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RESEARCH PAPER

Detection of some toxin genes in *Escherichia coli* and *Vibrio cholerae* from street foods by PCR amplification

[1]Minh, N.H., [2]Tue, N.H., [1]Hao, N.P., [3]Ngoc, L.M.T., [3]Quang, H.T. and [2,*]Loc, N.H.

[1]*University of Medicine and Pharmacy, Hue University, 6 Ngo Quyen St., Hue 49000, Vietnam*
[2]*University of Sciences, Hue University, 77 Nguyen Hue St., Hue 49000, Vietnam*
[3]*Institute of Biotechnology, Hue University, Nguyen Dinh Tu St., Hue 49000, Vietnam*

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Abstract

Diarrhea brought on by *Escherichia coli* and cholera brought on by *Vibrio cholerae* is two health issues that are of concern globally. These diseases are frequently prevalent in developing countries where hygienic standards are not always assured. One of the causes of diarrhea and cholera is unhygienic food, especially street food. The present study employed microbiological cultures to detect *E. coli* and *V. cholerae* in street food and PCR amplification to identify the toxin genes in these bacteria. The results showed that all 8 food samples tested were contaminated with bacteria, of which 6/8 samples were infected with *E. coli* and 5/8 samples were contaminated with *V. cholerae*. Among the samples contaminated with *E. coli*, only mussel rice contained *E. coli* carrying two toxin genes, *eltB* and *estA*. There were no samples contaminated with *V. cholerae* that had the bacterial *ctxA* toxin gene. PCR amplification has shown its potential in applications of this technique for detecting toxin genes in bacterial-contaminated street food.

1. Introduction

Cholera caused by *Vibrio cholerae* and diarrhea caused by *E. coli* is health problems of widespread concern worldwide. These are diseases transmitted through the gastrointestinal tract, often found in residential areas with unsanitary conditions, low education levels, and little knowledge about diarrhea prevention, especially in developing countries (Fleckenstein *et al.,* 2000; Kang *et al.,* 2004).

Although it can be severe, cholera infection is typically mild or symptomless. One in ten cholera patients will experience severe symptoms such as vomiting, cramping in the legs, and watery diarrhea (Centers for Disease Control and Prevention, 2023). Over the past few years, the World Health Organization (WHO) has continued to receive a high number of reports of cholera cases. In 2022, 44 countries reported 472,697 cases and 2,349 deaths to the WHO. Therefore, cholera remains a global threat to public health (WHO, 2023). Diarrhoeal disease is the second leading cause of death in children under five years old, is responsible for killing around 525,000 children every year, and mostly results from contaminated food and water sources. Diarrhea due to infection is widespread throughout developing countries (WHO, 2017). The cholera and diarrhea situation has improved a lot in recent years, but it is still a public health problem that needs attention. There are many causes of diarrhea and cholera, of which the use of contaminated foods is one of the most common, especially street foods (Rabbani and Greenough, 1999; WHO, 1993). Testing contaminated foods using traditional microbiological and biochemical methods is often time-consuming and expensive. Meanwhile, applying molecular biology methods, e.g., PCR technique, will yield faster, more accurate results at a lower cost (Whyte *et al.,* 2002).

The present study reports the first findings from employing PCR amplification to identify toxin genes in some street foods in Hue, a well-known Vietnamese cultural and tourism hub, such as *ctxA* from *V. cholerae*, which causes cholera, and *eltB* and *estA* from *E. coli*, which causes diarrhea.

2. Materials and methods

2.1 Food samples

Some traditional street foods in Hue, Vietnam, were used in the present study, such as cooked foods (fried tofu, fried fishcake, rice, and fried rib meat) were collected from some eateries in Ben Ngu Market, and sticky rice from eatery in Dong Da Street. Non-cooked foods such as mung bean sprouts and raw vegetables

*Corresponding author.
Email: *nhloc@hueuni.edu.vn*

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were collected from eateries in Ben Ngu Market, and mussel rice from an eatery in An Cuu Market. Mussel rice is a dish made from cold rice mixed with mussel juice, shrimp paste, raw vegetables, pork rinds, grilled rice paper, fried peanuts, chili, and mussels stir-fried in oil with spices.

