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PEVALANCE OF ENTEROTOXIC *E. COLI* IN RAW VEGETABLES AND SPROUTS SOLD IN MARKETS OF GOA

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Abstract

Background of the study: The infection of *E. coli* has been associated with the consumption of a number of raw vegetables and sprouts. Since last decade the raw vegetable and sprouts have been revealed as a potential source for the transmission of *E. coli*.

Method: A preliminary study was carried out to determine the incidences of *E. coli* in raw vegetables and sprouts sold in local markets of Goa. A total of 110 samples (raw vegetables, n=65 and sprouts, n=45) were collected from local vendors and investigated for prevalence of pathogenic *E. coli*. The samples were processed as per the method defined by ISO-16654 to isolate *E. coli* that has optimised particularly for leafy vegetables. Isolate were further studied for their in-vitro pathogenic potential by detecting the heat-labile enterotoxin (*lth*) gene.

Result: A total of 45 (40.90%) presumptive *E. coli* isolates were obtained which were further confirmed by their morphological and biochemical characterisation. Out of 45 *E. coli*, 21 (46.66%) showed haemolysis on 5% sheep blood agar. The haemolytic isolates were further tested for their pathogenic potential in-vitro, by screening the *lth* gene. The *lth* gene was present in 15 (71.42%) of the haemolytic *E. coli* isolates.

Conclusion: From this study it can be inferred that, potentially pathogenic *E. coli* are prevalent in raw vegetables and sprouts sold in local markets of Goa. It can be concluded that the marketed sprouts are of poor microbiological quality. Presence of such pathogens in day-to-day food is matter of concern.

Keywords: ETEC *E. coli*, vegetables, sprouts, heat labile enterotoxin

Introduction

Escherichia coli (*E. coli*) is a common intestinal bacteria of human and animals. Though majority of *E. coli* strains are harmless, some are pathogenic which cause fatal diarrhoeal diseases and urinary tract infection. Pathogenic *E. coli* strains are categorized into six pathotypes which collectively referred as diarrheagenic *E. coli*. Globally, *E. coli* is a leading foodborne pathogen and has caused several outbreaks [1]. The main source of infection of *E. coli* is

through contaminated water or food, contacts with animals or infected persons. Also, domestic healthy animals such as cattle, goats and sheep act as a reservoir for *E. coli* and through their faecal discharges this organism gets spread in the environment.

Infection with enterotoxigenic *E. coli* (ETEC) is the leading cause of traveller's diarrhea and a major cause of diarrheal disease in underdeveloped nations,

especially among children [2]. Study based on 15 population in low and medium human development index (HDI) countries from 1984 to 2005 reports incidence ranged from 39 to 4460 infections/1000 persons per year [3]. ETEC produces two types of toxins, heat-labile toxin (LT_h) and a heat-stable toxin (ST_h) which stimulates the lining of the intestines causing them to secrete excessive fluid, thus resulting in profuse watery diarrhea. Although different strains of ETEC can secrete either one or both of these toxins, the illness caused by each toxin is similar. Currently, identification of ETEC is based on their virulence markers -the *lth* or *sth* gene.

In India, the general or traditional practice to grow vegetables is mainly organic based. Such organic farming have been described as representing an increased risk to public health [4]. Vegetables and sprouts can become easily contaminated with foodborne pathogens at several steps such as - growing in fields, harvesting, postharvest handling, processing and distribution [5]. Depending upon the surface properties of vegetables, bacteria (including pathogens) adhere. To remove dust and soil, these vegetables or sprouts generally processed by simple water wash, which is insufficient to remove all the bacteria [6]. In addition, there is hardly any bactericidal procedure that is practiced so as to remove pathogens. Also, the water used to wash these vegetables itself could be the source of contamination [7]. Therefore, the occurrence of pathogens on vegetables and sprouts can't be denied. These food items then directly consumed or used in salads or similar preparations, hence, hazardous to public health. Some of the earlier studies from India reports the occurrence of *E. coli* in fruits, vegetables and sprouts sold locally [8, 9]. The objective of the present was to study the occurrence of enterotoxigenic *E. coli* in raw vegetables and sprouts sold in markets of Goa.

Material and Methods

Sampling:

The raw sprouts (n=65) and vegetables (n=45) were purchased from the local markets (Panjim, Mapusa, Vasco, Ponda and Margao) of Goa state, India.

