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Research Paper

Assessment of Enteropathogens from Fresh Vegetables Associated Serious Risks to Human Health

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Abstract: The general public has become increasingly interested in eating fresh vegetables all over the world; microbiologically unsafe vegetables are a persistent public health issue in developing countries, including India. The goals of this study were to assess infection risks from enteropathogens for raw vegetable consumers in different geographical locations in Madhya Pradesh, India, as well as to evaluate effectiveness, pathogenecity, and infection risks. To achieve the objectives, samples were collected from different locations in Madhya Pradesh, India. The findings indicate that regular consumption of fresh, raw vegetables sold on the street would be harmful due to the presence of enteropathogens. Though the immunity of Indians is considered high, regular intake of such contaminated vegetables would detoriate the individual's health.

Keywords: Enteropathogens, contaminated vegetables, Human health risk, medicinal and therapeutic agent.

1. Introduction

In the health conscious society of the 21st century, vegetables form an integral part of the human diet. Over the last three decades, the global consumption of fresh vegetables has increased significantly, thus expanding the market segment for fresh produce by more than 20%. Vegetables are included among the basic and nutritious foods for human beings. Vegetables are important protective foods and highly beneficial for the maintenance of health and the prevention of diseases. They contain valuable food ingredients that are essential for the proper function of the body. Vegetables contain various medicinal and therapeutic agents and are valued mainly for their high vitamin and mineral content. Studies have evaluated the association of fruit and vegetable consumption with the reduction of the risk of specific diseases. Being an edible part of plants, they are also full of vitamins and minerals [1].

For instance, the Food and Drug Administration (FDA) and World Health Organization (WHO) have recommended 5–9 servings of fruits and vegetables to be taken daily because correct fresh produce intake alone could save 2.7 million lives a year Johnston *et al.*, 2006 [2]. In general, freshly consumed vegetables are considered to be more vital than the cooked ones. Vegetables which are consumed raw or with minimal processing have fewer barriers (like salt and preservatives) against microbial growth and due to this lack survival and growth of microbial pathogen on fresh vegetables is expected to continue which leads to outbreaks of food borne illnesses [3].

The increased consumption of fruits and vegetables in recent years has been found to be accompanied by an increase in the number of human infections and outbreaks [4], as these can serve as reservoirs for pathogens or opportunistic pathogens [5]. Opportunistic pathogens can cause life-threatening infections mainly in immunocompromised people, but they may have positive effects on the health of immunocompetent individuals by stimulating immune functions and priming the immune system continuously [5]. Non-pathogenic microbes associated with fruits and vegetables may have a variety of effects on produce quality by influencing the rate of food spoilage.Fruits and vegetables appear to be sources of many microbes in food preparation areas.Leff and Fierer, 2013 [6].

Fruits and vegetables carry a pathogenic and non-pathogenic epiphytic microflora. The inner tissues of healthy plants and animals are free of microorganisms, however, the surfaces of raw vegetables are contaminated with a variety of microorganisms, and this depends on the microbial population of the environment from which the food was taken, the condition of the raw product, the method of handling, and the time and conditions of storage [7].

Vegetables have been associated with food borne outbreaks in many countries; they may be contaminated from the farm with human sewage, and from the irrigation water [8]. Unsafe water used for rinsing the vegetables and sprinkling to keep them fresh are other possible sources of contamination [9]. In Bhopal City, the capital of Madhya Pradesh and the heart of India, it has been observed that the local farmers are using municipal waste water discharged for irrigation and washing

purposes, which is the primary source of contamination of microorganisms in fresh fruits and vegetables [10].

Since the early 1990s, awareness about the potential of fresh products to cause food borne diseases has increased, and reported outbreaks associated with the consumption of fresh vegetables have grown steadily. As most of these products are eaten raw or with minimal cooking, their microbial content may represent a risk factor for the consumer's health [11]. Most of the reported outbreaks of gastrointestinal diseases are linked to the use of fresh products and have been associated with bacterial contamination, particularly with members of the Enterobacteriaceae family [12], [13],[14].

The Enterobacteriaceae is a large family of Gram negative bacteria that includes more familiar pathogens such as *Escherichia coli*, *Salmonella*, *Shigella, Klebsiella* and *Yersinia.* Most of the members of the family Enterobacteriaceae cause gastrointestinal problems in humans [16]. The diseases are caused by either toxins from the disease causing microbes or by the humans' reactions to the microbe itself [15]. Although usually regarded as human pathogens, members of this family have also been recognised as inhabitants of soil and plants. Thus, vegetables may serve as a reservoir from which these bacteria can colonise and infect a susceptible host [17].

