# Detection and Surveillance of Enterotoxigenic *E. coli* in Food Nature: A Review

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## ABSTRACT

With regard to a rapid development of knowledge in emerging and re-emerging pathogens, local microbiologists should be well-informed about new detection methods, characteristics of pathogenic microorganisms. In addition, surveillance through foodborne illness should be carried out as a responsibility to increase food security in local community. Enterotoxigenic E. coli is one major re-emerging pathogen that can cause a devastating outbreak that could lead to death. However, Enterotoxigenic *E. coli* is often mistakenly identified as *Salmonella* and *Shigella* because of their toxins are relatively similar. Therefore, this paper is addressed to give more information on specific characteristics, conventional and molecular detections of the pathogen. PCR detection is the recommended method. Hence, the skill should be acquired by local microbiologists. In a stark contrast to biochemical assays, PCR detection will result a better identification and a higher reliability. A general surveillance mechanism is introduced to reduce the number of possible outbreak in local community. That includes involving healthcare units to report foodborne incidence as well as encouraging people to file their stomach problem to local authority.

***Keywords: enterotoxin E. coli, PCR, molecular detection, surveillance***

## INTRODUCTION

In East Kalimantan, there is a high chance of food outbreak, especially in relation to source of water, food processing, as well as food handling. Unfortunately, there is less attention from local authority in documenting and establishing a good surveillance for foodborne illness. A false identification is prominent, since we lack of knowledge to emerging and re-emerging pathogens, such as *Cronobacter sakazakii*, *Bacillus cereus*, and Enterotoxigenic *E. coli*. With regard to current situation, more concise review emphasized on new detection methods and surveillance is increasingly important.

Enterotoxigenic *Escherichia coli* (ETEC) is also highly linked with traveller's diarrhoea as well as infant's weanling diarrhoea. Turner et al (2006) highlighted that ETEC infection is occurred at least 650 million cases around the world, with 800.000 casualties among children and infants, mostly in third world countries. It is also blamed for great financial losses in herding industry such as cattle and pigs. Putnam et al (2004) and Wood et al (1983) recorded several outbreaks of ETEC in military installations in Egypt as well as in the United States. The outbreaks of ETEC occur sporadically around

the world (Motarjemi and Adams, 2006). Thus, the detection of these bacteria becomes an issue in food safety (Bischoff, et al, 2005; Levine, 1987).

Some rapid methods such as enzyme linked immunosorbent assay (ELISA) may overcome the problem by providing more simplified test within hours. Nevertheless, according to some research, accuracy and sensitiveness of the test for detecting ETEC are still behind the molecular method (Blomen, et al, 1993). The DNA based detection is developed to address the problem of accuracy, reproducibility and sensitivity. One of the most popular methods is using polymerase chain reaction (PCR) followed by agarose gel electrophoresis (Wolcott, 1992; Yano et al, 2007). In addition to PCR method, multiplex approaches are designed to amplify several targeted DNA sequences in a single reaction (Gauthier and Blais, 2005).

# DEFINITIONS

## *Enterotoxigenic Escherichia coli*

Formerly, *E coli* strains are commonly recognized from the code of O, H, and K representing cytoplasmic, flagella, and polysaccharide antigens, respectively. Moreover, the O, H, K antigens numbering is based on biochemical reaction and does not have a correlation with the ability of the strain to produce toxin (Rademaker et al, 1992). To distinguish ETEC from other serovars requires an exhaustive biochemical assays as well as time consuming.

In addition to biochemical classification of *E. coli*, nowadays the bacteria can also be classified based on its activity to cause public health problem. From current segregation of *Escherichia coli*, they are divided into six categories with regard to the virulence factors and the way to induce diarrhoea, including entero-toxigenic (ETEC). This *E. coli* group is claimed as the most common cause of traveller's diarrhoea around the world (Turner et al, 2006). The major sources of ETEC infection are through contaminated foods and water with capability of causing sudden secretory diarrhoea. However, there are also other major types of *E. coli* regarding the virulence factors, which are entero-invasive (EIEC), entero-pathogenic (EPEC), entero-haemorrhagic (EHEC) and entero-agglutinative (EAggEC) (Gauthier and Blais, 2005). The distinction of enterotoxin strains with other types of *E coli* is the capability of such microorganism to produce either heat labile or heat stable toxins (Levine, 1987) after colonizing the surface of the small bowel mucosa (Bischoff, et al, 2005).

The toxins are differentiated based on the heat retention, in which for LT toxin is 30 min at 60°C, while ST toxin is still active at 100°C after heat treatment for 15 min. The LT toxin is divided into two subtypes which are LT1 and LT2 based on biological activity and serum test, while ST toxin is also segregated into two groups, ST1 and ST2 for the same reason (Adam and Moss, 2000). Some strains may produce only ST or LT group of toxins, while many of them are capable to produce both types (Candrian et al, 1991).

