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DEVELOPMENT AND APPLICATION OF MULTIPLEX PCR FOR THE DETECTION OF *ESCHERICHIA COLI* AND *VIBRIO CHOLERAE* IN STREET FOODS

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Abstract: PCR is a very useful method for detecting the presence of pathogenic microorganisms in foods and organisms. This study developed a multiplex PCR (mPCR) method for detecting *E. coli* and *V. cholerae* in some street foods in Dong Ha, Vietnam, through the housekeeping genes *phoA* and *sodB*, respectively. This study used methods such as single PCR, multiplex PCR, and bacterial culture. The study established appropriate mPCR conditions to detect *E. coli* and *V. cholerae* at a minimum concentration of 10^1 CFU/mL and 10^2 CFU/mL, respectively. The developed mPCR method successfully detected *E. coli* in 93.75% of the street food samples, including *nem chua*, *tôm chua*, and *bún hến*, whereas *Vibrio cholerae* was not detected in any of the tested samples.

Key words: *cholera, diarrhoea, pathogens, multiplex PCR, contamination, rapid screening*

INTRODUCTION

In developing countries, street food vendors significantly contribute to the economy and society by creating jobs, providing affordable food, and catering to diverse socioeconomic classes (Eromo, Tassew, Daka & Kibru, 2016; Mehboob & Abbas, 2019; Salamandane, Malfeito-Ferreira & Brito, 2023).

However, due to their informal nature, these businesses often suffer from poor infrastructure, lack of sanitation, and improper personal hygiene (Moloi, Lenetha & Malebo, 2021). This poses potential microbial hazards and chemical contamination, contributing to a high risk of foodborne diseases (Rane, 2011; Raza et al., 2021; Ahmed et al., 2022). To mitigate these risks, vendors require hygiene training and access to audited facilities to ensure food safety (FAO, 2009; Tang et al., 2018). In developing countries, government legislation and NGO collaboration are vital for enforcing health policies and safety standards (Sharma et al., 2024).

Some recent studies used PCR as a complementary approach to traditional techniques for assessing bacterial contamination in street foods (Eromo et al., 2016; Mehboob & Abbas, 2019; Moloi et al., 2021). For example, Tang et al. (2018) used an mPCR assay for the detection of *V. cholerae* in street foods (*satar* and *otak-otak*). Raza et al. (2021) employed multiplex PCR (mPCR) to detect contamination in ready-to-eat street foods from Quetta, Pakistan, identifying *Salmonella enteritidis* and *S. typhimurium*. Ahmed *et al*. (2022) also developed

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the mPCR method to detect three bacterial species, *E. coli*, *Shigella dysentery*, and *Salmonella typhi*, in street-vended *Um-Jingir* traditional food in Khartoum State, Sudan.

Some other studies in developing countries have also detected various bacteria in some street foods by PCR, such as *E. coli* (Godambe, Bandekar & Shashidar, 2017), *E. coli* and *Vibrio cholerae* (Minh et al., 2024), *Listeria monocytogenes* (Sharma et al., 2024), *Shigella dysentery*, and *Salmonella typhi* (Ahmed et al., 2022). This study developed an mPCR method for detecting *E. coli*, which causes diarrhoea, and *V. cholerae*, which causes cholera, two common intestinal diseases in areas with poor sanitation, in some street foods in Dong Ha, Vietnam, through the housekeeping genes *phoA* and *sodB*, respecttively.

Although numerous mPCR assays have been developed for pathogen detection, their use in complex fermented street foods such as *nem chua* and *tôm chua* remains limited, largely due to the high concentrations of organic acids and spices that can inhibit PCR amplification. This study aims to provide a validated, highly sensitive mPCR protocol (10^1-10^2 CFU/mL) specifically optimized for these unique food matrices in Central Vietnam, offering a rapid screening tool for local food safety authorities.

