

Article

# Microbial and Parasitic Contamination of Fresh Raw Vegetable Samples and Detection of the *Bla*<sub>TEM</sub> and *Bla*<sub>CTX-M</sub> Genes from *E. coli* Isolates

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**Abstract:** A total of 100 fresh-raw vegetable samples were collected from the Kathmandu, Lalitpur and Bhaktapur districts of Nepal to evaluate microbial and parasitic contamination, presence of Extended Spectrum Beta Lactamase (ESBL)-producing *Escherichia coli* and detect the *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes among the *Escherichia coli* isolates. This study revealed that the prevalence of *Giardia* cysts was highest (100%) and Hookworm and *Entamoeba coli* were lowest (1% each). Coliforms were isolated from every raw vegetable sample. A total of 178 bacterial isolates were isolated among which 57 isolates were identified as *E. coli*, out of which 33 were Multi-drug resistant (MDR) isolates. The high rate of resistance was found towards amoxicillin/clavulanate, tetracycline and cotrimoxazole. The 10 *E. coli* isolates tested positive in an ESBL screening, out of which 4 were confirmed as ESBL producers by a combined disc test. Out of these 4 confirmed ESBL *E. coli*, one was found to carry both the *bla*<sub>TEM</sub> gene and *bla*<sub>CTX-M</sub> genes by the Polymerase Chain Reaction (PCR) technique. One isolate has only the *bla*<sub>TEM</sub> gene, while other isolate harboured only *bla*<sub>CTX-M</sub> genes.

**Keywords:** raw vegetable; parasite; coliform bacteria; Kathmandu Valley; AST; MDR; ESBL

## 1. Introduction

Vegetables like carrot, radish, cucumber, tomato, cabbage, lettuce, coriander, etc., can be consumed without heat treatment. Sometimes incomplete washing and peeling can provide a reservoir for many microorganisms resulting in food borne diseases. Different kinds of pathogens can cause human infection through the oral route [1]. It was shown that washing minimizes the burden of bacteria on

vegetables but does not remove them completely [2]. As an example, lettuce does not undergo any inactivation or preservation treatment during processing; consumers may be exposed directly to all of the (resistant) bacteria present [3].

According to Beuchat et al. [4], during harvesting fecal materials, human handling, harvesting equipment, transport containers, wild and domestic animals, air, transport vehicles, ice or water can contaminate vegetables. Another major cause of vegetable contamination could be the unavailability of hygienic irrigation water and proper knowledge. Due to low sanitary practices and improper drainage systems as well as poor hygienic practices related to planting, harvesting, packaging, transportation and storage, fruits and vegetables get contaminated easily. Since fruits and vegetables are essential parts of our food that are consumed raw or sometimes inadequately cooked, consumption of those fruits and vegetables can be the major route of transmission of human pathogens [5].

Food-borne illness has been reported in an increasing number, mainly linked to eating fresh vegetables [6,7]. Among the various causes of diseases in humans, intestinal parasitic infection is one that is responsible for infecting more than two billion individuals globally [8]. Various species of protozoan parasites are linked with food-borne diseases and some of them can cause serious health problems and economic issues [9].

Antibiotic resistant bacteria and antimicrobial resistance genes can be exchanged between the animal reservoir and the human reservoir [10–12] due to direct contact with animals or their environment or through indirect contact through food [3].

Most ESBLs can be divided into four groups: TEM, SHV, OXA, and CTX-M types. Currently, CTX-Ms are the most prevalent type of ESBLs described [13,14]. ESBL genes may spread between bacterial isolates via the exchange of plasmids (and other mobile elements), which may harbor additional antimicrobial resistance genes [15].

Not many studies have investigated the presence of ESBL-producing *E. coli* on vegetables and among them, only few have found and described in detail ESBL-producing *E. coli* or have identified leafy salads or sprouts as a source [16,17]. In this work, we have determined the prevalence of Parasites, Coliform bacteria, and the resistance pattern of *E. coli*-producing B-lactamases in raw vegetables of the Kathmandu Valley.

## 2. Methodology

The study was conducted from June 2018 to December 2018 at Microbiology laboratory of Golden Gate international college, Battisputali, Kathmandu, Nepal. Random sampling was implemented for the collection of samples to study any variances in the contamination level of microbes with respect to the places. A total of one hundred raw salad vegetable samples including Cabbage, Carrot, Capsicum, Coriander and Lettuce were obtained from different open markets and grocery shop outlets in the Kathmandu, Lalitpur and Bhaktapur districts of Nepal over the period of 6 months.

Each sample was bought either from an open market or a grocery shop. Most of the shops were enclosed but vegetables were not packed in any type of packaging material. The vegetables selected for sampling were all placed in the open air and were handled by consumers. Usually in Kathmandu Valley, vegetables are brought directly from farms and sold in open markets. In Nepal, vegetables are grown in the traditional way.

### 2.1. Parasitological Analysis of Vegetable

A total of 100 gm of each vegetable was sampled from the skin and washed in a physiological saline solution (0.95%) and the washing water was left for 10 h for sedimentation to take place in a conical flask in a slightly tilted position. The top layer was removed, and the remaining washing water was centrifuged for 15 min at 2000 g speed. The supernatant was discarded and the residue tested using the Bailenger-modified Teleman Rivas technique [18].

Identification of Parasites

- i. Simple smear: A drop of sediment was applied on the center of a clean grease-free slide. A clean cover slip was placed gently to avoid air bubbles and overflowing. The preparation was examined under a light microscope using 10× and 40× objectives.
- ii. Iodine smear: A drop of sediment was mixed with a drop of lugol's iodine solution and examined in the same way as the simple smear.

The simple and iodine smear were used for the detection of parasitic eggs, cysts and larva. The process was systematically repeated until the mixer in each test tube was exhausted. Eggs, cysts and oocysts of parasites found under the light microscope were identified. *Entamoeba* spp. is differentiated based on the size of the cyst and the number of nuclei inside the cyst. The mature cyst of *Entamoeba coli* is larger and contains 8 nuclei in comparison to 4 nuclei inside the cyst of *Entamoeba histolytica* [19].

