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Rapid Simultaneous Detection of *Salmonella* and *Campylobacter* Bacteria Directly from Chicken Faeces

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ABSTRACT

Campylobacter and *Salmonella* are widely recognized as significant causes of foodborne diseases, with poultry and poultry products being the most frequent sources of infection in humans. Rapid, simultaneous detection of both pathogens can prevent contaminated food from entering the human food chain. The present study aimed to simultaneously detect *Salmonella* and *Campylobacter* spp. directly from faeces of broiler chickens aged 4 to 5 weeks by multiplex polymerase chain reaction (mPCR). The suitability of the PCR protocol using pairs of primers targeting the *invA* and *cadF* genes was evaluated to detect *Salmonella* and *Campylobacter* spp., respectively, from 15 samples. The specificity of the PCR assay was 100% for both pathogens, as no positive cross-reactions were detected with non-targeted bacteria. The limit of detection for pure culture of *Salmonella* was 1 CFU/mL, whereas for *Campylobacter* it was 10^1 CFU/mL. Incubation of spiked faeces in brain heart infusion broth for 24 to 48 hours maintained a detection limit of 1 CFU/mL for *Salmonella*; however, the sensitivity decreased, resulting in a detection limit of 10^2 CFU/mL for *Campylobacter*. Simultaneous detection from 15 chicken faecal samples revealed two samples co-carrying both *Salmonella* and *Campylobacter* spp., four samples positive for *Salmonella*, six samples positive for *Campylobacter*, and three samples were negative for both pathogens. The developed mPCR protocol in the present study was highly specific and sensitive for detecting *Salmonella* and *Campylobacter* spp. directly from chicken faeces, achieving results in under 36 hours compared to the conventional culture method. The mPCR protocol can benefit veterinary and public health authorities during epidemiological investigations and rapid diagnostic purposes, which require timely confirmation of the disease status in chickens during the grow period.

Keywords: *Campylobacter*, Faeces, Multiplex PCR, *Salmonella*, Simultaneous detection

INTRODUCTION

Campylobacter (*C.*) and *Salmonella* (*S.*) spp. are two of the top foodborne bacteria associated with diarrhoeal diseases in humans worldwide (WHO, 2015). Foodborne diseases pose a substantial threat to food safety and international trade (Gizaw, 2019; Tibebu et al., 2024). Although the poultry sector in Malaysia has exceeded local demand (Department of Veterinary Services Malaysia, 2017), efforts to increase the quality and safety of the food are necessary to sustain production in the long term.

In Malaysia, several studies have reported that *Campylobacter* and *Salmonella* spp. are prevalent in chicken farms (Osman et al., 2021; Wahab et al., 2021), as well as in chicken meat and retail outlets (Rejab et al., 2012). Sinulingga et al. (2020) found *Campylobacter* spp. in 50.9% of broiler chickens in farms and 26.6% of chicken meat in markets. *Salmonella* spp. was isolated from 46.3% of cloacal swabs collected from poultry farms (Osman et al., 2021) and in 35.0% of raw chicken meat sold in markets (Sukri et al., 2021).

Transmission of foodborne bacteria to humans most commonly occurs through consumption of contaminated food, including foods with animal origin (Rivera-Pérez et al., 2014; Heredia and García, 2018; Abebe et al., 2020), fruits and vegetables (Park et al., 2012; Abakpa et al., 2015; Nguyen et al., 2021), or from direct contact with animals (Ranjbar and Babazadeh, 2017; Sodagari et al., 2020; Babazadeh and Ranjbar, 2022), resulting in diarrhoea, abdominal pain, nausea, and fever. These transmission routes may reflect gaps in poultry husbandry practices, such as flock hygiene and biosecurity, as well as control measures throughout the poultry supply chain. Contamination of poultry products can occur during slaughtering and processing (Rivera-Pérez et al., 2014), as well as in markets and retail outlets (Sadek et al., 2023). Several studies have indicated higher rates of *Salmonella* spp. detected in retail markets compared to processing plants (Adeyanju and Ishola, 2014). Moreover, improper temperature control and inadequate hygiene practices at retail outlets can further promote bacterial growth and survival (Salama and Chennaoui, 2024).

The conventional method for identifying *Salmonella* and *Campylobacter* spp. involves bacterial isolation by a culture method, and biochemical tests for presumptive identification are time-consuming and laborious, requiring five to seven days to confirm the diagnosis (Strakova et al., 2021; Neyaz et al., 2024). Additionally, the inability to detect viable but non-culturable cells in the sample may result in false-negative outcomes. The development of rapid detection tests,

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such as multiplex polymerase chain reaction (mPCR), enables the simultaneous detection of multiple strains of pathogens (Settanni and Corsetti, 2007), allowing for early, rapid, responsive, and preventive measures to be taken. The direct detection of *Salmonella* and *Campylobacter* spp. as single organisms from faecal samples has been previously documented by Pathmanathan et al. (2003), Persson and Olsen (2005), and Al Amri et al. (2007); however, co-detection by mPCR directly from chicken faeces has yet to be established. Therefore, the present study aimed to simultaneously detect *Salmonella* and *Campylobacter* spp. directly from the faecal samples of 4- to 5-week-old broiler chickens in Malaysia, using mPCR.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia, Malaysia (AUP no: UPM/IACUC/AUP-R089/2019).

