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Forensic Identification and Isolation of Pathogenic Bacteria From Raw Vegetables and Fruits

Anuradha Sharma† and Sakshi Manhas

Department of Forensic Science, University Institute of Applied Health Sciences, Chandigarh University, Gharuan, Mohali-140413, Punjab, India

† Corresponding author: Anuradha Sharma; sanu3338@gmail.com

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ABSTRACT

The consumption of contaminated fruits and vegetables is the prime cause of outbreaks of various human diseases. Although fruits and vegetables have high nutritional value, today because of their contamination during handling while performing harvesting and post-harvesting techniques, they are harmful to human health. Most of them are eaten raw without being washed or without providing any treatment. Vegetables and fruits, being rich nutritional sources, can act as carriers or vectors of pathogenic microorganisms, which can create a serious issue for the health of the community targeted. This entire research is based on an emerging field of Forensic Microbiology. Various types of microbial agents can be utilized as bioweapons to conduct the bio crime or bioterrorism through food and water. This research also represents that the identification of microbial agents is very much necessary for the welfare of humans. Identification and isolation of different pathogenic bacteria from raw vegetables and fruits can also shed some light on the terms of the necessity of Forensic Microbiology.

INTRODUCTION

Consumption of fruits and vegetables that are fresh or minimally processed is causing different types of human infections. As some fruits and vegetables are eaten raw, they play the role of hosts for different types of pathogenic microorganisms. Pathogenic microorganisms may be a result of microcolonies spread over plants or plant tissues. The variation in the number of colonies may be due to the soil organisms, exposure of fruits and vegetables to air, dust, and water, along with handling during harvesting and post-harvesting techniques. The reason behind the contamination of fruits and vegetables is the use of untreated wastewater and the manure used for the cultivation of fruits and vegetables. Several types of plant and human pathogens are found in raw fruits and vegetables (Ghazali & Rashid 2019). Several outbreaks due to the consumption of raw fruits and vegetables have gone undetected and unnoticed due to the lack of inspection, supervision, and screening. Various types of causative agents, like Listeria monocytogens, Salmonella spp, Shigella spp, etc., are responsible for the outbreak of illnesses (Hussain et al. 2021). The pathogenic bacteria were found in large amounts in the research conducted on spoiled food products. Their amount of percentage of multi-drug resistant isolates was also high, causing harm to human health and resulting in food-borne illnesses. Microbial forensics has benefit in that its protocol holds out against the legal investigation. The results obtained from the microbial forensic investigation can also be utilized as proof evidence that might either support or neglect the allegations made in court (Kubba et al. 2021).

This complete research is going to be based on highlighting the techniques of identifying and isolating the microorganisms (Pathogens) present in raw fruits and vegetables, which are very harmful to consume for the human body. Various harmful effects of consuming contaminated raw vegetables and fruits are also going to be entertained by conducting this research.

MATERIALS AND METHODS

The samples of the raw vegetables and fruits were collected, and past years' datasets were also analyzed on behalf of conducting this research. New techniques were explored to achieve better results in identifying pathogens and bacteria on the food products so that the food items could be protected from contamination.

Research Design

The quantitative research design was used in the present study. This type of research design was used based on the analysis of data from past years, which is related to the pathogen's bacteria on the food products.

Study Location

The study was conducted in Nepal considering the collection of 50 samples, 5 of each fresh fruits and vegetables like cucumber, tomato, spinach, cabbage, carrot, apple, guava, grapes, strawberry, and cherry.

Study Population

The dataset of past years is analyzed as secondary data on behalf of conducting this present research.

Data Collection Method

A total of 50 samples, 5 of each fresh fruits and vegetables namely Cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), spinach (*Spinacia oleracea*), cabbage (*Brassica oleracea*), carrot (*Daucus carota*), apple (*Malus domestica*), guava *(Psidium guajava*), grapes (*Vitis vinifera*), Strawberry (*Fragaria ananassa*) and cherry (*Prunus avium*) were collected from 5 different localities, i.e., open market of Kathmandu, Nepal.

Sample Preparation

After collecting the samples, they were taken into the laboratories within an hour. They were washed with 100 mL of distilled water and were diluted fivefold serially. The 10 mL of the washed aqueous solution was mixed with 90 ml of LB (Luria Broth). The incubated mixture (37 degrees Celsius for 24 h) was then utilized for the isolation and identification of bacterial strains using the streaking plate technique (Yeung 2016).