## 2.2. Total Coliform Count

The Most Probable Number (MPN) method was used to detect and enumerate coliform bacteria present in the vegetable samples as described by Rompré et al. [20]

- I. Presumptive test Three single strength lactose broth tubes were labeled as "0.1", another 3 tubes "1" and 3 double strength broth tubes "10". Each "10" tube was aseptically inoculated with 10 mL of sample, the "1" tubes were aseptically inoculated with 1 mL of sample using a 1 mL sterile pipette and the "0.1" tubes were inoculated aseptically with 0.1 mL of sample using a sterile pipette. All of the nine-inoculated tubes were incubated at 37 °C for 24–48 h.
- II. Confirmed test All primary tubes showing any amount of gas or acid within 24–48 h of incubation were submitted to the confirmed phase. Primary tubes (positive) were gently shaken to re-suspend the organism. The EMB agar plate was inoculated with the positive culture with a sterile inoculating loop. The plate was incubated for 24–48 h at 37 °C in the inverted position.
- III. Completed test To establish definitely the presence of coliform bacteria and to provide quality control data, the completed test was used on all positive confirmed cases. The lactose-fermentation broth tube was inoculated with the isolated colony from an agar plate using an inoculated loop. The nutrient agar was streaked with the colony from an agar plate with an inoculated loop. The organism on the nutrient agar was tested for gram stain, and subjected to biochemical tests, and identified as coliform [21].

The culture media used in this study were from Hi-Media Laboratories Pvt. Limited, Bombay, India. All compositions are given in grams per liter, at 25 °C temperature and made as by the company procedure. Preparations of Eosin Methylene Blue (HiMedia, M317) and Nutrient Agar (HiMedia, MN012) have been used in the study.

## 2.3. Antimicrobial Susceptibility Testing

Susceptibility tests of the bacterial isolate (*E. coli*) towards Gentamycin, Chloramphenicol, Amoxicillin/Clavulanate, Ciprofloxacin, Tetracycline, Cotrimoxazole, Ceftriaxone, Cefotaxime, Ceftazidime antibiotics were performed by the modified Kirby–Bauer disc diffusion method and Mueller Hinton Agar. *E. coli* (ATCC 25922) was used in this study.

## 2.4. Phenotypic Characterization of the ESBL Producers

The *E. coli* isolates were screened for possible ESBL production using Ceftazidime (30 µg) and Cefotaxime (30 µg). The suspected ESBL-producing *E. coli* were subjected to Combined Disk (CD) assay using Ceftazidime (30 µg), Cefotaxime (30 µg), Ceftazidime plus Clavulanic acid (30/10 µg), and Cefotaxime plus Clavulanic acid (30/10 µg) for phenotypic confirmation.

### 2.5. Molecular Characterization of *Bla*<sub>TEM</sub> and *Bla*<sub>CTX-M</sub> Genes

The plasmid DNA was extracted from phenotypically confirmed ESBL-producing *E. coli* by the alkaline lysis method followed by the phenol: chloroform purification method [22]. A conventional linear PCR was used to amplify the *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes in the extracted plasmid DNA. The *bla*<sub>TEM</sub> gene was amplified by using a primer with forward nucleotide sequence 5'-GAGACAATAAGGGTGGTAAAT-3' and reverse nucleotide sequence 5'-AGAAGTAAGTTGGCAGCAGTG-3'.

Similarly the *bla*<sub>CTX-M</sub> gene was amplified by using a primer with forward nucleotide sequence 5'-TTT GCGATGTGCAGTACCAGTAA-3' and reverse nucleotide sequence 5'-CTCCGCTGCCGGTTTTAT-3'. The master mix containing 200 µM of dNTPs, 0.5 U/µL of Taq polymerase in 1X PCR buffer and 25 mM MgCl<sub>2</sub> from Qiagen was used.

The PCR was carried out in 25 µL volume, which was prepared by mixing the 13 µL of the master mix, 8 µL of the double-distilled water, 0.5 µL each of the forward and reverse primer and 3 µL of the template DNA. Amplification reactions were carried out using the reaction conditions: initial denaturation at 95 °C for 15 min; denaturation at 94 °C for 45 s, annealing at 55 °C for the *bla*<sub>TEM</sub> genes and 56 °C for the *bla*<sub>CTX-M</sub> gene for 30 s, extension at 72 °C for 3 min repeated for 35 cycles and final extension at 72 °C for 2 min. The PCR products were stained with the ethidium bromide solution and analyzed by the gel electrophoresis in 1.5% agarose gels in Tris–Acetate–EDTA buffer and then visualized by the UV-trans illuminator.

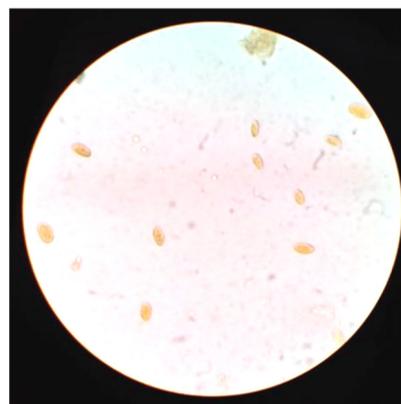
### 2.6. Data Analysis

All the data were analyzed by the Statistical Package for Social Science (SPSS) software (Version 22.0, IBM, New York, NY, USA) and MS Excel (Version 10, Microsoft, Washington, DC, USA).

## 3. Results

### 3.1. Distribution of Intestinal Parasitic Contamination

The prevalence of *Giardia* cysts was found highest (100%) followed by *Entamoeba histolytica* (24%), *Entamoeba coli* (1%) and Hookworm (1%). *Giardia* cysts stained with Lugol's iodine under microscope is shown in Figure 1.