Sample collection

To develop the protocol using field-isolated samples, 15 pooled chicken faecal samples were collected from three broiler farms in Selangor, Malaysia, by convenience sampling, following verbal consent from the farmers. The samples originated accidentally from physically healthy chickens aged four weeks, with no clinical signs of disease. Clean plastic sheets were placed on the floor in the chicken house for approximately 30 minutes to collect freshly voided faeces from live chickens. The faeces were then collected using a sterile spatula, with multiple droppings originating from different chickens piled together into a sterile 50 mL Falcon™ tube to ultimately collect five tubes of pooled faecal samples, and transported in an ice box to the laboratory. The samples were processed immediately upon arrival at the laboratory. To evaluate the developed protocol, all collected chicken faeces were analysed in parallel using both the conventional culture method and the mPCR assay.

Isolation and identification of *Salmonella* and *Campylobacter*

For isolation and identification of *Salmonella*, the method was conducted according to the terrestrial manual (WOAH, 2022). The faecal samples were placed in 10 mL of buffered peptone water broth (Oxoid, UK) and incubated for 18 to 24 hours at 37°C. Then, 1 mL of the overnight culture was inoculated into 9 mL of Rappaport-Vassiliadis (RV) enrichment broth (Oxoid, UK) and incubated for 18 to 24 hours at 42°C. A loopful of cultured RV broth was streaked onto selective media, which were xylose lysine deoxycholate (XLD) agar (Oxoid, UK) and brilliant green agar (BGA; Oxoid, UK), and incubated at 37°C for 24 hours. All presumptive colonies were identified from each plate, with the appearance of circular red colonies with a black center on XLD agar and pink with mucoid colonies on BGA agar (Figure 1A and B). The *Salmonella*-suspected colonies were subjected to a series of biochemical tests, namely oxidase (Remel Europe, UK), urease (Oxoid, UK), triple sugar iron (TSI; Oxoid, UK), citrate (Oxoid, UK), and further tested with slide agglutination using polyvalent O (A-G) and polyvalent H antisera (phase 1 and 2; Denka Seiken, Japan). The presumptive colonies were stored in glycerol at -40°C until further use.

For *Campylobacter*, the method was carried out based on ISO 10272-1:2017 (WOAH, 2018). The cotton swab containing the faecal sample was streaked directly onto modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid, UK) and incubated under microaerophilic conditions at 42°C for 48 hours. Colony morphology was examined before Gram staining and the motility test. Presumptive colonies were sub-cultured onto Columbia blood agar (CBA; Oxoid, UK) agar containing 5% defibrinated horse blood and incubated in microaerophilic conditions for an additional 48 hours (Figure 1C and D). Then, the presumptive colonies were further identified by biochemical tests, including oxidase, catalase, urease, indoxyl acetate hydrolysis (Remel, USA), and hippurate hydrolysis (Sigma Aldrich, USA).

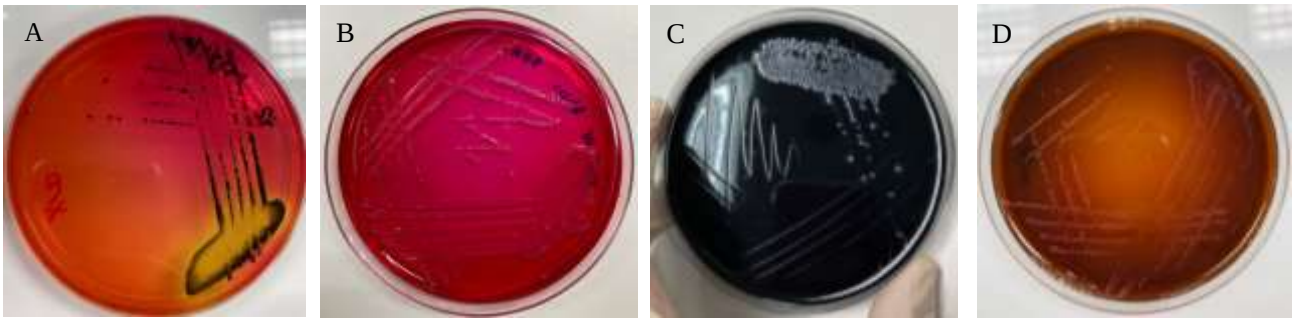

Figure 1. Presumptive isolates of Salmonella and Campylobacter spp. colonies on selective growth media agar plates. A: Presumptive growth with colony morphology typical of Salmonella on xylose lysine deoxycholate agar, B: Presumptive growth on brilliant green agar, C: Presumptive growth with colony morphology typical of Campylobacter spp. on modified charcoal cefoperazone deoxycholate agar, D: Presumptive growth on Columbia blood agar. Source: Authors of the present study.