Specifications for sampling, preservation, and handling of samples are carried out in accordance with Vietnamese National Standards, TCVN 7905-1:2008 for *V. cholerae* and TCVN 7924-2:2008 for *E. coli*. Standard bacteria (*E. coli* and *V. cholerae*) were provided by the Department of Biotechnology, University of Sciences, Hue University, Vietnam.

2.2 Bacterial isolation

Five grams of each food type were homogenized in 0.9% NaCl to a final volume of 50 mL using a Stomacher at a speed of 260 times per min for 1.5 mins. Approximately 100 µL of the homogeneous solution were spread on MacConkey agar medium and incubated at 37°C for 18-24 hrs for *E. coli*. For *V. cholerae*, the same volume was spread on TCBS (thiosulfate citrate bile salts sucrose) agar medium and incubated at 37°C for 24-48 hrs. Finally, the number of colonies formed on each medium was observed and counted.

2.3 Detection of toxin genes

Escherichia coli colonies growing on MacConkey medium were used directly for PCR to detect the *eltB* and *estA* genes, which encode heat-labile enterotoxin B subunit and heat-stable enterotoxin, respectively. *Vibrio cholerae* colonies growing on TCBS medium were cultured in alkaline peptone liquid medium supplemented with 1% NaCl (pH 8.6). Bacterial biomass was then harvested by centrifugation, resuspended in Tris-EDTA buffer, and boiled for 15 mins to disrupt cells. Centrifuge to obtain the supernatant for PCR to detect the *ctxA* gene, which encodes cholera toxin (Hue *et al.,* 2004).

The PCR component consists of an *E. coli* or *V. cholerae* DNA template as mentioned above, 6 µL of PCR Master Mix (Promega, USA), 10 pmol of each primer (Table 1), and ddH_2O added to a final volume of 12 µL. The PCR amplification was set up as follows: genomic denaturation at 95°C for 2 mins, followed by 30 cycles of 95°C for 1 min, 45-60°C for 1 min, and 72°C for 2 mins; and a final extension of 72°C for 10 mins. The amplicon was then examined by electrophoresis on a 1% agarose gel and sequenced by Sanger's method.

3. Results

3.1 Bacterial contamination in some street foods

Assays from 8 different street food samples collected in Hue showed that *E. coli* appeared in 6/8 samples, *V. cholerae* appeared in 5/8 samples, and other bacteria appeared in all 8 samples in quite large quantities. Generally, non-cooked foods (mung bean sprouts, raw vegetables, and mussel rice) have a higher rate of bacterial contamination than cooked foods (fried fishcake, rice, sticky rice, and fried rib meat), except fried tofu (Table 2). Isolated colonies of *E. coli* are red or pink, round, not mucoid, and are surrounded by a zone of precipitated bile. Other colonies on MacConkey medium range from white to pale pink, are surrounded by a white border and do not precipitate (Figure 1). *Escherichia coli* colonies appeared the most in two samples: mussel rice and mung bean sprouts, both with a density of 1.67×10^3 CFU/g.

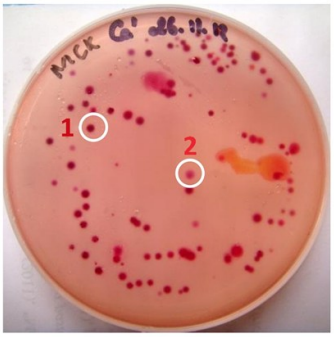

Figure 1. Contamination of *E. coli* and other bacteria in fried fishcake. 1: *E. coli*, 2: other bacteria.

On TCBS medium, *V. cholerae* colonies were large, yellow, round, and shiny. Colonies of *V. parahaemolyticus* had blue to green centers, and those of *V. vulnificus* were greenish blue (Loharikar *et al.,* 2012; Karunasagar *et al.,* 2018) (Figure 2). *Vibrio cholerae* appeared most frequently in mussel rice (5×10^3 CFU/g)

Table 1. Nucleotide sequence of specific primers for some indicator segments of toxin genes in *E. coli* and *V. cholerae*.

