals.
- **Gloves (KimTech™)**
Used to protect hands from chemicals.
- **Tissue paper (KimTech™)**
Used to clean various instruments and surfaces.
- **Safety Match Stick**
Used to ignite the spirit lamp.
- **Rabbit plasma**
Used for the coagulase test. It was obtained after centrifugation of blood obtained from a rabbit (Figure 1).
- **23G Butterfly needle**
Used to extract blood from rabbit for the preparation of plasma used in the coagulase test (Figure 1).

3.5 Methods

3.5.1 Sampling area

For the sampling, the Mirzapur district was divided according to tehsils. The Mirzapur district has four tehsils viz. Chunar tehsil, Lalganj tehsil, Madihan tehsil and Sadar tehsil. From each tehsil, three retail vegetable markets were selected where fresh vegetables were available. (Figure 2 and 3).



Figure – 1: Collection of blood from rabbit for the extraction of plasma (Used in Coagulase test)

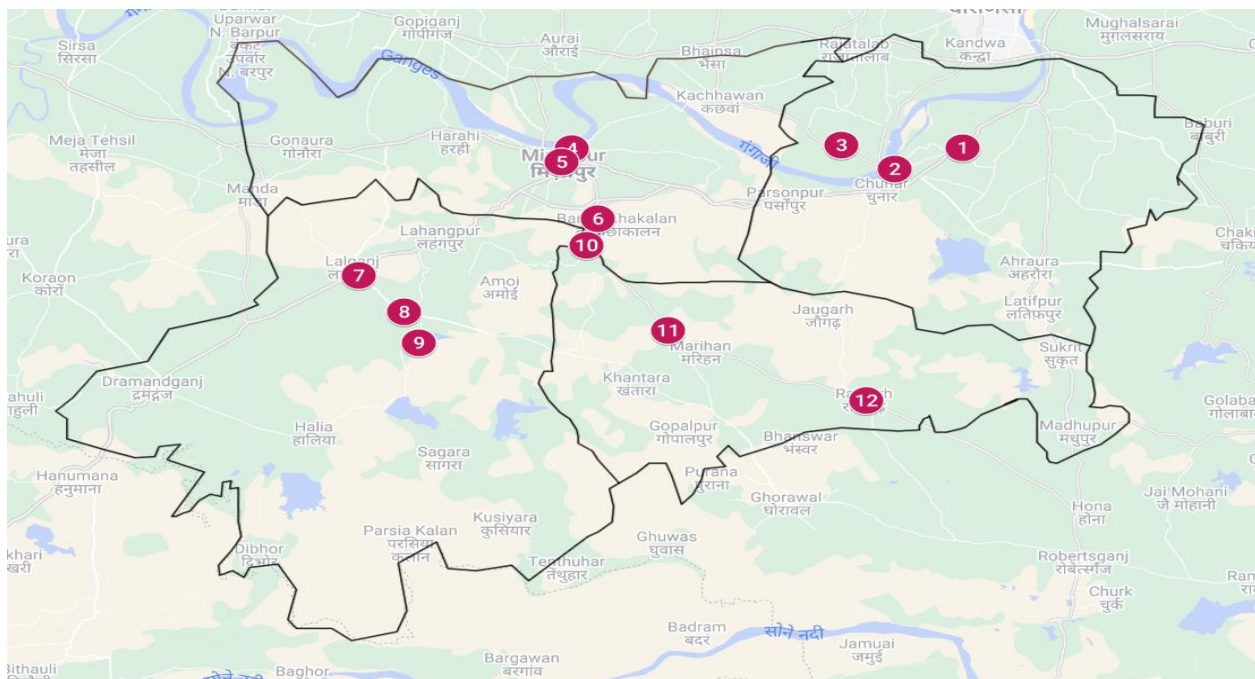


Figure - 2: Sampling area across four tehsils of Mirzapur district, Uttar Pradesh: Chunar Tehsil (1. Kailahat, 2. Gola Bazar mandi and 3. Magaraha). Sadar Tehsil (4. Ghantaghar mandi, 5. New Mandi Samiti and 6. Barkachha). Lalganj Tehsil (7. Lalganj tehsil office, 8. Dubar and 10. Barkach). Madihaan Tehsil (10. Belhara, 11. Madihaan tehsil office and 11. Rajgarh.)



A - Gola bazar Market (Chunar Tehsil)



B - Dubar Market (Lalganj Tehsil)



C - Rajgarh market (Madihaan Tehsil)



D - New Mandi Market (Sadar Tehsil)

Figure – 3: Retail vegetable markets in various tehsils of Mirzapur district. (A-D)

3.5.2 Collection of samples

Five vegetables from each fresh vegetable market, i.e., Cabbage, Carrot, Coriander, onion and radish as samples were collected. So a total of 60 samples were taken for this study. These samples were kept in well-labelled Ziplock bags till they were brought to the laboratory where the experiment was conducted. After removing the samples from the Ziplock bag they were washed in tap water and then eatable parts were taken for the experiment. The respective samples were processed as follows:

- **Cabbage** – 3 to 4 small pieces of inner leaves about 1 by 2 cm in were taken.
- **Carrot** – 3 to 4 rectangular pieces about 1 by 2 cm were taken after peeling and cutting the outer surface.
- **Coriander** – 5 to 6 leaves were taken from different positions in the bundle.
- **Onion** – 3 to 4 rectangular pieces about 1 by 2 cm were taken after peeling and cutting the outer surface.
- **Radish** – 3 to 4 rectangular pieces about 1 by 2 cm were taken after peeling and cutting the outer surface.

3.5.3 Labelling

Each sample was labelled properly so that it can be identified easily. Sample name first capital letter represents tehsil, second capital letter along with any small letter if present represents the area, third capital letter along with small letter if present represents vegetable the last 2 digits represents sample number.

Table 15: Sample labelling details

Sample No.	Sample Name	Tehsil	Area	Vegetable
1	MBCo01	Madihaan	Belahara	Coriander
2	MBCa02	Madihaan	Belahara	Carrot
3	MBCb03	Madihaan	Belahara	Cabbage
4	MBO04	Madihaan	Belahara	Onion
5	MBR05	Madihaan	Belahara	Radish
6	LBaCo06	Lalganj	Barkach	Coriander
7	LBaCa07	Lalganj	Barkach	Carrot
8	LBaCb08	Lalganj	Barkach	Cabbage

9	LBaO09	Lalganj	Barkach	Onion
10	LBaR10	Lalganj	Barkach	Radish
11	LTCO11	Lalganj	Tehsil office	Coriander
12	LTCa12	Lalganj	Tehsil office	Carrot
13	LTCb13	Lalganj	Tehsil office	Cabbage
14	LTO14	Lalganj	Tehsil office	Onion
15	LTR15	Lalganj	Tehsil office	Radish
16	LDCo16	Lalganj	Dubar	Coriander
17	LDCa17	Lalganj	Dubar	Carrot
18	LDCb18	Lalganj	Dubar	Cabbage
19	LDO19	Lalganj	Dubar	Onion
20	LDR20	Lalganj	Dubar	Radish
21	SBrCo21	Sadar	Barkachha	Coriander
22	SBrCa22	Sadar	Barkachha	Carrot
23	SBrCb23	Sadar	Barkachha	Cabbage
24	SBrO24	Sadar	Barkachha	Onion
25	SBrR25	Sadar	Barkachha	Radish
26	SGCo26	Sadar	Ghantaghar	Coriander
27	SGCa27	Sadar	Ghantaghar	Carrot
28	SGCb28	Sadar	Ghantaghar	Cabbage
29	SGO29	Sadar	Ghantaghar	Onion
30	SGR30	Sadar	Ghantaghar	Radish
31	SNCo31	Sadar	New Mandi	Coriander
32	SNCa32	Sadar	New Mandi	Carrot
33	SNCb33	Sadar	New Mandi	Cabbage
34	SNO34	Sadar	New Mandi	Onion
35	SNR35	Sadar	New Mandi	Radish
36	MTCO36	Madihaan	Tehsil office	Coriander
37	MTCa37	Madihaan	Tehsil office	Carrot
38	MTCb38	Madihaan	Tehsil office	Cabbage

39	MTO39	Madihaan	Tehsil office	Onion
40	MTR40	Madihaan	Tehsil office	Radish
41	MRCo41	Madihaan	Rajgarh	Coriander
42	MRCa42	Madihaan	Rajgarh	Carrot
43	MRCb43	Madihaan	Rajgarh	Cabbage
44	MRO44	Madihaan	Rajgarh	Onion
45	MRR45	Madihaan	Rajgarh	Radish
46	CKCo46	Chunar	Kailahat	Coriander
47	CKCa47	Chunar	Kailahat	Carrot
48	CKCb48	Chunar	Kailahat	Cabbage
49	CKO49	Chunar	Kailahat	Onion
50	CKR50	Chunar	Kailahat	Radish
51	CGoCo51	Chunar	Gola Bazar Mandi	Coriander
52	CGoCa52	Chunar	Gola Bazar Mandi	Carrot
53	CGoCb53	Chunar	Gola Bazar Mandi	Cabbage
54	CGoO54	Chunar	Gola Bazar Mandi	Onion
55	CGoR55	Chunar	Gola Bazar Mandi	Radish
56	CMCo56	Chunar	Magaraha	Coriander
57	CMCa57	Chunar	Magaraha	Carrot
58	CMCb58	Chunar	Magaraha	Cabbage
59	CMO59	Chunar	Magaraha	Onion
60	CMR60	Chunar	Magaraha	Radish

Another type of labelling was used in the Screening of ARB after DNA isolation. For the DNA samples used in ARB screening, sample number was used with the prefix of β for ESBL-E and M for MRSA.

3.5.4 Sterilization

All the glassware like measuring cylinders, beakers, and conical flasks were firstly washed with detergent and then wiped clean with tissue paper. They were then placed in a hot air oven for dry heat sterilization at 160°C for 2 hours.

For the sterilization of consumables like pipette tips, cryovials, etc., culture media like MSA, LB broth, MacC-A, etc., glycerol stock, and distilled water were vapour phase sterilized by vertical Autoclave at 15 lbs pressure and 121°C temperature for 15 min.

The inoculating loop was first cleaned with 70% ethanol and then sterilized by burning it till its red hot over the spirit lamp.

The work area in laminar airflow was first cleaned with 70% ethanol and then treated with U.V. radiation for 15 min by the U.V. lamp in the laminar airflow.

3.5.5 Preparation of mother culture or Primary enrichment

This step is required to increase the number of bacteria present in the samples. To the sterilized test tubes containing 10 ml LB broth added the processed small pieces (3.5.2) of each vegetable (i.e. Cabbage, Carrot, Coriander, onion and radish) of each location in the different test tubes thus a total of 60 test tubes were prepared to contain samples and also one negative control not containing any sample was prepared.

Incubated all these test tubes for 18 hours at 37°C. After 18 hours checked the turbidity of the test tubes was checked. The presence of turbidity meant the presence of bacteria.