Specifically, sprouts and vegetables that generally minimally processed or do not processed and consumed as it is, were chosen for the study. The samples were collected in sterile zip-lock sampling bags, transported in chilled condition processed immediately for the isolation of *E. coli*.

Isolation and identification of *E. coli*:

The isolation of *E. coli* was performed by method defined by "Bacteriological Analytical Manual, Chapter 4A: Diarrheagenic *Escherichia coli*" which is optimised for sample preparation for leafy vegetables and produces [10]. Accordingly, 5 g of sample was inoculated into 45 ml of Brain-Heart infusion (BHI) broth (dilution factor of 1:10) in stomacher bag. The bag was transferred to 'Seward' stomacher (Thermo Scientific) and sample was macerated for 10 min. The homogenate was incubated for 10 min. at room temperature with periodic shaking then samples were allowed to settle for 10 min. The medium was decanted carefully into a sterile container and incubate for 3 h at 35°C to resuscitate injured cells. The 5 ml content was mixed with 45 mL double strength tryptone phosphate broth and incubated for 20 h at 44.0 ± 0.2°C. After incubation, a loopful of enriched broth was streaked onto Levine's eosin-methylene blue (L-EMB) agar and MacConkey agar. The agar plates were incubated for 20 h at 35°C. After respective incubation, the plates were observed for typical *E. coli* colonies. Typical lactose-fermenting dark centred and flat colonies with or without metallic sheen on L-EMB agar and brick red colonies on MacConkey agar were considered as of *E. coli*. Such presumptive colonies were transferred to 1.5 ml BHI broth and incubated for 18 h at 35°C. The growth was transferred to sterile 2 ml screw cap tubes preserved at 4°C for further study.

All the presumptive isolates were identified by morphological and biochemical test defined previously [10]. Gram staining and batteries of typical biochemical tests to identify *E. coli* (Indol, Methyl red, Voges-Proskauer, Citrate utilisation, urease and catalase) were performed in order to confirm isolates. Haemolysis test was performed on 5% sheep blood agar.

Screening for the *lth* gene:

All the *E. coli* isolates that could exhibit haemolysis were further analysed for their potential pathogenicity by screening for heat-labile enterotoxin (*lth*) gene. The DNA was isolated by classical phenol: chloroform method. The quality and quantity of the obtained DNA was determined by nanodrop (Thermo-Scientific). Polymerase chain reaction detecting the *lth* gene was performed as described previously [2]. In brief, PCR assays was carried out in 25 µl reaction mixtures consisting of 50 ng template DNA, 12.5 µl of 2x ReadyMix™ Taq PCR reaction mix (Sigma, Cat. No. P4600), 15 pmol of each primer (F: 5'- AGC AGG TTT CCC ACC GGA TCA CCA -3'; R: 5'- CGT GCT CAG ATT CTG GGT CTC -3') adjusting final volume by molecular grade water. The reactions were performed in thermal cycler (Eppendorf, Germany) with the following cycling profile: 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1.5 min and primer extension at 72 °C for 2 min and a final extension at 72 °C for 5 min. Amplified products were resolved on 2 % agarose gel electrophoresis and visualized under UV transillumination after ethidium bromide staining. DNA templates from positive (*E. coli* ATCC 8739) and negative control (*L. monocytogenes* EGDe) strains were included in each PCR.

Results and Discussion

E. coli is one of the deadliest pathogen that gets transmitted through contaminated water, food and contact with animals or infected persons. In last few years, raw vegetables and sprouts is been revealed as one of the major source for pathogenic *E. coli*. In 2011, a large outbreak of the haemolytic-uremic syndrome associated with the rare *E. coli* serotype O104:H4 due to consumption of contaminated raw vegetables and sprouts occurred in Germany [11]. Also, *E. coli* is been responsible for several worldwide outbreaks, of which some are attributed to vegetables [1]. Worldwide studies also document the incidences of *E. coli* in sprouts and vegetables [12–15]. As an approach to 'clean' the vegetables, the only procedure used to reduce microbial load

is washing with water. Though this procedure is partly successful to remove dust and soil, it do not ensure the removal of all the bacteria [16]. Therefore, contamination of fresh vegetables and sprouts with pathogenic microbes may persist. In India, several vegetable and sprouts produced are sold locally. There are no monitoring or governing policies controlling such sale. Also, very few reports are available that access the prevalence of *E. coli* on such food products. Therefore, a study was carried out to access the prevalence of *E. coli* on the raw vegetables and sprouts sold in market.

