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Quantification of enteropathogenic *Escherichia coli* from retailed meats

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Abstract

Meats are apparently found to be the vehicle of several pathogenic bacteria. Although the existence of enteropathogenic *Escherichia coli* (EPEC) was reported to be reserved in certain meat types in various countries around the globe, insufficient informations about EPEC were documented in Thailand. In this study, the preliminary investigation of EPEC quantity in meats was carried out by Most probable number-polymerase chain reaction (MPN-PCR) method. In the screening process, a total of 30 meat samples purchased from various fresh market in Hat-Yai city, were investigated. The highest and lowest amounts of typical EPEC were found in chicken meat samples as 15 MPN/g and 3.6 MPN/g, respectively. However, the amount of *bfp*-habouring *E. coli* revealed relatively high (highest at 53 MPN/g). Atypical EPEC was found in one beef sample as 19 MPN/g. Typical and atypical EPEC were not found in pork samples. These results exhibited the baseline data on the EPEC quantity in meats sold in southern Thai area and may suggest the high prevalence of pathogenic *E. coli* carrying virulence genes which may be resulted in pathogenesis to human.

Introduction

Diarrheal disease plays a major role as the public health problem. Diarrheal diseases account for 1 in 9 child deaths worldwide, making diarrhea the second leading cause of death among children under the age of 5 (www.cdc.gov). Enteropathogenic *Escherichia coli* (EPEC) is one of the most common causes of infantile diarrhea in developing world (Nataro and Kaper, 1998; Wedley *et al*., 2012). *E. coli* which is classified as typical EPEC, possesses *bfp* and *eae* genes while atypical EPEC carries *eae* only (Nguyen *et al.*, 2006). Healthy asymptomatic animals probably carry pathogenic *E. coli* including EPEC to the food chain (Islam *et al.*, 2008). Upon the arrival of this *E. coli* pathotype in human intestine, it induces the pathological wound called attaching and effacing (A/E) lesion (Clarke *et al.*, 2003). The pathogenesis of EPEC infection has been proposed by four stages, engendered by several virulence genes. *In vitro* study reveals that EPEC binds to epithelial cells in a so-called localized-adherence (LA) pattern (Clarke *et al*., 2003). This phenomenon is created by a crucial adherent factor, bundle-forming pilli (Bfp) encoded by *bfp* gene. Bfp of EPEC was first described by Girón and colleagues (Girón *et al*., 1993). Its structure is 50 to 500 nm wide and 14 to 20 µm long. Three dimentional network of Bfp-producing bacteria is able to be formed by intertwining each other of individual Bfp from other Bfp-producing bacteria (Clarke *et al*., 2003). After the intimate adherence of EPEC to epithelial cell, type III secretion system (T3SS) injects effector proteins which trigger the alterations of cellular cytoskeletons, resulting in the elongation, vesiculation and destruction of microvilli (Hicks *et al*., 1998; Clarke *et al.*, 2003). Translocated intimin receptor (Tir) is also injected by T3SS, which subsequently modified by protein kinase A and tyrosine protein kinase. After modification, modified Tir is embedded in the cytoplasmic membrane. Bacterial protein, intimin, binds to the modified Tir, led to the intimate attachment, accumulation of actin beneath the site of bacterial attachment, resulted in the formation of pedestal structure. The integrity of tight-junction and mitochondrial function were lost, leading to the death of the cell.

Although the existence of EPEC contamination in foods especially meats, was reported from several countries (Chomvarin *et al.*, 2005; Farooq *et al.*, 2009; Bardiau *et al.*, 2010; Xia *et al*., 2010; Alonso *et al*., 2011), the outbreaks caused by EPEC were rarely reported. Retrospectively, in 1971, there were 107 outbreaks of gastroenteritis involving 387 people in the United States. The outbreaks were associated with the imported French cheese consumption. Enteropathogenic *E. coli* O124 was isolated from stool and French cheese samples and believed to be the aetiologic agent (Marier *et al*., 1973). In addition, one report of outbreak in Northern France in 1995, supporting the infections by EPEC with the

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consumption of foods, revealed that atypical EPEC serotype O111 was the aetiologic agent. Prawn Mayonnaise vol au vents was suspected to be the cause of outbreaks (Wight *et al.*, 1997). These outbreaks were shown to be linked to the consumption of foods at the restaurant. Focusing on outbreak by EPEC in Asia, one outbreak was reported from Japan in August 2004. Although the causative foods could not be determined in this outbreak, EPEC serotype O115: H19 led to the food poisoning in 103 individuals in the site of training camp in Miyagi prefecture (Saito *et al*., 2005).

