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Isolation and detection of enteroinvasive *Escherichia coli* from skewered meatballs by using *ipaH* gene

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Abstract

Nowadays, food safety has become an increasingly important aspect to be considered, particularly in developing countries such as Indonesia. Yogyakarta is known for its plethora of street foods, which are sold in public places and school environments. In the present work, the microbiological safety of such street foods, particularly skewered meatballs, was assessed. The present work aimed to detect enteropathogenic bacterial contaminations, particularly enteroinvasive *E. coli* (EIEC), using a combination of microbiological, biochemical assay API 20E, and molecular characterisation of virulence factor by *ipaH* primer pairs. The results confirmed the presence of bacterial contamination particularly from the most-common coliform group. The street food vendors must be educated by presenting information that the total bacterial count has exceeded the safety threshold. The microbiological examination confirmed the presence of suspected enteropathogenic colonies, based on the biochemical assays. Molecular identification of three isolates yielded positive results containing *ipaH* gene, which is a strong indication of EIEC-type of bacteria, most probably *E. coli* and *Shigella* spp.

Introduction

Yogyakarta is the "Mecca" of students indulged by larger-than-life street foods. Street foods are ready-to-eat foods and beverages prepared and/or sold by vendors or hawkers, especially in the streets and other similar places. One of the most favourite street foods is skewered meatballs. Most of the people are very fond of this savoury snack, particularly students. Skewered meatballs are indeed delicious, popular, appetising, and relatively affordable for people living in Yogyakarta. Skewered meatballs are a type of snack prepared from flour and meat, which then are formed into a ball shape, and boiled (Tahya *et al.,* 2018). Although this snack is a crowd pleaser, the preparation and serving process prove otherwise; it is unhygienic and lacks of quality standards, which can cause foodborne illnesses especially diarrhoea (Rosida and Windyasmara, 2017; Tahya *et al.,* 2018). *Escherichia coli* is one of the normal flora found in the human body, and it can be pathogenic, which can cause significant morbidity and mortality worldwide (Clements *et al.,* 2012). Data in Indonesia show that the level of morbidity and mortality caused by diarrhoea escalated between the year 2000 to 2010, and remains a public health problem (Indonesian Ministry of Health, 2011).

Diarrhoea is caused by the ingestion of *E. coli.* *Escherichia coli* is a commensal bacterium found in the intestine of warm-blooded animals. Based on this fact, if it is found in food, it could be used as a faecal contamination indicator. The pathogenic characteristics and virulence factors confirm that *E. coli* causes infection, and is grouped into: (i) enteroinvasive *E. coli* (EIEC); (ii) enterotoxigenic *E. coli* (ETEC); (iii) enteropathogenic *E. coli* (EPEC); (iv) enteroaggregative *E. coli* (EAggEC), and (v) verotoxin *E. coli* (VTEC) (Løbersli et al., 2016; Miri et al., 2017). Several studies have reported about EIEC contamination in processed beef products, poultry products, vegetables, and fruit salads (Godambe *et al.,* 2017; Tehrani *et al.,* 2018). The EIEC is one of the pathogenic *E. coli* that can cause diarrhoea due to its ability to invade and penetrate the intestinal epithelium. Thus, EIEC is one of the main factors of child morbidity and mortality in developing countries. Detecting EIEC in clinical and non-clinical samples remains perplexing as they share similar properties with other *E. coli* strains. The present work focuses on detecting EIEC in skewered meatballs samples using microbiological, biochemical, and molecular confirmation tests using API 20E and PCR with EIEC specific primers for *ipaH* gene.

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Materials and methods

Sample collection

Skewered meatballs samples were obtained from ten different street vendors in various locations in Yogyakarta, e.g. schools and public places. Samples taken were temporarily stored in sterile plastic bags then placed in an ice-packed cooling container to maintain the temperature at 0 - 4°C. The samples were then taken to the Microbiology Laboratory of Universitas Kristen Duta Wacana for analysis in less than 2 h, according to the standard method (Akbar *et al.,* 2014; Rouger *et al.,* 2017; Andrews and Hammack, 2018).