In this study we have covered major markets of the Goa state. A total of 110 food samples (Raw vegetables and sprouts) samples were collected that would be consumed directly without any processing. A total of 45 (40.90%) of 110 were found to be positive for *E. coli*. All the isolates were confirmed by morphological and biochemical test. When all the isolates tested for haemolytic activity on 5% sheep blood agar, 21 (46.66%) isolates could exhibit haemolysis. Sporadic reports from India shows occurrence of *E. coli* and other pathogens on vegetables and sprouts. Vishwanathan and Kaur (2001) has reported 31.9% of vegetables and 66.7% of sprouts samples contaminated with *E. coli*, *S. aureus*, *Enterobacter* sp., *Klebsiella* sp., *S. typhi*, *Serratia* sp., *Providencia* sp. and *P. aeruginosa* sold in local market of Mumbai, India [17]. Another study by Saroj et al. (2006) documents the occurrence of *Staphylococci*, *Salmonella*, *Listeria monocytogenes*, *Escherichia coli*, and coagulase-positive *Staphylococcus aureus* in sprouts [18]. Of the six pathotype, ETEC *E. coli* is mainly responsible for the diarrhea in the developing world. ETEC *E. coli* has been responsible for approximately 210 million cases and 380,000 deaths, particularly in developing countries [3]. Also previous studies describes the relative frequency of toxin phenotypic subgroups during three different ETEC outbreaks in India [19–21]. All ETEC isolates from each outbreak were positive for heat labile toxin and negative for heat stable toxin. Therefore, we focused our further study particularly with respect to the heat labile toxin containing *E. coli*. Those

isolate which were haemolytic on 5% sheep blood agar, were further screened for presence of the *lth* gene encoding heat-labile toxin. Presence of this gene also distinguishes *E. coli* pathotype ETEC. Of the 21 haemolytic strains 15 (71.42%) were found to possess the *lth* gene. Overall 33.33% of the *E. coli* isolates were of ETEC pathotype. In Goa, approximately 12,000 diarrheal cases are reported per year [22]. Occurrences of such potential pathogenic *E. coli* in ready to eat food must be resulting in disease, however due lack of surveillance system such disease or outbreak do not get noticed and therefore underestimated.

The contamination by *E. coli* is generally considered due to faecal discharges in water. In addition to human, domestic healthy animals are potential carrier of *E. coli*, including ETEC strains [23–25]. Due to lack of proper sewage treatment in most of the part of India, this pathogen gets easily mixed with the local water bodies [26]. Such water then gets used for agricultural activities, which lead to contamination of the crops that being grown [27, 28]. Use of such untreated water for germination of sprouts or to wash the vegetables increases the surface microbial load on this food items. As there is no regulation or monitoring system to check locally sold vegetables or sprouts, the pathogens must be getting spread through such daily food items. Several antimicrobial washing solutions, O₃, UV-C radiation, intense light pulses, super high O₂, N₂O and noble gases, alone or in combination have been suggested for promising treatments. However, change from use of conventional to innovative sanitizers requires knowledge of the benefits and restrictions as well as a practical outlook [16]. Thus, there is need to provide basic education for the farmers to use this innovative sanitizer, which can help to reduce the transfer of pathogens from field to market. Also, government bodies should

enforce some regulations over sale of raw and readily consumed food items.

Conclusion

This preliminary study shows prevalence of potentially pathogenic *E. coli* ETEC strains in vegetables and sprouts that get sold in local markets of Goa. Occurrence of such pathogens on readily consumed food could be hazardous from public health point of view. From this study we came to conclusion that the marketed sprouts are of poor microbiological quality and hence there is a need to carry out further studies to trace the source of contamination. It is suggested that necessary precautions taken at individual level to minimize the degree of contamination of vegetables during production, harvesting, processing, marketing and preparation. The vegetables should be washed thoroughly with clean water containing 100 - 120 ppm chlorine as suggested by FDA [29]. Government regulations for microbial contamination at local markets are necessary to prevent spread of pathogens for public safety.

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Author's contribution

VM carried out sampling, isolation and identification; SK performed PCR. AR performed biochemical. SD participated in design of the study, its analysis and written the manuscript. SB supervised the work and reviewed the manuscript.

Competing interests - None

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