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Antibiotic-resistant bacterial pathogens associated with raw vegetables from Nanded city

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Abstract:

Raw vegetables are beneficial to health as they are a good source of essential nutrients. Many vegetables including salads are consumed in raw form rather than a cooked form to retain the natural taste and heat-labile nutrients. The poor hygiene and improper washing, leads to the safety issues of raw vegetables. The microorganisms has acquiring resistance against several antimicrobial drugs which became a serious issue. We reported the microbial quality of salad vegetables sold in local markets in Nanded city, Maharashtra. A total of 15 bacterial isolates were obtained from salad vegetables viz. beetroot, carrot, and tomato. Isolated microorganisms were identified as diarrhoea, enteric fever, gastrointestinal diseases, typhoid, etc. causing *Bacillus spp., E. coli, Salmonella spp., Shigella spp., Staphylococcus spp.,* and *Streptococcus spp.* based on morphological and biochemical characteristics as mentioned in Bergey's Manual of Systemic Bacteriology. The important finding is the isolated bacterial pathogens showed resistance against Amikacin, Amoxycillin, Ceftadime, Kanamycin, and Rifampicin. The data generated will be used for enhancing awareness about raw vegetable safety and safeguarding public health.

Keywords: Raw vegetables, Pathogens, Identification, Antimicrobial resistance.

Introduction:

Fresh raw vegetables are considered to be an essential component of a healthy diet of human . Raw vegetables such as tomato, carrot, radish, cucumber, beetroot, etc. are a good source of proteins, vitamins, and micronutrients (Hornick et al., 2011). Vegetables are commonly consumed in raw salad form or cooked form , however cooking vegetables reduces the nutrition values (Kate et al., 2018), hence consumption of salads has increased throughout the globe including India. Despite this, infectious diseases have been reported the consumption of raw vegetables due to contamination with human pathogenic microorganisms. The microbial contamination in vegetables became a hotspot for illness and diseases, it has been evidenced by reports of various public health organizations, enhanced epidemiological and surveillance techniques. Numerous outbreak-associated with consumption of raw vegetables has been reported. (Murray, 2005, Argud et al., 2010, Callejon et al., 2015 and Kyung et al., 2018).

Fresh vegetables are known to harbour large bacterial populations (Leff and Fiere, 2013), which are both plant pathogenic and human pathogens viz enteric fever-typhoid causing *Salmonella spp*., gastrointestinal pathogen *E. coli*, *Staphylococcus aureus*, and several other opportunistic pathogens (Uzeh et al., 2009). Some vegetables have shown a load of 10^3 to 10^5 microorganisms/cm^3 or 10^4 to 10^7 microorganisms/g (Beuchat, 1995). The microorganisms may transmit on vegetables due to poor hygienic practices and consumption of such raw vegetables are responsible for several diseases (Sia et al., 2012, Angeles-Núñez, 2014). The disease-causing microorganisms are showing resistance to the prescribed drugs.

The Nanded district from Marathwada region have good irrigation facilities, therefore Nanded city has a very good market for vegetables and fresh produce. Therefore in the present study, attempts have been made to analyze the microbiological quality of raw vegetables and antibiotics resistance in associated microorganisms.

Materials and Methods:

Raw vegetables (Beet-root, Carrot & Tomato), 1% $HgCl_2$, Nutrient Broth, Luria-Bertani Broth, Nutrient Agar (HI Media Pvt. Ltd.), Antibiotic Discs: Amikacin 30µg, Amoxycillin 30µg, Ceftadime 30µg, Chloramphenicol 30µg, Erythromycin 15µg, Gentamycin 120µg, Kannamycin 30µg, Rifampicin 05µg, and Tetracycline 30µg)

***Sample Collection*:** The raw consumable vegetable samples such as beetroot, carrot, and tomato were bought at random from the area of local markets of Nanded. The salad vegetables were placed in pre-sterilized food-grade bags and transported to the laboratory.

Sample Processing: Salad vegetables were distributed into three groups–Untreated (U): Used as it is, Treated with distilled water (D): Washed with distilled water for 10 minutes and Treated with HgCl2 (H): Deep in 1% HgCl2 solution for 10 minutes. After processing, vegetable samples were crushed and homogenized in saline

Enrichment: 2ml of homogenized mixed vegetable samples were inoculated in conical flasks containing Nutrient broth and Luria Bertani broth. Inoculated flasks were incubated at 37°C for 24hrs. Flasks were shaken at regular intervals to obtain maximum growth.

Isolation: One loop-full of enriched broth was streaked on sterile nutrient agar plates for isolation of bacterial species and plates were incubated at 37°C for 24hrs. After incubation, well isolated colonies were studied for cultural characteristics and well isolated colony was transferred on an agar slant. After the growth of bacterial isolates, the slants were preserved in the refrigerator at 4°C.