**Figure 1.** *Giardia* cysts stained with Lugol's iodine under microscope (40×).

### 3.2. Parasitic Contamination in Vegetable Sample

The parasites detected in different samples were *Giardia* cysts, *Entamoeba histolytica*, *Entamoeba coli* and Hookworm. Among the positive samples for parasites, prevalence of *Giardia* was found to be highest and Hookworm and *Entamoeba coli* were lowest. Coriander and lettuce were found to be contaminated with multiparasite.

### 3.3. Distribution of Parasites According to Districts in Vegetable Sample

Every examined sample from Kathmandu, Lalitpur and Bhaktapur was contaminated with a high percentage of *Giardia* cysts (100%). Maximum parasitic contamination from *Giardia*, *Entamoeba coli* and Hookworm has been detected in the Kathmandu district (75.47%, 22.64%, and 1.89%). Neither of the samples taken from the Lalitpur district showed any contamination with *Entamoeba coli* and Hookworm.

### 3.4. Multiple Parasites Contamination in Vegetables

Polyparasitic contamination was observed in green raw vegetables examined in this study. Single parasitic contamination was detected in 90% of both the Capsicum and Lettuce samples. Two species of parasites were mainly found in 40% of cabbage and coriander samples. Lettuce showed contamination with three different types of parasites, namely *Giardia* spp. *Entamoeba histolytica* and *Entamoeba coli*.

### 3.5. Coliform Bacteria in Fresh Vegetables, Five Type of Sample

Five different types of raw vegetable were tested for the presence of Coliform bacteria. Coliforms were isolated in 100% of the samples analyzed. The isolates identified were *E. coli* (*n*:57), *Citrobacter* spp. (*n*:42), *Enterobacter* spp. (*n*:4) and *Klebsiella* spp. (*n*:75). As shown in Table 1, in some cases (64 out of 100), the bacterial burden (NMP) of the samples was over the detection limit of the method (<2400 coliforms). Lettuce were the vegetables where (16 out of 20) samples showed the detection limit of MPN (>2400 coliforms/g).

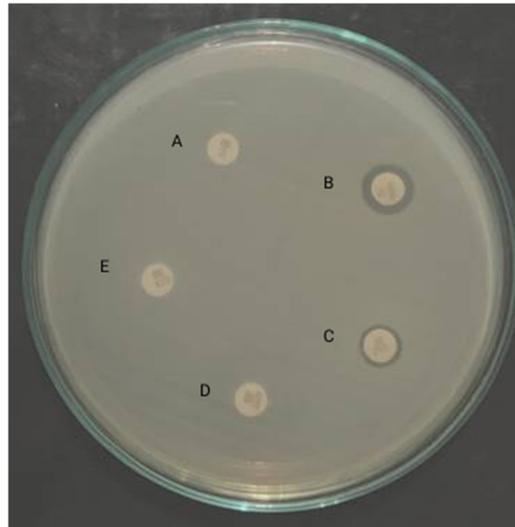
**Table 1.** Most Probable Number (MPN) table of Coliform bacteria isolated from raw vegetable samples from the Kathmandu Valley.

Sample	MPN	Bacterial Species			
<b>Cabbage (n:20)</b>	>2400 (14/20)	<i>Citrobacter</i> (9/20)	<i>E. coli</i> (7/20)	<i>Klebsiella</i> spp. (9/20)	
	1100 (4/20)	<i>Citrobacter</i> (1/20)	<i>E. coli</i> (3/20)	<i>Klebsiella</i> spp. (2/20)	
	210 (1/20)	<i>Citrobacter</i> (1/20)		<i>Klebsiella</i> spp. (1/20)	
	53 (1/20)		<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	
<b>Carrot (n:20)</b>	>2400 (10/20)	<i>Citrobacter</i> (6/20)	<i>E. coli</i> (6/20)	<i>Klebsiella</i> spp. (10/20)	
	1100 (5/20)	<i>Citrobacter</i> (1/20)	<i>E. coli</i> (2/20)	<i>Klebsiella</i> spp. (3/20)	
	460 (1/20)	<i>Citrobacter</i> (1/20)			
	290 (1/20)	<i>Citrobacter</i> (1/20)		<i>Klebsiella</i> spp. (1/20)	
	210 (1/20)			<i>Klebsiella</i> spp. (1/20)	
	150 (1/20)	<i>Citrobacter</i> (1/20)		<i>Klebsiella</i> spp. (1/20)	
<b>Cori-ander (n:20)</b>	>2400 (11/20)	<i>Citrobacter</i> (7/20)	<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	
	1100 (6/20)	<i>Citrobacter</i> (1/20)	<i>E. coli</i> (4/20)	<i>Klebsiella</i> spp. (8/20)	<i>E. cloacae</i> (1/20)
	93 (1/20)		<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	
	15 (1/20)		<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	
	6 (1/20)			<i>Klebsiella</i> spp. (1/20)	
<b>Capcicum (n:20)</b>	>2400 (13/20)	<i>Citrobacter</i> (6/20)	<i>E. coli</i> (7/20)	<i>Klebsiella</i> spp. (9/20)	<i>E. cloacae</i> (2/20)
	1100 (3/20)		<i>E. coli</i> (2/20)	<i>Klebsiella</i> spp. (2/20)	
	460 (1/20)		<i>E. coli</i> ((1/20)		
	240 (2/20)		<i>E. coli</i> (2/20)	<i>Klebsiella</i> spp. (1/20)	
	210 (1/20)	<i>Citrobacter</i> (1/20)	<i>E. coli</i> ((1/20)		
<b>Lettuce (n:20)</b>	>2400 (16/20)	<i>Citrobacter</i> (5/20)	<i>E. coli</i> (10/20)	<i>Klebsiella</i> spp. (14/20)	<i>E. cloacae</i> (1/20)
	1100 (1/20)		<i>E. coli</i> (1/20)		
	460 (1/20)		<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	
	29 (1/20)	<i>Citrobacter</i> (1/20)	<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	
	28 (1/20)		<i>E. coli</i> (1/20)	<i>Klebsiella</i> spp. (1/20)	

The bacterial isolates isolated within the limit (>2400 coliforms/g) for lettuce are *Citrobacter* spp. (5/20), *E. coli* (10/20), *Klebsiella* spp. (14/20), *Enterobacter cloacae* (1/20).