Identification and Isolation of Bacterial Strains

The isolation and identification of bacterial strains were carried out using different selective and non-selective mediums, i.e., Nutrient agar for Total Viable Bacterial Count (TVBC). For *E. coli*, Eosin Methylene Blue (EMB) and for *Salmonella*, Xylose Deoxycholate Agar (XLD) was used. For Identification confirmation, IMViC tests were performed for every species (Zambelli & Brasil 2022).

Bacterial Enumeration

For bacterial enumeration spread plate count technique was used to determine the number of colony-forming units (CFUs).

The list of materials and methods is summarized in Table 1.

Table 1: List of methods and materials.

Universe	Nepal
Research design	Quantitative research design
Collection of data	Samples are collected
Primary data	Documents, Online journals, datasets, and articles of last years are used as well as analyzed as secondary data.
Duration	45 days

RESULTS

Bacterial Load in Fruits and Vegetables

All the samples of fruits and vegetables were examined microbiologically. The results were obtained which gave a huge number of bacterial count, and also E. coli and Salmonella were detected.

Total Viable Count

The total viable bacterial count is represented in Table 2. Ten different samples were collected from five different locations, and the bacterial load was examined.

From the table, it can be observed that the maximum number of bacteria were found in guava and grapes (TMTC), i.e., too many to count (Fig. 1), followed by carrots. Spinach, cucumber, strawberry, cherry, and tomato were moderately contaminated. Apple was the least contaminated among all the fruits and vegetables.

Selective Culture

Culture of pathogenic bacteria, namely E. coli and Salmonella was done on selective media (Table 3), i.e., Eosin Methylene Blue (EMB) Agar and Xylose Lysine Deoxycholate (XLD) Agar, respectively. The washed water was first enriched in Luria Broth (LB) for 24 h at 37 degrees Celsius and then was cultured on the selective media respectively. From location 1, samples A, B, C, E, F, G, H, I, and J showed a greenish metallic sheen (Fig. 2), which indicated the presence of E. coli, while Salmonella was only present in samples B, C, and E, and I and J by formation of black, mucoid colonies as displayed in Fig. 3. Location 2, which was taken from the farm directly showed the formation of a greenish metallic sheen in samples E, F and I while black colonies formation was present in samples E, F, and J. Similarly, the samples obtained from location 3, gave E. coli colonies in samples A, B, D, E, H, I, and J while Salmonella was only obtained in sample D. For location 4, E. coli was present in samples E, F, H and I with Greenish metallic colonies meanwhile sample E, F and J gave black colonies resulting in Salmonella formation. Finally, sample A, taken from location 5, gave no growth at all on the plates which concluded in the absence of E. coli as well as Salmonella. Samples B, C, and D gave a Greenish metallic sheen, confirming the presence of E. coli. At the same time, Salmonella presence

Table 2: Total viable bacterial count.

Sample	Location 1	Location 2	Location 3	Location 4	Location 5
A (Spinach)	TMTC	$2.89*10^8$	TMTC	$2.52*10^8$	$2.21*10^8$
B (Cucumber)	$4.8*10^8$	$1.64*10^8$	TMTC	$2.24*10^8$	TMTC
C (Cabbage)	$2.78*10^8$	$2.40*10^8$	$2.13*10^8$	$2.13*10^8$	$1.46*10^8$
D (Carrot)	$1.96*10^8$	TMTC	TMTC	$2.02*10^8$	TMTC
E (Tomato)	$1.28*10^8$	$1.78*10^8$	$1.74*10^8$	$2.56*10^8$	TMTC
F (Strawberry)	TMTC	$2.44*10^8$	TMTC	TMTC	$1.66*10^8$
G (Apple)	$1.42*10^8$	$2.12*10^8$	$2.15*10^8$	$1.55*10^8$	$2.76*10^8$
H (Guava)	TMTC	TMTC	TMTC	1.88*108	TMTC
I (Grapes)	TMTC	TMTC	TMTC	TMTC	TMTC
J (Cherry)	TMTC	$1.83*10^8$	$1.58*10^8$	$1.22*10^8$	TMTC

Table 3: Selective media and growth.

S. No.	Selective Media	Growth
1.	Eosin Methylene Blue Agar (EMB)	Greenish metallic sheen, shiny colonies
2.	Xylose Lysine Deoxycholate Agar (XLD)	Black, mucoid colonies

was shown by samples B, H and I with black, mucoid colonies.