Bacterial strain and DNA extraction

The reference bacterial strains used in the present study are listed in Table 1. The *S. typhimurium* ATCC 14028 and *C. jejuni* ATCC 29428 were cultured in brain heart infusion (BHI) broth (Oxoid, UK) at 37°C for 18 hours and 42°C, respectively. These strains served as positive controls for the development of mPCR. For direct detection of bacteria, the chicken faeces were placed in 20 mL of BHI and incubated for 24 hours at 37°C for *Salmonella* and in microaerophilic conditions at 42°C for *Campylobacter*. Then, 1 mL of the faecal mixture was transferred into a 1.5 mL microcentrifuge tube for DNA extraction. The DNA extraction was performed using a modified method described by Saeki et al. (2013). A 1 mL bacterial suspension was centrifuged for 10 minutes at 13,000 g, and the resulting pellet was resuspended in 200 µL of Triton X-100 2% lysis solution. After heating at 96°C for 10 minutes and cooling down on ice for two minutes, the mixture was centrifuged again at 13,000 g. The supernatant containing DNA was collected and stored at -20°C.

Table 1. The bacterial strains used for optimisation of the mPCR assay in the present study

Bacteria strains	Origin
Salmonella typhimurium ATCC 14028	Remel*
Salmonella enteritidis ATCC 13076	Remel*
Salmonella pullorum ATCC	Remel*
Escherichia coli ATCC 25922	Remel*
Klebsiella pneumonia ATCC 700603	Remel*
Campylobacter coli ATCC 33559	Remel*
Campylobacter jejuni ATCC 29428	Remel*
Pseudomonas spp.	FPV**
Staphylococcus spp.	FPV**
Enterococcus faecalis	FPV**
Enterobacter spp.	FPV**

Remel*: Strains manufactured by Remel Inc., USA, FPV**: Strains obtained from the Bacteriology Laboratory, Veterinary Laboratory Services Unit, Faculty of Veterinary Medicine, University of Putra Malaysia.

Multiplex PCR

The genus-specific genes, *invA* and *cadF*, were used for the development of mPCR to simultaneously detect *Salmonella* spp. and *Campylobacter* spp., respectively. The primer sequences are listed in Table 2. The final volume of the reaction mixture for the protocol was 50 µL, consisting of 8 µL RNAse-free water, 25 µL of REDiant PCR master mix 2X (1st BASE, Singapore), 3 µL each of the *invA* primer, 3 µL of each *cadF* primer, and 5 µL of the DNA template. The DNA amplification was performed using a lab cycler gradient (SensoQuest GmbH, Germany) under the following cycling conditions; Two minutes of initial denaturation at 95°C, 35 cycles of denaturation at 94°C for one minute, annealing for one minute at 52°C, one minute of extension at 72°C, and later finished by final extension for two minutes at 72°C. The finished PCR products were visualised on a 1.5% (w/v) agarose gel (1st BASE, Singapore). The product size was estimated by comparing with the positive control using the ExcelBand™ 100 DNA Ladder (Smobio, Taiwan).

Table 2. Primers used in mPCR for the simultaneous detection of the *Salmonella* and the *Campylobacte*r genus in broiler chicken

Species	Primer (Gene)	Sequence (5'-3')	Product size (bp)	Reference
Salmonella genus	*invA*	(F) AAA CGT TGA AAA ACT GAG GA (R) TCG TCA TTC CAT TAC CTA CC	119	Saeki et al. (2013)
Campylobacter genus	*cadF*	(F) TTG AAG GTA ATT TAG ATA TG (R) CTA ATA CCT AAA GTT GAA AC	400	Al Amri et al. (2007)

PCR specificity and sensitivity

The PCR specificity test was conducted using *Salmonella*, *Campylobacter* spp., and other bacterial species mentioned in Table 1. The specificity of PCR was assessed by determining the reactivity between genus-specific primers for *Salmonella* and *Campylobacter* and bacterial reference strains. The test was considered highly specific if the PCR protocol could accurately identify *Salmonella* and *Campylobacter* spp. The sensitivity of the PCR was evaluated by determining the ability of the protocol to detect *Salmonella* and *Campylobacter* DNA at the lowest dilution. The sensitivity of the mPCR assay was evaluated using a bacterial suspension containing 100 to 10^7 CFU of *Salmonella* and *Campylobacter* per mL of BHI broth (Saeki et al., 2013). The overnight bacterial suspensions were serially diluted in 9 mL of BHI broth at a 1:10 ratio. One mL of each dilution was extracted, and the DNA template was used to conduct the mPCR assay. The test was repeated three times to ascertain replicability. A PCR sensitivity test was conducted using DNA from *Salmonella* and *Campylobacter* spp., testing both species individually and in combination within a single reaction tube.

