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Anticipatory surveillance for detection of *Escherichia coli* from fresh fruits and vegetables using DuPont BAX System

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Abstract

Escherichia coli is a foodborne pathogen that causes severe illnesses in humans worldwide. Cows are a source of *E. coli* O157:H7, and raw products contaminated with cattle faeces are typical carriers of the pathogen. The consumption of unhygienic fresh fruits and vegetables provides a potential risk factor for microbial contamination-related infections. In this context, the present work was performed to investigate the prevalence rate of *E. coli* in fresh fruits and vegetables that were commonly distributed and consumed in Zakho City, Iraq. In addition, the present work also aimed to detect the incidence rate of pathogenic strain *E. coli* O157:H7, thereafter suggesting the best and most efficient sanitiser for the decontamination of fruits and vegetables. A total of 172 samples, comprising various types of fruits and vegetables, were randomly collected for this cross-sectional study from retail markets in Zakho City. A number of laboratory tests, including DuPont BAX System PCR, microbiological, and biochemical-based techniques were performed for the isolation and identification of *E. coli* microbial contamination. Out of the 172 samples collected, 32 (18.6%) tested positive for *E. coli* using traditional enrichment and selective media. The DuPont BAX system technique confirmed that all isolates were *E. coli*, and none of the isolates were identified as *E. coli* O157:H7 strain. Furthermore, peracetic acid was found to be a more effective sanitiser than aqueous chlorine for cleaning leafy green vegetables. Despite the absence of the pathogenic strain *E. coli* O157:H7, the present work highlighted the potential health risk to the community due to *E. coli* contamination of leafy green vegetables.

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Introduction

Escherichia coli is a typical microorganism found in both human and animal digestive tracts. Most strains of *E. coli* do not harm people; however, certain strains can result in diarrhoea or more severe illnesses. According to Erickson *et al*. (2019), Shiga toxin-producing *E. coli* O157:H7 has evolved into a significant water- and food-borne pathogen, affecting people and leading to conditions such as haemorrhagic colitis, diarrhoea, acute renal failure, and haemolytic uremic syndrome (HUS). As reported by Ameer *et al*. (2022), the pathogenic *E. coli* O157:H7 strain contaminates the digestive system, resulting in symptoms such as haemorrhagic diarrhoea and abdominal pain. The faecal-oral mode of transmission of *E. coli* O157:H7 occurs following the ingestion of contaminated, undercooked foods, and even fresh vegetables (Erickson *et al*., 2019). Furthermore, *E. coli* O157:H7 can be transmitted from person to person through faecal shedding, and is responsible for an estimated 11% of illnesses (Thomas and Elliott, 2013).

Enterohaemorrhagic *E. coli* (EHEC) O157:H7 causes illness by producing the Shiga toxin, which can result in various gastrointestinal symptoms, including watery diarrhoea and haemorrhagic colitis. The EHEC O157:H7 serotype has been associated with outbreaks related to various foods, such as spinach leaves and apple juice, as noted by Pennington (2010). Furthermore, the Shiga toxin-producing *E. coli* (STEC) O104:H4 serotype has

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gained significant public attention due to its association with the German outbreak related to sprout consumption, as highlighted by Muniesa *et al.* (2012). Various strains of *E. coli* can lead to acute, potentially fatal illnesses, as well as chronic and lifelong health conditions (Thomas and Elliott, 2013; Atnafie *et al.*, 2017; Erickson *et al.*, 2019).

Today, people are increasingly adopting healthy diets that include fresh fruits and vegetables as they are rich in vitamins and essential nutrients (Liu, 2003). For example, salads, which are made from minimally processed, pre-packaged, and ready-to-eat fruits and vegetables (Everis, 2004) can create favourable conditions for the growth and survival of pathogens, as noted by Heaton and Jones (2008). Recent studies have established a link between dietary supply and the presence of numerous infectious pathogens (Heaton and Jones, 2008; Muniesa *et al.*, 2012).