3.5.6 Phenotypic screening of *E. coli* and *S. aureus*

For the identification, isolation, and purification of *E. coli* and *S. aureus* present in the sample, EMB agar plates and MSA plates were used respectively. The mother culture containing samples was streaked on both the plates and then incubated. One negative control plate was also prepared on which nothing was streaked for both types of Agar plates. Thus, a total of 61 EMB plates and 61 MSA plates were prepared. All the agar plates were incubated for 18 hours at 37°C.

After incubation checked the colony characteristics of both the bacteria on their respective plates. *E. coli* and *S. aureus* colonies were thus isolated by picking up their colonies and they can be purified by streaking them on their respective plates again and again.

Eosin methylene blue agar (EMB) is a selective and differential medium used to isolate faecal coliforms. Eosin Y and methylene blue are pH indicator dyes

that combine to form a dark purple precipitate at low pH, they also serve to inhibit the growth of most Gram-positive organisms. Sucrose and lactose serve as fermentable carbohydrate sources which encourage the growth of faecal coliforms and provide a means of differentiating them. *E. coli*, a vigorous fermenter, often produces a green metallic sheen. Slow or weak fermenters will produce mucoid pink colonies. Normally-coloured or colourless colonies indicate that the organism ferments neither lactose nor sucrose and is not a faecal coliform.

The MSA will select organism as *Staphylococcus* species which can live in areas of high salt concentration. The differential ingredient in MSA is the sugar mannitol. Organisms capable of using mannitol as a food source will produce acidic by-products of fermentation that will lower the pH of the media. The acidity of the media will cause the pH indicator, phenol red, to turn yellow. *S. aureus* is capable of fermenting mannitol.

3.5.7 Secondary enrichment/Subculture

This step was required to increase the number of bacteria isolated and purified in the previous step. To the sterilized test tubes containing 10 ml LB broth streaked the previous *E. coli* and *S. aureus* isolates in different test tubes according to the samples. Incubated the test tubes containing isolates for 18 hours at 37°C. After 18 hours checked the turbidity of the test tubes was checked. The presence of turbidity meant the presence of bacteria.

3.5.8 Confirmatory biochemical test for *E. coli* and *S. aureus*

These tests were used to confirm *E. coli* and *S. aureus* biochemically. For this LB agar plates were prepared by streaking 1° subculture on them and incubating them for 18 hours at 37°C. The growth on these LB agar plates was used for further biochemical tests. All the controls were obtained from the Veterinary public health department.

Biochemical tests for *E. coli*

For confirming *E. coli* IMViC tests along with TSI and urease tests were carried out.

- **Indole test**

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophan into indole, which accumulates in the medium. Indole production test is important in the identification of Enterobacteria. Most strains of *E. coli* break down the amino acid tryptophan with the release of indole. This is performed by a chain of a number of different intracellular enzymes, a system generally referred to as tryptophanase. It is a test designed to distinguish among members of the family Enterobacteriaceae. *E. coli* is positive for this test.

Procedure of Indole test

- 1) Took sterilized test tubes containing 4 ml of tryptophan broth.
- 2) Inoculated the tube aseptically by taking the growth.
- 3) Incubated the tube at 37°C for 24-28 hours.
- 4) Added 0.5 ml of Kovac's reagent to the broth culture.
- 5) Observed for the presence or absence of a ring.

Result Interpretation of Indole Test

Positive: Formation of a pink to red colour ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent.

Negative: No colour change even after the addition of the appropriate reagent.

Quality Control for Indole Test

Positive Control: *E. coli* (ATCC25922)

Negative control: *Proteus mirabilis* (field isolate)

- **Methyl Red (MR) test**

The methyl red (MR) test detects the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5, as shown by a change in the colour of the methyl red indicator which is added at the end of the period of incubation. *E. coli* is positive for this test.

Procedure of MR Test

- 1) Before inoculation, allowed the medium to equilibrate to room temperature.
- 2) Lightly inoculated the medium.

- 3) Incubated aerobically at 37 degrees C. for 24 hours.
- 4) Following 24 hours of incubation, aliquoted 1ml of the broth to a clean test tube.
- 5) Reintubated the remaining broth for an additional 24 hours.
- 6) Added 2 to 3 drops of methyl red indicator to the aliquot.
- 7) Observed red colour immediately

Result Interpretation of MR test

Positive Reaction: A distinct red colour

Negative Reaction: A yellow colour

Quality Control for MR Test

Positive Control: *E. coli* (ATCC25922)

Negative control: *Klebsiella pneumoniae* (ATCC13883)

- **Voges–Proskauer (VP) Test**

Voges-Proskauer or VP test is a test used to detect acetoin in bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth, which is a glucose-phosphate broth that has been inoculated with bacteria. *E. coli* is negative for this test.

Procedure of VP Test

- 1) Before inoculation, allowed the medium to equilibrate to room temperature.
- 2) Using organisms taken from an 18–24-hour pure culture, lightly inoculated the medium.
- 3) Incubated aerobically at 37°C. for 24 hours.
- 4) Following 24 hours of incubation, aliquoted 2 ml of the broth into a clean test tube.
- 5) Re-incubated the remaining broth for an additional 24 hours.
- 6) Added 6 drops of 5% alpha-naphthol, and mixed well to aerate.
- 7) Added 2 drops of 40% potassium hydroxide and mixed well to aerate.
- 8) Observed a pink-red colour at the surface within 30 min. Shook the tube vigorously during the 30 min period.

Result Interpretation of Voges–Proskauer (VP) Test

Positive Reaction: pink-red colour on the surface

Negative Reaction: lack of a pink-red colour

Quality Control for VP Test

Positive Control: *Enterobacter aerogenes* (ATCC13048)

Negative control: *E. coli* (ATCC25922)

- **Citrate Utilization test**

Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) as the sole nitrogen source. *E. coli* is negative for this test.

Procedure of Citrate Utilization Test

- 1) Streaked the slant back and forth with a light inoculum picked from the centre of a well-isolated colony.
- 2) Incubated aerobically at 35 to 37°C for up to 4-7 days.
- 3) Observed a colour change from green to blue along the slant.

Result interpretation of Citrate Utilization Test

Positive Reaction: Growth with colour change from green to intense blue along the slant.

Negative Reaction: No growth and no colour change; Slant remains green.

Quality Control for citrate utilization Test

Positive Control: *Klebsiella pneumoniae* (ATCC13883)

Negative control: *E. coli* (ATCC25922)

- **Triple Sugar Iron (TSI) Test**

Triple Sugar Iron Agar is used for the presumptive identification of Enterobacteriaceae based on the fermentation of glucose, lactose, sucrose, and the production of gas and H_2S . For *E. coli* the results are yellow slant, yellow butt, gas present but no H_2S is produced i.e., no blackening of media.

Procedure of TSI Test

- 1) With a straight inoculation needle, touched the top of a well-isolated colony.
- 2) Inoculated TSI by first stabbing through the centre of the medium to the bottom of the tube and then streaking the surface of the agar slant.
- 3) Left the cap on loosely and incubated the tube at 35°-37°C in ambient air for 18 to 24 hours.

- 4) Examined the reaction of the medium.

Result interpretation of TSI Test

An alkaline/acid (red slant/yellow butt) reaction: It is indicative of dextrose fermentation only.

An acid/acid (yellow slant/yellow butt) reaction: It indicates the fermentation of dextrose, lactose and/or sucrose.

An alkaline/alkaline (red slant, red butt) reaction: Absence of carbohydrate fermentation results.

Blackening of the medium: Occurs in the presence of H₂S.

Gas production: Bubbles or cracks in the agar indicate the production of gas (formation of CO₂ and H₂)

- **Urease Test**

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. *E. coli* is negative for this test.

Procedure of Urease test

- 1) Streaked the surface of a urea agar slant with a portion of a well-isolated colony or inoculated slant with 1 to 2 drops from an overnight LB broth.
- 2) Left the cap on loosely and incubated the tube at 35°-37°C in ambient air for 48 hours to 7 days.
- 3) Examined for the development of pink colour for as long as 7 days.

Result interpretation of Urease Test

Positive reaction: Development of an intense magenta to bright pink colour in 15 min to 24 hours.

Negative reaction: No colour change.

Quality Control for Urease Test

Positive Control: *Klebsiella pneumoniae* (ATCC13883)

Negative control: *E. coli* (ATCC25922)

Biochemical test for *S. aureus*.

The coagulase test helps to identify *S. aureus* which produces the enzyme coagulase (coagulates) plasma from coagulase-negative staphylococci (CONS). Coagulase is also the virulence factor of *S. aureus*, it converts soluble fibrinogen in

plasma to insoluble fibrin, and the fibrin coat the bacterial cells making protecting them from opsonization and phagocytosis.

Procedure of Coagulase test (A slide coagulase test is done to detect bound coagulase)

- 1) Placed a drop of physiological saline on each end of a slide.
- 2) With the loop, emulsified a portion of the isolated colony in each drop to make two thick suspensions.
- 3) Added drop rabbit plasma to one of the suspensions, and mixed gently.
- 4) Looked for clumping of the organisms within 10 seconds.
- 5) No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

Result interpretation of Coagulase Test

Positive: Clumping present

Negative: Clumping absent

Quality Control for Coagulase Test

Positive Control: *S. aureus* (ATCC25923)

Negative control: *E. coli* (ATCC25922)

3.5.9 Phenotypic screening of the ARBs (ESBL-E and MRSA)

For the identification, isolation and purification of ESBL-E and MRSA present in the sample, Selective media with antibiotics were used. In this method, bacteria are grown on their media agar plates (Selective or differential) in which antibiotics are already added according to the CLSI standards for the screening of completely resistant bacteria.

For ESBL-E and MRSA, EMB plates added ceftazidime and MSA plates added with oxacillin were used respectively. Both had the antibiotics were added in 4 mg/L conc. as per the CLSI standards.

The secondary enriched culture was streaked on both the plates and then incubated. One negative control plate was also prepared on which nothing was streaked for both types of Agar plates.

The growth that came on the EMB plate containing ceftazidime was ESBL-E colonies whereas the growth that came on the MSA plate containing oxacillin

was MRSA. The absence of any growth meant that the isolates of *E. coli* and *S. aureus* were not ARB. The Positive ARB samples were used for preparing tertiary enriched culture.

3.5.10 Tertiary enrichment/subculture

This step was required to increase the number of ARBs isolated and purified in the previous step. To the sterilized test tubes containing 10 ml LB broth streaked the previous ESBL-E and MRSA isolates in different test tubes according to the samples. Incubated test tubes were checked. The presence of turbidity meant the presence of bacteria.

3.5.11 Validation of ARB found by detecting ARGs through PCR method

It was used to confirm ARB by the presence of ARG and screening the type of ARG present in the samples. For this DNA was isolated, the integrity of DNA was checked, ARGs were amplified and results were seen by Gel electrophoresis.