### *Heat-labile toxin*

The LT toxin is very similar with cholera toxin, which is holotoxin (Bischoff, et al, 2005; Fan et al, 2004). According to Spangler (1992), the LT toxin causes a severe dehydration, symptomatic cramps, as well as rapid and significant loss of fluid and electrolytes.

Subsequently, LT toxin group is divided into two types, LT1 and LT2 based on the relationship of antigens (Candrian et al, 1991). The additional suffix of h (human) or p (porcine) in the LT1 subtype is attached to define the source of the strain (Candrian et al, 1991). Therefore, LT1p means the strain is capable to produce LT1 type of toxin and originated from human. The LT2 subtype is commonly isolated from bovine (Nardi, 2005).

### *Heat-stable toxin*

On the other hand, Saeed et al (1986) highlighted that ST toxin will activate intestinal mucosal guanyl cyclase and fluid secretion in the body. As like as LT toxin, the ST has two types, ST1 or STA and ST2 or STB (Candrian et al, 1991). In the subtype of ST1h, isolated from human, there are two different alleles but remarkably homologous, *est* A2 and *est* A3/4. Another subtype, ST1p, obtained from porcine, has *est* A1 alleles which has 70% similarity with those from human origin (Candrian, 1991).

### *Gene sequences*

The alleles that expressed each of the toxins are characterized in many subsequent researches (Candrian et al, 1991; Meyer and Karch, 1989; Olive, 1989; Rademaker et al, 1992; Sommerfelt et al. 1988). Instead of using a single primer to detect a subtype of toxin gene in a strain, some variety of primers can be used as shown in Table 1. Further, two inosine-containing primers were used by Candrian et al (1992), which are ST1-EC01 and ST1-EC02 oligonucleotides. The other primers to detect LT1 and ST1, which are used by Yano et al (2007), may be obtained from GenBank with accession number K01995 and M34916, respectively.

Table 1 Primers for ETEC detection

| PCR Assay | Oligonucleotides (5'-> 3') | PCR fragment | Fragment length |
|---|---|---|---|
| LT1 | GCAGGTTTCCCACCGGAT | | |
| LT1-01 | TTACGGCGTTACTATCCTCTCTA | LT-01,LT-02 | 275 BP |
| LT1-03 | CATTTCAGGTCGAAGTCCCG | | |
| LT1-02 | GGTCTCGGTCAGATATCTCATTC | LT-02,LT-03 | 101 BP |
| LT2 | GTGCTCAGATTCTGGGTCT | | |
| ST1 | ATTTTTATTTCTGTATTGTCTTT | | |
| ST1-EC01 | TTITTTCTGTATTITCTTTIICIICTTTIIITCAG | ST1-EC01,ST1-EC02 | 175 BP |
| ST1-EC02 | GCAGGATTACAACAIATTCACAGC | | |
| ST1-EC05 | CATTAGAGACTAAAAAGTGTGAT | ST1-EC05,ST1-EC02 | 103 BP |
| ST1-EC06 | GCTACTATTCATGCTTTCAGGA | ST1-EC01,ST1-EC06 | 143 BP |
| ST2 | GATTACAACACAGTTCACAG | | |

Adapted from Yano et al (2007) and Candrian et al (1992).

# ETEC INVESTIGATION

## *Epidemiological investigation*

As soon as a number of victims are hospitalized or reported by practitioners, the investigation should be conducted to find the similar pattern of source of illness. Some set of questions are asked to find any association with foods, particular places, drink, or water. For some groups, such as army personnel, airplane or cruise ship passengers, the source of outbreak can be narrowed relatively easy. However, if the outbreak is not associated with any special group of people, the investigation should be able to find a connection between each of the cases. In Wood et al (1983) case, telephone interviews were conducted randomly to check the remaining personnel in the same area of outbreaks.

## *Laboratory assay*

### Isolation

Further, a representative number of samples are collected from the victims and assessed in laboratory. Wood et al (1983) collected samples from 58 patients, 22 healthy individuals in the same group, 3 food handlers, and water at the suspected cafeteria. A broad range of assay such as total plate count, coliform *E. coli, Shigella, Salmonella, Campylobacter*, and *Vibrio* were done using conventional isolation techniques. Some biochemical assays can be conducted using rapid test kits and automated systems (Wood et al, 1983).

### Enterotoxin assay

After a collection of *E. coli* isolates are obtained, the enterotoxin assays is carried out to confirm the outbreak. In conventional method, Y-1 adrenal cell test is conducted to detect heat-labile toxin (LT), whereas positive strains are confirmed with anti cholera toxin (Wood et al, 1983). According to Bischoff et al (2005) and Fan et al (2004), the LT toxin is very similar with cholera toxin, which is in a group of holotoxin. For heat-stable (ST) toxin, biochemical reaction is observed from intestine of the suckling mouses (Wood et al, 1983).