Pathogens like *E. coli* O157:H7 can infect fruits and vegetables at various stages of their growth and consumption. This can occur due to a range of factors, including soil quality, the presence of manure (both human and animal excrement), water sources, insect interactions, environmental conditions, post-harvest handling practices, washing, cutting, and shipping procedures (Beuchat, 2002). Eating raw and leafy vegetables is a common source of *E. coli* infection, as demonstrated by Heaton and Jones (2008). Outbreaks linked to the leafy greens are common, and can have serious health consequences, impacting inhabitants of several countries (Herman *et al.*, 2015). Diarrheal outbreaks related to STEC are frequently linked to livestock, particularly cattle (Thomas and Elliott, 2013). In feedlot settings, approximately 30% of cattle excrete *E. coli* O157:H7, as reported by Callaway *et al.* (2009). Several previous studies have revealed that the outbreaks related to *E. coli* O157:H7 originate from many different sources including salad sprouts (Fukushima *et al.*, 1999) and green lettuce (Ackers *et al.*, 1998). Another noteworthy example is an outbreak that occurred in Germany in 2011 where over 4,000 cases of severe gastroenteritis with more than 850 cases of HUS, together with 49 fatalities, were caused by the STEC infection epidemic (EFSA, 2011). Therefore, we conducted the present work utilising a novel molecular-based technique, the DuPont BAX system, to identify common strains of *E. coli* in fresh fruits and vegetables available in Zakho City's marketplaces, Iraq.

Materials and methods

Materials

Buffered peptone water (BPW), tryptone bile X-glucuronide agar, and sorbitol MacConkey agar (supplemented with tellurite-cefixime) were purchased from HiMedia laboratories (HiMedia, India). Two different reference strains of *E. coli*, ATCC 13216 and ATCC 43888, were purchased from American Type Culture Collection (ATCC; Manassas, VA, United States). A real-time polymerase chain reaction (PCR) kit for detecting *E. coli* O157:H7, catalogued as KIT2000, was purchased from Hygiena (USA). The remaining chemicals were of analytical grades. Biochemical food systems, reference number 71680, were purchased from Liofilchem (Italy).

Sample preparation

In this cross-sectional study, 172 samples of fresh fruits and vegetables were randomly selected from several local retail markets in Zakho City, Iraq between March and November 2022 for pathogen isolation. The collected samples were promptly placed in an ice box, and transported to the laboratory. The protocols followed were based on the Association of Official Agricultural Chemists (AOAC) and Performance Tested Methods (PTM) number 031002. Additionally, validation studies were conducted in accordance with the International Organization for Standardization (ISO), ISO 16140-2. QUA 18/07-07/10 of Association Française de Normalisation (AFNOR; or French Standardization Association) was also referenced. It is worth noting that the AOAC research institute has validated and approved the BAX System for detecting *E. coli* O157:H7 in spinach and lettuce, and the AFNOR has validated the BAX System for detecting *E. coli* O157:H7 in raw vegetables. However, these methods have not been tested on any kind of fruit to detect such a pathogen.

Sample enrichment

The protocols were following those laid out by AOAC and AFNOR with minor modifications. The samples were handled aseptically, and 25 g of chosen raw fruits and vegetables were measured and coarsely chopped in a stomacher (Stomacher 400 Circulator Lab Blender, Seward, UK). The weighed samples were then placed in a bottle containing 225 mL pre-warmed sterile BPW. Subsequently from this, 1 mL

of the BPW was diluted ten times. The mixture was then incubated at 42°C for 8 h. The procedure was conducted following the ISO 16649-2-2001 method. Next, 1 mL of the test sample was transferred using a sterile pipette into a sterile Petri plate. Then, 15 mL of tryptone bile X-glucuronide (TBX) medium, previously equilibrated in a water bath at 44 - 47°C, was added to the Petri dish. The agar was then allowed to solidify, and subsequently incubated at 44°C for 18 - 24 h. The typical colonies of *E. coli* on the TBX agar plates were subjected to further analysis to identify the *E. coli* O157:H7 strain.