In addition to culture on selective media, for confirmatory test biochemical test was performed, which includes the Gram Staining, Indole test, MR/VP test, and Citrate test.

According to Table 4, bacterial strains performing Gram-staining displayed a rod-shaped morphology and exhibited Gram-negative staining. The fruits and vegetables gave a Positive test for the Indole test for the first strain by producing a red ring on the top of the media when Kovach's reagent was added to it, while it gave a negative Indole test for the second strain. Additionally, both strains tested positive for the MR test as the red color appeared in the tube when methyl red was added. However, both the first

Table 4: Various tests performed.

Organism	Gram Staining/ morphology	I (Indole test)	M (MR test)	V (VP test)	C (Citrate test)
Organism 1 (*E. coli*)	Gram-ve/rod-shaped morphology	Positive	Positive	Negative	Negative
Organism 2 (*Salmonella*)	Gram-ve/rod-shaped morphology	Negative	Positive	Negative	Negative

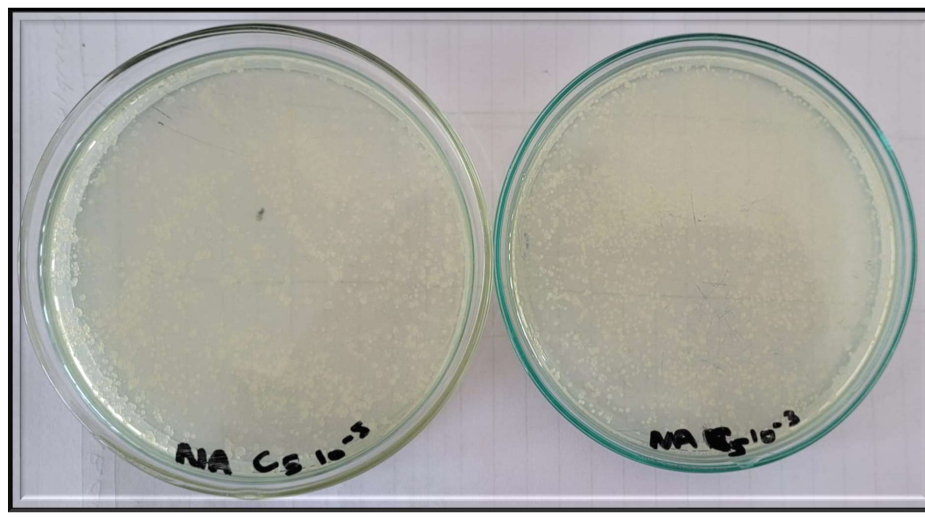

Fig. 1: Total viable bacterial count (TVBC) at different dilutions.

and second strains tested negative for the VP and Citrate tests.

DISCUSSION

The use of different biological and chemical agents in warfare, their production, and their development were recognized in the 1960s (Ranbir et al. 2022). The importance of microbial forensics along with molecular epidemiology needs to be recognized to answer the legal questions that are related to the anthropogenic incidents affecting society through outbreaks as well as biological attacks (Roselló-Soto et al. 2018). In the year 1984, an outbreak affected almost 751 people in Dalles, Oregon, out of which 45 were also hospitalized because of Salmonella. Similarly, in May 2000,

