ORIGINAL ARTICLE

Isolation of *E. coli* in chicken bishop noses using microbiology and molecular methods

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Abstract:

Escherichia coli is an organism commonly used as a fecal indicator to assess and monitor the quality of food product to prevent gastrointestinal infection in humans. This study was conducted to detect and isolate the presence of *E. coli* in poultry products using conventional and molecular detection methods. A total of 10 chicken bishop noses were obtained from two supermarkets in Bandar Puncak Alam. Conventional cultural techniques were performed to screen the presence of *E. coli*. The amplification of the *lamB* gene from bacterial isolates detected a single band at 309 bp, confirming the presence of *E. coli*. In total, 6 out 10 chicken bishop noses were positive for *E. coli* following screening using standard microbiological techniques coupled with PCR. In summary, conventional methods of *E. coli* detection should be used in conjunction with molecular based tests to provide optimal diagnostics. Additional work is warranted to further characterize the bacterial isolates to determine its clinical significance.

Keywords: Chicken bishop noses, *E. coli*, *lamB* gene

1. INTRODUCTION

Food hygiene and safety has become an important public health concern worldwide, and food-borne outbreaks caused by toxigenic bacteria is on the rise. Chicken bishop nose otherwise known as '*tongkeng ayam'* or '*buntut ayam*' are local delicacies that is popular among Malaysians. Nevertheless, it also serves as a natural medium for bacterial growth, depending on its preparation and cooking practices. *E. coli* is a normal flora that can become pathogenic following ingestion of contaminated food products [1]. It can act as an indicator for the presence of other pathogenic bacteria and can be detected in foods including poultry [2] Microbiological methods to detect *E. coli* typically involves culturing the organism on selective media and identifying the isolates based on morphological and biochemical characteristics [3]. This traditional method often necessitates 5-11 days to execute [4]. In contrast, molecular based detection techniques such as Polymerase Chain Reaction (PCR) provides a more sensitive, specific and rapid mode of microorganism detection.

PCR is a significant tool that can be used to identify pathogens and food-borne outbreaks [5]. The specificity of PCR depends on several factors including the quality of DNA testing material and the optimized conditions using during the PCR cycles [6]. The purity of the starting material plays an important role towards the successful amplification of the desired product. Various studies had been conducted to compare DNA extraction methods that could yield quality DNA for subsequent analysis [7].

This study aims to describe and compare detection methods that can be used to isolate and identify *E. coli* using conventional microbiology and molecular techniques. Specifically, *E. coli* were screened using the *lamB* gene which encodes for the protein surface of the organism [8] and could be used as a marker for rapid *E. coli* detection.

2. MATERIALS AND METHODS

2.1 Sampling

Ten chicken bishop noses were purchased from two supermarkets in Bandar Puncak Alam. All samples were transported on ice to the Medical Laboratory, Centre of Medical Laboratory Technology, Faculty of Health Sciences and processed within 24 h. Samples were used to extract DNA from tissue and bacterial isolate.

2.2 Sample isolation and identification

One g of chicken bishop noses were homogenized, pre-enriched with 5 mL LB broth and incubate at 37ºC for 24 h. Samples were isolated on Nutrient agar and subcultured on Nutrient, MacConkey and Eosin-Methylene Blue (EMB) agars. Colonies were then subjected to Gram staining and biochemical testing.

2.3 DNA extraction

2.3.1 Boiling method

One mL of the culture grown overnight at 37ºC in 5 mL LB broth was centrifuged at 2,500 x g for 4 min. The supernatant was discarded, the pellet was washed three times in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the cells were resuspended in 50 µL of TE buffer. The suspension was placed in 100ºC of boiling water for 10 min and centrifuged for 30 sec at 9,000 x g at 4ºC.

2.3.2 Boiling plus purification method

One mL of the culture grown overnight at 37ºC in 5 mL LB broth was centrifuged at 4,000 x g for 5 min. The supernatant was removed, the pellet was resuspended in 2 mL iced-cold 70% (v/v) ethanol and incubated on ice for 30 min. The suspension was re-centrifuged at 12,400 X g for 5 min and the pellet was re-suspended in 150 µL TE buffer (pH 8.0). Ten µL of 10 mM lysozyme (SIGMA, US) was added to the suspension and incubated for 30 min at 37ºC. The lysate was boiled at 100ºC in boiling water for 10 min and re-centrifuged at 12,400 X g for 5 min before removing the supernatant. Ten µL of 20 mg/mL proteinase K was added to the supernatant. The suspension was incubated for 30 min at 37ºC and boiled for 15 min.

2.3.3 DNA extraction kit

The procedure was conducted using standard instructions as stated in the RTP® Bacteria DNA Mini Kit (Invitek, Berlin).