### 3.6. Antibiotic Susceptibility Pattern of *E. coli*

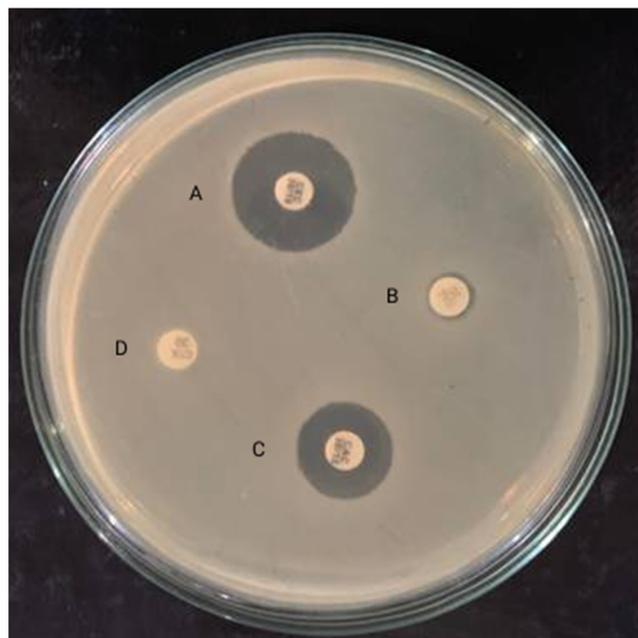
A total of 57 *Escherichia coli* isolates were subjected to AST. In the AST pattern of *E. coli* isolates, Gentamycin (GEN) and Ceftriaxone (CTR) showed maximum sensitive results compared to other antibiotics i.e., (96.50%), whereas 94.74% of total isolates showed resistance to Amoxicillin/clavulanic acid (AMC), being the most resistant of all antibiotics used for the study (Figure 2).



**Figure 2.** Antibiotic susceptibility pattern for *E. coli* (A): Gentamycin; (B): Ceftazidime; (C): Cotrimoxazole; (D): Cefotaxime; (E): Chloramphenicol (Resistant to all antibiotics).

### 3.7. ESBL-Producing MDR *E. coli*

Out of 57 *E. coli*, 57.89% isolates were MDR, all of which were screened as suspected ESBL producers. Among these 33 ESBL suspected cases, the total number of ESBL confirmed cases were ( $n = 4$ ) (Table 2). Phenotypic confirmation of ESBL was performed by Combined Disk (CD) test method (Figure 3).



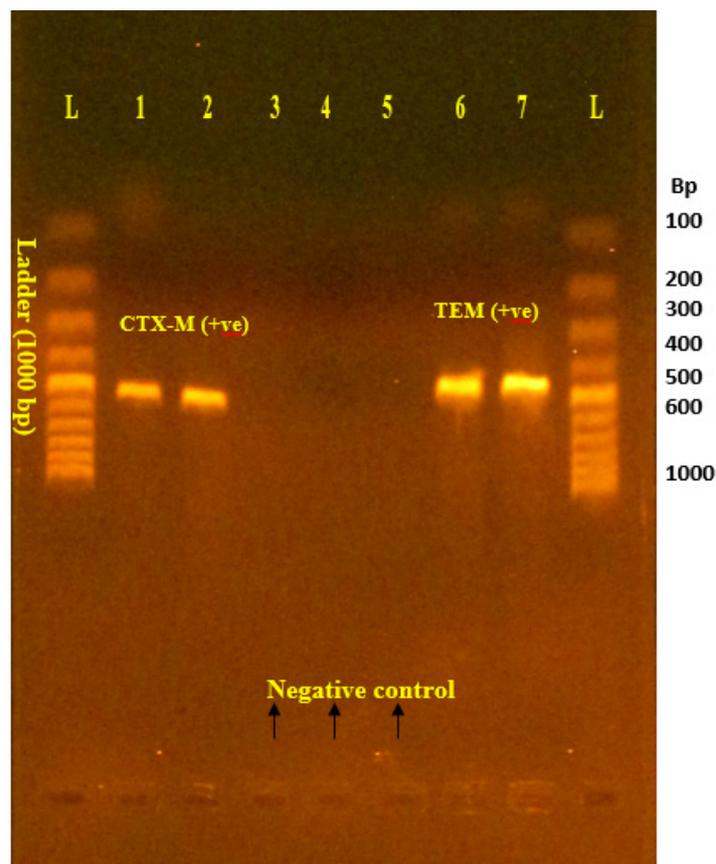
**Figure 3.** Phenotypic confirmation of ESBL by Combined Disk (CD) test method. (A): Ceftazidime; (B): Ceftazidime + clavulanic acid; (C): Cefotaxime; (D): Cefotaxime + clavulanic acid.

**Table 2.** Multi-drug resistant (MDR) *E. coli*-producing Extended Spectrum Beta Lactamase (ESBL).

Total <i>E. coli</i> Isolates (%)	No. of MDR Strains (%)	No. of Suspected ESBL Producers (%)	No. of Confirmed ESBL Producers (%)
57	33	10	4

### 3.8. Detection of ESBL Genes

Out of the four phenotypically confirmed ESBL-producing isolates of *E. coli*, the *bla*<sub>TEM</sub> gene was detected in two isolates during the amplification process by PCR. Similarly, the *bla*<sub>CTX-M</sub> gene was detected in two isolates (Figure 4).



**Figure 4.** Electrophoresis of Polymerase Chain Reaction (PCR) products of the *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes of *E. coli* on agarose gel.

Both the *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes were detected in one isolate only. One strain harboured only the *bla*<sub>TEM</sub> gene and one isolate harboured only the *bla*<sub>CTX-M</sub> gene. Both the genes were absent in one isolate (Table 3).