DNA extraction

DNA was isolated from colonies grown on agar plates, and for this purpose, a previously reported protocol was followed with minor modifications (Said-Ahmed *et al.*, 2017). Briefly, a loop of growth colonies from the initial inoculation streak was suspended in 0.5 mL of purified water, and heated for 10 min. The suspension was vortexed, and then centrifuged at 15,000 rpm for 5 min. The supernatant was carefully transferred into a clean fresh tube, and utilised in PCR as a DNA. This step of the PCR was used to identify the Shiga toxin (Stx) and eaeA (intimin, an outer membrane protein) genes for the confirmation of STEC and enteropathogenic *E. coli* (EPEC) strains, respectively.

Polymerase chain reaction

PCR was used to detect and confirm the EPEC isolates. The PCR tablets were hydrated by transferring 30 µL of the previously extracted lysate DNA into each strip containing the tablets of the lyophilised master mix. The tubes were securely tightened by placing new optical caps on top of the strips. The rack of the tubes was placed into the BAX system Q7 instrument that was previously operated for pre-warming. The PCR technique was carried out following the instruction on the instrument's screen.

Enterohaemorrhagic E. coli testing

The biochemically verified isolates were spread onto the sorbitol MacConkey agar in the presence of potassium tellurite and cefixime, and incubated overnight at 37°C. This was done to confirm that the isolates were EHEC based on their ability to ferment sorbitol. Because EHEC strains are unable to digest sorbitol, the colonies appear colourless when culturing (Tang *et al.*, 2014).

Biochemical identification of E. coli isolates

Liofilchem biochemical food systems kits with reference number 71680 were used for the biochemical identification of *E. coli* strains. Following the manufacturer's instructions, the samples were homogenised in a 1:10 ratio (25 g of samples mixed with 225 mL of prewarmed BPW). From this, 10 mL was transferred into a sterile tube, and incubated for 18 h at 37°C. Subsequently, 0.5 mL from this suspension was added to the physiological solution provided with the kit. Afterward, 0.2 mL was distributed into each well of the system. The system was then covered with its lid, and incubated for 18 - 24 h at 37°C. The following day, the results were interpreted by adding two drops of Kovac's reagent to well 8-IND and two drops of hydrogen peroxide (H_2O_2) reagent to well 11-CAT, respectively. The appearance colours were observed, and the results were recorded (Smith and Selby, 2017).

Sample decontamination

The samples were artificially contaminated with 3.86 ± 0.4 $\log_{10}$ colony forming unit (CFU)/g of *E. coli*. After that, the samples were soaked in previously prepared aqueous sanitary cleaning agents for 10 min (Table 1). Autoclaved distilled water was used as a control for comparison with the result obtained from the cleaning agents. The following reagents were prepared as agents for decontamination of leafy greens: aqueous chlorine in concentration of 50 mg/L or part per million (ppm), peracetic acid (50 ppm), and H_2O_2 of 3 - 5%.

Colony screening

The PCR-positive samples were subjected to colony isolation. A total of 20 - 30 colonies were subjected to DNA extraction and PCR. This procedure was repeated until pure colonies responsible for eaeA-positivity of PCR in the first culture were isolated. The pure bacterial cultures were kept at temperatures ranging from -20 to -70°C. All positive isolates for the eaeA gene were subjected to the biochemical identification of *E. coli*, including triple sugar iron experiment, sulphide indole motility medium, Simmon's citrate agar, and methyl red-Voges-Proskauer broth.

Table 1. Residual levels and reduction of *E. coli* on green leaves after soaking for 10 min.

Concentration	Sanitiser	Microorganism load (3.86 ± 0.4 $\log_{10}$ CFU/g) *E. coli* residual ($\log_{10}$ CFU/g)	Reduction ($\log_{10}$ CFU/g)
50 ppm	Control	3.86 ± 0.4	No reduction
	Aqueous chlorine	3.86 ± 0.4	2.05 ± 0.50
50 ppm	Control	3.86 ± 0.4	No reduction
	Peracetic acid	3.86 ± 0.4	1.27 ± 0.11
3 - 5%	Control	3.86 ± 0.4	No reduction
	H_2O_2	3.86 ± 0.4	2.9 ± 0.1

Values are mean ± standard deviation. Control: autoclaved distilled water; ppm: part per million; and CFU: colony forming unit.