Identification of Isolates: The bacterial isolates were tentatively identified by using colony and morphological features viz. size, shape, colour, margin, opacity, gram's nature, and biochemical analysis such as Indole, MR-VP, Citrate utilization, Oxidase, Catalase, etc. as given in Bergey's Manual of Systemic Bacteriology (Clauss D and Berekeley RCW, 1986 and Hemraj V et al., 2013).

Antimicrobial resistance: Antimicrobial resistance spectrum of *Staphylococcus aureus, Salmonella spp., Escherichia coli, Streptococcus spp., Bacillus spp.* and *Shigella spp.* were determined against antibiotics with the concentration of- Amikacin 30µg, Amoxycillin 30µg, Ceftadime 30µg, Chloramphenicol 30µg, Erythromycin 15µg, Gentamycin 120µg, Kannamycin 30µg, Rifampicin 05µg, and Tetracycline 30µg. Antibiotic spectrum was determined by disc diffusion method: Nutrient agar (soft and hard) were prepared. Soft agar was inoculated with 2% of active microbial culture isolated from raw vegetables was prepared and poured into previously prepared hard agar plates. The plates were incubated at room temperature for 10 minutes. After incubation, standard discs of antibiotics mentioned above were placed on inoculated agar plates aseptically. All plates were incubated at 37°C for 24 to 48 hrs. After incubation plates were observed for the zone of inhibition to check the drug sensitivity and resistance pattern of isolates. The zone of inhibition formed around discs was measured with a zone measuring scale in millimeters (Lucas AE et al., 2017).

Result:

A total of three raw vegetables: beetroot, carrot, and tomato were selected for experiments. Vegetables were distributed in three parts (unwashed, washed with distilled water, and cleaned with 1%$HgCl_2$ solution) vegetable samples were processed (Fig.1).

Turbidity after incubation in enrichment broth confirmed microorganisms had successfully enriched in media broth. Enriched nutrient and LB broth by crushed vegetable homogenized mixture when streaked on nutrient agar plates, shown presence of distinct colonies of bacteria. A total of 16 isolates were obtained from the vegetable samples which was purified on agar media plates as shown in fig.2

The well-isolated colonies range in size of 2mm to 5mm in diameter in which six were white and nine were pale in color. Among the bacterial isolates, eleven were gram's positive bacteria and the rest were negative. Tentative identification by biochemical tests viz. Indole test (2 positive and 13 negatives), Methyl Red test (14 positives and 1 negatives), VP test (10 positive and 5 negatives), Citrate utilization (11 positives and 4 negatives), Oxidase test (all 15 negative), and Catalase (13 positive and 2 negative).

As the isolated bacteria are pathogenic, their response to various antibiotics was checked. Disc diffusion method was used to check the antimicrobial susceptibility of the isolates. Zone of inhibition developed around the antibiotic disc as shown in fig.3 & Table.1

Discussion:

Vegetables are a important source of many nutrients. The pathogenic microorganisms associated with raw vegetables are cause of foodborne diseases (Callejón et al., 2015) such as enteric fever, typhoid, dysentery, diarrhea,

etc (Zahra SK et al., 2016). Leff and Fierer, (2013) found that the diverse microbial community of phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria are associated with vegetables are dominated. The raw form of vegetables contains less count of microorganisms, the increased microbial count by laboratory enrichment technique of samples in liquid media; in the form of turbidity were recorded. The turbidity was observed in the vegetable samples washed with $HgCL_2$ also, which indicates that the microorganisms had developed resistance against sterilizing agents too. Law JW-F et al., (2015) used the enrichment method for the isolation of *Listeria monocytogens* from food and environmental samples. Hence for obtaining the isolates from samples, enrichment is an important technique to increase the number of microorganisms up to a detectable level.

The streak plate method is suitable for cultural isolation that gives pure isolated colonies. The four-quadrant technique has used by most researchers to get well-isolated colonies (Nithya A and Babu S, 2017). In this investigation, several pure colonies with distinct morphologies were obtained from raw vegetables by the quadrant streak method. It reveals that more than one type of microorganism is associated with the samples. With the help of morphological characteristics such as size, shape, colour, margin, elevation & opacity of isolated colonies and microscopic features such as gram's nature as well as cell shape of isolates, microorganisms can be differentiated. Using biochemical tests Indole, MR-VP, Catalase, Oxidase, etc, (Beuchat LR, 2002, Hassan NA and Zulkahar NA, 2018 and Ferone M et al., 2020).