MATERIALS AND METHODS

Samples and reference bacteria

Samples of traditional street foods, including (1) *nem chua* (fermented pork rolls), (2) *bún hến* (mussel noodle), (3) *tôm chua* (sour shrimp), and (4) *bún thịt nướng* (rice noodles with barbecue), were collected from popular eateries and local markets in Dong Ha city (Quang Tri province, Vietnam). Sampling sites were strategically selected from high-density residential areas and major commercial hubs, including Ward 3, Ward 4, Ward 5, and Dong Ha Market. The collection was conducted between June 2022 and September 2022. All procedures for sampling, preservation, and handling were carried out in accordance with Vietnamese National Standards (TCVN) 6404:2016 (MOST, 2016).

Bún hến is served with raw vegetables, mussel juice, shrimp paste, pork rinds, and mussels stir-fried in oil. *Tôm chua* is consumed with boiled pork and pickled mung bean sprouts. *Bún thịt nướng* is often accompanied with raw vegetables. *Nem chua* is a fermented pork roll typically eaten raw with garlic, chili, and herbs. Reference bacteria, *E. coli* ATCC 8739 and *V. cholerae* ATCC 14035, were used as positive controls.

Determination of reference bacteria

Bacteria *E. coli* was detected by the pour-plate method (Humphrey & Gawlera 1986). Bacteria grow at 44°C to form a typical blue colony on tryptone bile X-glucuronide (TBX) medium for 18 h under aerobic conditions. The colony-forming units (CFU) of β-glucuronidase-positive *E. coli* were calculated per milliliter of the sample.

Bacteria *V. cholerae* was detected by the method of Donovan and van Netten (1995). Bacterial colonies were obtained on thiosulfate citrate bile sucrose (TCBS) agar medium after incubation under aerobic conditions at 37 °C for 24 h. The CFU of *V. cholerae* were calculated per millilitre of the sample.

DNA extraction and quality control

Total genomic DNA was isolated from *E. coli* ($1.8x10^8$ CFU/mL) and *V. cholerae* ($1.3x10^8$ CFU/mL) cultures - obtained from street food samples - using the Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocol. These CFU values represent the bacterial concentrations post-enrichment. DNA quantity and purity were assessed using an Evolution 60S UV-Visible Spectrophotometer (Thermo Scientific, USA); only samples exhibiting an A_{260}/A_{280} ratio of 1.8-2.0 were selected for further analysis.

Target selection and primer design

The housekeeping genes *phoA* (for *E. coli*) and *sodB* (for *V. cholerae*) were selected for species-level identification due to their high genetic stability, which is essential for a reliable detection assay. While virulence markers (e.g., *stx*, *ctx*) provide risk assessment, this study aimed to establish a robust baseline detection method. Primers were adapted from Peng et al. (2021) and Tarr et al. (2007). Notably, sequences were modified to optimize them for a multiplex format, producing smaller amplicons (622 bp for *phoA* instead of the original 1416 bp and 248 bp for *sodB* instead of the original 585 bp) to increase amplification efficiency and reduce extension time.

PCR amplification and analysis

Multiplex PCR (mPCR) was performed in a total volume of 20 µL, containing 10 µL of GoTaq® 2x Master Mix (Promega, USA), 10 pmol of each primer (equimolar, yielding a final concentration of 0.5 µM), and 30 ng of template DNA. Amplification was conducted in a T100™ Thermal Cycler (Bio-Rad, USA) with the following program: initial denaturation at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at an optimized temperature of 60 °C for 30 s (determined via gradient PCR), and extension at 72 °C for 30 s; with a final extension at 72 °C for 10 min. This 30-cycle protocol was optimized to ensure robust amplification of all targets while avoiding plateau-phase artifacts and background noise.

Amplicons (622 bp for *phoA* and 248 bp for *sodB*) were visualized on a 1.5% agarose gel stained with RedSafe™ (iNtRON, South Korea) using a UV transilluminator (Wealtec, Taiwan). Following visualization, the PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purified products were then sent to Phu Sa Biochem Co. (Vietnam) for Sanger sequencing. To ensure accuracy and reproducibility, sequencing was performed in triplicate for both single and multiplex PCR products. Finally, DNA sequences were checked for quality using BioTrace software (BioEdit version 7.2.5) and analysed using CLC Sequence Viewer software (Qiagen).