Results

Incidence rate of E. coli

Table 2 shows the incidence rate and the percentage of positive samples from the collected items of different kinds of fruits and vegetables. The contamination rates for watercress and spinach were 100%. Following watercress and spinach, parsley, celery, and lettuce had *E. coli* incidence rates of 50, 40, and 30%, respectively. Surprisingly, out of a total of 62 fruits included in the present work, none of them were contaminated with *E. coli.*

Table 2. Incidence rate of *E. coli* in fruits and vegetables.

Number	Sample type	Local name	Sampling size	Positive (%)
1	Cabbage	Kelem	10	n.d. (0.0%)
2	Carrot	Gêzer	10	n.d. (0.0%)
3	Cucumber	Xiyar	10	n.d. (0.0%)
4	Tomato	Bacanê sor	10	n.d. (0.0%)
5	Lettuce	Kahu (Xes)	10	3 (30%)
6	Radish	Tivir	10	n.d. (0.0%)
7	Paprica	Bîber	10	n.d. (0.0%)
8	Watercress	Pîz (Kizi)	10	10 (100%)
9	Spinach	Spînax (Silqok)	10	10 (100%)
10	Parsley	Gêjnok (Kerefis)	10	5 (50%)
11	Celery	Kêrefiz	10	4 (40%)
12	Grape	Tirî	10	n.d. (0.0%)
13	Pomegranate	Hinar	10	n.d. (0.0%)
14	Peaches	Xox	10	n.d. (0.0%)
15	Orange	Pirteqal	10	n.d. (0.0%)
16	Apple	Sêv	10	n.d. (0.0%)
17	Mango	Mengo	6	n.d. (0.0%)
18	Avocado	Evokado	6	n.d. (0.0%)
	Total		**172**	**32 (18.6%)**

n.d.: not detected.

Confirmation of isolates

Figure 1 shows the results from the PCR experiment. The PCR assay was performed for all the isolates. None of the isolates tested positive for *E. coli* O157:H7 using the DuPont BAX System KIT2000. Furthermore, biochemical tests were done on the isolates, and the results from this assay are shown in Figure 2.

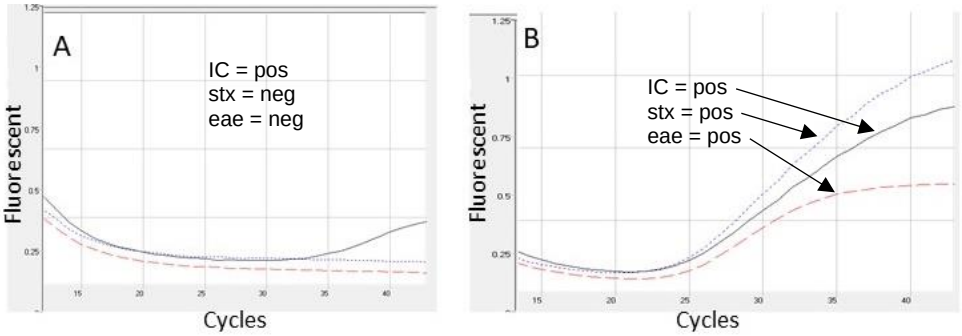

Figure 1. DuPont BAX system screens for detection of *E. coli* O157:H7 from the tested samples. **(A)**: negative sample; and **(B)**: reference strain of *E. coli* O157:H7 (ATCC 43888) as a positive control. IC: internal control; stx: Shiga toxin (STEC) gene target; eae: Enteropathogenic *E. coli* (EPEC) strains; pos: positive; and neg: negative.