Spiked faeces experiment

The experiment was conducted using a modified method described by Alves et al. (2012). *Salmonella* and *Campylobacter*-negative chicken faeces samples were used in this experiment. One gram of chicken faeces was suspended in 20 mL of sterile BHI broth. The faecal suspension was inoculated with different concentrations of *Salmonella*, then incubated for 24 hours at 37°C. Additionally, *Campylobacter* was incubated at 42°C under microaerophilic conditions using CampyGen 2.5 L (Oxoid, UK) with oxygen levels maintained at 8-9% and carbon dioxide at 7-8%. For species detection, 1 mL of spiked faeces was transferred into a 1.5 mL microcentrifuge tube. For co-detection, 500 µL of *Campylobacter*-spiked faeces was mixed with 500 µL of *Salmonella*-spiked faeces in a 1.5 mL microcentrifuge tube. The spiked faecal mixture was spun at 13,000 g for 10 minutes. The pellet was washed twice with 900 µL of BHI broth, spun at 13,000 g for 10 minutes, and resuspended in 200 µL of Triton X-100 1%. The boiling method was conducted to extract DNA. The experiment was conducted in three independent replicates.

Data analysis

The diagnostic performance of the mPCR assay for detecting *Salmonella* and *Campylobacter* spp. in broiler faecal samples was evaluated using culture as the reference method. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated using the data presented in Table 5, according to standard definitions and the following formulas (Parikh et al., 2008).

$$Sensitivity = \frac{100 \times True\ positive}{(True\ positive + False\ negative)}$$

$$Specificity = \frac{100 \times True\ negative}{(False\ positive + True\ negative)}$$

$$Positive\ predictive\ value = \frac{100 \times True\ Positive}{(True\ positive + False\ positive)}$$

$$Negative\ predictive\ value = \frac{100 \times True\ Negative}{(True\ negative + False\ negative)}$$

RESULTS AND DISCUSSION

A multiplex PCR protocol was developed for the co-detection of *Salmonella* and *Campylobacter* spp. directly from chicken faeces. In the present study, both genus-specific primers targeting the *invA* and *cadF* genes for *Salmonella* and *Campylobacter* spp., respectively, were included in the protocol. Before specificity and sensitivity tests, the PCR protocol was optimised to determine the most suitable primer concentrations and PCR cycling conditions. All *Salmonella* and *Campylobacter* strains tested were amplified and yielded 119 bp and 400 bp, respectively (Figure 2). Other non-targeted bacterial strains representing common gastrointestinal bacteria found in chicken indicated no amplification for both primer pairs. In alignment with prior studies conducted by Alves et al. (2012) and Saeki et al. (2013), the Styinva-JHO-2 primer pair demonstrated 100% specificity, supporting its reliability for detecting *Salmonella* serovars. The primer pair targeting the *invA* gene was a protein located in *Salmonella* pathogenicity island 1 (SPI-1) that plays a role in the invasion of host intestinal cells. The SPI-1 activates the Type III secretion system (T3SS) to assist *Salmonella* in invading epithelial cells and recruiting host immune cells (Singh et al., 2018). Amplification of the *invA* gene has been widely used in numerous studies to detect *Salmonella* from different types of samples, such as animal faeces (Deguenon et al., 2019), poultry meat (Shanmugasamy et al., 2011; Sharma, 2016), beef (Thung et al., 2018), and foods (Heymans et al., 2018).

Another virulence gene targeted in the present study was the *cadF* gene, which facilitates bacterial attachment to the host fibronectin matrix and subsequent invasion of epithelial cells during *Campylobacter* colonization (Ngobese et al., 2020). Casabonne et al. (2016) demonstrated high prevalence (100%) of *cadF*, *flaA*, and *cdtB* virulence genes compared to *ciaB* (50%), *docC* (23.3%), *wlaN* (20%), and *cgtB* (6.7%) genes when tested with different *Campylobacter* strains isolated from human stool samples. A high prevalence of the *cadF* gene was discovered in faecal samples from different livestock animals, including cattle, chickens, goats, sheep, and pigs (Ngobese et al., 2020), as well as in broiler chickens (Khoshbakht et al., 2013). Additionally, Reddy and Zishiri (2018) detected the *cadF* gene in 100% of broiler faeces and human clinical isolates, which could be suggestive of its pathogenic potential to humans. Additionally, Ayaz et al. (2016) reported 100% specificity for the detection of the *hipO* gene by PCR compared to the *ceuE* gene, which detected only 43.9% of 41 *C. jejuni* isolates. Although different genes were targeted to identify *Campylobacter* by PCR, *cadF* has a higher specificity rate compared to the other genes, such as *ciaB* and *ceuE*.