Method validation

The assay was validated following ISO 16140-2:2016 principles. Specificity was confirmed using a panel of six non-target strains (ATCC), showing no cross-reactivity. Accuracy was determined by the percentage of agreement between mPCR results and the gold-standard culture method (ISO 16649 for *E. coli* and ISO 21872 for *Vibrio*). Linearity and Limit of Quantitation (LOQ) were established using a semi-quantitative approach by measuring band intensity via ImageJ software (v.1.54). A standard curve was plotted using the log of band intensity versus the log of bacterial concentration (CFU/mL). The LOQ was defined as the lowest concentration within the linear range with a Relative Standard Deviation (RSD) < 20%.

Due to the endpoint nature of mPCR, the densitometric analysis of band intensity using ImageJ software was employed as a semi-quantitative approach. Linearity was established across a five-point calibration curve of the mixed standard DNA, covering the range from 10^1 to 10^5 CFU/mL. A standard curve was plotted by correlating the log of band intensity with the log of the bacterial concentration. The LOQ was defined as the lowest concentration within this linear range that achieved a RSD < 20%, following the validation criteria for alternative microbiological methods outlined in ISO 16140-2:2016.

Total DNAs that were isolated from the bacteria were diluted from 10^{-1} to 10^{-5} and used as templates for PCR amplifications, as mentioned above. The limit of detection (LOD) was defined as the lowest dilution of DNA at which the target PCR product could be detected in the gel.

Precision (intra-day, n = 6 replicates; inter-day, repeated over three days) was evaluated using DNA templates from standard bacterial cultures at 10^4 and 10^2 CFU/mL, with results expressed as Relative Standard Deviation (RSD) of the detection signals. Accuracy was established by calculating the percentage of agreement between the qualitative mPCR results (positive/negative) and the results from the standard culture method (10^1 to 10^5 CFU/mL). Robustness was tested by intentionally introducing minor variations in the critical PCR parameters. The effect of varying the annealing temperature (±1°C: 59 °C and 61 °C) and primer concentration (±10%: 9 pmol and 11 pmol) on the amplification quality of a standard sample (10^3 CFU/mL) was evaluated.

Table 1.
Specific primers were used for the detection of *E. coli* and *V. cholerae* isolates from some street foods.

Target gene	Primer	Nucleotide sequence (5' - 3')	Amplicon size (bp)*
phoA	phoA_F	TAC AGG TGA CTG CGG GCT TAT C	622
	phoA_R	CTT ACC GGG CAA TAC ACT CAC TA	
sodB	sodB_F	AAG ACC TCA ACT GGC GGT A	248
	sodB_R	GAA GTG TTA GTG ATC GCC AG AGT	

*Indicator fragments for *phoA* (*E. coli*) and *sodB* (*V. cholerae*) genes designed by Peng et al. (2021) and Tarr et al. (2007)

RESULTS

PCR amplification and characterization

Single PCR amplification of indicator fragments of the *phoA* and *sodB* genes, which will be used as positive controls, from reference *E. coli* and *V. cholerae* with specific primers obtained two very clear and single DNA bands on the agarose gel with sizes of about 540 and 200 bp, respectively (Fig. 1). The nucleotide sequences of these PCR products (verified in triplicate for both sPCR and mPCR as described in the Methods section) were searched by BLAST in the GenBank database and revealed that they have a high similarity of 99.82% with that of *E. coli* (NCBI: CP047658.1) and 98.51% with that of *V. cholerae* (NCBI: CP053796.1). The specificity of the developed mPCR method was confirmed by obtaining only two target DNA bands, 622 bp for the *phoA* gene (*E. coli*) and 248 bp for the *sodB* gene (*V. cholerae*), when tested with the reference bacterial mixture (Fig. 2, lane 1), similar to those in single PCR (Fig. 2, lanes 2 and 3). The non-target bacterial strains (negative controls, Fig. 2, lanes 4-9) yielded no DNA bands, demonstrating the high specificity of the primers for *E. coli* and *V. cholerae*. Minor, stochastic low-molecular-weight smears (~100 bp) observed in some non-target lanes were identified as primer-dimer artifacts. These were easily distinguished from the specific diagnostic bands of *phoA* (622 bp) and *sodB* (248 bp) due to their diffuse appearance and significant size difference.