Figure 2. Common biochemical experiments in accordance with the food system Liofilchem (reference 71680). *E. coli* shows positive indole and catalase reaction. **(A)**: isolate from sample; and **(B)**: reference strain.

Decontamination of fruits and vegetables

The results from decontamination revealed that the autoclaved distilled water (control) had no effect on reducing the microbial load on leafy green vegetables. The other detergent-based agents used were able to reduce the microbial load from 3.86 ± 0.4 $\log_{10}$ CFU/g to 2.9 ± 0.1, to 2.05 ± 0.50, and to 1.27 ± 0.11 $\log_{10}$ CFU/g for H_2O_2, aqueous chlorine, and peracetic acid, respectively (Table 1).

Discussion

Foodborne diseases could be caused by potential sources of contamination, including pathogenic microorganisms such as bacteria (Alam *et al.*, 2015). Contamination can originate from various sources, including soil, sewage, animal manure, water, and wildlife animals (Liu *et al.*, 2013). Therefore, it should be mentioned that foodborne illnesses have not only critical effects on the individuals' health but also on the economy. Additionally, there are societal costs, including decreased work performance, research expenses related to outbreaks, revenue loss due to food company closures, legal expenses for disease-related lawsuits, and public medical care charges (Luna-Guevara *et al*., 2019).

Some pathogens may be transferred to the environment during preharvest by the application of insufficiently composted animal manure. As a result, it is crucial to use properly stabilised fertilisers. Composting is one method of stabilisation in which

organic matter is decomposed by microorganisms for a period, typically ranging from 3 to 15 days at a specific temperature (131°F), followed by curing stage under colder conditions (Luna-Guevara *et al*., 2019). These circumstances reduce the levels of pathogenic microorganisms, promote cellulose and lignin decomposition, and stabilise their composition. Human waste sewage cannot be used to fertilise vegetables and crops for human consumption unless it meets the biosolid's specifications (Luna-Guevara *et al*., 2019).

For all types of salad vegetable samples, the incidence of biochemically confirmed *E. coli* isolates was 18% (n = 32), with 100% (n = 10) for watercress and spinach, 40 to 50% (n = 10) for parsley and celery, 30% (n = 10) for lettuce, and 0.0% (n = 10) for all other isolates listed in Table 2. Watercress and spinach had the highest incidence rate, followed by parsley, celery, and lettuce. This indicated that green leafy vegetables were the most commonly associated with *E. coli* infections. The low prevalence of *E. coli* contamination found in the present work was consistent with previous research data. A published study has revealed that out of 91 fruits and vegetable samples, 24 (26.4%) were contaminated with *E. coli*. Of these, 3 (11.1%) of the imported vegetables were found to be carrying the eaeA gene (Skočková *et al*., 2013). On the other hand, another cross-sectional study carried out in Tabuk City, Saudi Arabia had an *E. coli* prevalence rate of 14.3% (Abu-Duhier, 2015). Furthermore, this type of contamination may be attributed to water contamination during plant irrigation. Consequently, a number of outbreaks have been reported with many deaths (Luna-Guevara *et al*., 2019).

Even though this investigation did not find any prevalence of *E. coli* O157:H7, microbiological quality is still a concern when handling and storing vegetables before and after harvest, because EHEC can infect people even at very low infectious doses (Adebayo-Tayo *et al*., 2012). *E. coli* O157:H7 is associated with faecal contamination and may be a sign of the presence of other enteric pathogens linked to food-borne illnesses such as diarrhoea and gastroenteritis (Pennington, 2010; Erickson *et al*., 2019; Ameer *et al*., 2022).

The three cleaning agents were tested at a fixed concentration in this investigation. This exploratory analysis revealed that the chosen concentrations which did not affect the taste and flavour of lettuce leaves, spinach, celery, parsley, and watercress were aqueous chlorine and peracetic acid at 50 ppm (Chinchkar *et al*., 2022).