The two genes *bla*TEM and *bla*CTX both were checked for positive samples of ESBL-E and one *mecA* gene was checked for positive samples of MRSA, obtained from the Phenotypic screening of ARB.

- **DNA isolation**

DNA isolation was done by boiling lysis method in which 1 µl of Tertiary enriched sample was taken in 1.5 ml microcentrifuge tube, then centrifuged at 15,000 × g for 15 min. the supernatant was discarded and cell pellets were resuspended in 0.1 ml of molecular biology-grade water and were centrifuged at 15,000 × g for 10 min. Pellets were resuspended in 40 µl of water, subjected to boiling at 100°C in a water bath for 10 min, cooled on ice, and centrifuged at 15,000 × g for 10 s. It was used as a template during PCR and these were stored at -20°C.

- **Quantitative and qualitative analysis of isolated DNA**

It was done after DNA isolation. Nanodrop was used for it. It was done by placing 3 µl drops of each sample in the spectrometer. It operates on Beer's law.

- **Amplification of ARGs by PCR**

Before gene amplification standardization of primer annealing temperature was conducted for all the primers to get the best annealing temperature to be used during PCR. PCR was used to amplify ARGs, if they were present, they would form bands

during gel electrophoresis according to their size (bp). As mentioned in the table of genes in Table 14. *bla*TEM and *bla*CTX primers were used in ESBL-E whereas *mecA* primer was used in MRSA for the respective ARGs identification.

Prepared 0.2 µl PCR tubes according to Table 16 for all the samples and negative control.

PCR program was set according to Table 17 thermo-cyclic conditions.

Table 16: Composition reaction mixture of Sample and negative control used in PCR

	Sample	Negative control
Taq Mixture	12.5 µl	12.5 µl
Forward Primer	1.0 µl	1.0 µl
Reverse Primer	1.0 µl	1.0 µl
Template	5.0 µl	-
Molecular grade water	5.5 µl	10.5 µl
Total	25.0 µl	25.0 µl

Table 17: PCR thermocycling conditions

Gene	Thermocycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
		Repeat for 30 cycles			
<i>bla</i> TEM	95°C – 1 min	95°C – 20 sec	55°C – 20 sec	72°C – 20 sec	72°C – 5 min
<i>bla</i> CTX	95°C – 1 min	95°C – 20 sec	55°C – 20 sec	72°C – 20 sec	72°C – 5 min
<i>mecA</i>	95°C – 5 min	95°C – 20 sec	60°C – 15 sec	72°C – 20 sec	72°C – 4 min

- **Gel electrophoresis**

For observing the presence of DNA band firstly used the gel electrophoresis with 1% conc. of Agarose gel, in which added 0.5 µl/ml EtBr and 1xTBE running buffer. The mixture of loading dye and amplified DNA sample by PCR was prepared in which they were 2 µl and 5 µl respectively which gives a total of 7µl. In the first well loaded 7µl of the ladder, then in subsequent wells added different mixture of

loading dye and samples, including control. A constant supply of current at 100V was used for 1 hour for the separation of DNA bands.

Secondly, after 1 hour the bands were observed with the help of GelDoc Go imaging system. If DNA bands were formed at their respective position i.e., *bla*TEM at 843 bp, *bla*CTX at 570 bp and *mecA* at 232 bp, then these ARGs were present otherwise absent. If in control DNA bands were formed that means contamination, then that batch of samples was discarded.

3.5.12 Preservation of samples

All positive samples were preserved by the cryopreservation method in which samples are stored at -84°C with the help of 25% Glycerol.

Procedure of Cryopreservation

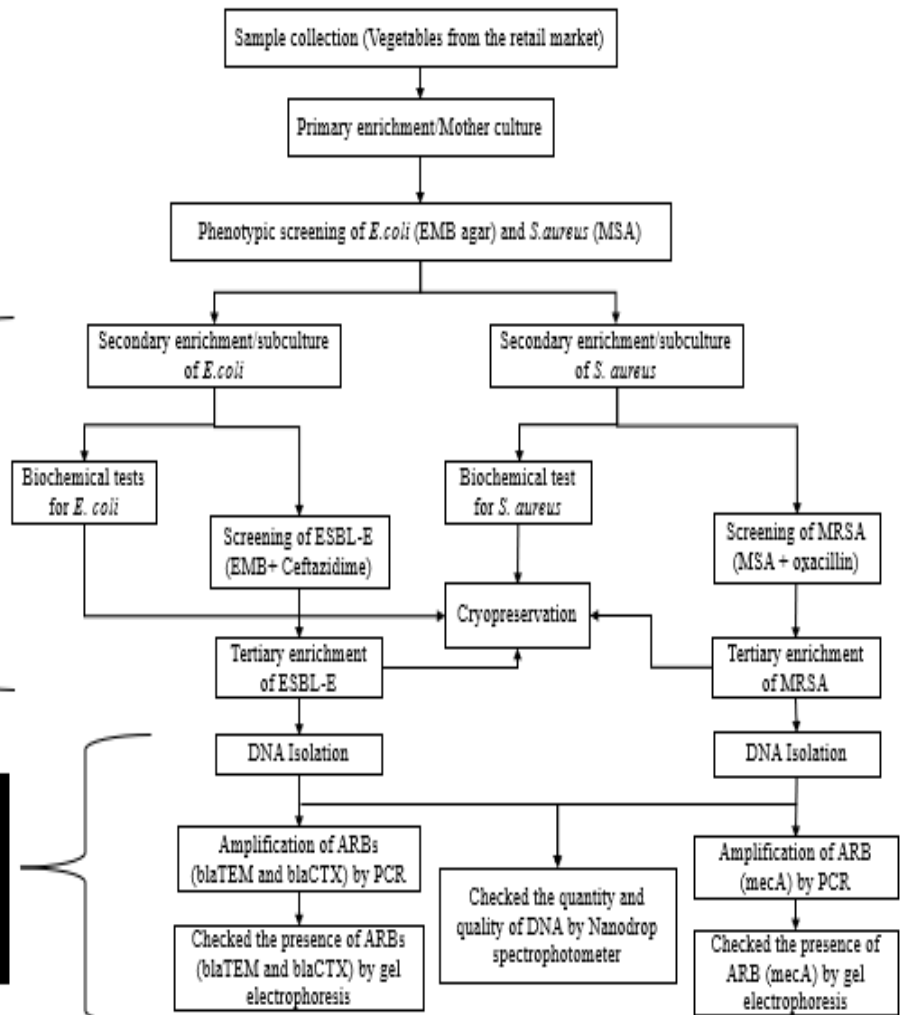
- 1) A stock of 50% glycerol was prepared by mixing 50 ml of glycerol with 50 ml distilled water in a 100 ml glass bottle with a cap.
- 2) Sterilized by autoclaving it at 15lbs (121°C) for 15 min.
- 3) In a 2 ml Cryovial took 500 μ (0.5ml) of overnight grown culture and added 500 μ (0.5ml) of sterilized 50% glycerol stock so we got a final 25% final glycerol concentration.
- 4) Gently shook the cryovial 5 to 6 times till no different layers were present in between glycerol and culture, and got one uniform solution.
- 5) Placed the cryovials in refrigerator at -84°C.

Figure 4: Overview of the technical plan

OBJECTIVE 1 : To study the prevalence of *E. coli* and *S. aureus* bacteria in the fresh vegetable from the retail markets in the Mirzapur district.

OBJECTIVE 2 : To study the prevalence of ARB (ESBL-E and MRSA)

OBJECTIVE 3 : To validate the ARB through PCR detection of ARGs (*bla*TEM, *bla* CTX and *mecA*)



RESULTS

The present investigation entitled “Study on the presence of antibiotic-resistant bacteria in the raw vegetables of Mirzapur district” was performed in the Veterinary Physiology and Biochemistry laboratory in the Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary and Animal Sciences, Rajiv Gandhi South Campus, Banaras Hindu University, Mirzapur, Uttar Pradesh, India. This study was used to monitor the prevalence of pathogenic bacteria viz. *E. coli* and *S. aureus*, their AMR forms viz. ESBL-E and MRSA, and ARB genes viz. *bla*TEM, *bla*CTX and *mecA*. All these aspect’s results are presented in the following manner –

4.1 Phenotypic screening of *E. coli* and *S. aureus*

Five different types vegetables of were selected from 3 different markets of each tehsil and altogether 4 tehsils are present in the Mirzapur district so a total of 60 samples were taken. Selective and differential agar media were used for the screening of bacteria, forming different types of colonies on these media which are used as the basics of identification.

For screening of *E. coli*, EMB agar was used. On EMB agar *E. coli* forms colonies with a distinctive metallic green sheen that reflects light (Figure 7). So, on this basis out of 60 samples, 10 samples were positive for the presence of *E. coli* (Table 18) i.e., 16.67%. For screening of *S. aureus*, MSA was used. On MSA agar *S. aureus* forms yellow colonies with yellow zones (Figure 9). So, on this basis out of 60 samples, 25 samples were positive for the presence of *S. aureus* i.e., 41.67%, (Table 18). Out of a total of 60, altogether contamination of either *E. coli* or *S. aureus* was found in 30 samples (50.0%).

Table 18: Screening of *E. coli* and *S. aureus* based on phenotypic appearance

Sample No.	Sample name	<i>E. coli</i>	<i>S. aureus</i>
1	MBCo01	+	+
2	MBCa02	-	-
3	MBCb03	-	+
4	MBO04	-	-
5	MBR05	+	-

6	LBaCo06	+	+
7	LBaCa07	-	+
8	LBaCb08	-	+
9	LBaO09	-	+
10	LBaR10	-	+
11	LTC011	+	+
12	LTCa12	+	-
13	LTCb13	-	+
14	LTO14	-	+
15	LTR15	-	-
16	LDCo16	-	+
17	LDCa17	-	+
18	LDCb18	-	-
19	LDO19	-	-
20	LDR20	+	+
21	SBrCo21	+	-
22	SBrCa22	-	-
23	SBrCb23	-	-
24	SBrO24	-	-
25	SBrR25	-	+
26	SGCo26	-	+
27	SGCa27	-	-
28	SGCb28	+	+
29	SGO29	-	-
30	SGR30	-	-
31	SNCo31	-	-
32	SNCa32	-	-
33	SNCb33	-	-
34	SNO34	-	-
35	SNR35	-	-

36	MTCo36	-	-
37	MTCa37	-	-
38	MTCb38	-	-
39	MTO39	-	-
40	MTR40	-	-
41	MRCo41	-	-
42	MRCa42	-	+
43	MRCb43	-	-
44	MRO44	-	-
45	MRR45	-	-
46	CKCo46	-	+
47	CKCa47	-	+
48	CKCb48	-	+
49	CKO49	-	-
50	CKR50	+	-
51	CGoCo51	-	+
52	CGoCa52	-	-
53	CGoCb53	-	+
54	CGoO54	+	-
55	CGoR55	-	+
56	CMCo56	-	+
57	CMCa57	-	-
58	CMCb58	-	+
59	CMO59	-	-
60	CMR60	-	-
	Total positive samples	10 out of 60	25 out of 60
	Percentage of positive samples	16.67%	41.67%