2.3.4 High salt method

A total of 0.5 cm^2 of chicken bishop nose tissue were placed overnight at 50ºC in 600µL of TNES buffer (10 mM Tris pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS) and 35 µL of 20 mg/mL Proteinase K (Qiagen GmbH, Germany). A total of 166.7 µL of 6 M NaCl was added to the suspension and centrifuged at 14, 000 rpm for 10 min at room temperature. Four hundred µL of absolute ethanol was added to the 400 µL supernatant. The sample was centrifuged at 14, 000 rpm for 20 min at 4ºC and the pellet was washed in 500 µL of 100% (v/v) ethanol. The sample was air dried at 30 min and re-suspended in 100 µL sterile distilled water.

2.4 Concentration and purity of DNA extraction

The concentration and purity of 1 µL DNA was checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific, US) at 260 nm and 280 nm.

2.5 Oligonucleotide primer

A set of primers targeting the *lamB* gene was synthesized and its oligonucleotide sequences are shown in Table 1.

Table 1: Sequences for the *lamB* gene used and amplified product size.

Gene	Primer	Oligonucleotide sequence (5'-3')	Size of amplified product
lamB	Forward	CTGATCGAATGGCTGCCAGGCTCC	309 (bp)
	Reverse	CAACCAGACGATAGTTATCACGCA	

2.6 PCR amplification

Two µL of DNA was used as a template for PCR amplification. The PCR cocktail mixture used were as follows: 0.25 µM 2x Taq Master Mix (Philekorea Technology, PKT, Korea), 10 µM of each primer and 8.5 µL sterilized distilled water. PCR reaction was performed with an initial denaturation at 94ºC for 3 min, followed by annealing at 64ºC for 30 sec and extension at 72ºC for 2 min.

2.7 Agarose gel electrophoresis and gel documentation

All PCR products were visualized in a 2.0% (w/v) agarose gel in TBE buffer (89 mM Tris-borate and 2 mM EDTA pH 8.3) stained with ethidium bromide following electrophoresis for 90 min at 60 V. Amplified products were visualized by UV illumination using a gel documentation system.

3. RESULTS

3.1 Microbiological analysis

Gram negative organisms (Figure 1) were isolated from the tissue samples. The following characteristics were observed: creamy-white colonies on Nutrient Agar, lactose-fermenting colonies on MacConkey agar and green-metallic sheen on EMB (Figure 2). Citrate, SIM and TSI test showed negative citrate test, positive indole reaction and motile organisms. TSI results revealed acid slant/acid butt reaction with gas with no H_2S.

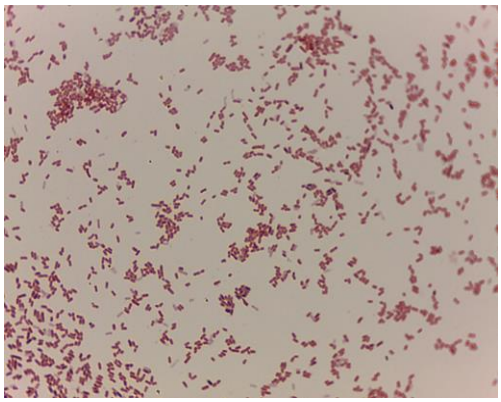
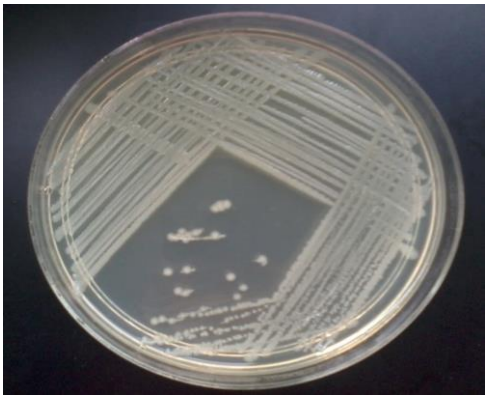


Figure 1: Gram stain showing Gram-negative organisms.

(a)



(b)

(c)

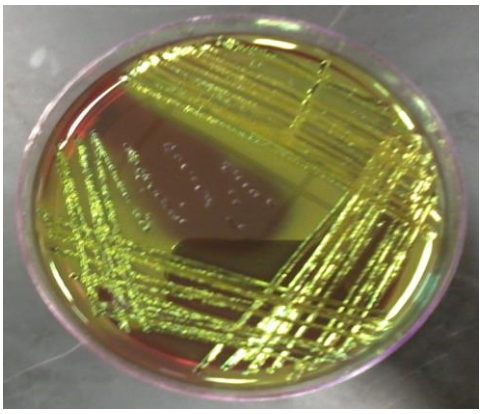


Figure 2: Colonies on (a) Nutrient, (b) MacConkey and (c) EMB agar following overnight incubation.

3.2 Concentration and purity of DNA extraction

Four different types of DNA extraction were tested namely boiling method, boiling method plus purification and the use of a commercially available DNA extraction kit (RTP Mini Kit) and high-salt method. The boiling method yielded the best results in terms of DNA yield and purity with A_{260}/A_{280} at ratio of 1.7 to 2.0.