Target gene	Primer	Nucleotide sequence (5'-3')	Amplicon size (bp)	Ref./NCBI
eltB	LT_F	TCTCTATGTGCATACGGAGC	322	Hien *et al.* (2008)/ AF242418.1
	LT_R	CCATACTGATTGCCGCAAT		
estA	ST_F	GCTAAACCAGTAGAGTCTTCAAAA	147	Hien *et al.* (2008)/ M29255.1
	ST_R	CCCGGTACAAGCAGGATTACAACA		
ctxA	CT_F	CGGGCAGATTCTAGACCTCCTG	564	AF175708.1
	CT_R	CGATGATCTTGGAGCATTCCCAC		

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Table 2. Level of bacterial contamination of some tested street foods.

No	Sample	Bacterial concentration (×10^3 CFU/g sample)		
		E. coli	*V. cholerae*	Other bacteria
A	Cooked foods			
1	Fried fishcake	0.67	-	13.33
2	Rice	-	-	6.67
3	Fried tofu	1.0	3.33	66.67
4	Sticky rice	-	-	6.67
5	Fried rib meat	0.67	0.67	16.67
B	Non-cooked foods			
6	Mung-bean sprouts	1.67	1.67	51.7
7	Raw vegetables	1.33	1.67	102.1
8	Mussel rice	1.67	5.0	66.67

and fried tofu (3.33×10^3 CFU/g). In general, in the present study, mussel rice was the food most contaminated with *E. coli* and *V. cholerae* bacteria, including other intestinal bacteria that also account for a large amount (6.67×10^3 CFU/g), equivalent to fried tofu, and only after raw vegetables (102.1×10^3 CFU/g).

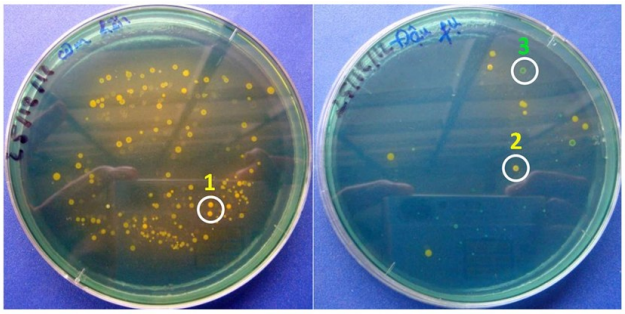

Figure 2. Contamination of *V. cholerae* and other bacteria in mussel rice (left) and fried tofu (right). 1 and 2: *V. cholerae*, 3: other bacteria.

3.2 Identification of toxin genes in some street foods

First, the annealing temperature for PCR was optimized based on standard bacteria. The PCR amplifications of all three toxin genes, *eltB*, *estA*, and *ctxA*, had positive results at different annealing temperatures from 45 to 60°C. The PCR products have sizes equivalent to the gene segments being searched: about 320 bp for *eltB*, 150 bp for *estA*, and 560 bp for *ctxA* (Figures 3A-C). However, the optimal temperature was found to be 55°C, where the DNA bands were intense and specific. At 45°C, besides the main lighter DNA band, there were also some other non-specific DNA bands. At 50°C and 60°C, the PCR product was more than specific (only one DNA band) but lighter than the DNA band at 55°C. PCR products obtained from an annealing temperature of 55°C for the *eltB*, *estA*, and *ctxA* genes were cloned into a pGEM T-Easy vector for sequencing. The results revealed that these sequences were 100% similar to those of the corresponding genes, *eltB* (NCBI: AF242418.1), *estA* (M29255.1), and *ctxA* (AF175708.1). From these results, we found that we could apply an annealing temperature of 55°C to the PCR assay to find the toxin genes *eltB*, *estA*, and *ctxA* in some street foods in Hue.

Next, PCR amplification with an annealing temperature of 55°C was employed to detect toxin genes in foods contaminated with *E. coli* (6 samples) and *V. cholerae* (5 samples). The results showed that only mussel rice was contaminated with *E. coli* carrying the *eltB* gene (Figure 4A) and the *estA* gene (Figure 4B). There were no foods contaminated with *V. cholerae* carrying the *ctxA* gene (Figure 4C). It can be observed that the PCR amplification was carried out successfully since the PCR products were visible in the positive control and absent in the negative control.