- **Tehsil-wise prevalence of *E. coli* and *S. aureus***

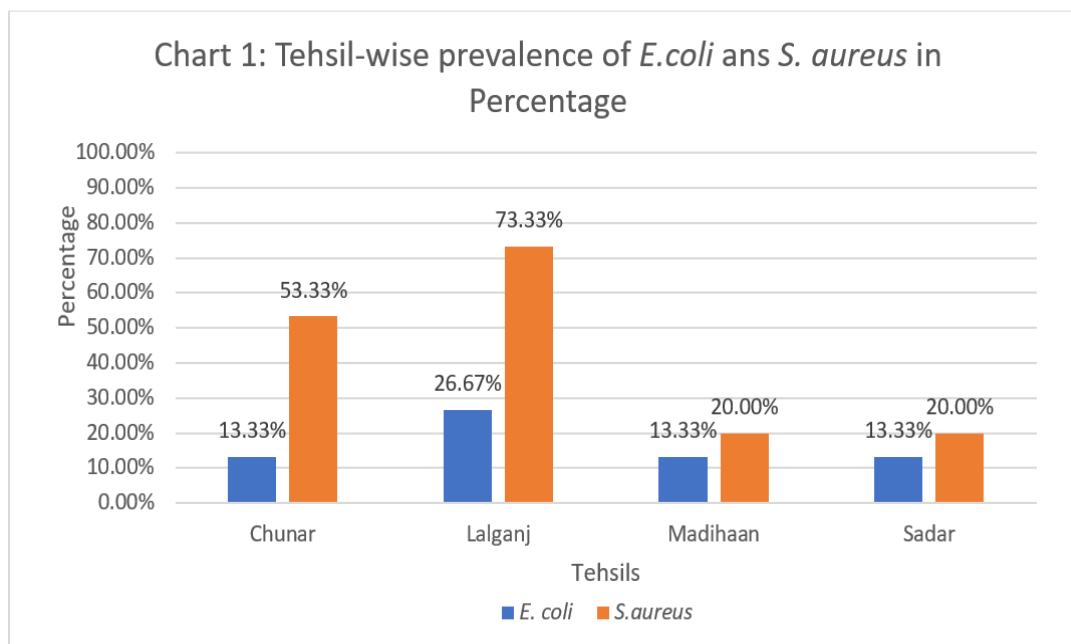
From each tehsil, a total of 15 samples were collected, out of which for *E. coli*, in three tehsils viz Chunar, Madihaan, and Sadar, 2 (13.33%) samples were found positive and 4 (26.67%) samples were found positive in Lalganj (Table 19). *S. aureus*, in Madihaan and Sadar 3 (20%) samples were found positive, in Chunar 8 (53.33%) samples were positive and 11 (73.33%) samples were positive in Lalganj (Table 20). Both *E. coli* and *S. aureus* contamination was highest in Lalganj while *E. coli* was lowest in Chunar, Madihaan, and Sadar, whereas *S. aureus* was lowest in Madihaan and Sadar (Chart 1).

Table 19: Tehsil-wise prevalence of *E. coli*

Tehsils	Total number of samples collected	Positive samples of <i>E. coli</i>	Percentage of positive samples
Chunar	15	2	13.33%
Lalganj	15	4	26.67%
Madihaan	15	2	13.33%
Sadar	15	2	13.33%
Total	60	10	16.67%

Table 20: Tehsil-wise prevalence of *S. aureus*

Tehsils	Total number of samples collected	Positive samples of <i>S. aureus</i>	Percentage of positive samples
Chunar	15	8	53.33%
Lalganj	15	11	73.33%
Madihaan	15	3	20.00%
Sadar	15	3	20.00%
Total	60	25	41.67%



- **Vegetable-wise prevalence of *E. coli* and *S. aureus***

From each vegetable, a total of 12 samples were collected, out of which for *E. coli*, only 1(8.33%) sample was found positive in Carrot, Cabbage, and Onion, 3(25%) samples were positive in Radish and 4(33.33%) samples were positive in Coriander (Table 21). For *S. aureus*, Coriander, Carrot, Cabbage, Onion, and Radish had positive samples as 8 (66.67%),4 (33.33%),7 (58.33%),2 (16.67%) and 4 (33.33%) respectively (Table 22). *E. coli* and *S. aureus* contamination was highest in Coriander while *E. coli* was lowest in Carrot, Cabbage, and Onion, and *S. aureus* was lowest in Carrot and Radish (Chart 2).

Table 21: Vegetable-wise prevalence of *E. coli*

Vegetables	Total number of samples collected	Positive samples of <i>E. coli</i>	Percentage of positive samples
Coriander	12	4	33.33%
Carrot	12	1	8.33%
Cabbage	12	1	8.33%
Onion	12	1	8.33%
Radish	12	3	25.00%
Total	60	10	16.67%

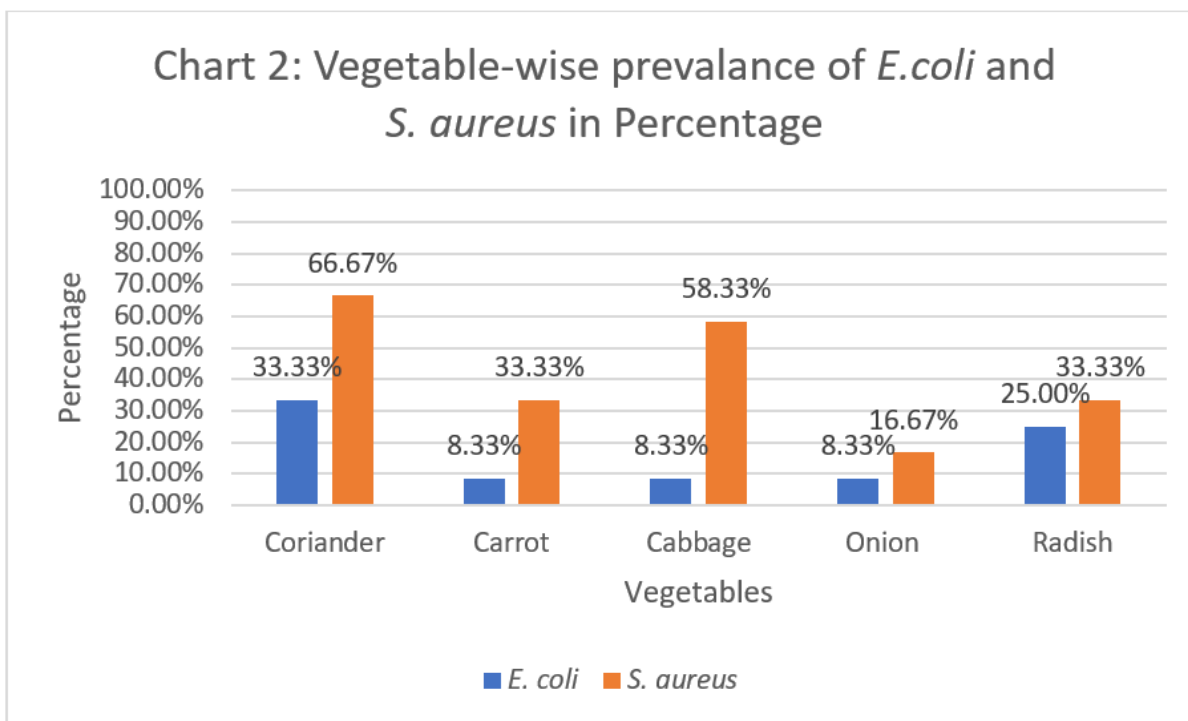
Table 22: Vegetable-wise prevalence of *S. aureus*

Vegetables	Total number of samples collected	Positive samples of <i>S. aureus</i>	Percentage of positive samples
Coriander	12	8	66.67%
Carrot	12	4	33.33%
Cabbage	12	7	58.33%
Onion	12	2	16.67%
Radish	12	4	33.33%
Total	60	25	41.67%

4.2 Confirmatory biochemical tests

- **In *E. coli***

All the positive samples of *E. coli* obtained from phenotypic screening were subjected to confirmatory biochemical tests. For this IMViC tests were carried out along with additional TSI and urease tests. Other bacterial colonies observed on EMB agar plates that did not have the characteristics of *E. coli* colonies were also used for comparison.



The results of biochemical tests of phenotypically *E. coli* positive samples are as follows (Table 23) (Figure 10).

1. **Indole test:** Positive – Cherry red ring formed in the reagent layer on top of the medium within seconds of adding the reagent.
2. **Methyl red (MR) test:** Positive – distinct red colour was formed.
3. **Voges-Proskauer (VP) test:** Negative – Pink colour was not formed at the surface.
4. **Citrate test:** Negative - No growth and no colour change; Slant remained green.
5. **Tri Sugar Iron (TSI) test** – Acidic yellow slant, yellow butt and gas was present but no H₂S is produced i.e., no blackening of media.
6. **Urease test:** Negative - No colour change was found.

The results of biochemical tests of phenotypically *E. coli* negative samples are as follows (Table 23) (Figure 11).

1. **Indole test:** Negative – No colour change even after the addition of the reagent
2. **Methyl red (MR) test:** Negative – yellow colour.
3. **Voges-Proskauer (VP) test:** Negative – Pink colour was not formed at the surface.
4. **Citrate test:** Positive - Growth with colour change from green to intense blue along the slant.
5. **Tri Sugar Iron (TSI) test** - yellow slant, yellow butt, gas was present but no H₂S is produced i.e., no blackening of media.
6. **Urease test:** Negative - No colour change was found.

By comparing the results of biochemical tests of 10 phenotypically positive *E. coli* by their respective positive and negative controls, it was confirmed that they were *E. coli* colonies.

Table 23: Testing *E. coli* in the sample through biochemical tests

Tests	<i>E. coli</i>	other bacteria
Indole	+	-
Methyl red (MR)	+	-
Voges-Proskauer (VP)	-	-
Citrate	-	+
Urease	-	-

- **In *S. aureus***

The 25 positive samples of *S. aureus* obtained from phenotypic screening were subjected to the confirmatory biochemical test. A coagulase test for bound coagulase was conducted for *S. aureus* which produces the coagulase enzyme (Coagulase positive *S. aureus*). Clumping was found in all the slides containing *S. aureus* samples and in negative control in which samples have not been added no clumping was found (Figure 12) (Table 24). Thus, by comparing the results with positive and negative controls it was confirmed that the 25 phenotypically screened samples were colonies of Coagulase-positive *S. aureus*.

Table 24: Testing *S. aureus* in the sample through biochemical test

Test	<i>S. aureus</i>	Negative control
Coagulase	+	-

4.3 Phenotypic screening of ARB (ESBL-E and MRSA)

All the 10 samples of *E. coli* and 25 samples of *S. aureus* were used for the presence of Antibiotic-Resistant bacteria i.e., ESBL-E and MRSA respectively. For this, selective/differential media along with antibiotics were used.