Different sources like air, soil, water used for irrigation or washing of vegetables, transportation containers, packaging material, and vegetable handling persons through which vegetables get contaminated. In this investigation, we got *Staphylococcus aureus*, *Salmonella spp.*, *Escherichia coli*, *Streptococcus spp.*, *Bacillus spp.*, *Shigella spp.* which are responsible for diseases like dysentery, diarrhea, gastrointestinal infections, enteric fever, typhoid, . The environment and factors such as water used for irrigation may be contaminated with enteric pathogen, the hygiene of person handing the vegetable, hence above mentioned microorganism was dominant in the samples obtained from the local market

The organisms isolated from fresh vegetables were found to be partial or completely resistant to several antibiotics (Zahra SK et al., 2019). It was found that *Salmonella spp.* and *Bacillus spp.* was completely resisted to Amikacin 30µg, Amoxycillin 30µg, Ceftadime 30µg, and Kanamycin 30µg, *Staphylococcus aureus* was completely resisted to Amikacin 30µg, Amoxycillin 30µg, Ceftadime 30µg, Kannamycin 30µg, and Rifamycin 5µg, *Streptococcus spp.*.

From these results, it can be concluded that vegetables available in the Nanded market with microorganisms are not safe for consumption by infants, the elderly, and immunocompromised patients. Rather than raw vegetables, boiled or cooked vegetables can be safe for consumption by healthy as well as persons with weak immunity.