Pre-enrichment and enumeration of enteric bacteria

Twenty-five grams of samples were mashed and then inoculated in buffered peptone water medium for pre-enrichment culture. The medium was then stored at 37°C in a rotary shaker for 16 - 22 h. Pre-enrichment culture will nourish cells damaged during the cooking process and yield high cell concentrations (Sangadkit *et al.,* 2016). Cell cultures were serially diluted to 10-7 with 0.1% peptone water. Next, 0.1 mL cell cultures were inoculated from the 10-5, 10-6, and 10-7 dilutions using the spread plate technique on the surface of chromocult coliform agar (CCA) medium (Merck, Germany), and then incubated for 24 - 48 h at 37°C. The suspected colonies of enteric bacteria grown were selected based on their respective colonies. The CCA medium is a chromogenic medium that can detect distinctive bacterial colonies based on their ability to use the substrate present in the medium. Members of the family Enterobacteriaceae, particularly coliform and *E. coli*, will have different typical colonies based on their ability to produce β-galactosidase and β-glucuronidase enzymes. Members of enteric bacteria that are positive for β-galactosidase and β-glucuronidase will have dark blue coloured colonies, which are generally from the *Escherichia coli* group. Bacteria positive for β-galactosidase and negative β-glucuronidase will have red coloured colonies, generally from the bacterial groups of *Citrobacter, Enterobacter*, and *Klebsiella*, while bacteria positive for β-glucuronidase will have bright blue coloured colonies. The CCA medium can be used to distinguish between *E. coli* suspected colony and other family Enterobacteriaceae members (Rattanabumrung *et al.,* 2012; Sangadkit *et al.,* 2016).

Selection of enteropathogenic bacterial candidates

Typical dark blue coloured colonies of suspected *E. coli* bacteria growing on CCA medium were then streaked to obtain pure isolates. *Escherichia coli* suspected isolates were selected through a series of tests, based on biochemical properties according to the Bergey's Manual (Brenner and Farmer, 2015). The biochemical tests to identify *E. coli* suspected isolates are Gram-staining, motility, IMViC, urease, carbohydrate, acid, and H_2S fermentation tests. All test samples were incubated at 37°C for 24 - 48 h (Chong *et al.,* 2017; Patel *et al.,* 2017; Cappuccino and Welsh, 2018).

Biochemical characterisation

The confirmation step was carried out to confirm the species from the suspected isolates using API 20E kit (Julian *et al.,* 2015). The suspected isolates were selected using CCA medium, which were then transferred to BHI medium with incubation time of 18 - 24 h at 37°C. The initial culture was taken using a loop, and placed into 5 mL of 0.85% NaCl. Standardisation of the turbidity level of bacterial suspension was carried out using a McFarland 0.5 solution. Next, the suspension was dropped on 20 wells using a sterile drop pipette, and a sterile aquadest was introduced at the bottom of the kit to maintain moisture during incubation, based on API 20E test procedures. The results were confirmed using API web software (BioMérieux, French).

Molecular confirmation of the presumptive positive enteroinvasive E. coli

Molecular identification was carried out only for EIEC with true pathogenic characteristics of invasion plasmid antigen H (*ipaH*) (Løbersli *et al.,* 2016). The isolation of DNA was performed using Wizard® Genomic DNA Purification Kit from overnight grown culture. The obtained DNA was sequentially amplified using *ipaH* F (5'GTT CCT TGA CCG CCT TTC CGA TAC CGT C'3) and *ipaH* R (5'GCC GGT CAG CCA CCC TCT GAG AGT AC3') (Dias *et al.,* 2016), producing amplicon of 619 bp by the following PCR program: pre-denaturation at 95°C for 5 min, denaturation at 94°C for 60 s, annealing at 58°C for 60 s, extension at 72°C for 60 s and 30 cycles, and final extension at 72°C for 5 min (Ranjbar *et al.,* 2016).