For screening of ESBL-E, EMB agar plates were used in which ceftazidime antibiotic was added. Out of 10 samples, *E. coli* growth was present on 4 samples which indicated that they were ESBL-E (Table 25) i.e., 40% whereas when compared to the total 60 samples it was 6.67%. For screening of MRSA, MSA plates were used in which oxacillin antibiotic was added. Out of 25 samples, *S. aureus* growth was present on 6 samples which indicated that they were MRSA (Table 26) i.e., 24% whereas when compared to the total 60 samples it was 10%. Out of all contaminated 35 samples from either *E. coli* or *S. aureus*, a total of 9 samples of ESBL-E or MRSA were present i.e., 25.71%

Table 25: Phenotypic screening results of ESBL-E

S.N.	Sample name	<i>E. coli</i>	ESBL-E
1	MBCo01	+	+
2	MBR05	+	+
3	LBaCo06	+	+
4	LTCo11	+	-
5	LTCa12	+	-
6	LDR20	+	+
7	SBrCo21	+	-
8	SGCb28	+	-
9	CKR50	-	-
10	CGoO54	+	-
		Total Positive ESBL-E samples	4 out of 10 <i>E. coli</i> samples i.e., 40%

Table 26: Phenotypic screening results of MRSA

S.N.	Sample name	<i>S. aureus</i>	MRSA
1	MBCo01	+	+
2	MBCb03	+	+
3	LBaCo06	+	-
4	LBaCa07	+	-
5	LBaCb08	+	-
6	LBaO09	+	-
7	LBaR10	+	+
8	LTCo11	+	+
9	LTCb13	+	-
10	LTO14	+	-
11	LDCo16	+	-
12	LDCa17	+	-
13	LDR20	+	-
14	SBrR25	+	-

15	SGCo26	+	-
16	SGCb28	+	-
17	MRCa42	+	-
18	CKCo46	+	-
19	CKCa47	+	-
20	CKCb48	+	+
21	CGoCo51	+	-
22	CGoCb53	+	-
23	CGoR55	+	+
24	CMCo56	+	-
25	CMCb58	+	-
		Total Positive MRSA samples	6 out of 25 <i>S. aureus</i> samples i.e., 24%

- **Tehsil-wise prevalence of ESBL-E and MRSA**

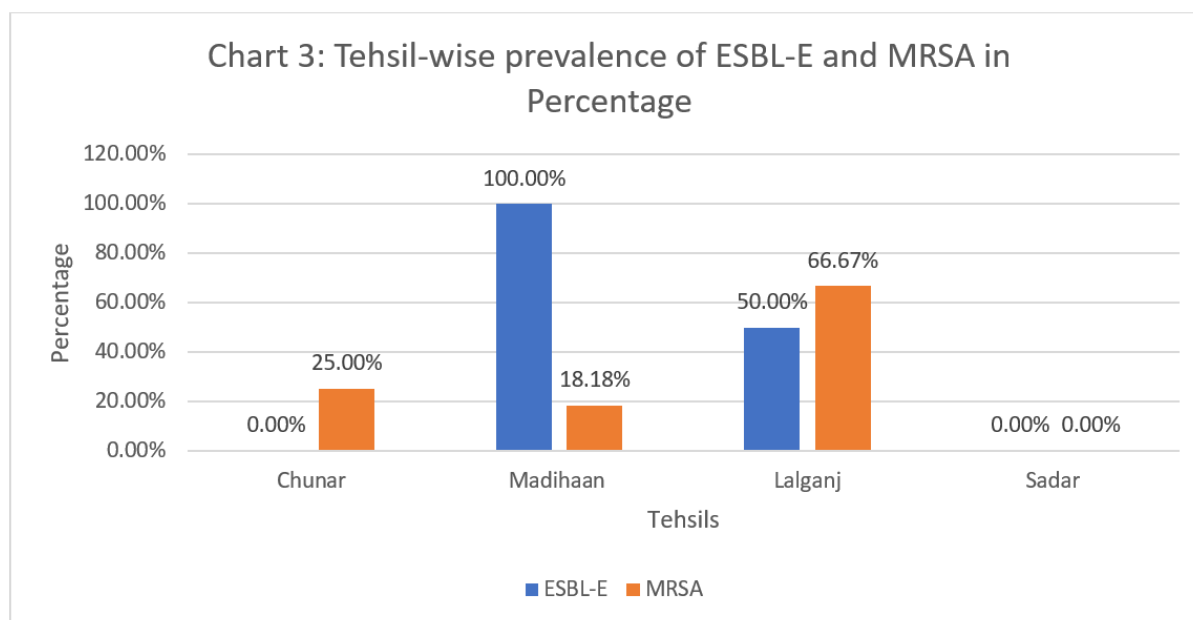
When the positive samples of ESBL-E and MRSA isolated from *E. coli* and *S. aureus* samples respectively, were separated tehsil-wise it was found that ESBL-E was absent in Chunar (out of 2 *E. coli* samples) and Sadar (out of 2 *E. coli* sample), present in 2 samples in Lalganj (out of 4 samples of *E. coli*) and Madihaan (out of 2 samples *E. coli*) (Table 27) whereas MRSA was absent in Sadar (out of 2 samples of *S. aureus*), present in 2 samples in Chunar (out of 8 samples of *S. aureus*), Lalganj (out of 3 samples of *S. aureus*) and Madihaan (out of 11 samples of *S. aureus*) (Table 28). ESBL-E was found highest in Madihaan and MRSA was found highest in Lalganj while both were lowest in Sadar (Chart 3).

Table 27: Tehsil-wise prevalence of ESBL-E

Tehsils	Total number of <i>E. coli</i> samples collected	Positive samples of ESBL-E	Positive samples in Percentage
Chunar	2	0	0.00%
Madihaan	2	2	100.00%
Lalganj	4	2	50.00%
Sadar	2	0	0.00%
Total	10	4	40%

Table 28: Tehsil-wise prevalence of MRSA

Tehsils	Total number of <i>S. aureus</i> samples collected	Positive samples of MRSA	Positive samples in Percentage
Chunar	8	2	25.00%
Madihaan	11	2	18.18%
Lalganj	3	2	66.67%
Sadar	3	0	0.00%
Total	25	6	24%



- **Vegetable-wise prevalence of ESBL-E and MRSA**

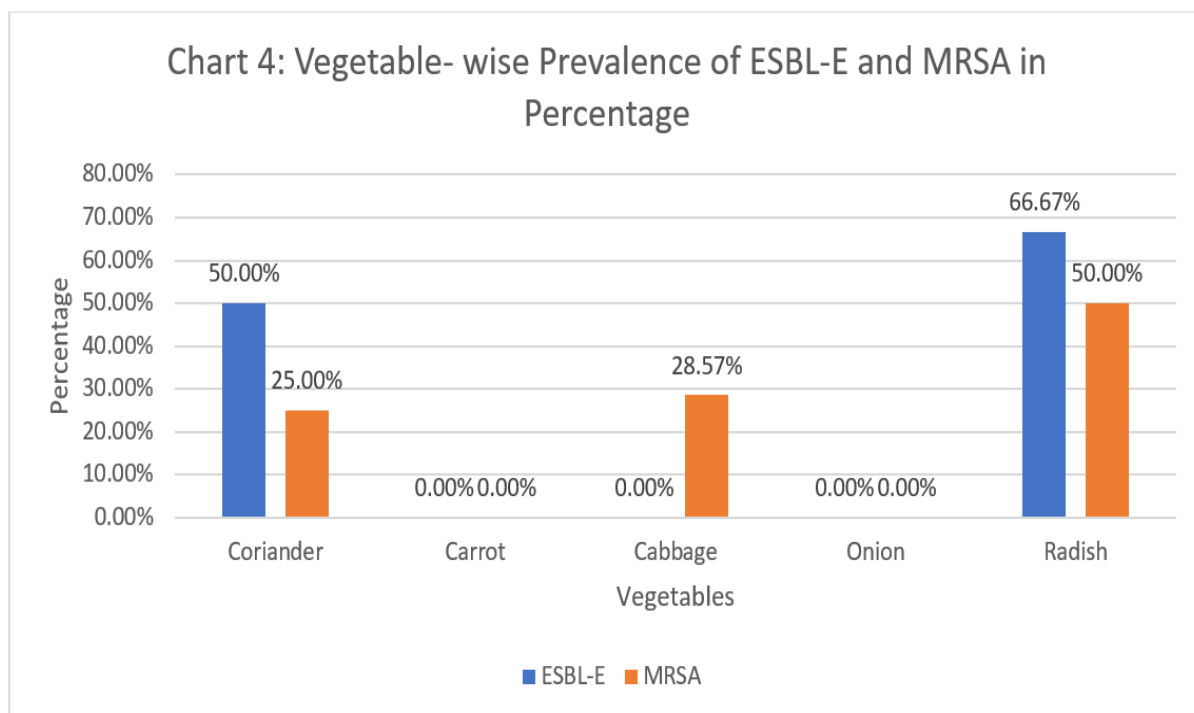
When the positive samples of ESBL-E and MRSA isolated from *E. coli* and *S. aureus* samples respectively, were separated vegetable-wise it was found that ESBL-E was absent in Carrot (out of 1 *E. coli* sample), Cabbage (out of 1 *E. coli* sample) and Onion (out of 1 *E. coli* sample), present in 2 samples in Coriander (out of 4 samples of *E. coli*) and 2 samples of Radish (out of 3 samples *E. coli*) (Table 29) whereas MRSA was absent in Carrot (out of 4 samples of *S. aureus*) and onion (out of 2 samples of *S. aureus*), present in 2 samples in Coriander (out of 8 samples of *S. aureus*), Cabbage (out of 7 samples of *S. aureus*) and Radish (out of 4 samples of *S. aureus*) (Table 30). ESBL-E and MRSA contamination were highest in Radish while lowest in Carrot and Onion (Chart 4).

Table 29: Vegetable-wise prevalence of ESBL-E

Vegetables	Total number of <i>E. coli</i> samples collected	Positive samples of ESBL-E	Positive samples in Percentage
Coriander	4	2	50.00%
Carrot	1	0	0.00%
Cabbage	1	0	0.00%
Onion	1	0	0.00%
Radish	3	2	66.67%
Total	10	4	40%

Table 30: Vegetable-wise prevalence of MRSA

Vegetables	Total number of <i>S. aureus</i> samples collected	Positive samples of MRSA	Positive samples in Percentage
Coriander	8	2	25.00%
Carrot	4	0	0.00%
Cabbage	7	2	28.57%
Onion	2	0	0.00%
Radish	4	2	50.00%
Total	25	6	24%



4.5 Genotypic screening of ARB

- **Results of quantitative and qualitative analysis of isolated DNA**

DNA was isolated by the boiling method. The DNA yield and quality were low (Table 31 and 32).