Bacterial pathogenicity is defined as all the biochemical mechanisms that allow the microorganisms to cause infection [18]. Pathogenicity is a complex characteristic that depends on a large number of factors, some related to the host and its immunity, and others related to the microorganism: infectious doses, virulence factors (adhesins, invasins, toxins, etc.), and resistance to the host defence mechanisms [19].

The present study focuses on the superficial extraction of microbes (particularly Gram negative Enterobacteriaceae) from fresh raw salad vegetables, which can help in observing common causes of contamination of vegetables and also the presence of any pathogenic microbe associated with serious human diseases.

2. Materials and Methods

Study sites and collection of samples

The study sites were selected in different locations in Madhya Pradesh, India. The study sites and samples are listed in **Table-1** The bacteriological studies were carried out on eight types of fresh vegetables from local markets. The fresh vegetables, like Capsicum, Cucumber, Spinach, Carrot, Onion, Lemon, Tomato and Chili were collected. Samples were purchased from different local markets in Bhopal, and they were selected depending on their availability in the market during that period of sampling. The samples were collected aseptically and refrigerated until analyzed within 24 hours [20].

Sample preparation and Isolation

For the preparation of serial dilution, a 25 gm of subsample of each vegetable was aseptically weighed and vigorously shaken in 225mL of sterile saline for 3 minutes separately to homogenise the samples. Serial dilutions were prepared from the original homogenate in saline according to Prescott, 2002 [21]. The samples were inculcated using the spread plate technique, in which a volume of 0.1 mL of an appropriate dilution was spread-plated on nutrient agar media and incubated for 24 hours at 37 °C [21].For the counting of bacterial cells calculated by the method of colony forming units, the number of microorganisms was expressed as the number of colony forming units CFU/ml and was calculated using the formula, Prescott, (2002) [21]. CFU/mL = [(number of colonies multiplied by dilution factor) / volume of inoculums].

Table 1: Study sites of different location of Madhya Pradesh

S. No.	Sample Code	Sample Name	Location
1	A	Capsicum	Ashoka Garden
2	B	Cucumber	Piplani
3	C	Spinach	Anna Nagar
4	D	Carrot	Kolar
5	E	Onion	Chetak
6	F	Lemon	Prabhat
7	G	Tomato	MP Nagar
8	H	Chilli	Jahangirabad

Characterization and Identification of Bacteria

The colonies which grew solitarily were selected and then individually evaluated for size, shape, surface, edge, structure and color [21].

Microscopic observation of bacteria Gram's staining:

This is the most important widely used procedure for characterizing bacteria. It was first described by Christian Gram. [21].

Selective-cum-differential Agar media-based identification:

Gram-negative isolates were considered to belong to the family Enterobacteriaceae, The count of Enterobacteriaceae was performed on MacConkey agar and EMB agar media by the streak plate method and incubation at 35◦C for 24 hrs. The pure isolated colonies were identified by their distinct growth appearance [21].For each selective medium, one bacterial isolate showing the typical morphology was selected from each sample for identification.

Biochemical Tests:

The isolated Enterobacteriaceae bacterial colonies were confirmed by Biochemical kit (Hi25^{TM} Enterobacteriaceae Identification Kit KB003) and the results of biochemical tests were interpreted to determine the presumptive nomenclature of the potential pathogenic enteric bacteria isolates through ABIS online (Advanced Bacterial Identification Software).

Pathogenecity testing – Haemolysis Test:

Hemolysin production is associated with pathogenecity of the organism [22]. Blood Agar is a general purpose enriched medium often used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties. To sterile Blood Agar Base which has been melted and cooled to 45 to 50°C, add 5% (vol/vol) sterile defibrinated blood that

has been warmed to room temperature. Swirl the flask to mix thoroughly, avoiding the formation of bubbles, and dispense into sterile plates. The excretion of hemolytically active compounds resulted in either α- or β-hemolysis around the bacterial colonies on blood agar plates [23].