Serial dilution analysis demonstrated that the mPCR protocol could detect *Salmonella* spp. via the *invA* gene (119 bp) and *Campylobacter* spp. via the *cadF* gene (400 bp) in pure culture at concentrations as low as 1 CFU/mL and 10^1 CFU/mL, respectively. The assay proved effective for both the individual and simultaneous detection of the genes (Figures 3 and 4). After 24 hours of enrichment, both *Salmonella* (119 bp) and *Campylobacter* (400 bp) could be detected directly from chicken faeces at 1 CFU/mL and 10^2 CFU/mL, respectively (Figure 5). The mPCR assay presented in the present study was able to detect as low as 10^1 CFU/mL of *Salmonella* and *Campylobacter* spp. (Figure 6), which is more sensitive compared to the study reported by Alves et al. (2012). A pure culture of both *Salmonella* and *Campylobacter* spp. at 10^4 CFU/mL was detected during the study. The comparable sensitivity levels observed between the direct detection of spiked chicken rinses reported by Alves et al. (2012) and the present study, using chicken faecal

samples, highlighted the robustness of the mPCR assay, suggesting its suitability for reliable detection across different poultry matrices and potential application in broader food safety surveillance.

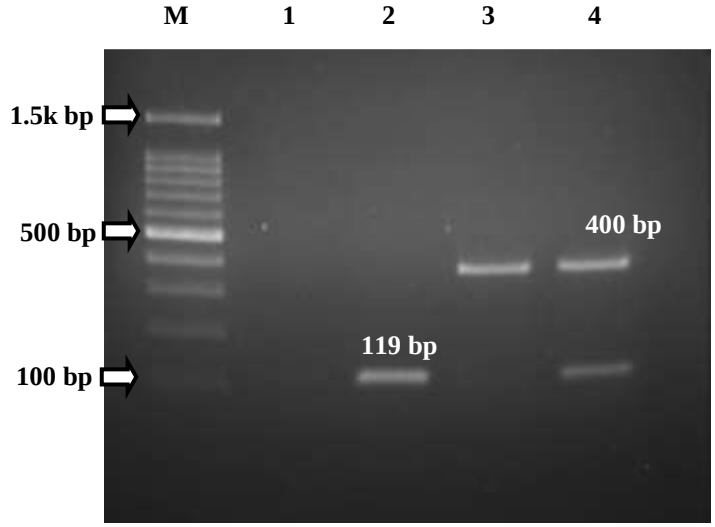

Figure 2. Multiplex PCR of positive controls used for the detection of *Salmonella* and *Campylobacter* spp. isolates from broiler chicken faeces. Lane M: Molecular size marker (100 bp). Lane 1: *Klebsiella pneumonia* ATCC 700603 (Negative control), Lane 2: *Salmonella* spp. (119 bp), Lane 3: *Campylobacter* spp. (400 bp), Lane 4: *Salmonella* spp. (119 bp) and *Campylobacter* spp. (400 bp).

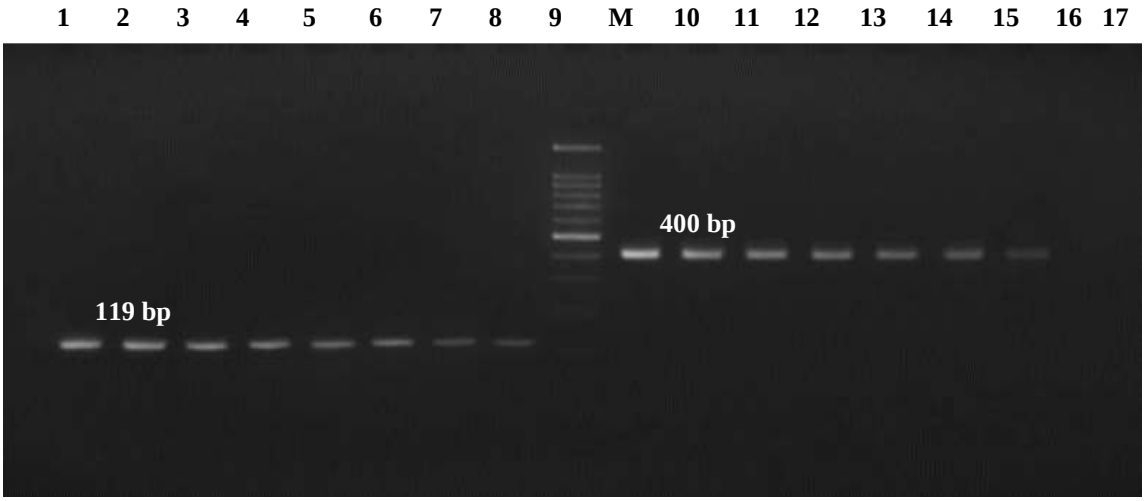

Figure 3. Sensitivity detection of *invA* and *cadF* genes from pure cultures of *Salmonella* and *Campylobacter* spp. from broiler chicken faeces by detecting each gene individually. Lane 1: *Klebsiella pneumonia* ATCC 700603, Lane 2-9: 10-fold serial dilution containing 10^7 to 1 CFU/mL of *Salmonella* (119 bp), M: molecular size marker (100 bp), Lane 10-17: 10-fold serial dilution containing 10^7 to 1 CFU/mL of *Campylobacter* spp. (400 bp).