3.3 PCR amplification of the *lamB* gene

Six out of 10 samples of chicken bishop noses were positive for *E. coli* using both conventional and PCR based methods. Optimization of the cycling conditions using gradient PCR revealed 64ºC to be the most optimum annealing temperature. All the 6 positive amplified products revealed a single band at 309 bp (Figure 3).

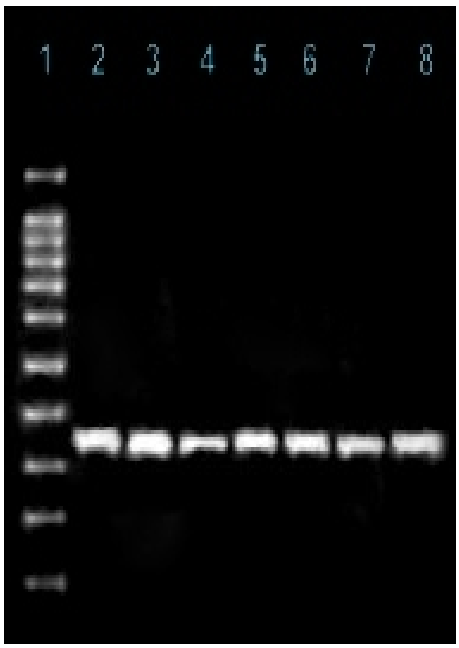


Figure 3: Amplification on 2.0 % (w/v) agarose gel stained with ethidium bromide showing: Lane 1: 100bp of ladder, lane 2: Positive control (ATCC 4157) Lane 3, 4, 5, 6, 7 and 8: positive bands of samples A1, A3, A5, A1, A2 and A5 with amplified products at 309 bp.

4. DISCUSSION

Foodborne pathogens usually cause mild to moderate self-limiting gastroenteritis. Nevertheless, invasive diseases and complications may occur, resulting in more severe cases if left undiagnosed or untreated [9]. It is therefore essential to establish an appropriate method to detect possible *E. coli* infection to circumvent incidences of food poisoning. In this present study, both types of detection methods were able to detect the presence of *E. coli* in chicken bishop noses. Conventionally, was isolated and identified using Gram Stain, culture on selective media and biochemical testing. Pre-enrichment in LB facilitated the enhancement of bacterial growth and aided the detection of *E. coli* [10]. The

use of enrichment broth had been documented in literature to improve *E. coli* detection efficiency [11, 12]. Although valuable, traditional methods such as microbial culture are time-consuming and less specific.

PCR amplification of regions within the *lamB* gene was tested for the identification of *E. coli.* PCR is a sensitive tool that can be rapidly used to for detection purpose due to its sensitivity and specificity. Nevertheless, several factors will determine the final output of PCR. The choice of primer, the cycling conditions and quality of starting material are integral parameters that needs to be carefully selected and optimized to ensure the validity and reproducibility of test results. Undesired reaction conditions can lead to the generation of unwanted products and even no amplification at all [13]. For instance, there are several methods that can be used to extract bacterial DNA. Methods vary according to principles and the concentration and quality of the DNA obtained by each one of these methods is variable and microorganism dependent [14]. In this study, the boiling method yielded the most optimum results in terms of concentration and purity. This method is easy, inexpensive and relatively rapid. The use of RTP extraction kit was equally as good in terms of time and purity but yielded less amount of DNA in this study.

Following extraction, gradient PCR was used to enable optimization of the cycling conditions. DNA amplified directly from tissue samples initially resulted in several non-specific bands, requiring additional optimization. Following several runs, the most optimum annealing temperature was found to be at 64ºC for the *lamB* gene. In contrast, amplification of the *lamB* gene from bacterial isolates detected a single band at 309 bp. The *lamb* gene was able to amplify the targeted gene from the extracted DNA from tissue and bacterial samples indicating the presence of *E. coli* in the chicken bishop noses. Results of this study indicated that 6 out of 10 samples were positive for *E. coli* with 309 bp visualized on agarose gel. The PCR results validated the initial screening using microbiological culture.

This preliminary study provides a platform to investigate the presence of *E. coli* in chicken bishop noses in Malaysia. The gene of interest is a suitable marker that can be used to assess the presence for fecal contaminant not only in food products but also fecal pollution in water [15]. Further work should determine the relevance of the *E. coli* isolated in terms of pathogenicity and virulence. Further investigation is warranted using a larger sample size to corroborate this finding. It is suggested that the amplified product be purified, cloned and sequenced in forthcoming studies.

5. CONCLUSION

E. coli were present in 6 out of 10 chicken bishop nose samples detected using both conventional and PCR based methods. Results from this study suggests that although *E. coli* can be detected by bacteriological, biochemical and serological tests, it should ideally be tested in conjunction with molecular techniques to provide optimal diagnostics. Additional work is warranted to further characterize the bacterial isolates to determine its clinical significance.

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