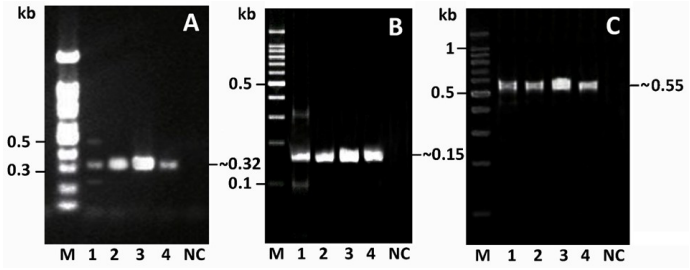

Figure 3. PCR amplification of toxin gene segments from standard *E. coli* and *V. cholerae*. A: *eltB* gene, B: *estA* gene, and C: *ctxA* gene. M: standard DNA scale, 1: annealing temperature 45°C, 2: annealing temperature 50°C, 3: annealing temperature 55°C, 4: annealing temperature 60°C. NC: Sterile distilled water used as a negative control.

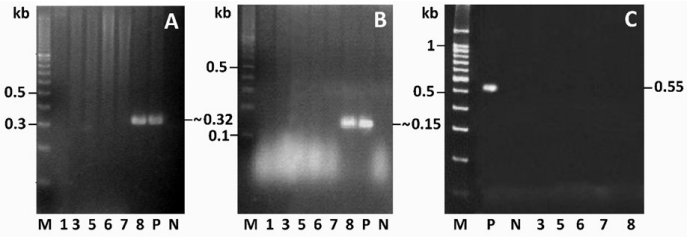

Figure 4. PCR amplification of toxin gene segments from some street foods. A: *eltB* gene, B: *estA* gene, and C: *ctxA* gene. M: standard DNA scale, 1: fried fishcake, 3: fried tofu, 5: fried rib meat, 6: mung-bean sprouts, 7: raw vegetables, 8: mussel rice, P: standard *E. coli* and *V. cholerae* used as positive controls, N: sterile distilled water used as a negative control.

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4. Discussion

This study showed that even when tofu is fried, it still contains significant levels of bacteria, which may be because this food is past its expiration date. The bile precipitate that appeared in *E. coli* was due to a local pH drop around the colony from lactose fermentation. The growth of colonies with bile precipitates strongly suggests the presence of *E. coli* (Condalab, 2022). Although there were many street foods contaminated with *E. coli* and *V. cholerae*, they did not necessarily cause diarrhea or cholera. The decisive factor in these diseases is the presence of toxin genes in the bacteria.

There are many factors that can affect the efficiency of PCR amplification, such as the quality, composition, and concentration of reagents (template DNA, primers, Taq polymerase, dNTPs, and Mg^{2+}), and PCR conditions (temperature and reaction time). Among them, one of the most important factors is the annealing temperature to attach the primer to the template DNA (Balne *et al.,* 2014).

Therefore, PCR amplification needs to be standardized before identifying target genes. Contamination of *E. coli* and *V. cholerae* in street foods, drinks, and some other foods has also been studied by different methods, such as microbiological and biochemical assays (Sultana *et al.,* 2021) or PCR (Koch *et al.,* 1993; Islam *et al.,* 2015; Waturangi *et al.,* 2015; Bonyadian *et al.,* 2018; Ling *et al.,* 2018; Tang *et al.,* 2018). However, most tests for bacterial contamination in food and beverages use PCR amplification because it has many advantages over traditional assays, such as rapidity, low cost, high sensitivity, and accuracy (Toze, 1999).

5. Conclusion

The level of contamination with bacteria that can cause diarrhea and cholera in some street foods in Hue was not high in the winter. Of the 8 tested food samples, 6 had *E. coli* (0.67-1.67×10^3 CFU/g) and 5 had *V. cholerae* (0.67-5.01×10^3 CFU/g). However, because the study period was in the winter, pathogens rarely appeared. Thus, PCR amplification found only one *E. coli* colony carrying the *eltB* gene and another colony carrying the *estA* gene, both in the mussel rice sample. No *V. cholerae* colonies were found carrying *ctxA* genes.

Conflict of interest

The authors declare no conflict of interest.

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