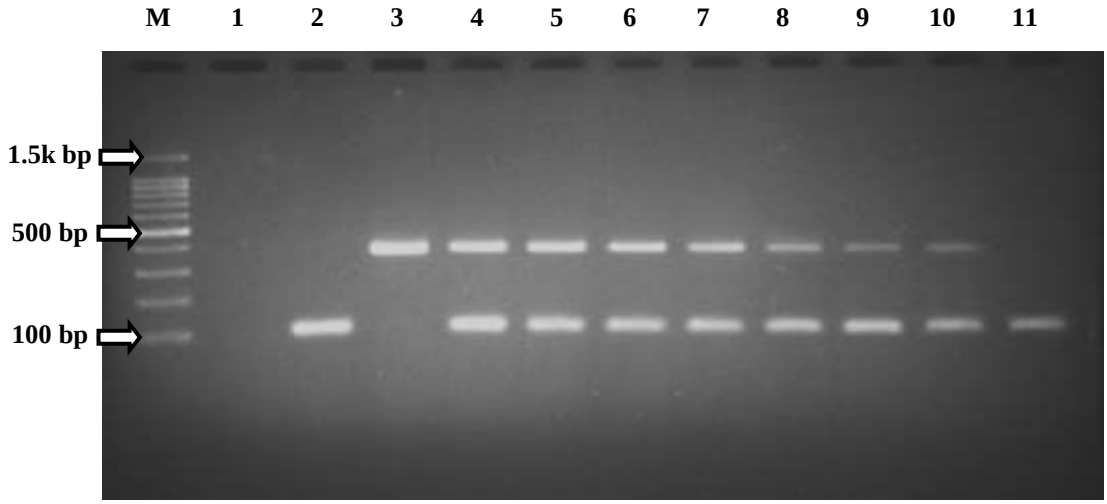

Figure 4. Sensitivity detection of the *invA* and *cadF* genes from pure cultures of *Salmonella* and *Campylobacter* spp. from broiler chicken faeces by detecting both genes simultaneously in one PCR tube. Lane M: Molecular size marker (100 bp), Lane 1: *Klebsiella pneumonia* ATCC 700603, Lane 2: *Salmonella* (119 bp), Lane 3: *Campylobacter* spp. (400 bp), Lane 4-11: 10-fold serial dilution of pure culture containing 10^7 to 1 CFU/mL of both *Salmonella* and *Campylobacter* spp.

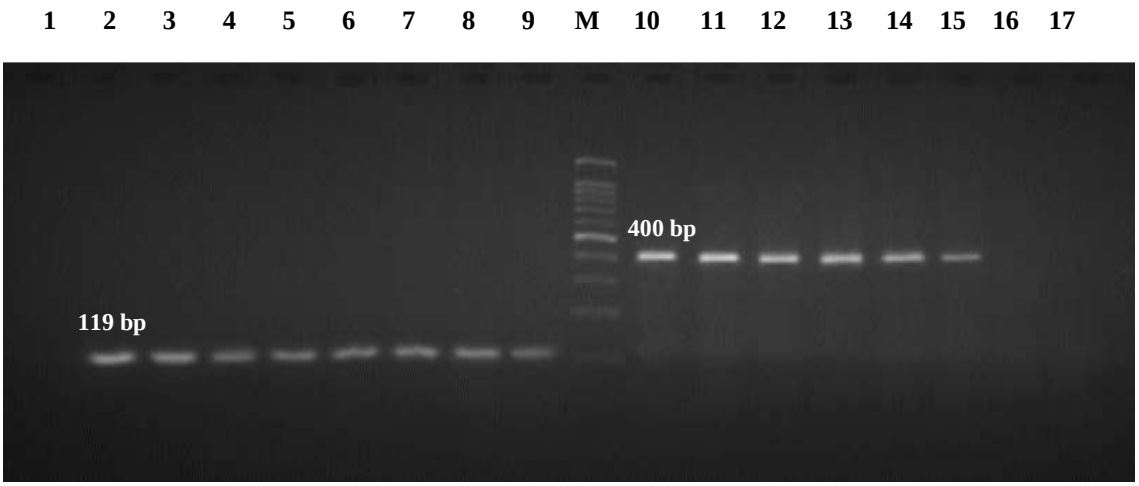

Figure 5. Sensitivity detection of *invA* and *cadF* genes from spiked broiler chicken faeces containing *Salmonella* and *Campylobacter* spp. after overnight incubation by detecting each gene individually. Lane 1: *Klebsiella pneumonia* ATCC 700603, Lane 2-9: 10-fold serial dilution containing 10^7 to 1 CFU/mL of *Salmonella* (119 bp), M: molecular size marker (100 bp), Lane 10-17: 10-fold serial dilution containing 10^7 to 1 CFU/mL of *Campylobacter* spp. (400 bp).