Table 30: ESBL-E DNA samples results

S.N.	ESBL-E DNA samples	ng/ μ l	A260/A280
1	β 01	2422.80	1.67
2	β 05	2132.10	1.61
3	β 06	2095.00	1.63
4	β 20	1572.10	1.62

Table 32: MRSA DNA samples results

S.N.	MRSA DNA samples	ng/μl	A260/A280
1	M01	1166.80	1.71
2	M03	1668.90	1.66
3	M08	1118.40	1.70
4	M10	1216.40	1.64
5	M11	1103.50	1.69
6	M26	1273.30	1.61
7	M48	683.40	1.63
8	M56	1123.50	1.67

- ARGs results**

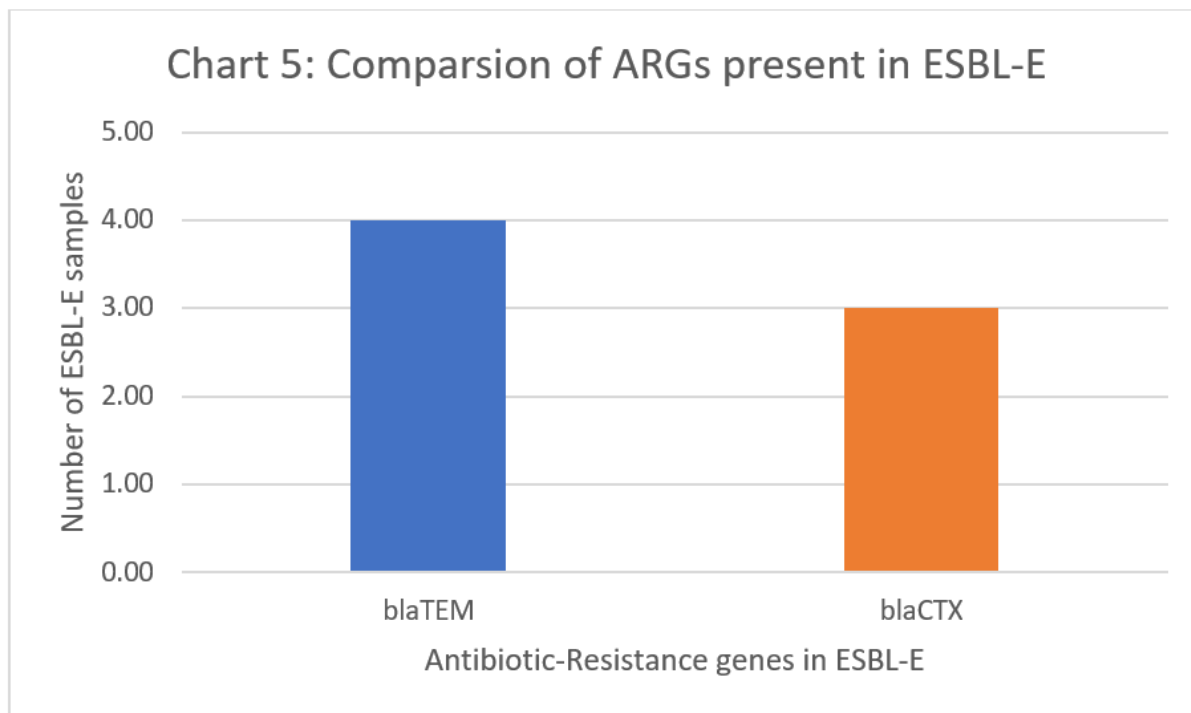
The two genes *bla*TEM and *bla*CTX both were checked for the 4 positive samples of ESBL-E and one *mecA* gene was checked for the 8 positive samples of MRSA, obtained from the phenotypic screening of ARB. In the 4 *bla*TEM samples, all contained prominent DNA bands at 843 bp position (Figure 13) which meant they contained the *bla*TEM gene whereas out of 4 *bla*CTX gene samples only 2 samples showed mild DNA bands at 570 bp (Figure 13) position which meant that only 3 samples contained *bla*CTX gene (Table 33). So, in comparison *bla*TEM is present in all samples whereas *bla*CTX gene is only present in 3 samples (Chart 5). In the 8 samples of the *mecA* gene, no DNA bands were found (Figure 14) (Table 34).

Tables 33: ARGs results in ESBL-E samples

S.N.	ESBL-E DNA Samples	<i>bla</i> TEM gene	<i>bla</i> CTX gene
1	β01	+	+
2	β05	+	+
3	β06	+	+
4	β20	+	-
	Total ARG gene present	4 out of 4 samples	3 out of 4 samples

Table 34: ARG results in MRSA samples

S.N.	MRSA DNA samples	mecA gene
1	M01	-
2	M03	-
3	M08	-
4	M10	-
5	M11	-
6	M26	-
7	M48	-
8	M56	-
	Total ARB gene present	None out of 8 samples



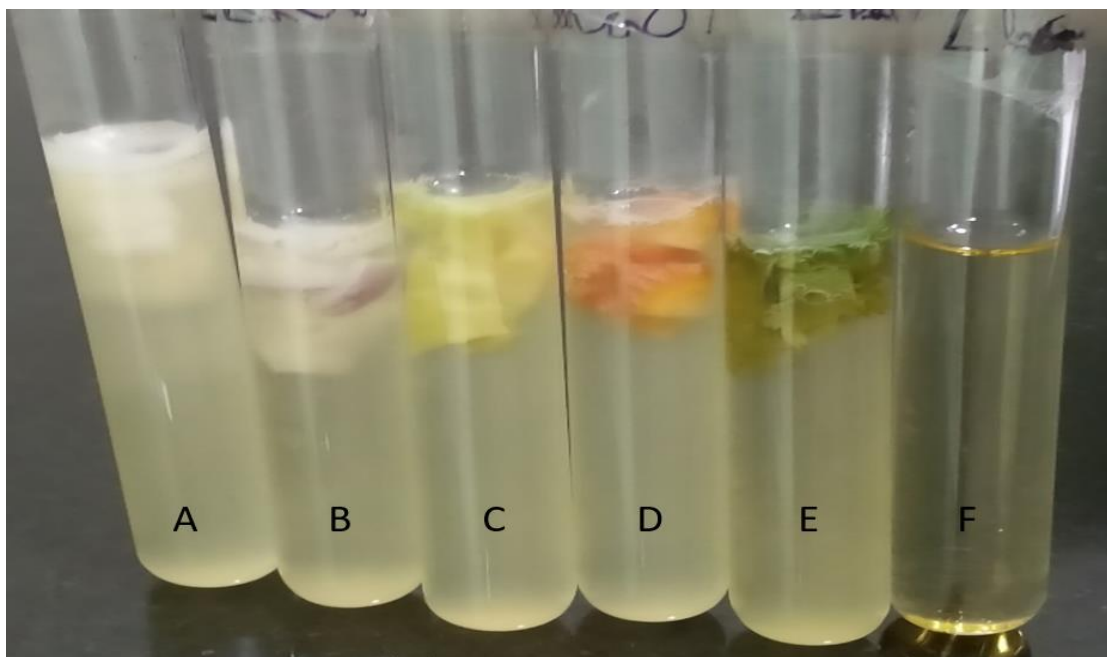


Figure – 5: Primary enrichment of samples - A to E [(A - Radish, B – Onion, C – Cabbage, D – Carrot and E – Coriander) (turbidity showing bacterial growth)] and F (negative control)

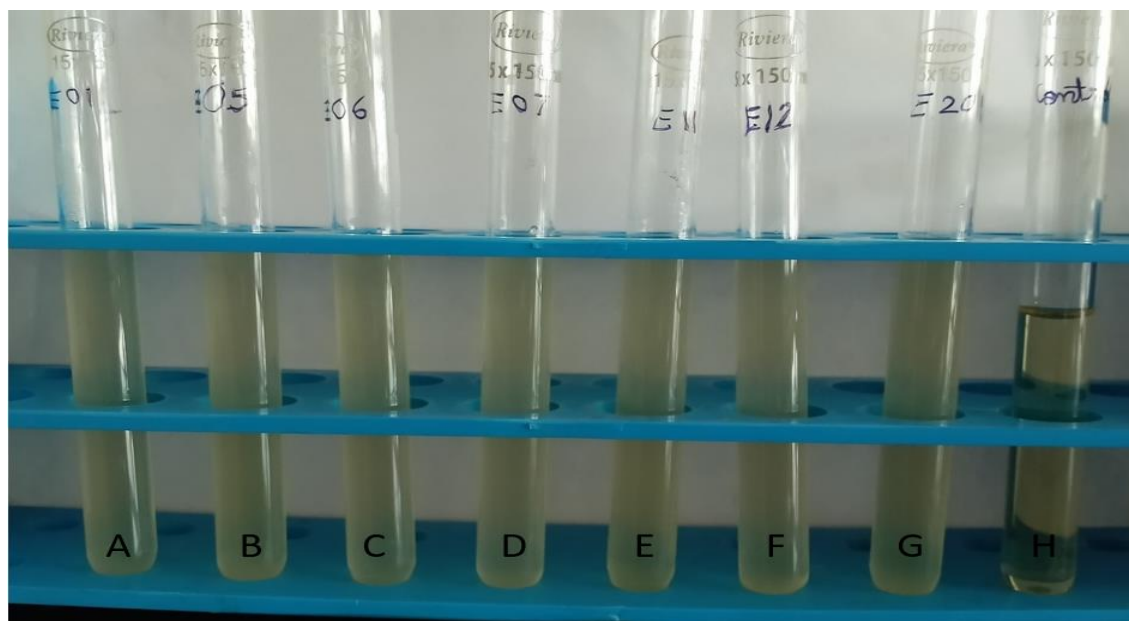


Figure – 6: Secondary enrichment of isolated *E. coli* - A to G (turbidity showing bacterial growth) and H (negative control)

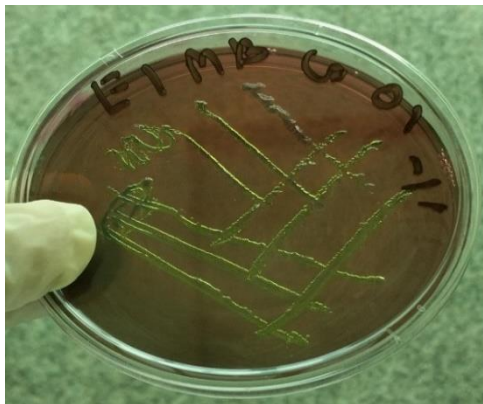


Figure – 7: *E. coli* colony on EMB agar (Metallic green sheen)