**Table 3.** Distribution of the *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes among *E. coli*

ESBL Genotypes	TEM	CTX-M
Positive	2	2
Negative	2	2
Total	4	4

Lane Lis DNA ladder. Lane 1 and 2 are bands of positive PCR products of the *bla*<sub>CTX-M</sub> gene (521 bp). Lane 6 and 7 are bands of positive PCR products of the *bla*<sub>TEM</sub> gene (459 bp). Lanes 3–5 are the Negative control.

#### 4. Discussion

The study showed high prevalence of *Giardia* cysts (100%) followed by *Entamoeba histolytica* (24%), *Entamoeba coli* (1%), and Hookworm (1%). Eraky et al. [23] also reported that *Giardia lamblia* cysts were the most prevalent parasite followed by *Entamoeba histolytica* cysts. During the study period, intense rainfall had taken place causing the Hanumante river to swell up and flood. Places like Jagati, Barahasthan, Radhe-Radhe and Thimi, from where almost most of vegetables are supplied to Kathmandu Valley, were engulfed in flood. Due to the flood, transportation was disturbed and vegetables from the Bhaktapur district were distributed to different outlets in an unhygienic condition. Kathmandu also had to face vegetable shortages at that period of time.

The broad range in prevalence could be attributed to many factors. These may include geographical location, type and number of samples examined, methods used for detection of the intestinal parasites, type of water used for irrigation, and post-harvesting handling methods of such vegetables, which are different from one country to another. Other factors that can affect parasitic transmission may also include population-related hygienic habits, sanitary facilities, climatic conditions, and a range of food-borne parasites native in certain countries [23]. Although contamination of vegetables may occur in a variety of ways, it is mainly associated with the water used for irrigation. The use of sewage water plays an important role in the epidemiology of transmission of parasitic diseases to humans through consuming of such vegetables [24].

The study has demonstrated the contamination of vegetables with *Giardia* cysts, *E. histolytica* cysts, *E. coli* cyst and Hookworm. These parasites are considered as pathogenic agents for man and the consumption or manipulation of such contaminated agricultural crops is considered unsafe and might constitute a risk for farmers and the whole population [25]. The presence of the *Entamoeba* spp. in the vegetable samples could be due to inappropriate agricultural practices during cultivation, with cultivated vegetables coming into direct contact with soil and water that is contaminated with human and animal faeces [26]. In this study, Cabbage and Coriander showed maximum contamination with intestinal parasites. *Giardia* spp. (71.43%) was present in both samples. A total of 28 isolates was found in Cabbage with *Giardia* spp. (71.43%) and 28.57% of *Entamoeba histolytica*. The least occurring parasite was found to be hookworm (3.57%). This result contradicts with [27] (Brooker et al., 2004), where Hookworm (*Ankylostoma deudonale*, *Necatrus americanis*) was the second most abundant (10.8%) parasite in the vegetables examined. Cabbage, lettuce and other green leafy vegetables had uneven surfaces that make parasitic eggs, cysts and larvae attach to their surface more easily, when washed with contaminated water either in the farm or market [28,29].

Isolation of more than one parasite per sample in this work reflects the possibility of more than one fecal contamination of vegetables, which most probably results in multiple parasitic infections in people. The high occurrence of these parasites reflects a high level of contamination and persistence of human infection. The study showed that 24% of the total sample were contaminated with two species of parasites while only 1% of the sample with three species of parasites. The result obtained by Tefera et al. [30] showed that 37.5% of the total samples were contaminated with two species of parasites, while 6.25% of the samples with three species of parasites and quadruple parasitic contamination was observed in two samples.

The high percentage of incidence of *Klebsiella* spp. and *E. coli* in raw vegetable samples may be due to one of these factors; Fecal contamination from animal manures, irrigated water, cross contamination by food handlers through poor hand washing, or contamination of utensils. Coliform were isolated in 100% out of 100 samples analyzed. The identified species included *Klebsiella* spp., *E. coli*, *Citrobacter* spp. and *Enterobacter cloacae*. A case was found where (64 out of 100) the bacterial burden (MPN) of the samples was over the detection limit of the method (>2400 Coliforms/g). Lettuce were the vegetables

most contaminated, followed by the Coriander, Carrot, Cabbage and Capsicum. *E. coli*-positive samples (34/57) had total coliform counts of  $\geq 2400$  MPN/g, and *E. coli*-positive samples (12/57) had total coliform counts  $\geq 1100$  MPN/g. Similar findings were reported by Valentin-bon et al. [31]. The presence of coliforms is often associated with foods grown close to the ground or human handling during harvesting and processing. *Escherichia coli* is commonly present in the gastrointestinal tract of warm-blooded animals and is used as an indicator of faecal contamination [32]. Consumers, in turn, cannot visually assess all safety aspects when they purchase food. Bacteriological contamination levels are invisible and can only be determined by laboratory testing.

In this study, 9 common antibiotics were used for a total of 57 isolates for Antibiotic Susceptibility testing. Among 57 total isolates of *E. coli*, 55 isolates were sensitive to Gentamicin (GEN) i.e., 96.50% and Ceftriaxone (CTR) i.e., 55 (96.50%), except amoxicillin/clavulanic acid (AMC), which shown most resistance to the organism 54 (94.74%). A study by Hassan et al. (2011) showed that the isolates exhibited resistance in decreasing order for aminoglycosides (21.9%), tetracycline (17.2%), amoxicillin-clavulanic acid (13.3%), and chloramphenicol (7.8%). According to the report of Kwaku et al. (2016), of a study conducted in Ghana, 54.5% of *E. coli* isolates were found to be resistant to Tetracycline, Cefotaxime and Ceftriaxone, 27.3% were resistant to Cotrimoxazole, 18.2% were resistant to Gentamycin. Similarly, several studies have documented the drug-resistant *E. coli* and other coliforms in vegetables [33]. Fresh raw vegetables may explain this anomaly, as epiphytic bacteria may develop antibiotic resistances as a consequence of the large amount of antibiotics used in agriculture, and also treating soil with organic fertilizers, such as sewage sludge and manure, and contaminated irrigation water, may lead to vegetable contaminations with resistant bacteria from animal origins and/or human sources [34].