Results and discussion

The present work sampled skewered meatballs from ten various street vendors in Yogyakarta. The enteropathogenic contamination in skewered meatballs was detected using selective differential CCA medium. The total coliform colonies and total enteric bacterial colonies are shown in

Table 1. All skewered meatball samples yielded total count of bacteria of more than 109 CFU/g. This concurs with the findings of Ferawati *et al.* (2017), stating that the average total number of bacterial colonies found on meatballs was more than 108 CFU/g. The findings indicate that the heat treatment introduced to meatballs by steaming evidently failed to kill the *E. coli.* Haslia *et al.* (2015) claimed that for hygiene, meatballs must be prepared and heated at a temperature of ≥ 90°C for 20 min. This treatment will control bacterial pathogen contamination, including *E. coli.*

Table 1. The number of total coliform and colonies on CCA medium.

No.	Sample ID	Total coliforms (CFU/g)	Total colonies (CFU/g)
1	S1	2.6×10^9	3.5×10^9
2	S2	1.5×10^9	2.5×10^9
3	S3	1.6×10^9	2.5×10^9
4	S4	3.1×10^9	3.4×10^9
5	S5	2.5×10^9	3.5×10^9
6	S6	1.0×10^9	1.9×10^9
7	S7	1.2×10^9	2.8×10^9
8	S8	1.5×10^9	2.2×10^9
9	S9	2.0×10^9	3.9×10^9
10	S10	1.1×10^9	1.7×10^9

The possible source of *E. coli* contamination in skewered meatballs assessed in the present work could be raw chicken meat. These findings confirm the study conducted by Akbar *et al.* (2014). The study found that the level of *E. coli* contamination from 152 chicken samples was 25%. The coliform colonies found in skewered meatball samples in the present work exceeded the safe threshold for processed meat products of 104 - 106 CFU/g. Contamination of human or animal faecal and non-faecal matter may cause bacterial coliform contamination in skewered meatballs. This might indicate the poor food hygiene, safety, and sanitation among the meatballs handlers. Coliform bacteria are also an indicator for other pathogenic bacteria (Martin *et al.,* 2016). All suspected enteropathogenic bacterial isolates were characterised biochemically using API 20E test (Table 2). In the previous test, 37 isolates were identified to the genus level, from which the samples were taken for the API 20E test.

Based on the test, the dark blue coloured colonies isolates were identified as *E. coli.* This confirms the typical colony selection and biochemical identification results. *E. coli* identification results showed the highest ID percentage of 99.6% for the S4BG1 isolate. *Shigella* spp. was identified in typical bright blue coloured as non-coliform colonies with the ID percentage of 72.8% (S8BT1), while the S10BT1 isolate was identified as *E. coli* with the ID percentage of 86.2%, which in the previous biochemical characterisation, was suspected of being the genus *Shigella* spp. Based on the API 20E results, there are differences in the sorbitol test, such as the *Shigella* spp. isolates are known for its inability to ferment sorbitol, whereas *E. coli* isolates can ferment sorbitol. In other test tubes, it is known that the two bacteria share the same result, meaning that they have almost similar biochemical properties.

E. coli contamination is commonly found in each sample. Enteropathogenic bacteria (*Escherichia coli, Enterobacter cloacae, Klebsiella pneumonia, Shigella* spp., and *Proteus mirabilis*) that have been identified may contaminate skewered meatballs from dirt, air, and food handler's hands, and even raw materials (Akbar *et al.,* 2014). These bacteria can survive heating temperature (under 60°C) in the skewered meatballs preparing process, high heat

Table 2. Biochemical characterisation of isolates based on API 20E test.