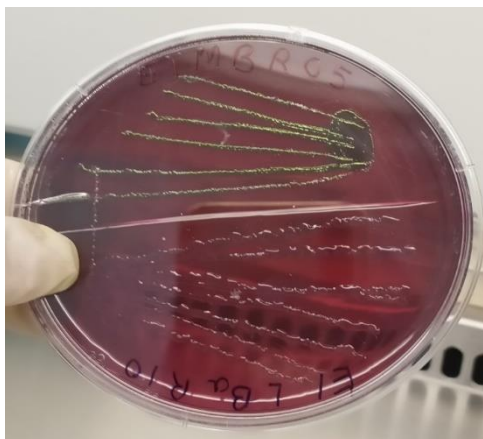


Figure – 8: Different bacterial colonies on EMB agar, A – *E. coli* (Metallic green sheen) and B – other bacteria (Purple colour)

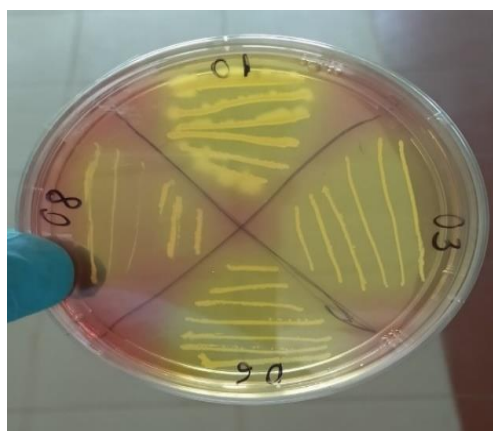


Figure – 9: *S. aureus* colonies on MSA (yellow colonies with yellow)

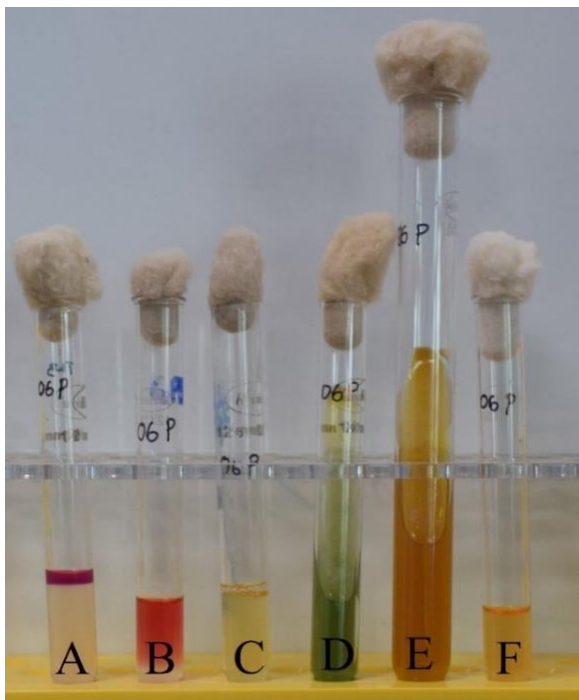


Figure – 10: Biochemical test results of *E. coli* (A - Indole positive, B – MR negative, C – VP negative, D – Citrate negative, TSI – yellow slant, yellow butt, no blackening of media, F – Urease negative)



Figure – 11: Biochemical test results of the other bacteria (A - Indole negative, B – MR negative, C – VP negative, D – Citrate positive, TSI – yellow slant, yellow butt, no blackening of media, F – Urease negative)

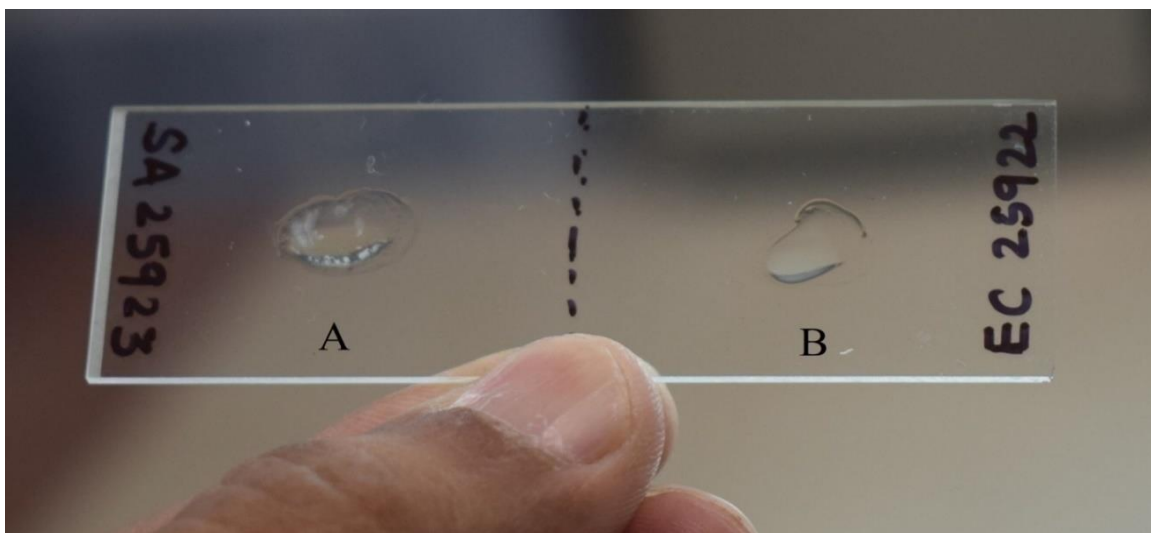


Figure - 12: Coagulase Test Result of *S. aureus*, A - Sample (Clumping) and B - Negative control (No clumping)

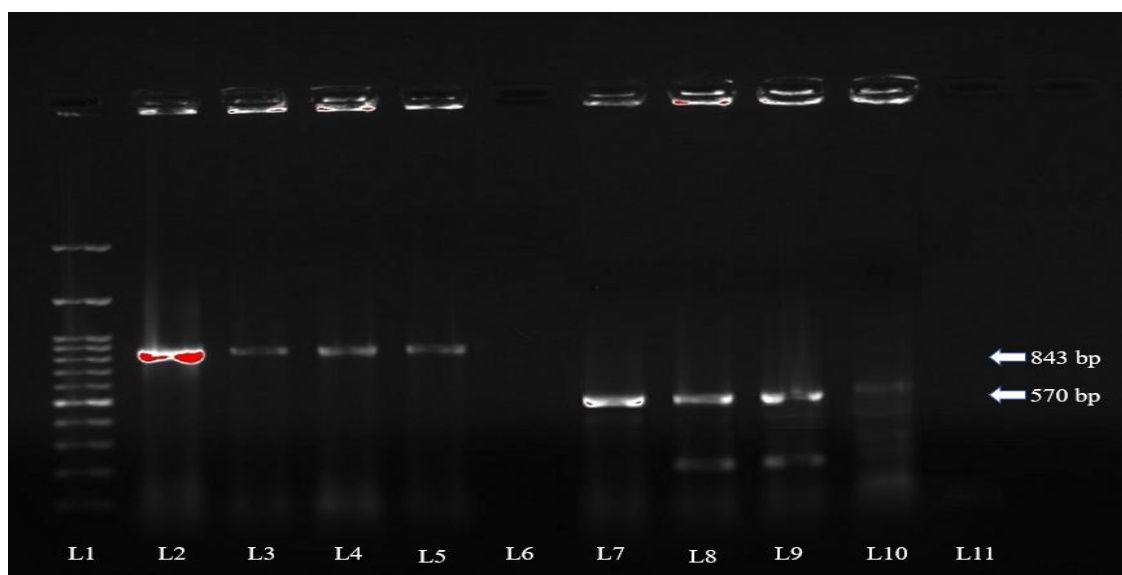


Figure - 13: Gel Electrophoresis results for *bla*TEM and *bla*CTX genes in ESBL-E: For *bla*TEM gene [L1 (Ladder), L2 (β 01), L3 (β 02), L4 (β 03), L5 (β 04), L6 (negative control)]. For *bla*CTX gene L7 [(β 01), L8 (β 02), L9 (β 03), L10 (β 04), L11 (negative control)]

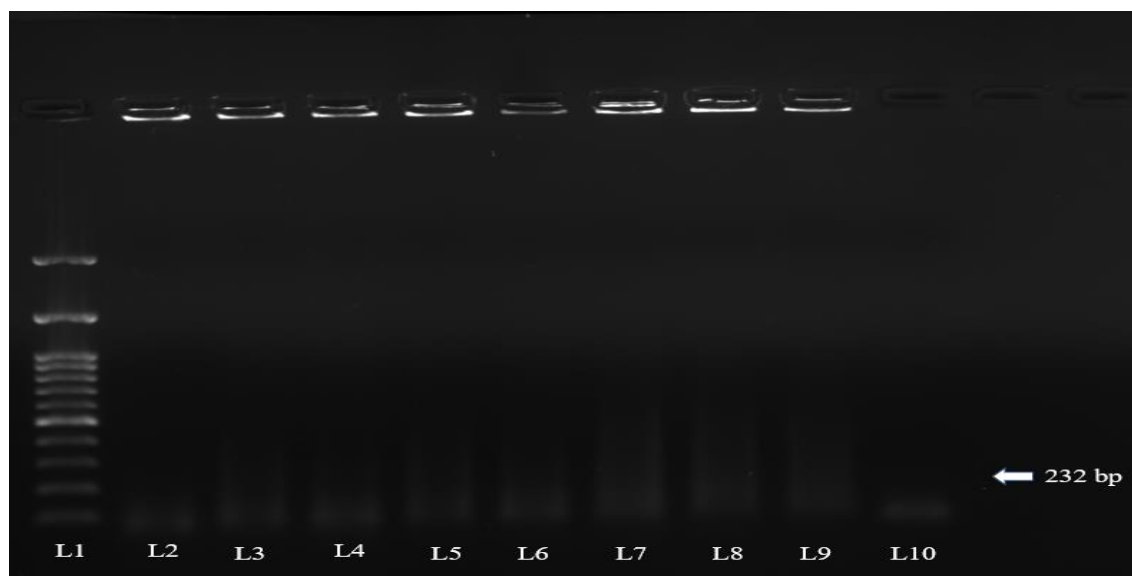


Figure – 14: Gel Electrophoresis results for *mecA* gene in MRSA: L1 (Ladder), L2 (M01), L3 (M02), L4 (M03), L5 (M04), L6 (M05), L7 (M06), L8 (M07), L9 (M08), L10 (negative control)

DISCUSSION

As a remarkable dietary supply of nutrients, micronutrients, vitamins, and fibre for people, vegetables are essential for human health and wellness. Vegetable-rich, well-balanced meals are especially important for vitamin C and vitamin A deficiency as well as for lowering the risk of numerous diseases (Kalia *et al.*, 2006). Despite the fact that fresh or minimally processed vegetables have many health and nutritional advantages but the epidemics of human illnesses are on the rise (Keene *et al.*, 1994; Altekruse *et al.*, 1996; Beuchat and L.R., 1996, Beuchat and L.R., 2002). Various unhygienic practices used during vegetable growth and marketing may lead to microbial contamination of the produce (Khan *et al.*, 1992). These veggies may pick up harmful bacteria directly through contaminated soil, farmers, equipment, transit containers, wild and domestic animals, and human handling. Another study revealed a connection between salad veggies' bacterial contamination and the fact that they are typically eaten raw with simple washing without any heat treatment. As a result, the infectious agent from human, animal, and environmental sources easily mixed with and contaminate the food that is being served to the people (Tambekar *et al.*, 2006).