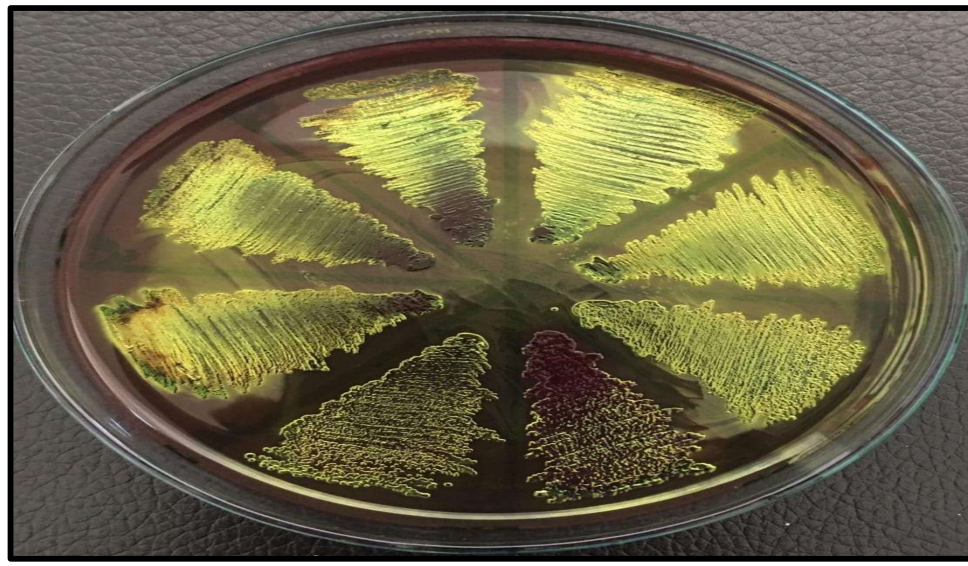

Fig. 2: Showing a greenish metallic sheen indicating the presence of *E. coli*.

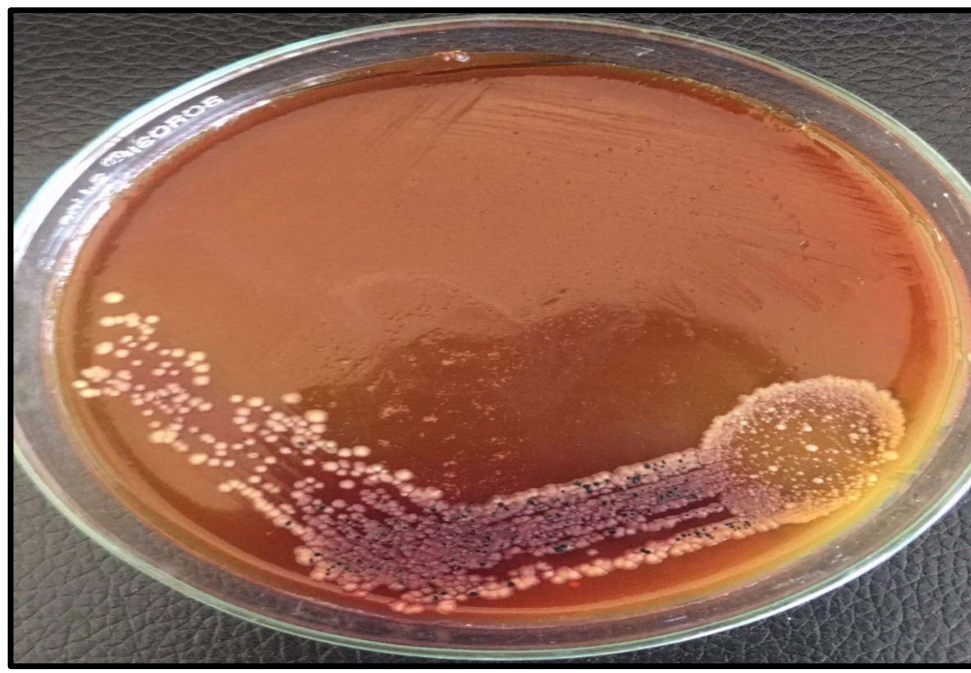

Fig. 3: Showing black colonies indicating the presence of *Salmonella*.

an outbreak due to the E. coli strain was identified due to which 2300 cases were reported and 7 deaths were recorded in Walkerton, Canada (Putnik et al. 2018). Microbial forensics was closely associated with the identification of perpetrators involved in biological attacks. Microbial agents can be used as bioweapons to take part in biocrime and bioterrorism (Abd Elkarim et al. 2022). Forensic investigation, with the help of Microbiology, can help to narrow down the type of crime that has taken place using microbial agents and also the person or group that may be involved in the crime (Burcham & Jordan 2017). Hence, to determine the type of crime committed, microbial forensics should be an integral part of a forensic investigation, i.e., bio crime, biowarfare, or bioterrorism. Biocrime is a crime that affects a specific individual using a microbial agent, while bioterrorism is the use of a microbial agent to harm a targeted community (Al-Jassani 2023).