Similarly, Antibiotic susceptibility patterns of ESBL *E. coli* showed 100% resistance to amoxicillin/clavulanate, cefotaxime and ceftazidime. The study showed 33 Multidrug resistance strains out of which 4 were confirmed as ESBL producers. Rasheed et al. [34] also found two ESBL producers from vegetable. The excessive and inappropriate use of the antibiotics, particularly the broad spectrum antibiotics prescribed empirically, has led to the emergence of MDR strains [22]. A potential health hazard to consumers can be expected from resistant bacteria. If the organism is resistant to antibiotics, then initial treatment may be ineffective both in man and animals and an alternative treatment would need to be applied [35].

The Non-ESBL *E. coli* isolates ( $n = 53$ ) showed high resistance to amoxicillin/clavulanate (94.34%), tetracycline (69.81%) and cotrimoxazole (47.16%), whereas the ESBL *E. coli* ( $n = 4$ ) showed 100% resistance to amoxicillin/clavulanate, cefotaxime and ceftazidime, and showed 25% resistance to tetracycline, cefotaxime, ceftazidime. Not many studies have investigated the presence of ESBL-producing *E. coli* on vegetables and among them, only few have found and described in detail ESBL-producing *E. coli* [16,17,36] or have identified leafy salads or sprouts as a source [16,17].

A total of 33 MDR *E. coli* were detected, out of which 10 were suspected as ESBL producers and 4 were confirmed as ESBL producers. Similar multi resistance phenotypes of bacteria populating fresh vegetables have been reported worldwide [33].

The study confirmed one strain showing both the *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes. The presence of these genes is also favoured by other studies. The study conducted by Zurfluh [37] showed that, of the 26 *E. coli* isolates, 17 (65.8%) *E. coli* strains produced CTX-M group 1 ESBLs and 8 (30.8%) produced CTX-M group 9 ESBLs. Ten (38.5%) harbored *bla*<sub>CTX-M-15</sub>, six (23%) *bla*<sub>CTX-M-55</sub>, five (19.2%) *bla*<sub>CTX-M-14</sub>, and three (11.5%) *bla*<sub>CTX-M-65</sub>. One isolate (3.8%) tested positive for *bla*<sub>CTX-M-1</sub>, and one (3.8%) harbored SHV-12. Another study by Reuland et al. [38] showed that four of the 15 vegetable types were contaminated with ESBL-E. Seven samples (6%) yielded ESBL-E. Three *bla*<sub>CTX-M-15</sub>, one *bla*<sub>CTX-M-1</sub>, two genes of the CTX-M-9 group and one SHV ESBL-encoding gene were found. The ESBL genes were similar to what is found in enterobacterial strains from human origin. Therefore, raw vegetables might be a source of resistance genes for the enterobacterial strains found in humans. Different pathways might be relevant to explain why these resistance genes were found on raw vegetables [34,39]. Knapp et al. (2010) showed that the levels of antibiotic

resistance genes in soil have increased considerably over the past 70 years in the Netherlands [40]. Other reservoirs of antibiotic resistance genes are the aquatic system and sewage, created by antibiotic use and waste disposal [41]. Fresh produce can also become contaminated during processing with the loss of valuable nutrients like flavonoids [42–46].

## 5. Conclusions and Recommendations

### 5.1. Conclusion

Out of 100 samples, almost all samples were heavily contaminated with high numbers of coliform bacteria and parasites. Parasites isolated were *Giardia* cysts, *Entamoeba histolytica*, *Entamoeba coli* and Hookworm. Prevalence of *Giardia* cysts was highest among the detected parasites. Coliform detected include *Citrobacter* spp., *Klebsiella* spp., *E. coli* and *Enterobacter cloacae*. The occurrence of ESBL among *E. coli* isolates was found. The *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes in the ESBL *E. coli* were found in equal proportion.

### 5.2. Recommendations

High rates of contamination with parasites and coliform bacteria were found in the raw vegetable samples, so good agricultural practice should be reviewed and applied in every agricultural farm, and health authorities should focus on implementing legislation that forbids irrigation with untreated sewage water of both root and leafy vegetables. This type of study, at a large scale, can reflect the overall health risks to people from the consumption of vegetables, and compel the authorities to make policies on vegetable markets.

Thorough washing and disinfection of raw vegetables is highly recommended prior to selling and consumption. Moreover, consumption of vegetables after peeling can reduce the risk of parasitic infections. Proactive and practical education programs are needed at all steps in the process, i.e., from farm to fork. Since Multi Drug Resistance organisms are seen from the tested vegetable samples, there may be contamination from industrial effluents and waste disposal from the hospital. So, this kind of contamination should be avoided and the fresh produce should be handled with care.

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## References

1. Amoah, P.; Drechsel, P.; Abaidoo, R.C.; Klutse, A. Effectiveness of common and improved sanitary washing methods in selected cities of West Africa for the reduction of coliform bacteria and helminth eggs on vegetables. *Trop. Med. Int. Health* **2007**, *12*, 40–50. [[CrossRef](#)] [[PubMed](#)]
2. Hamilton-Miller, J.M. Identity and antibiotic susceptibility of enterobacterial flora of salad vegetables. *Int. J. Antimicrob. Agents* **2001**, *18*, 81–83. [[CrossRef](#)]
3. Holvoet, K.; Sampers, I.; Callens, B.; Dewulf, J. Moderate Prevalence of antimicrobial resistance in *Escherichia coli* isolates from lettuce, irrigation water, and soil. *Appl. Environ. Microbiol.* **2013**, *79*, 6677–6683. [[CrossRef](#)] [[PubMed](#)]
4. Beuchat, L.R. Pathogenic microorganisms associated with fresh produce. *J. Food Prot.* **1996**, *59*, 204–216. [[CrossRef](#)] [[PubMed](#)]