Method validation parameters

Limit of detection (LOD): Initial single PCR (sPCR) analysis using bacterial DNA from reference strains, as positive control, showed detection at a 10^{-4} dilution (10^{1} CFU/mL) for both targets (Fig. 3). For multiplex PCR (mPCR), the detection threshold was established using total DNA isolated from a mixed culture of *E. coli* and *V. cholerae* (diluted 10^{-1} to 10^{-6}, corresponding to 10^{5} to 10^{0} CFU/mL). Figure 4 demonstrated that *E. coli* was detected at a 10^{-5} dilution and *V. cholerae* at 10^{-4}. The final established LODs were 10^{1} CFU/mL for *E. coli* and 10^{2} CFU/mL for *V. cholerae*.

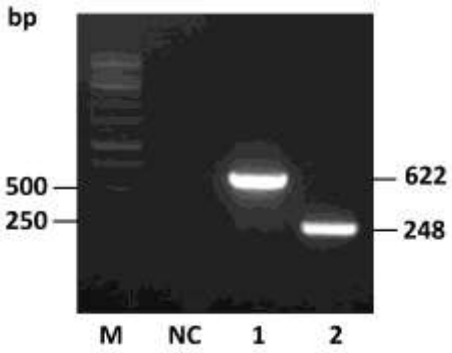

Figure 1. Single PCR amplification of reference bacterial strains. M: 1kb DNA Ladder (Thermo Fisher Scientific). NC: nuclease-free water (phoA primers), **1:** *phoA* gene in *E. coli* ATCC 8739, 2: *sodB* gene in *V. cholerae* ATCC 14035

Figure 2. Multiplex PCR amplification of reference bacterial strains. M: 1kb DNA Ladder (Thermo Fisher Scientific). 1: mixture of *E. coli* ATCC 8739 and *V. cholerae* ATCC 14035, 2: *E. coli* ATCC 8739, 3: *V. cholerae* ATCC 14035, 4 - 9: negative controls were *Salmonella* sp. ATCC 14028, *Bacillus pumilus* ATCC 14884, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, and *Pseudomonas aeruginosa* ATCC 9027, respectively

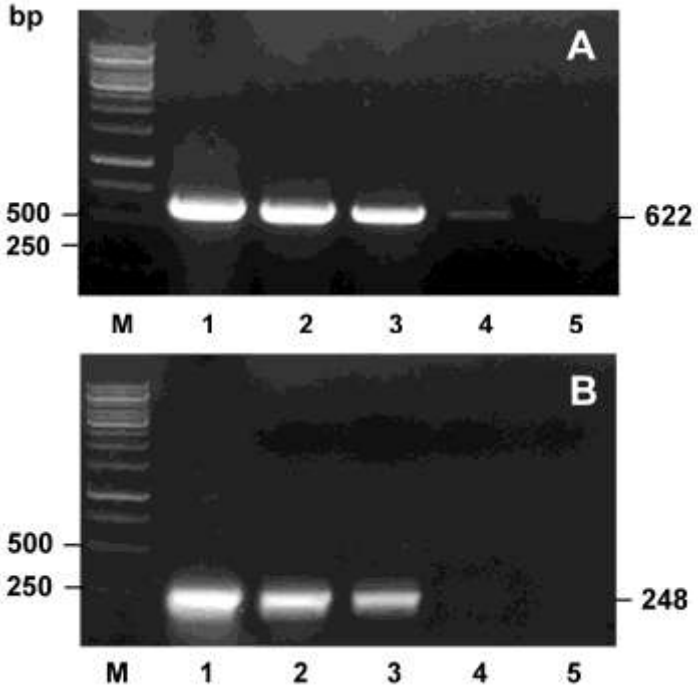

Figure 3. Detection threshold of *E. coli* (A) and *V. cholerae* (B) by single PCR amplification. M: 1kb DNA Ladder (Thermo Fisher Scientific). 1-5: various dilutions of bacterial density from 10^0-10^4 CFU/mL