In the present investigations, we mainly focused to find out the prevalence of *E. coli* and *S. aureus*, also their resistant forms, as well as the presence of antibiotic-resistant genes responsible for their resistance in raw vegetables collected from a different tehsil of the Mirzapur district.

Phenotypic screening of *E. coli* and *S. aureus* results

In a phenotypic screening of *E. coli*, we have confirmed that out of 60 samples 10 samples were positive which is around 16.67% (**Table 18**). Some researchers also reported the similar results i.e., 16.7% *E. coli* positive in their study (Saksena *et al.* 2020) whereas Mritunjay *et al.*, (2017) and Rasheed *et al.*, (2014) reported around 66.0% *E. coli* positive in different studies. However, studies from different countries such as Korea, Germany, and United States reported a lower rate of *E. coli* contamination with 6 to 14% (Fiedler *et al.*, 2017; Korir *et al.*, 2016; Seo *et al.*, 2010). In our study, we found that coriander and radish had a higher percentage of contamination of *E. coli* followed by the rest of the vegetables in present study. Tambekar *et al.*, (2006) also reported similar results in their study. Although *E. coli* is considered the normal flora

of the human gut, some strains of *E. coli* have been linked to diarrhoea, gastroenteritis and urinary tract infections (Aycicek *et al.*, 2006). *E. coli* is a well-known coliform bacterium that is frequently isolated from soil and water in the environment. (Al-Mohizea *et al.*, 1996).

Very few studies have been conducted to find out the *S. aureus* contamination or presence in the raw vegetables as compared to the other food ingredients. In a phenotypic screening of *S. aureus*, we found 25 samples were positive out of 60 samples, that is 41.67% sample were positive presented in Table 18. Almost similar results were reported by Kabir *et al.*, 2014 in which *S. aureus* was found in 35.7% in vegetables. Whereas Wu *et al.*, (2018) reported a higher percentage of *S. aureus* contamination i.e., about 76.7% in raw vegetables. Workers involved in the handling of food at different places are a potent source of contamination particularly if they did not follow hygienic procedures. Especially nasal discharge of food handlers who are having *staphylococcal* infections is a potent source of infection. The capacity of the bacteria to produce food-borne illnesses and food-borne intoxication are responsible for the severe public health hazard (Tambekar *et al.*, 2006).

When data were compared among the four different tehsils of Mirzapur district we observed that Lalganj tehsil had the highest contamination of both *E. coli* and *S. aureus* as shown in Chart 1, such studies have not been conducted earlier in Mirzapur district as per our search, so we can say that this is the first study in Mirzapur district.

Vegetable-wise comparison indicated that Coriander has the highest contamination of both bacteria i.e., *E. coli* (33.33%) and *S. aureus* (66.67%), whereas cabbage showed *S. aureus* (58.33%) and *E. coli* (8.33%), radish *S. aureus* (33.33%) and *E. coli* (25.00%), carrot *S. aureus* (33.33%) and *E. coli* (8.33%) and onion *S. aureus* (16.67%) and *E. coli* (8.33%) (Chart 2). When we studied the data from other researchers, we found that a report from Northern India has similar findings related to the *E. coli* contamination such as 15% in tomatoes and cucumbers, and 20% in carrots (Mritunjay *et al.*, 2017). Whereas report from Aizawl (North-east India) showed that only 4.88% of fresh vegetables (tomato, potato, and cabbage) were positive for *E. coli* (Chellapandi *et al.*, 2015). Another report from Maryland, Delaware, and New Jersey of United States have showed 5.4% (n = 259) of tomatoes were positive for generic *E. coli* (Pagadala *et al.*, 2015). Similarly, Seo *et al.*, (2010) also found that 4.7% of vegetables in mixed salads were contaminated with *E. coli* in Korea. Possible sources of contamination include the use of contaminated irrigation

water, manure, unhygienic post-harvesting processes at the farms, unhygienic storage and transportation (Mathur *et al.*, 2014; Mritunjay *et al.*, 2015).

Confirmatory Biochemical tests results for *E. coli* and *S. aureus*

To confirm the phenotypically positive sample we have performed various biochemical tests, for *E. coli*, a total of 6 biochemical tests were performed viz. IMViC tests, Urease test and TSI test of all *E. coli* positive samples. Indole test and Methyl red test were positive, while Voges-Proskauer test, Citrate test and Urease test were negative (Table 23). Tri Sugar Iron (TSI) test had an acidic slant with the presence of gas but H₂S gas was absent, all the 10 phenotypically *E. coli* positive samples showed similar results. These results were constant for all 10 positive *E. coli* isolates. Similarly, we performed a coagulase test to confirm the presence of *S. aureus* in the phenotypically positive samples and clumping was present in all the 25 samples which indicated coagulase-positive *S. aureus* (Table 24). Our results are in agreement with the previous studies done by various researchers earlier for both *E. coli* and *S. aureus* (Alizadeh *et al.*, 2021; Dash *et al.*, 2012; Garg and A., 2022; Sahebnaasagh *et al.*, 2014).

Phenotypic screening of ESBL-E and MRSA results

Out of 10, *E. coli* isolates we could find only 4 (40%) samples were positive for ESBL-E (Table 25), when we compared them to the total sample collected i.e., 60 samples it gives only about 6.67%. Similar results were reported in another study by Reuland *et al.*, (2014) i.e., 6% of ESBL-E isolates compared to the total vegetable sample collected. In our study, we could find only 8 MRSA positive samples out of 25 *S. aureus* isolates (Table 26), whereas other researchers have reported 4 MRSA positive out of 5 isolates of *S. aureus* samples (Wu *et al.*, 2018). Furthermore, we found that Madihaan Tehsil had 100% contamination of ESBL-E whereas MRSA was highest at about 66.67% in Lalganj Tehsil (Chart 3). In our study, we found that Radish had the highest contamination of both ESBL-E (66.66%) and MRSA (50%) (Chart 4). A growing food safety concern is the role of food in human exposure to bacteria that are resistant to antibiotics, including zoonotic pathogens and commensal and ambient bacteria that act as a reservoir for resistance genes (Barud *et al.*, 2013; Zurfluh *et al.*, 2015).

Genotypic Screening of ARB results

The presence of ARGs confirmed the ARB. *bla*TEM and *bla*CTX genes were used for ESBL-E confirmation and it was found that *bla*TEM was present in all the samples of ESBL-E (4/4) whereas *bla*CTX was present in 3/4 samples of ESBL-E (Table 33).

mecA gene was used for MRSA confirmation but no sample out of 8 samples of MRSA showed the presence of the *mecA* gene (Table 34). The negative result that was obtained may be due to the degradation of *mecA* a primer before the experiment.

Like our study, Bajpai *et al.*, (2017), also targeted ESBL by using *bla* TEM, *bla*CTX and *bla*SHV genes, whereas Sahebnaasagh *et al.*, (2014), targeted the *mecA* gene and *nucA* for MRSA studies.

SUMMARY AND CONCLUSION

The present investigation entitled “Study on the presence of antibiotic-resistant bacteria in the raw vegetables of Mirzapur district” was performed in the Veterinary Physiology and Veterinary Biochemistry laboratory in the Department of Veterinary Physiology and Biochemistry, Institute of Agricultural Sciences, Rajiv Gandhi South Campus, Banaras Hindu University, Mirzapur, Uttar Pradesh, India. The current study was conducted with the aim to monitor the prevalence of Antimicrobial-resistant pathogenic bacteria viz. *E. coli* (ESBL) and *S. aureus* (MRSA), and validate it by detecting ARGs such as *bla*TEM, *bla*CTX, and *mecA*,

For our investigation, we collected 5 types of ready-to-eat vegetables viz. Coriander, Carrot, Cabbage, Onion, and Radish from twelve different vegetable retail markets which are distributed across four tehsils (Chunar, Lalganj, Madihaan and Sadar) in the Mirzapur district. Altogether 60 samples were collected. The edible part of the samples was enriched and the bacteria were isolated and identified with selective media such as Eosin Methylene Blue agar for *E. coli* and Mannitol salt agar for *S. aureus*. Out of 60, 10 samples and 25 samples were found positive for *E. coli* and *S. aureus*, respectively. All the positive isolates were further confirmed by biochemical tests such as the IMViC tests, TSI test, and Urease test for *E. coli* and Coagulase test to differentiate Coagulase positive and coagulase-negative staphylococcus aureus. On analysis, we found that the Coriander collected from Lalganj Tehsil of Mirzapur district had the highest contamination of *E. coli* and *S. aureus*.

Further, the positive isolates were screened for antibiotic resistance using the phenotypic and genotypic techniques, using we isolated Antibiotic-Resistant bacteria such as ESBL-E and MRSA, from our contaminated samples of *E. coli* and *S. aureus* respectively. The selective media was added with antibiotics such as Ceftazidime (4 mg/L) for ESBL and Oxacillin (4mg/L) for MRSA, and the plates were incubated at 37 C for 24-48 hours. Bacterial growth on the media was considered positive for ESBL and MRSA. Out of 10, 4 isolates were found to be positive for ESBL while 8 out of 25 were found positive for MRSA phenotypically. Based on the phenotypic screening of ARBs, Madihaan tehsil was found to be significantly higher ESBL while Lalganj tehsil had MRSA contamination over other tehsils of Mirzapur district. Among the samples, radish had higher antibiotic-resistant positive isolates. The colonies of phenotypic positive isolates were

used to isolate DNA to confirm the presence of Antibiotic-Resistant Genes (ARGs) such as *bla*CTX and *bla*TEM for ESBL and *mecA* for MRSA using Polymerase Chain Reaction (PCR) and gel electrophoresis technique. We found that 100% of samples were found to possess the *bla*TEM gene whereas 75% of samples had the *bla*CTX gene. Altogether 75% of samples possess both genes. But no samples were found to have the *mecA* gene.

Unhygienic handling and storage may have an impact on the overall rise in *E. coli* and *S. aureus* isolates from retail vendors compared to wholesale markets. This study emphasizes the requirement to implement strict regulatory measures to enhance hygienic product availability and halt the spread of foodborne illnesses. To meet the requirements set forth by the Food Safety and Standards Authority of India (FSSAI) for acceptable levels of microbial contamination, products at various stages of manufacturing and sale must be periodically inspected. Vegetables that contain ESBL and MRSA are a sign of the environmental spread of antibiotic resistance. Once digested, these could serve as means of spreading resistance to harmful bacteria and gut microbiota. Therefore, it is crucial to control the inappropriate use of antibiotics, not just in humans but also in agricultural and animal husbandry practices.

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