Fig.1 Raw vegetable samples

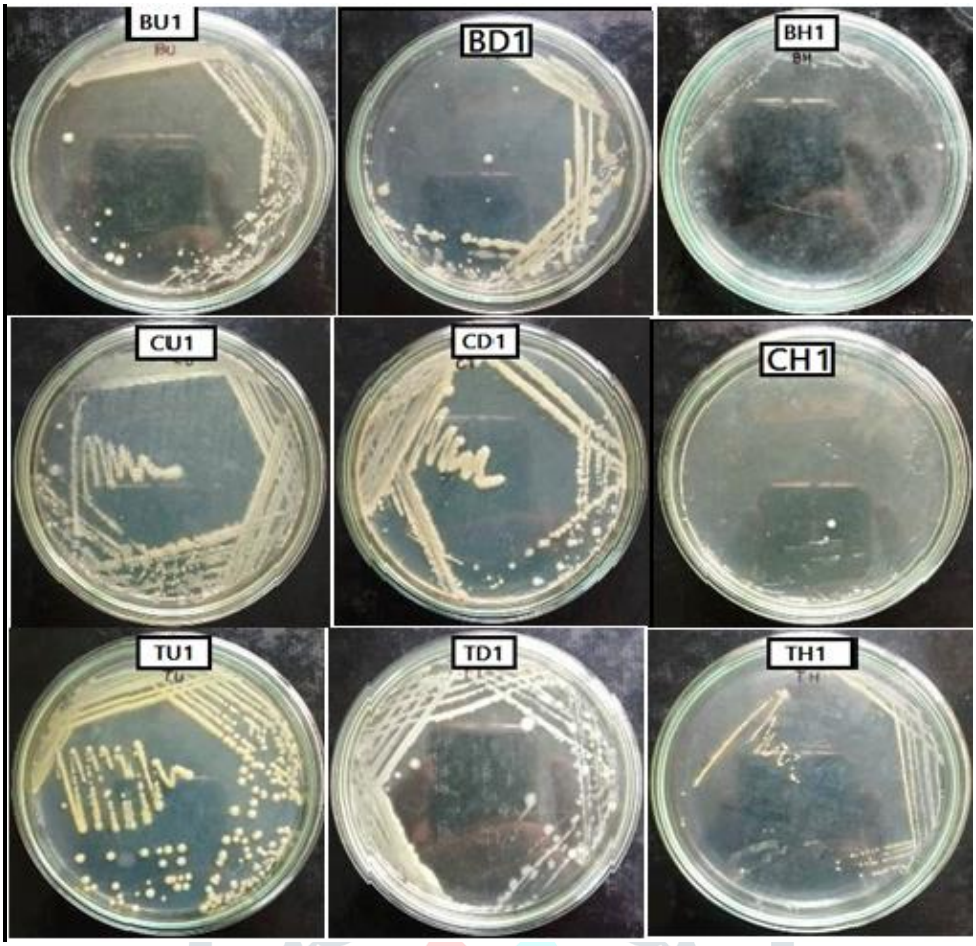

Fig. 2 Isolated bacterial isolates from raw vegetables

Table. 1 Antimicrobial resistance in pathogenic bacteria

Sr. No	Isolate	Antibiotics (Zone of inhibition in mm)								
		AK30	AM30	CA30	C30	E15	G120	K30	RIF5	TE30
01	*Bacillus* spp.	R	R	R	15	11	18	R	10	12
02	*Escherichia coli*	03	07	09	21	15	10	04	02	05
03	*Salmonella* spp. 1	R	R	R	23	11	18	R	14	10
04	*Salmonella* spp. 2	R	R	R	20	05	16	R	03	10
05	*Staphylococcus* spp. 1	07	09	06	12	14	11	07	11	12
06	*Staphylococcus* spp. 2	R	R	R	12	08	17	R	R	13
07	*Staphylococcus* spp. 3	R	04	06	11	06	12	R	R	05
08	*Staphylococcus* spp. 4	R	11	R	12	12	12	R	R	11
09	*Staphylococcus* spp. 5	R	09	R	10	13	12	R	R	08
10	*Staphylococcus* spp. 6	R	R	R	15	05	14	R	05	08
11	*Staphylococcus* spp. 7	22	27	R	20	23	26	20	19	22
12	*Staphylococcus* spp. 8	10	08	R	22	15	18	16	12	13
13	*Staphylococcus* spp. 9	16	21	R	22	12	19	12	10	12
14	*Streptococcus* spp.	25	22	R	36	29	28	27	25	27
15	*Shigella* spp.	NA	NA	NA	NA	NA	NA	NA	NA	NA

(AK-Amikacin 30µg, AM-Amoxycillin 30µg, C-Ceftadime 30µg, CA-Chloramphenicol 30µg, E-Erythromycin 15µg, G-Gentamycin 120µg, K-Kanamycin 30µg, RIF-Rifampicin 05µg, TE-Tetracycline 30µg, NA-Data Not Availabe, R-Resistant)

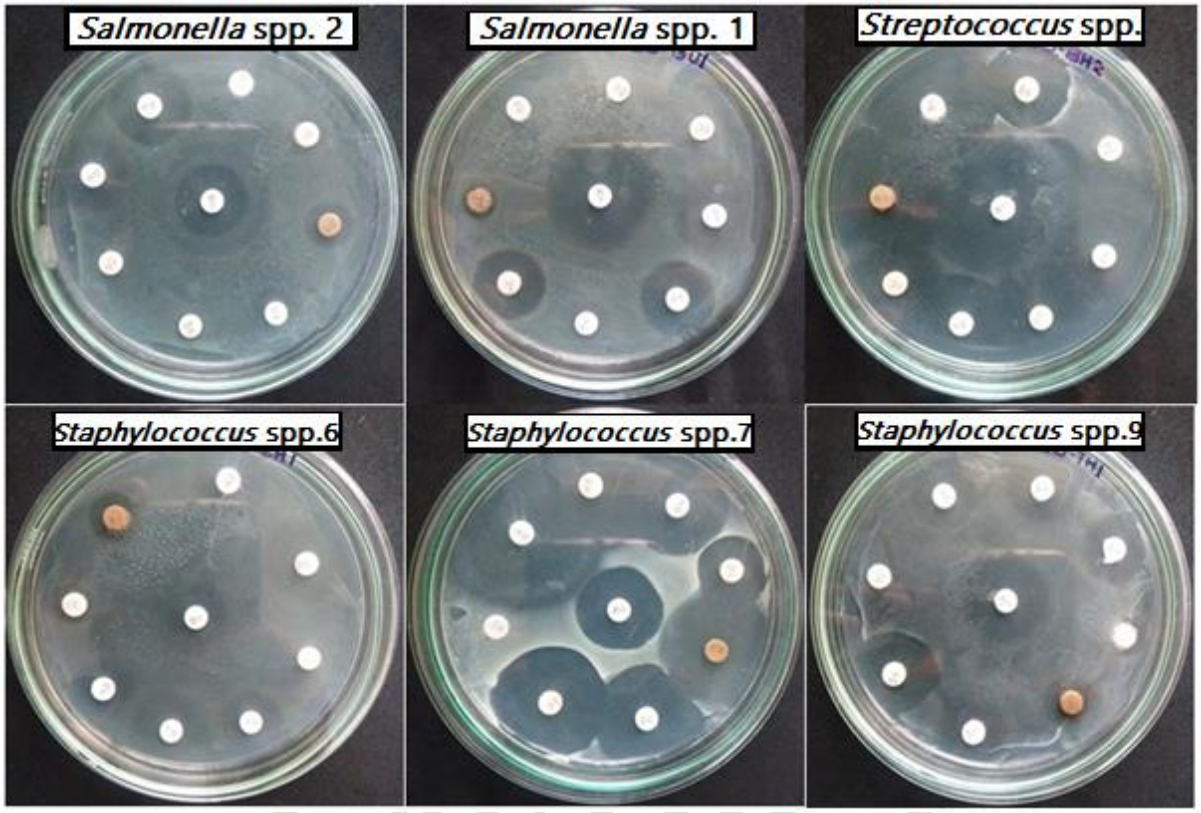

Fig. 3 Antibacterial activity of selected antibiotics against pathogenic bacteria

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