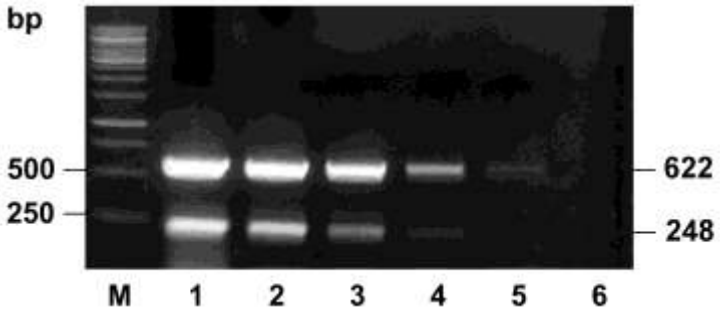

Figure 4. Detection threshold of *E. coli* (~540 bp) and *V. cholerae* (~200 bp) by mPCR amplification. M: 1kb DNA Ladder (Thermo Fisher Scientific). 1-6: various dilutions of bacterial density from 10^5-10^0 CFU/mL

Table 2.
Identification of *E. coli* and *V. cholerae* from street food samples using sPCR and culture-based methods.

Food samples (n=4 for each type)	**sPCR**				**Culture-based methods**			
	E. coli		***V. cholerae***		***E. coli***		***V. cholerae***	
	Positive	**(%)**	**Positive**	**(%)**	**Positive**	**(%)**	**Positive**	**(%)**
Nem chua	4	100	0	0	4	100	0	0
Tôm chua	4	100	0	0	4	100	0	0
Bún hến	4	100	0	0	4	100	0	0
Bún thịt nướng	3	75	0	0	3	75	0	0
Sample total: 16	15	93.75	0	0	15	93.75	0	0

Linearity and limit of quantification (LOQ): A strong linear relationship was observed between the logarithm of the band intensity and the logarithm of the bacterial concentration (CFU/mL) across the range of 10^2 to 10^5 CFU/mL, with a high correlation coefficient (R^2) of 0.995 for *E. coli* and 0.991 for *V. cholerae*. The LOQ was thus established as 10^2 CFU/mL for both *E. coli* and *V. cholerae*.

Accuracy and precision (reproducibility): The mPCR method exhibited high accuracy, showing detection results for street food samples