In Songklanagarind hospital, Hat-Yai, Thailand, Kalnauwakul *et al.* (2007) reported several diarrheal cases by various *E. coli* pathotypes. EPEC was found in 13 of 530 (2.5%) stool samples. This supports the potentiality of EPEC in causing infection in human. Moreover, in our previous investigation of enterohemorrhagic *E. coli* from beef in 2008, atypical EPEC serotype O157:H7 carrying *eae* was accidentally found (Sukhumungoon *et al*., 2011). This provides the possible existence of enteropathogenic *E. coli* in retailed meats and owing to the lack of informations about the occurrence and quantity of EPEC in foods in southern Thailand. This encouraged us to screen and quantify the amount of EPEC in retailed meat samples in this area to gain the preliminary informations which are useful for further investigation of EPEC in the future.

Materials and Methods

Sample collection

In order to obtain the baseline data on the EPEC quantity, a total of 30 samples comprising three types of meats, beef (n = 10), pork (n = 10), chicken (n = 10), were purchased from various fresh markets in Hat-Yai city, Songkhla, Thailand between April 2013 and September 2013. All samples were processed immediately upon the arrival to the laboratory.

Most Probable Number-polymerase chain reaction (MPN-PCR) method

MPN-PCR was performed as described by Chang *et al.* (2013) with slight modifications. Briefly, twenty five grams of meat were homogenized with 225 ml of tryptic soy broth (TSB). The liquid portion was used to perform the three-tube MPN, 100 fold and 1,000 fold dilutions of the stomacher fluids were prepared. One ml of the aliquot from each dilution was transferred into triplicate MPN tubes, and then incubated at 37ºC for 24 h. One milliliter of culture from the turbid tube was then subjected to PCR template preparation by boiling method. Briefly, the boiled bacterial culture were immersed on ice for 10 min prior to be centrifuged at 11,000 × *g* for 5 min. Ten-fold dilution of boiled supernatant was used as PCR template. Three virulence genes, *bfp*, *eae* and *escV*, were investigated for the detection of typical and atypical EPEC by simplex PCR (*GoTaq* Flexi system, Promega) (Figure 1). The 326 bp *bfp* amplicon was amplified by primers, EP-1 (5'AATGGTGCTTGCGCTTGCTGC3') and EP-2 (5'GCCGCTTTATCCAACCTGGTA3') (Gunzburg *et al.*, 1995). The *eae* amplicon (1,087 bp) was detected using primers, AE-19 (5'CAGGTCGTCGTGTCTGCTAAA3') and AE-20 (5'TCAGCGTGGTTGGATCAACCT3') as described by Gannon *et al.* (1993). The *escV* amplicon (534 bp) was investigated using primers, escV-F (5'GGCTCTCTTCTTCTTTATGGCTG3') and escV-R (5'CCTTTTACAAACTTCATCGCC3') (Müller *et al*., 2006). PCR amplification was carried out in 25 µl reaction mixture comprising 1X *GoTaq* Flexi Green buffer, 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM each primer pair, 0.5 unit of *GoTaq* DNA polymerase and 2 µl of DNA template. After 35 amplification cycles, the amplicons were visualized by 1% agarose gel electrophoresis and stained by ethidium bromide. All PCR profiles were indicated in Table 1. For amplification of *bfp* and *eae* genes, typical EPEC strain PE-27 was used as a positive strain (Reid *et al*., 1999). *E. coli* O157:H7 strain EDL933 was used as a positive strain for the detection of *escV* gene.

Table 1. PCR profiles for amplification of *bfp*, *eae* and *escV* genes

Profile name	PCR conditions				
	Pre-heat	Denaturation	Annealing	Extension	Final extension
bfp	95ºC, 3 min	94ºC, 1 min	55ºC, 1 min	72ºC, 50 sec	72ºC, 5 min
eae	95ºC, 3 min	94ºC, 1 min	55ºC, 1 min	72ºC, 1.15 min	72ºC, 5 min
escV	95ºC, 3 min	94ºC, 1 min	45ºC, 1 min	72ºC, 50 sec	72ºC, 5 min

Results and Discussion

In order to quantify the amount of EPEC, MPN-PCR method was performed. For the circumspectly observation of typical and atypical EPEC, the classificatory criteria based upon the works from Nguyen *et al*. (2006) and Müller *et al.* (2006), were applied. Although the presence of *bfp* simultaneously with the *eae* or *escV* in the same turbid tube, could not be interpreted directly that there were any *E. coli* cells which carry these genes together, in this study, it was attributed that the samples which exhibited these genes pattern, were typical EPEC and the samples that displayed only *eae* or *escV* gene, were atypical EPEC (Nguyen *et al.*, 2006; Müller *et al.*, 2006). In this investigation, the results revealed that the highest amount of typical EPEC was15 MPN/g and the lowest

Table 2. Quantity (MPN/g) of enteropathogenic *E. coli* in retailed meats

Type of meat	Pathotype	No. of positive sample	MPN/g	
			[a]min	max
Beef	typical EPEC	0	<3	<3
	atypical EPEC	1	<3	19
	bfp-habouring *E. coli*	1	<3	29
Pork	typical EPEC	0	<3	<3
	atypical EPEC	0	<3	<3
	bfp-habouring *E. coli*	7	<3	38
Chicken	typical EPEC	2	3.6	15
	atypical EPEC	0	<3	<3
	bfp-habouring *E. coli*	7	<3	53