5. Bekele, F.; Tefera, T.; Biresaw, G.; Yohannes, T. Parasitic contamination of raw vegetables and fruits collected from selected local markets in Arba Minch town, Southern Ethiopia. *Infect. Dis. Poverty* **2017**, *6*, 1–7. [[CrossRef](#)]
6. Alhabbal, A.T. The prevalence of parasitic contamination on common sold vegetables in alqalamoun region. *Int. J. Pharm. Sci. Rev. Res.* **2016**, *30*, 94–97.
7. Olyaei, A.; Hajivandi, L. Parasitological contamination of markets and farms in vegetables consumed in Southern Iran. *Glob. Vet.* **2013**, *10*, 327–331.
8. Orok, A.; Ajibaye, O.; Babasola, A.J.; Cheng, P. IPs in archetypal African urban slum in Nigeria. *J. Microbiol. Immunol. Infect.* **2016**, *52*, 106–113.
9. Yusof, A.M.; Mohammad, M.; Abdullahi, M.A.; Mohamed, Z.; Zakaria, R. Occurrence of intestinal parasitic contamination in select consumed local raw vegetables and fruits in Kuantan, Pahang. *Trop. Life Sci. Res.* **2017**, *28*, 23–32. [[CrossRef](#)]
10. Aarestrup, F.M.; Wegener, H.C.; Collignon, P. Resistance in bacteria of the food chain: Epidemiology and control strategies. *Expert Rev. Anti-Infect. Ther.* **2008**, *6*, 733–750. [[CrossRef](#)]
11. Van Den Bogaard, A.E.; Stobberingh, E.E. Antibiotic usage in animals impact on bacterial resistance and public health. *Drugs* **1999**, *58*, 589–607. [[CrossRef](#)] [[PubMed](#)]
12. Wooldridge, M. Evidence for the circulation of antimicrobial-resistant strains and genes in nature and especially between humans and animals and transmission of antibiotic. *Rev. Sci. Tech.* **2012**, *31*, 231–247. [[CrossRef](#)]
13. Cantón, R.; González-Alba, J.M.; Galán, J.C. CTX-M enzymes: Origin and diffusion. *Front. Microbiol.* **2012**, *3*, 110. [[CrossRef](#)]
14. Naseer, U.; Sundsfjord, A. The CTX-M conundrum: Dissemination of plasmids and Escherichia coli clones. *Microb. Drug Resist.* **2011**, *17*, 83–97. [[CrossRef](#)]
15. Michael, G.B.; Freitag, C.; Wendlandt, S.; Eidam, C.; Feßler, A.T.; Lopes, G.V.; Kadlec, K.; Schwarz, S. Emerging issues in antimicrobial resistance of bacteria from food-producing animals. *Future Microbiol.* **2015**, *10*, 427–443. [[CrossRef](#)] [[PubMed](#)]
16. Egea, P.; Navarro, M.D.; Pascual, A. Assessment of the presence of extended-spectrum beta-lactamase-producing Escherichia coli in eggshells and ready-to-eat products. *Eur. J. Clin. Microbiol. Inf. Dis.* **2011**, *30*, 1045–1047. [[CrossRef](#)] [[PubMed](#)]
17. Kim, H.; Chon, J.; Kim, Y.; Kim, D.; Kim, M.; Seo, K. International Journal of Food Microbiology Prevalence and characterization of extended-spectrum- $\beta$ -lactamase-producing Escherichia coli and Klebsiella pneumoniae in ready-to-eat vegetables. *Int. J. Food Microbiol.* **2015**, *207*, 83–86. [[CrossRef](#)] [[PubMed](#)]
18. Bailenger, J. Mechanisms of parasitological concentration in coprology and their practical consequences. *J. Am. Med. Technol.* **1979**, *41*, 65–71.
19. Fotedar, R.; Stark, D.; Beebe, N.; Marriott, D.; Ellis, J.; Harkness, J. Laboratory diagnostic techniques for Entamoeba species. *Clin. Microbiol. Rev.* **2007**, *20*, 511–532. [[CrossRef](#)]
20. Rompré, A.; Servais, P.; Baudart, J.; de-Roubin, M.R.; Laurent, P. Detection and enumeration of coliforms in drinking water: Current methods and emerging approaches. *J. Microbiol. Methods.* **2002**, *49*, 31–54. [[CrossRef](#)]
21. Falomir, M.P.; Gozalbo, D.; Rico, H. Coliform bacteria in fresh vegetables: From cultivated lands to consumers. *Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol.* **2010**, *2*, 1175–1181.
22. Thapa, P.; Parajuli, K.; Poudel, A.; Thapa, A.; Manandhar, B.; Laudari, D.; Malla, H.; Katiwada, R. Causative agents and susceptibility of antimicrobials among suspected females with urinary tract infection in tertiary care hospitals of Western Nepal. *J. Chitwan. Med. Coll.* **2013**, *3*, 16–19. [[CrossRef](#)]
23. Eraky, M.A.; Rashed, S.M.; Nasr, M.E.S.; El-Hamshary, A.M.S.; Salah El-Ghannam, A. Parasitic contamination of commonly consumed fresh leafy vegetables in Benha, Egypt. *J. Parasitol. Res.* **2014**, *2014*, 613960. [[CrossRef](#)] [[PubMed](#)]
24. Gupta, N.; Khan, D.K.; Santra, S.C. Prevalence of intestinal helminth eggs on vegetables grown in wastewater-irrigated areas of Titagarh, West Bengal, India. *Food Control* **2009**, *20*, 942–945. [[CrossRef](#)]
25. Amahmid, O.; Asmama, S.; Bouhoum, K. The effect of waste water reuse in irrigation on the contamination level of food crops by Giardia Giardia cysts and Ascaris Ascaris eggs. *Int. J. Food Microbiol.* **1999**, *49*, 19–26. [[CrossRef](#)]