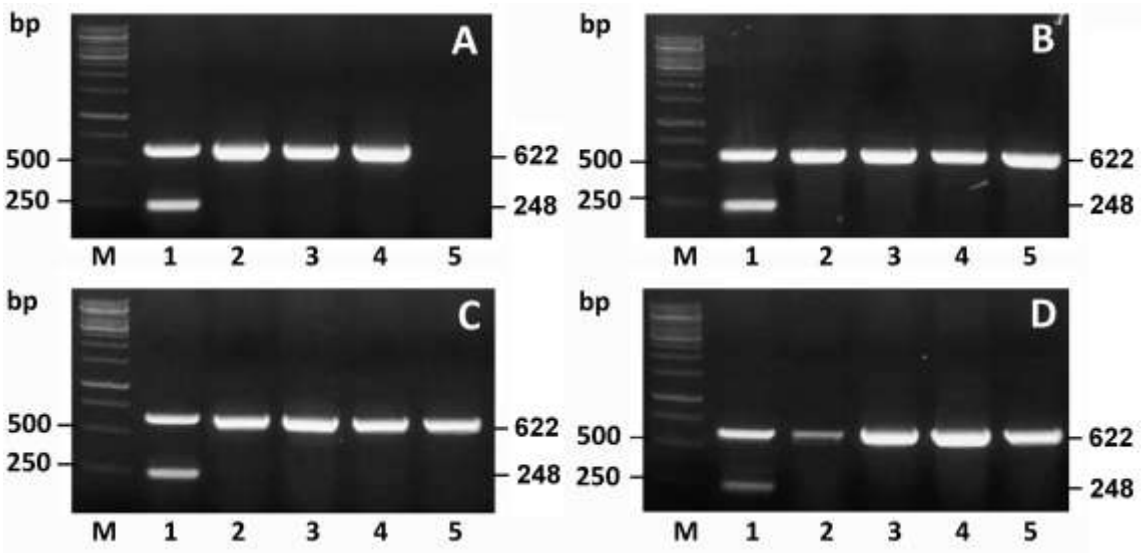

Figure 5. Detection of *E. coli* and *V. cholerae* in some foods in Dong Ha city by mPCR amplification. A: eatery in the Ward 3 market, B: eatery in the Ward 4 market, C: eatery in Dong Ha market, D: eatery in the Ward 5 market. M: DNA marker 1 kb ladder, 1: positive control (500 bp DNA band: *E. coli*, 250 bp DNA band: *V. cholerae*), 2: *nem chua*, 3: *tôm chua*, 4: *bún hến*, 5: *bún thịt nướng*

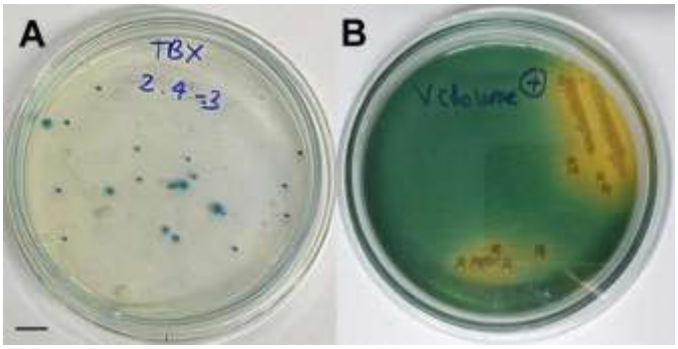

Figure 6. *E. coli* (A) and *V. cholerae* (B) colonies on screening medium

were completely consistent with culture-based methods (Table 2). Precision was high: the intra-day relative standard deviation (RSD) was < 5%, and the inter-day RSD was < 8%, both confirming high reproducibility for *E. coli* and *V. cholerae* at tested concentrations.

Robustness: The mPCR method proved to be robust. Minor variations in the annealing temperature (±1 ^{o}C) or the primer concentration (±10%) did not cause any loss of signal for the target bacteria or the appearance of non-specific bands, indicating the reliable stability of the method under small parameter fluctuations.

Application of mPCR to street food samples

Using the mPCR method for testing street food samples contaminated with *E. coli* and *V. cholerae*, we obtained the same results as with culture-based methods and the sPCR method (Figs. 5 and 6, Table 2). Although *E. coli* was detected in most of the street foods tested in all four locations (except *bún thịt nướng* at the market in Ward 3), no foods were contaminated with *V. cholerae*.

DISCUSSION

The PCR assay provides a simple, rapid, and reliable tool for the identification of the pathogens in food samples (Morin, Gong & Li, 2004; Ngamwongsatit, Chaturongakul & Aunpad, 2023). Peng et al. (2021) used the *phoA* gene, a nucleotide sequence that encodes alkaline phosphatase (Chang, Kuang & Chen, 1986; Kamitani, Akiyama & Ito, 1992), to identify drug-resistant *E. coli* in animal farms. PCR amplification of the *sodB* gene, which encodes iron–superoxide dismutase (Tarr et al., 2007), has also been applied to the identification of *Vibrio* species in both clinical and environmental samples.

The PCR results in this study demonstrated high specificity, with distinct and well-defined DNA bands, confirming that the PCR conditions and template DNA dilution were optimal for successful amplification. Some studies have also used the mPCR technique to simultaneously test various types of bacterial contamination in street foods (Sarker et al., 2013; Tang et al., 2018; Raza et al., 2021; Ahmed et al., 2022). The PCR assay in this study demonstrated high sensitivity, with a LOD of 101 CFU/mL for *E. coli* and 102 CFU/mL for *V. cholerae*. These results indicate that the method is highly effective for monitoring bacterial contamination in street foods. Our optimized mPCR protocol demonstrates a competitive detection limit compared to more expensive molecular methods, providing a cost-effective alternative for rapid screening in resource-limited settings.