Isolate ID	Isolate identity	% ID
S1P2	*Proteus mirabilis*	99.0%
S2BG1	*Escherichia coli*	97.9%
S3M1	*Klebsiella pneumoniae*	97.3%
S4BG1	*Escherichia coli*	99.6%
S6P1	*Bordetella/Alcaligenes/Moraxella* spp.	93.4%
S7P1	*Bordetella/Alcaligenes/Moraxella* spp.	93.4%
S8BT1	*Shigella* spp.	72.8%
S9M1	*Enterobacter cloacae*	98.6%
S10M2	*Klebsiella pneumoniae*	97.3%
S10BT1	*Escherichia coli*	86.2%

treatment might not have been achieved by steaming. Coupled with meat and starch which can function as a protective coating and heterogeneous structure, bacteria may acquire heat resistance (Icier *et al.,* 2014; Sengun *et al.,* 2014). Leaving the skewered meatballs uncovered can also increase the risk of substances contaminating the skewered meatballs. Therefore, safe and hygienic food handling practices are called for, since such a savoury snack is sold in the school environment.

There were three isolates identified as *E. coli,* and one isolate as *Shigella* sp. based on the biochemical identification using API 20E. Based on the molecular pathogenic identification using PCR amplification with the primer *ipaH* gene (Figure 1) as an EIEC invasive plasmid marker, there were three isolates positive for *ipaH* gene (Dutta *et al.,* 2014).

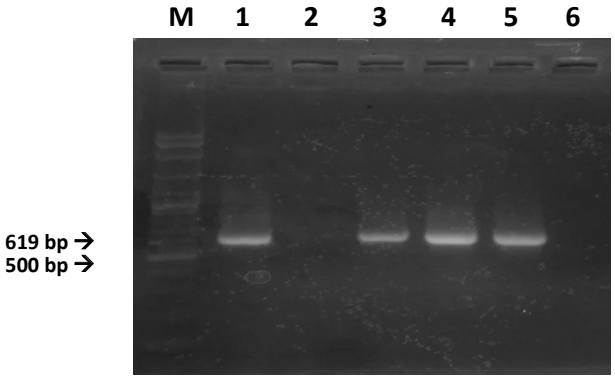

Figure 1. Agarose gel electrophoresis of PCR products using *ipaH* primer. M = 100 bp DNA ladder; Lane 1 = *Shigella boydii* ATCC 9207 (positive control); Lane 2 = *Escherichia coli* ATCC 25922 (negative control); Lane 3 = Isolate S4BG1; Lane 4 = Isolate S8BT1; Lane 5 = Isolate S10BT1; and Lane 6 = Isolate S2BG1.

S4BG1 and S10BT1 isolates, which were identified as *E. coli*, had ID percentage of 99.6 and 86.2%, respectively, and were found positive for *ipaH* gene. S2BG1 isolate, which was also identified as *E. coli*, had ID percentage of 97%, and was found negative for *ipaH* gene. S8BT1 isolate, which was identified as *Shigella* sp., was also found positive for *ipaH* gene. This proves that not all isolates were EIEC. The two *E. coli* isolates which were positive for *ipaH* gene can be grouped into EIEC. They share similar pathogenicity characteristics to *Shigella* spp. Due to its pathogenic nature, EIEC can cause acute diarrhoea, such as shigellosis. This bacterium can invade or penetrate the intestinal mucosa and multiply on the large intestinal epithelial cells, which can cause diarrhoea or red diarrhoea (Parsot, 2005; Løbersli *et al.,* 2016; Ranjbar *et al.,* 2016).

Conclusion

The skewered meatball samples obtained from ten different locations in Yogyakarta were contaminated by bacteria. The number of contaminants exceeded the standard of 109 CFU/g. The type of contaminant bacteria found were *E. coli, Klebsiella, Enterobacter, Proteus,* and *Shigella*. The results of molecular confirmation using PCR to detect the enteroinvasive nature of the *ipaH* gene found that three out of four positive *E. coli* isolates were enteroinvasive.

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