3. Results and Discussion

The present study, entitled Bacteriological Analysis and Identification of Enteropathogens from Fresh Vegetables, was carried out to study the enteric microflora associated with fresh raw vegetables. Eight fresh vegetable samples were collected from the local market, and a bacterial population was isolated from them and presented in **Fig-1**. The bacterial isolates were characterised on the basis of various morphological and biochemical tests. The identified enterobacteriaceae isolates were screened for pathogenicity. The results of the present study are summarised as follows: Data presented in **Table-2** showed that among vegetable samples, the highest CFU (colony forming units) count was obtained from carrots, i.e. 7.0 x10^9 on NAM. Similar findings were observed by Weldezgina and Muleta 2016 [26]. Ehimemen *et al.,* 2019 [27] who observed high contamination and a high number of pathogens on the surface of fresh vegetables and fruits in the case of local market samples.

Bacterial genera were confirmed on the basis of 25 biochemical tests and parameters using the Himedia Biochemical test kit (Hi25^{TM}Enterobacteriaceae Identification Kit KB003). The most prevalent genera confirmed in different isolates were *Enterobacter aerogenes* >*Proteus myxofaciens* > *Serratia entomophila* > *Yersinia aldovae* > *Proteus vulgaries* listed in **Table-3.**

A total of twenty three bacterial isolates were isolated in the present study. We did not set out to investigate the total epiphytic flora. The organisms that we considered to be of potential importance were those capable of surviving in the human intestine, i.e. enteropathogens. Gram-negative strains were considered to belong to the family Enterobacteriaceae, and only these were included in testing, Results shown in **Table-4**.

On the basis of morphological and biochemical tests, five isolates, namely B1, B2A, E2, H1 and H3 isolated shown in Fig-2 and 3 were identified as *Enterobacter aerogenes* Al-Kharousi et al., 2016; and Kape et al., 2016 [28], [29] discovered the presence of Enterobacteriaceae members among the bacterial population isolated from fruits and vegetables.

Serratia entomophila was identified as A1, C1A, and F1, and Proteus vulgaries was identified as D1C, G1, and C5 (Table 4).Kaur and Rai (2015) [20] also reported the presence of *Serratia entomophila* and *Proteus vulgaries* in vegetables and fruits.

E1 and H2 were identified as *Proteus myxofaciens.* Several other workers have also detected *Serratia entomophila* and *Proteus vulgaries* in fresh fruits and vegetables and ready-to-eat vegetable salads [10],[24],[25].

D2B and G2 were identified as *Yersinia aldovae*. MacDonald, *et al* (2012) [30] observed the presence of *Yersinia aldovae* while working with a ready-to-eat salad mix. It was notable that we did not isolate *Escherichia coli* or enterococci from any specimen, indicating a lack of contamination with human or animal faeces.

Hemolysin production is associated with the pathogenicity of the organism. In the present study, most isolates produced β hemolysin. It was observed in (12%) of *Proteus myxofaciens*, (7%) of *Serretia entomophila*, (3%) of *Yersinia aldovae*, (5%) of *Proteus vulgaries* and (18%) of *Enterobacter* aerogenes,results shown in **Table-5.** A function of hemolysins is that the bacteria can utilize hemolysis to release and utilise nutrients from the host animal cells. Iron, e.g., is essential to many pathogenic bacteria. Thus, it can be stated that most of the enteropathogens isolated on the surfaces of the fresh raw vegetables are pathogenic and will exert pathogenecity on human consumption results shown in **Fig-4.**

Many bacteria isolated in this study, such as *Enterobacter aerogenes, Serratia entomophila and Proteus vulgaries* are considered opportunistic pathogens; if they cause infection, their resistance to antibiotics can complicate treatment outcomes. Therefore, it is essential to study antibiotic resistance in bacteria isolated from food and fresh fruits and vegetables.

A β-hemolytic reaction implies complete lysis of the red blood cells, causing a clear zone on the agar surrounding the colony, and it is referred to as true hemolysis. An -hemolytic reaction, on the other hand, occurs when haemoglobin in red blood cells is reduced to methemoglobin, resulting in a greenish discoloration of the agar surrounding the colonies. Finally, the absence of hemolysis or discoloration is referred to as γ-hemolysis Similarly, the results found by many members of the family Enterobacteriaceae are among the most potent and prevalent pathogens [23], and they are also common in fresh fruits and vegetables [27] that are most often eaten raw [25].

Figure 1: Different vegetables collected for microbial analysis, A-Capsicum, B-Cucumber,C-Spinach, D- Carrot, E- Onion, F-Lemon, G-Tomato, H-Chilli.