The pathogenic bacteria were found in large amounts in the research conducted on spoiled food products (Venkatramanan et al. 2020). Their percentage of multi-drug resistant isolates was also high, causing harm to human health and resulting in food-borne illnesses (Vidic et al. 2019). Microbial forensics has an advantage: its protocol holds out against legal inquiry (Aljohni et al. 2021). The results obtained from the microbial forensic investigation can be used as evidence that might either support or neglect the allegations made in front of the court. 600 million cases with 4,20,000 deaths per year have been estimated by WHO (Callejón et al. 2015). Around 879 million tons of vegetables and 513 million tons of fruits are produced in Asia. The consumption of raw or partially cooked vegetables and fruits is high in Asian countries. Even though the consumption of organic fruits and vegetables is higher in Asian countries, the rate of contamination is great in organically grown crops, as animal manure is used as fertilizer. Contamination with pathogens can be introduced at different stages of the production of fruits and vegetables (De Giglio et al. 2017).

Different researchers carried out many studies to identify the contamination of fruits and vegetables by pathogenic microorganisms (El Sheikha & Mahmoud, 2016). A study was carried out by Al-Kharousi et al. (2016) to examine the presence of pathogenic bacteria in fresh fruits and vegetables, which concluded that the microbial communities found in the fresh produce of fruits and vegetables can act as opportunistic pathogens and cannot be ruled out instead. It should be provided with better management to improve the microbial quality of the fresh produce (Al-Kharousi et al. 2016).

In developing countries like Nepal, poverty along with poor sanitation is very common, leading to multiple outbreaks of human gastroenteritis caused by of ingestion of faecally infected food (Shuping & Eloff, 2017). In 2020, conducted research on street food and found that these food items which are inexpensive and easily accessible, contained a large number of pathogenic bacteria, which was causing high health problems in their consumers (Rudhy 2017). From their research, it was concluded that creating food safety and public awareness is very important for reducing foodborne illnesses. As fruits and vegetables are consumed without any thermal treatment, it cause bacterial contamination (Płotka-Wasylka et al. 2017). A study carried out in 2020, concluded that fresh fruits and vegetables are mostly eaten raw without providing heat treatment or washing them, which act as the best vector for transmitting different pathogenic microorganisms and are having great risk to human health. Hence, different awareness programs should be conducted to provide safety measures for buying and selling fruits and vegetables with hygienic practices (Piombo et al. 2021).

Vegetables and fruits are sold in open markets among which most of them are eaten raw, which allows bacterial contamination, providing a suitable environment (Bwambok et al. 2020). In 2022, research will be carried out to identify bacteria present in vegetable salads found in different local markets (Oliveira et al. 2020). Researchers identified different pathogenic bacteria from the salad, along with the detection of their resistant genes (Vaishnav et al. 2016). Researchers concluded that the safety and the microbial quality of ready-to-eat salads can be challenging for the human population (Asgari 2022). To understand the extent and transmission of the microbial agent across the infected community, the study of outbreak events is required, which will provide light on the emerging field of Forensics, i.e., Microbial forensics, a combination of microbiology and forensics (Umapathi et al. 2022). Identification of accurate microbial agents by applying different physical, biochemical, and molecular tests can help provide information about the person responsible for the attack (Al-Zaidi et al. 2022).

CONCLUSION

It is concluded that the investigation of outbreaks of infectious diseases can be done by identifying the microbial agent. Microbial agent gives us important information about their drug vulnerability, their sources, their transmission routes or pathways, and the factors enhancing their transmission. By determining the presence of microbial agents and their characteristics, it can be estimated whether the contamination of food is intentional and can be redefined as a bio-crime or bioterrorist attack. Samples of fruits and vegetables were examined microbiologically. The results were obtained, which gave a huge number of bacterial count, and also E. coli and Salmonella were detected. Strain under

investigation compared with reference sources, assumptions made, examined, and the person or group that could have intentionally caused the outbreak can be narrowed down. It can be observed that the maximum number of bacteria were found in guava and grapes (TMTC), i.e., Too Many to Count, followed by carrots. Spinach, cucumber, strawberry, cherry, and tomato were moderately contaminated. Apple was the least contaminated among all the fruits and vegetables. E. coli and Salmonella displayed rod-shaped morphology and exhibited Gram-negative staining. The fruits and vegetables gave a positive test for the Indole test for the first strain by producing a red ring on the top of the media when Kovach's reagent was added to it, while it gave a negative Indole test for the second strain. Additionally, both strains tested positive for the MR test as the red color appeared in the tube when methyl red was added. However, both the first and second strains tested negative for the VP and Citrate tests. This research can be directly connected with the field of microbial forensics as well as forensic epidemiology.

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