[a]Min, Minimum MPN value; Max, Maximum MPN value

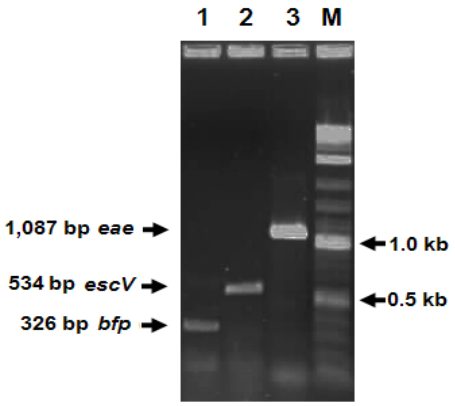

Figure 1. PCR assay detecting *bfp*, *escV*, and *eae* genes. Lane 1 and 3, typical EPEC strain PE-27; lane 2, *E. coli* O157:H7 strain EDL933; lane M, 2-log DNA ladder.

quantity of typical EPEC was 3.6 MPN/g. Both were found in chicken meat samples. While atypical EPEC was detected in one beef sample as 19 MPN/g (Table 2).

Poultry was found to be frequently related to the presence of EPEC. In the slaughtering processes, chickens are killed, scalded and plucked, followed by several steps that bear the risk in EPEC contamination such as an evisceration, internal and external surface washing and the removal of fecal materials. These steps contribute to the transfer of EPEC to the meat retailers. Kagambèga *et al.* (2012) investigated the diarrhegenic *E. coli* in raw meats and found that chicken meat showed the highest rate of EPEC contamination as 25%. One study from Alonso *et al.* (2011) in which they studied the contamination of EPEC in cloacal swabs, unwashed eviscerated carcasses and washed carcasses. The results exhibited the high atypical EPEC contamination rate as 6 to 28% in cloacal swabs, 39 to 56% in unwashed eviscerated carcasses, and 4 to 58% in washed carcasses, In addition, Farooq *et al.* (2009) investigated 212 faecal samples collected from chickens, ducks and pigeons. They demonstrated that 33 pathogenic *E. coli* strains (15.56%) were classified as atypical EPEC. Thus, avian species are evidenced to play a role as an important EPEC reservoir.

Our study exhibited 15 samples comprised the sole *bfp* gene (*bfp*-habouring *E. coli*) (Table 2). This phenomenon was consistent with the work from Rúgeles *et al.* (2010). They demonstrated the existence of *bfp*$^+$ *E. coli* in meats (12.5%) and vegetables (8.3%) but not in clinical samples. These strains possessed a sole *bfp* gene. Bfp belonged to the type IV pilus, based on the amino terminal sequence of the major structural protein of Bfp (Girón *et al*., 1991). This type of pilus revealed the similarity to the ones expressed by other bacteria such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholerae* (Stone *et al*., 1996). In this study, it was not doubtful that the presence of *bfp* gene in the sample reflected the presence of *bfp*-habouring *E. coli* since the EPEC *bfp* specific primers based on the work from Gunzburg *et al.* (1995) was employed. These primers were demonstrated to amplify EPEC-specific *bfp* only. Thus, we classified that the *bfp*-habouring bacteria in our experiment belonged to EPEC pathotype.

In the course of our investigation, certain samples showed the existence of *escV* but not *eae.* We hypothesized that, in this case, the lack of *eae* amplicon, possibly, resulted from the inability of PCR reaction to amplify the large target gene. In the situation that the template for PCR was prepared from various bacterial species like by MPN-PCR method, the amplification of large DNA fragment might be difficult. The primers AE-19 and AE-20 specific to *eae* gene in this study, were designed to amplify a 1,087 bp fragment which is considered to be relatively large. Thus, it was most likely that the lack of eae amplicons in these samples was plausibly resulted from the incapability of amplification. The supporting data were seen in the work from Müller *et al.* (2006). They observed the 100% *eae-escV* detection agreement in clinical *E. coli* strains. Moreover, in the investigation of diarrheagenic *E. coli* from Ouagadougou, Burkina Faso, the control EPEC and Shiga toxin-producing *E. coli* (STEC) also showed *eae-escV* detection agreement (Kagambèga *et al.*, 2012). Thus, it was thought that the primers used for detecting target gene in MPN-PCR method, should be designed to amplify small-size amplicon to increase the sensitivity in detection.

Conclusion

The presence of EPEC in retailed meats is considered important because the bacteria are capable of infecting human body through oral route either the contamination on skin surface or utensils. This study demonstrated the presence of typical, atypical EPEC, including the *bfp*-habouring *E. coli* in meats. Chicken meats were thought to be an important vehicle in EPEC transfer to human. Therefore, the

high surveillant frequency of EPEC in retailed meats especially in chicken and beef is encouraged to be performed to prevent the outbreaks by this *E. coli* pathotype in this area.

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