Table 2: Colonial vegetable samples' morphological traits and total viable count

S. No.	Isolate Code	CFU/ml	Form	Elevation	Margin	Color	Appearance	Shape	Gram Reaction
1	A1	4.2 X 10^9	circular	convex	entire	Creamy white	translucent	Short rods	Negative
2	A2	5.8 X 10^9	circular	raised	entire	white	opaque	Cocci	Positive
3	A3	5.0 X 10^9	irregular	flat	curled	white	opaque	Long rods	Positive
4	B1	7.0 X 10^9	circular	raised	entire	white	translucent	Short rods	Negative
5	B2	3.8 X 10^9	circular	raised	entire	white	opaque	Long Rods	Negative
6	B3	6.0 X 10^9	irregular	flat	entire	white	opaque	Cocci	Positive
7	C1	6.6 X 10^9	circular	flat	entire	Creamy white	opaque	Short rods	Negative
8	C2	3.0X 10^9	irregular	flat	erose	Greenish	transparent	cocci	Positive
9	C3	7.0 X 10^9	circular	flat	entire	orange	translucent	Short rods	Positive
10	C4	3.8 X 10^9	circular	Convex	entire	white	opaque	cocci	Positive
11	C5	6.0 X 10^9	circular	raised	entire	yellow	opaque	Long Rods	Negative
12	D1	6.6 X 10^9	circular	raised	entire	white	opaque	Short rods	Negative
13	D2	3.0X 10^9	irregular	flat	undulate	white	opaque	Short rods	Negative
14	E1	4.2 X 10^9	Punchi form	raised	filamentous	white	translucent	Short rods	Negative
15	E2	5.8 X 10^9	circular	convex	entire	Creamy white	opaque	Short rods	Negative
16	F1	5.0 X 10^9	circular	flat	entire	Light yellow	opaque	Short rods	Negative
17	F2	7.0 X 10^9	circular	raised	erose	Creamy white	translucent	cocci	Positive
18	G1	3.8 X 10^9	circular	flat	entire	Creamy white	opaque	Long rods	Negative
19	G2	6.0 X 10^9	circular	raised	entire	white	translucent	Short rods	Negative
20	G3	6.6 X 10^9	filamentous	convex	erose	white	translucent	Short rods	Positive
21	H1	3.0X 10^9	circular	convex	entire	yellow	opaque	Long Rods	Negative
22	H2	4.2 X 10^9	filamentous	flat	erose	Creamy white	translucent	Short rods	Negative
23	H3	5.8 X 10^9	circular	umbonate	undulate	Creamy white	opaque	Long rods	Negative

Table 3: On selective-cum-differential media, growth Characteristic

Sample	Bacteria Identified	Selective Media
A	*Serratia entomophila*	Growth of pink colour colony of *Serratia entomophila* as on MacConkey Agar
B	*Proteus myxofaciens*	Growth of colourless colony of *Proteus myxofaciens* on MacConkey Agar
C	*Yersenia aldovae*	Growth of colourless colony of *Yersenia aldovae* on MacConkey Agar
D	*Proteus vulgaris*	Growth of colorless colony of *Proteus vulgaris* on EMB Agar.
E	*Enterobacter aerogenes*	Pink-colored colony without sheen over EMB Agar indicates the growth of *Enterobacter aerogenes*

Biochemical Identification

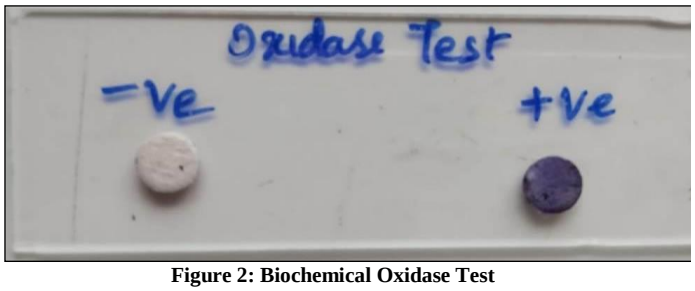

Figure 2: Biochemical Oxidase Test

Figure 3: Biochemical tests and parameters tested using Himedia Biochemical test kit (Hi25TMEnterobacteriaceae Identification Kit KB003).