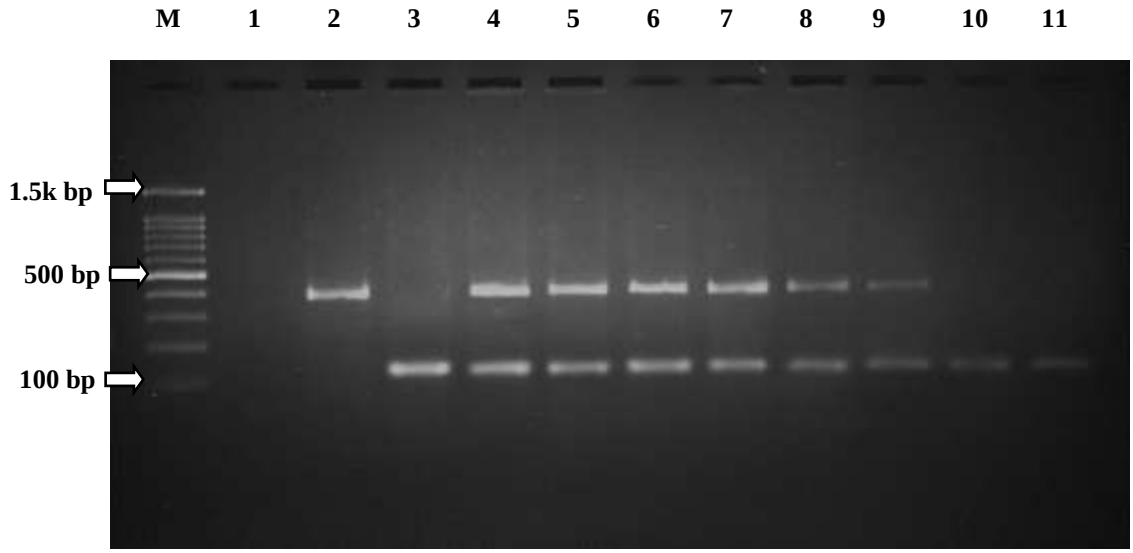

Figure 6. Detection of *invA* and *cadF* genes simultaneously from spiked broiler chicken faeces containing *Salmonella* and *Campylobacter* spp. in multiplex PCR in one PCR tube. Lane M: Molecular size marker (100 bp), Lane 1: *Klebsiella pneumonia* ATCC 700603, Lane 2: *Campylobacter* (400 bp), Lane 3: *Salmonella* (119 bp), Lane 4-11: 10-fold serial diluted spiked chicken faeces containing 10^7 to 1 CFU/mL of both *Salmonella* spp. and *Campylobacter* spp.

In the present study, the detection limit of the PCR for *Campylobacter* spp. in chicken faeces was 10^2 CFU/mL, indicating low analytical sensitivity compared to other studies. Lund et al. (2004) demonstrated a detection limit of 100 to 150 CFU/mL in chicken faecal suspension. The higher detection limit observed in the present study may be attributed to the nature of the faecal matrices that often contain PCR inhibitors such as bilirubin, bile salts, other organic and inorganic substances, which might interrupt the amplification of the targeted gene and, as a result, reduce the PCR sensitivity (Yan et al., 2020). Several studies suggested incorporating an enrichment step to supply essential nutrients, promote bacterial recovery and growth, and ultimately improve sensitivity and selectivity (Gwida and Al-Ashmawy, 2014; Lee et al., 2015). Additionally, Park and Ricke (2015) demonstrated that the optimized multiplex PCR and qPCR could detect approximately 2.2 CFU of *Salmonella* per gram following an 18-hour enrichment period in spiked chicken breast meat samples.

The mPCR protocol developed was compared to conventional culture methods using 15 pooled faecal samples from different broiler farms. The mPCR method detected more positive samples compared to the culture method, including some that the culture method failed to identify (Table 3). These results could be due to the higher sensitivity of mPCR, suggesting that conventional culture methods may underestimate the actual prevalence of the bacteria. In addition, this may reflect the ability of PCR to detect DNA from viable but non-culturable bacteria and samples with low bacterial loads. Among the culture-positive samples, *Salmonella* spp. was detected in 1/15 (6.7%) samples, *Campylobacter* in

6/15 (40%) samples, and co-detection of both bacteria was obtained in 2/15 (13.3%) samples. The mPCR assay detected *Salmonella* in 4/15 (26.7%) samples, *Campylobacter* in 6/15 (40.0%) samples, and both pathogens in 2/15 (13.3%) samples (Table 4). To determine the sensitivity, specificity, and predictive values, a two-by-two contingency table was constructed (Table 5). Table 5 indicates that the mPCR assay estimated sensitivity for classifying a sample as infected (Positive) was 100%. At the same time, 50% of samples were accurately identified as non-infected (Negative) by the mPCR. The mPCR approach had a 75% positive predictive value and a 100% negative predictive value. In the present study, some samples that were positive by mPCR but negative by culture were likely due to the presence of sub-lethally injured cells that fail to grow on selective media. This highlighted the greater sensitivity of PCR compared to conventional culture methods, rather than representing true false-positive results (Liang et al., 2018). Other comparative studies on detection methods using mPCR assay and conventional culture method to detect common diarrhoea causative bacteria, such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp., and *Escherichia coli* O157, indicated similar results to those in the present study (Gwida and Al-Ashmawy, 2014; Bonilauri et al., 2016; Huang et al., 2018).