Many studies showed that most ready-to-eat street foods were contaminated due to poor hygiene conditions during processing (Eromo et al., 2016; Mehboob & Abbas, 2019; Moloi et al., 2021). Of the two types of bacteria, *E. coli* and *V. cholerae*, the present study only detected *E. coli* in most foods tested in all locations (except *bún thịt nướng* at the market in Ward 3). The higher prevalence of *E. coli* (93.75%) compared to the absence of *V. cholerae* likely reflects the ubiquitous nature of *E. coli* as an indicator of fecal contamination, whereas *V. cholerae* is typically more sporadic and may be affected by the specific pH or salinity of food matrices.

While the developed mPCR assay provides a rapid, reliable, and cost-effective screening tool for the simultaneous detection of *E. coli* and *V. cholerae* in complex food matrices, certain limitations must be acknowledged. The target genes used in this study, *phoA* and *sodB*, are essential housekeeping genes for species-level identification; however, they do not provide information regarding the toxigenic potential of the isolates. Nevertheless, from a regulatory and public health perspective, the mere presence of these species in ready-to-eat street foods is a critical indicator of poor hygiene and potential health risks. Under most international food safety standards, there is a "zero tolerance" policy for pathogens like *E. coli*, meaning their detection - regardless of toxigenic profile - renders the food unfit for human consumption (Tokarskyy & Marshall, 2008). Therefore, while our method serves as an effective primary screening barrier, further characterisation integrating key virulence markers (e.g., *ctx*, *stx*) is necessary for a more comprehensive risk assessment and to distinguish commensal strains from highly pathogenic variants.

CONCLUSION

Recognising the urgent need for efficient pathogen detection in street foods, this study developed and validated a multiplex PCR (mPCR) method for the simultaneous detection of *E. coli* (*phoA* gene) and *V. cholerae* (*sodB* gene). The validation confirmed the method's high specificity, robustness, and sensitive detection limits (LODs) of 10^1 CFU/mL and 10^2 CFU/mL, respectively. Testing results showed that most street food samples (93.75%) were contaminated with *E. coli*, but no contamination of *V. cholerae* was detected. This mPCR method is proven to be a simple, rapid, and reliable tool that can be effectively applied in hygiene and food safety control.

AUTHOR CONTRIBUTIONS

Conceptualization and supervision, N.H.L.; Methodology, N.H.L. and N.Q.D.T.; Investigation, formal analysis, validation, N.H.L. and H.K; Writing-original draft preparation, N.Q.D.T.; Writing-review and editing, N.H.L.

DATA AVAILABILITY STATEMENT

Data contained within the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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RAZVOJ I PRIMENA MULTIPLEKS PCR ZA DETEKCIJU *ESCHERICHIA COLI* I *VIBRIO CHOLERAE* U HRANI KOJA SE PRODAJE NA ULICI

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Apstrakt: PCR je veoma korisna metoda za otkrivanje prisustva patogenih mikroorganizama u hrani i drugim uzorcima (iz okoline ili organizama). U ovoj studiji je razvijena multipleks PCR (mPCR) metoda za otkrivanje *E. coli* i *V. cholerae* u nekim vrstama hrane koja se prodaje na ulicama u Dong Hau, Vijetnam, putem detekcije gena za *phoA* i *sodB*, respektivno. Ova studija je koristila metode kao što su jednostruki PCR, multipleks PCR i gajenje bakterijskih kultura. Studija je uspostavila odgovarajuće mPCR uslove za otkrivanje *E. coli* i *V. cholerae* pri minimalnoj koncentraciji od 10^1 CFU/mL i 10^2 CFU/mL, respektivno. *E. coli* je otkrivena u većini testiranih uličnih namirnica na sve 4 lokacije, osim u bún thịt nướng (pirinčani rezanci sa roštiljem) na pijaci Vard 3. Međutim, nijedna od testiranih namirnica nije bila kontaminirana *V. choleare*. mPCR metoda se može koristiti za otkrivanje kontaminacije *E. coli* i *V. cholerae* u hrani koja se prodaje na ulici. Tri od četiri testirane takve hrane (nem čua, tom čua i kom hen) bile su kontaminirane *E. coli*, ali ni jedna nije bila kontaminirana *V. cholerae*.

Ključne reči: *kolera, dijareja, patogeni, multipleks PCR, kontaminacija, brzo određivanje*

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