Table 4: Biochemical tests for bacteria identification

S. No.	Code	Test performed																								Organism identified
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	
1	A1	-	+	+	+	-	+	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	*Serretia entomophila*
2	B1	-	-	+	+	-	+	-	+	-	+	-	+	+	-	+	-	-	-	+	-	-	-	+	-	+	*Enterobacter aerogenes*
3	B2A	-	+	+	+	-	+	-	+	-	+	-	+	-	+	+	-	-	-	+	-	-	-	+	-	+	*Enterobacter aerogenes*
4	C1A	-	-	-	+	-	+	-	+	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+	-	+	*Serretia entomophila*
5	C5	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	-	-	-	+	-	-	-	+	-	+	*Proteeus vulgaries*
6	D1C	-	-	+	-	-	+	+	+	-	+	-	+	-	+	+	-	+	-	-	-	-	+	+	+	+	*Proteeus vulgaries*
7	D2B	-	-	+	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	*Yesinia aldovae*
8	E1	-	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	*Proteeus myxofaciens*
9	E2	-	+	+	+	-	-	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	*Enterobacter aerogenes*
10	F1	-	-	-	+	-	+	-	+	-	+	-	-	-	+	+	-	-	-	+	-	-	-	+	+	+	*Serretia entomophila*
11	G1	-	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	*Proteeus vulgaries*

12	G2	-	-	+	+	-	+	-	+	-	+	-	+	-	+	+	-	-	-	-	-	-	+	-	+	*Yersinia aldovae*
13	H1	-	-	-	-	-	+	-	+	-	+	-	+	-	+	+	+	-	-	+	-	-	-	+	-	+	*Enterobacter aerogenes*
14	H2	-	-	-	+	-	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	+	+	-	+	*Proteeus myxofaciens*
15	H3	-	-	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	+	-	+	*Enterobacter aerogenes*

+ = Positive, - = negative

***A-** ONPG, **B-** Lysine, **C-** Ornithine, **D-** Urease, **E-** Phenylalanine deamination**, F-** Nitrate reduction, **G-** H_2S production**,H-** Citrate utilization, **I-** Voges-Proskauer's, **J-** Methyl Red, **K-** Indole, **L-** Malonate utilization, **M-** Esculine Hydrolysis, **N-** Arabinose, **O-** Xylose, **P-** Adonitol, **O-** Rhamnose**, Q-** Cellobiose, **R-** Melibiose, **S-** Saccharose, **T-** Raffinose, **U-** Trehalose, **V-** Glucose, **W-** Lactose**, X-** Oxidase

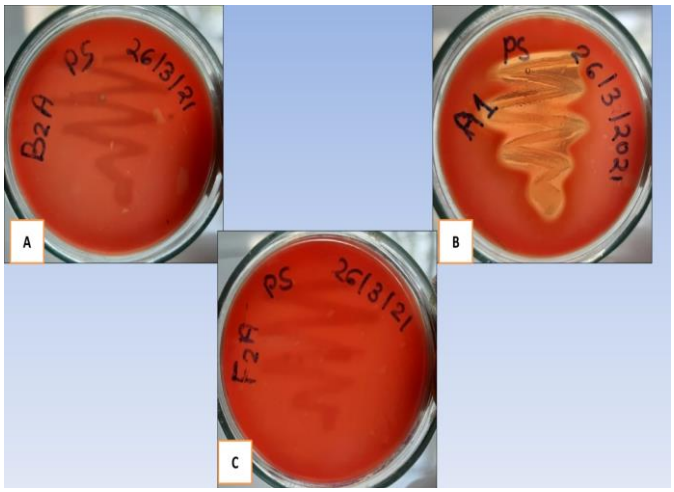

Figure 4: Haemolytic activity on Blood Agar Plates, A- γ Haemolysis ,B- β Haemolysis C- α Haemolysis

Table 5: Haemolysis test results

S.No.	Isolate code	Hemolysis
1	H2	β
2	A1	β
3	E2	β
4	G2	β
5	D2B	β
6	B1	β
7	C1A	β
8	D1C	β
9	F1	β
10	E1	β
11	G3A	α
12	H3	γ
13	F2A	γ
14	B2A	γ
15	C5	γ

4. Conclusion

From the above data, it can be concluded that regular consumption of fresh raw vegetables vended on the street would be harmful due to the presence of enteropathogens. Though the immunity of Indians is considered high, regular intake of such contaminated vegetables would detoriate the individual's health. The sources of contamination may even be the personal hygiene of vendors apart from the water and utensils used. So, regular checks and monitoring would be a good idea to minimize the contamination. Additionally, additional authority should be granted to FSSAI and other highly active administrative organizations like Mobile Court to conduct preventative investigations to evaluate the microbiological and chemical quality of vegetables. In addition to governmental and non-governmental groups, the public should also be made aware of the contamination of vegetables through the media, so that they can be cautious before consuming street-vended vegetables.

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