In most of the food industry, as well as in many supermarkets and catering companies, the most widely used method for washing fruits and vegetables is to soak them in a sanitiser solution for 10 min. Due to variations in the initial microbial load on the watercress and lettuce samples, it was necessary to standardise the load. This was achieved by washing the samples with running water before artificially inoculating them with the target microorganisms. This step aimed to achieve a standardised initial load of 3.8 $\log_{10}$ CFU/g in all the examined samples. This is important since the efficacy of the three sanitisers, particularly hypochlorous acid, is microbial-load dependent (Keeratipibul *et al*., 2011).

With respect to hypochlorous acid, the aqueous chlorine levels of 50 ppm hypochlorous acid treatment was slightly less effective at reducing the number of viable *E. coli*. When washing fresh products such as fruits and vegetables, it is recommended that the concentration of these agents should not exceed 80 ppm (FDA, 1998). This could be because using high concentrations of these agents on fruits and vegetables may have some drawbacks. For example, a study conducted by Gadelha *et al*. (2019) claimed that the high acidity of hypochlorous acid led to the nutritional degradation of leafy greens. Furthermore, a high volume of this agent has been found to cause a significant reduction in vitamin C in vegetables. However, more research is needed to understand its effects on vitamin B_{12} or other components (Kamoto *et al*., 2020).

Hydrogen peroxide had less effect on reducing microbial load at a specific concentration of 3 - 5% (Table 1). This data were in agreement with results from a previous study conducted in Thailand where they recorded 4.49 ± 0.65 to 2.47 ± 0.81 $\log_{10}$ CFU/g post-sanitisation for peracetic acid, whereas aqueous chlorine's initial load, recorded as 5.67 ± 0.48 CFU/g, declined to 1.29 ± 0.49 $\log_{10}$ CFU/g post treatment (Keeratipibul *et al*., 2011).

Study limitation

One of the limitations of the study on anticipatory surveillance for the detection of *E. coli* from fresh fruits and vegetables using the DuPont BAX System was the potential for false positives and false negatives. While the DuPont BAX System is a widely used and reliable method for detecting pathogens, it is not infallible. False positive results

may occur due to cross-contamination during sample preparation or issues with the specificity of the molecular markers used. Conversely, false negatives can arise from the presence of *E. coli* strains that do not match the system's target markers or from limitations in the detection threshold. Furthermore, the study's accuracy was dependent on the quality and representativeness of the sampled produce, which can vary due to factors like geographic location, seasonality, and agricultural practices. Therefore, the study's findings should be interpreted with the awareness that they may not capture the full extent of *E. coli* contamination in fresh produce, and that complementary surveillance methods may be necessary to mitigate these limitations.

Future directions

The future of anticipatory surveillance in food safety relies on the synergistic application of innovative technologies, multidisciplinary collaboration, and a proactive approach to mitigating the risks associated with *E. coli* contamination in fresh produce.

A promising future direction for research in anticipatory surveillance is the integration of advanced data analytics and machine learning techniques. By analysing these multifaceted datasets, it may become possible to predict potential *E. coli* outbreaks in specific regions or during particular seasons, allowing for proactive risk mitigation strategies and targeted interventions. Additionally, future research can focus on the refinement and expansion of the molecular markers used in the DuPont BAX System. This includes the incorporation of markers specific to emerging or highly virulent *E. coli* strains. Moreover, advancements in genomics and bioinformatics can facilitate the rapid identification of genetic variations in *E. coli* populations, aiding in the development of more accurate and versatile detection assays.

Conclusion

The present work was based on the analysis of 172 fruit and vegetable samples which revealed that 32 of them (18.6%) were contaminated with *E. coli*. However, none of the tested samples showed contamination with *E. coli* O157:H7. These findings underscored the widespread presence of *E. coli* across various fruits and salad vegetables in the Zakho Market, Iraq. Additionally, peracetic acid exhibited superior performance as a potential sanitiser compared to aqueous chlorine. The importance of ensuring the safety and cleanliness of ready-to-eat fruits and vegetables cannot be overstated, as they should be free from contaminants such as metals, insects, and soil residues.

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