The rapid identification of the common foodborne pathogens is beneficial for preventive and control measures, as it enables early intervention to reduce the dissemination of bacteria and potential contamination of poultry products (Zendrini et al., 2021). Comparable to the findings of Benlashehr et al. (2024), who demonstrated that pre-enrichment and selective enrichment were essential for the successful recovery of *Salmonella enteritidis* from poultry and murine faecal samples, the present study also indicated that a culture-based method with enrichment can detect *Salmonella* and *Campylobacter* spp. from the faecal samples. However, both culture and PCR approaches were able to identify the pathogens; culture required at least five days to obtain a result, whereas PCR yielded outcomes within a much shorter time frame. For *Campylobacter*, an additional 24 hours of incubation is required if no growth was observed on the plate, as recommended by ISO 10272-1:2017 (WOAH, 2018). This additional incubation period can delay reporting and the implementation of appropriate control measures, potentially allowing the foodborne pathogens to persist longer in the food production chain. In the current study, *Salmonella* and *Campylobacter* spp. were detected simultaneously directly from chicken faeces by reducing the detection time to less than 36 hours compared to the standard culture method as described in the terrestrial manual and ISO 10272-1:2017 (WOAH, 2018; 2022).

The values were used to calculate the sensitivity, specificity, positive predictive value, and negative predictive value of the mPCR assay in comparison to the culture method for detecting *Salmonella* and *Campylobacter* spp. in broiler faeces, using the formulas described by Parikh et al. (2008). The protocol developed in the present study successfully identified all (100%) true positive samples (sensitivity), with a positive predictive value of 75%, and 50% of the true negative samples (specificity), with a negative predictive value of 100%.

Table 3. Culture and mPCR assay results for detecting *Salmonella* and *Campylobacter* spp. from broiler chicken faeces

	Faeces samples (Number)	
	Culture	**mPCR**
Negative	6	3
Positive	9	12

mPCR: Multiplex polymerase chain reaction.

Table 4. Comparison of culture method and mPCR assay for detecting *Salmonella* and *Campylobacter* spp. from broiler chicken faeces

Targeted bacteria	Culture	mPCR
Salmonella spp.	1/15 (6.7%)	4/15 (26.7%)
Campylobacter spp.	6/15 (40.0%)	6/15 (40.0%)
Both *Salmonella* spp. and *Campylobacter* spp.	2/15 (13.3%)	2/15 (13.3%)

mPCR: Multiplex polymerase chain reaction.

Table 5. Two-by-two contingency table comparing the mPCR and culture results for *Salmonella* and *Campylobacter* spp. detection from broiler chicken faeces

		Culture		Total
		Positive	**Negative**	
mPCR	Positive	9	3	12
	Negative	0	3	3
Total		9	6	15

CONCLUSION

The use of cultivation techniques to isolate and detect pathogens may help explain the lack of surveillance data on *Salmonella* and *Campylobacter* infections in Malaysia. The mPCR protocol developed in the present study enabled the rapid, simultaneous detection of *Salmonella* and *Campylobacter* spp. directly from chicken faeces. The protocol developed allowed the detection of *Salmonella* and *Campylobacter* spp. simultaneously at the detection limit of 10^1

CFU/mL and 10^2 CFU/mL of spiked chicken faeces, respectively, after 24 hours of enrichment. Compared to conventional culture methods, this approach substantially reduced detection time by eliminating the need for bacterial isolation and separate confirmatory assays. The DNA extraction and multiplex amplification steps minimized labour and reagent use, while optimized primer sets maintained high sensitivity and specificity. These advantages make the protocol a practical tool for routine surveillance, enabling the detection of pathogens at an early stage of the food chain to prevent the spread and reduce the risk of transmission to humans. Since *Salmonella enteritidis*, *Salmonella typhimurium*, *C. jejuni*, and *C. coli* pose a public health threat, a simultaneous detection method for these species directly from chicken faeces is recommended for future studies.

DECLARATIONS

Acknowledgments

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Authors' contributions

Sherryl Nurfarhanim Abd Ghafar has contributed to the sample collection, processing, and cultivation of the bacteria and the development of the mPCR protocol. Nur Indah Ahmad supervised and approved the final edition before submission. Saleha Abdul Aziz participated in the sample collection process and cultivation of the bacteria. Zunita Zakaria was involved in the molecular analysis. All authors read and approved the final manuscripts.

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Competing interests

The authors declared no conflict of interest.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. All authors have verified the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results. The authors confirmed that no AI tools, including generative AI models, were used in the writing or preparation of the present study.

Availability data and materials

All datasets used in the current study are available from the corresponding author on reasonable request.

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