26. Silva, S.; Maldonade, I.R.; Ginani, V.C.; Lima, S.A.; Mendes, V.S.; Lidiane, M.; Azevedo, X. Short Communication Detection of intestinal parasites on field-grown strawberries in the Federal District of Brazil. *J. Braz. Soc. Trop. Med.* **2014**, *47*, 801–805. [[CrossRef](#)]
27. Brooker, S.; Bethony, J.; Hotez, P.J. Human hookworm infection in the 21st century. *Adv Parasitol.* **2004**, *58*, 197–288.
28. Amoah, P.; Drechsel, P.; Abaidoo, R.C.; Ntow, W.J. Pesticide and pathogen contamination of vegetables in Ghana's urban markets. *Arch. Environ. Contam. Toxicol.* **2006**, *50*, 1–6. [[CrossRef](#)]
29. Avcioglu, H.; Soykan, E.; Tarakci, U. Control of Helminth contamination of raw vegetables by washing. *Vector Borne Zoonotic Dis.* **2011**, *11*, 189–191. [[CrossRef](#)]
30. Tefera, T.; Biruksew, A.; Mekonnen, Z.; Eshetu, T. Parasitic contamination of fruits and vegetables collected from selected local markets of Jimma Town, Southwest Ethiopia. *Int. Sch. Res. Not.* **2014**, *2014*, 1–7. [[CrossRef](#)]
31. Valentin-bon, I.; Jacobson, A.; Monday, S.R.; Feng, P.C.H. Microbiological quality of bagged cut spinach and lettuce mixes. *Appl. Environ. Microbiol.* **2008**, *74*, 1240–1242. [[CrossRef](#)] [[PubMed](#)]
32. Oliver, D.M.; van Niekerk, M.; Kay, D.; Heathwaite, A.L.; Porter, J.; Fleming, L.E.; Kinzelman, J.L.; Connolly, E.; Cummins, A.; McPhail, C.; et al. Opportunities and limitations of molecular methods for quantifying microbial compliance parameters in EU bathing waters. *Environ. Int.* **2014**, *64*, 124–128. [[CrossRef](#)] [[PubMed](#)]
33. Pensala, O.; Heleniusec, H.; Huovinen, P.; Osterblad, M.; Peterzéns, M. Antimicrobial susceptibility of Enterobacteriaceae isolated from vegetables. *J. Antimicrob. Chemother.* **1999**, *43*, 503–509.
34. Davies, J.; Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **2010**, *74*, 417–433. [[CrossRef](#)] [[PubMed](#)]
35. Rasheed, M.U.; Thajuddin, N.; Ahamed, P.; Teklemariam, Z.; Jamil, K. Resistência microbiana a drogas em linhagens de Escherichia coli isoladas de fontes alimentares. *Rev. Inst. Med. Trop. Sao Paulo* **2014**, *56*, 341–346. [[CrossRef](#)]
36. Mesa, R.J.; Blanc, V.; Blanch, A.R.; Cortés, P.; González, J.J.; Lavilla, S.; Miró, E.; Muniesa, M.; Saco, M.; Tórtola, M.T.; et al. Extended-spectrum  $\beta$ -lactamase-producing Enterobacteriaceae in different environments (humans, food, animal farms, and sewage). *J. Antimicrob. Chemother.* **2006**, *58*, 211–215. [[CrossRef](#)]
37. Zurfluh, K.; Nüesch-Inderbinen, M.; Morach, M.; Hächler, H.; Stephan, R. Extended-Spectrum-B-Lactamase-Producing Enterobacteriaceae Isolated from Vegetables Imported from the Dominican Republic, India, Thailand, and Vietnam. *Appl. Environ. Microbiol.* **2015**, *81*, 3115–3120. [[CrossRef](#)]
38. Reuland, E.A.; al Naiemi, N.; Raadsen, S.A.; Savelkoul, P.H.M.; Kluytmans, J.A.J.W.; Vandembroucke-Grauls, C.M.J.E. Prevalence of ESBL-producing Enterobacteriaceae in raw vegetables. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 1843–1846. [[CrossRef](#)]
39. Heuer, H.; Schmitt, H.; Smalla, K. Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* **2011**, *14*, 236–243. [[CrossRef](#)]
40. Knapp, E.T. Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940. *Environ. Sci. Technol.* **2010**, *44*, 580–587. [[CrossRef](#)]
41. Hoogenboom, L.A.P.; Bokhorst, J.G.; Northolt, M.D.; van de Vijver, L.P.L. Contaminants and microorganisms in Dutch organic food products: A comparison with conventional products. *Food Addit. Contam. Part A* **2008**, *25*, 1195–1207. [[CrossRef](#)] [[PubMed](#)]
42. Aryal, S.; Baniya, M.K.; Danekhu, K.; Kunwar, P.; Gurung, R.; Koirala, N. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants* **2019**, *8*, 96. [[CrossRef](#)] [[PubMed](#)]
43. Koirala, N.; Dhakal, C.; Munankarmi, N.N.; Ali, S.W.; Hameed, A.; Martins, N.; Sharifi-Rad, J.; Imran, M.; Arif, A.M.; Hanif, M.S.; et al. Vitex negundo Linn.: Phytochemical composition, Nutritional Analysis, and Antioxidant and Antimicrobial Activity. *Cell Mol. Biol.* **2020**, *66*, 1–7. [[CrossRef](#)] [[PubMed](#)]
44. Panthi, M.; Subba, R.K.; Raut, B.; Khanal, D.P.; Koirala, N. Bioactivity evaluations of leaf extract fractions from young barley grass and correlation with their phytochemical profiles. *BMC Complement. Med. Ther.* **2020**, *20*, 64. [[CrossRef](#)] [[PubMed](#)]

45. Schwaiger, K.; Helmke, K.; Hölzel, C.S.; Bauer, J. Antibiotic resistance in bacteria isolated from vegetables with regards to the marketing stage (farm vs. supermarket ). *Int. J. Food Microbiol.* **2011**, *148*, 191–196. [[CrossRef](#)] [[PubMed](#)]
46. Upadhyaya, J.; Raut, J.K.; Koirala, N. Analysis of nutritional and nutraceutical properties of wild-grown mushrooms of Nepal. *EC Microbiol.* **2017**, *12*, 136–145.



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