### Hemagglutination test

Wood et al (1983) explained that the cultures were grown in Ca-amino acids yeast extract agar at 37 °C before testing with erythrocytes from human, bovine, adult chicken, monkeys, and pig in the presence and absence of mannose. During the agglutination test, the colonisation factor antigens (CFA) were observed using electron microscopy method.

## *Molecular Detection*

In East Kalimantan, there is no information if this sophisticated method is already acquired by local authority. Therefore, in order to protect people from a disastrous outbreak of Enterotoxigenic E. coli, we need to expert this molecular method as well as

to establish the equipment in a certified laboratory. To give more information about DNA identification method, a summary of a popular detection mechanism is provided in this paper.

Wolcott (1992) reported that the first description of DNA and its function to store information of cell function and reproduction was in 1953. Adenosine, thymine, guanine, and cytosine are the base nucleotides to form DNA sequence or strand (ssDNA). Further, a strand of DNA performs hydrogen bonds with another complementary strand, producing a double strand (dsDNA). Adenosine bonds thymine, while guanine is a pair for cytosine. An ssDNA is relatively stable to such heat or pH that can unbound the double helix, therefore re-annealing happened by removal of heat or extreme pH. If a synthesized ssDNA is inserted and annealed, the process, then, called hybridization. The ability of DNA to realign by the function of heat or pH is the basic principle that enables DNA-based detection.

**Polymerase Chain Reaction**

The PCR, which was invented by Mullis and Falona (1987), is the most popular molecular detection technique. It is based on three reiteration steps, dsDNA denaturation to ssDNA, annealing with synthetic oligonucleotides, and elongation by the help of thermostable enzyme (Wolcott, 1992). There are advantages of using PCR to rapidly detect ETEC. PCR detection method performed a very accurate result, with 100% and 80% successful detection for LT1 and ST1 toxins, respectively. Moreover, another molecular method, which is alkaline-phosphatase conjugated oligonucleotide of DNA hybridization, can only detected up to 92% in accuracy compared with PCR (Blomen, et al, 1993). PCR method is one of the most sensitive detection methods for ETEC, which can detect as small as 40 CFU/test (Yano et al, 2007). Nevertheless, regarding current development of molecular detection, Yano et al (2007) found that loop-mediated isothermal amplification (LAMP) is by 10-fold more sensitive than PCR assay. Tamanai-Shacoori et al (1996) reported that there is no difference in result for amplification of targeted DNA using PCR. However, the researchers noticed that a slight difference may occur as the changing of salinity on the process.

Despite the fact that PCR method is highly popular, it has some drawbacks compared to current rapid detection method, namely LAMP. The PCR test requires many sophisticated equipments, such as thermal cycler, thus make it not available in smaller laboratories (Yano, et al, 2007). Although PCR method to detect ETEC is already simpler than that with conventional biochemical assay, the process remains complicated compared with more recent method such LAMP (Yano et al, 2007). PCR requires further confirmation, such as electrophoresis and southern blot. However, in interpreting data, it is not practically easy to obtain a correct reading (Yano et al, 2007).

Several steps are required to detect ETEC using combined PCR and agarose electrophoresis, which are isolation of ETEC, bacterial lysis, oligonucleotides primers, and synthesis of oligonucleotides, PCR amplifications, and DNA analysis (Olive, 1989; Candrian et al 1991, Tamanai-Shacoori et al, 1996).

**E coli strains isolation.** Candrian et al (1991) used standard isolation and purification methods from Swiss Food Manual. The abilities of *E coli* to ferment lactose, to reduce 4-methyl-umbelliperyl-β-glucorinide and to produce indole from tryptophan at 44 °C were tested to confirm all presumptive *E. coli* strains.

**Bacterial lysis.** Lysozyme and proteinase K is used to breakdown bacterial cell wall, according to Candrian et al (1991).

**Oligonucleotides preparation and synthesis.** The prepared primers are shown in table 1, and there are some variations of primers as well in other research (Olive, 1989; Candrian et al, 1991; Candrian et al, 1992; Tamanai-Shacoori et al, 1996, Yano et al, 2007). DNA synthesizer is used to synthesize nucleotides primers by β -cyanoethyl phosporamidite techniques (Candrian et al, 1991; Tamanai-Shacoori et al, 1996). The process is followed by purification of primers using commercial columns (Candrian et al, 1991).

**Amplification of DNA.** Synthesized primers, DNA extracted from bacterial lysis process are dissolved in reaction buffer prior the amplification process. According to Tamani-Shacoori et al (1996), the buffer is a mixed solution of 0.02 M $(NH_4)_2SO_4$, 0.08 M Tris HCl at pH 9, and 2.5 mM $MgCl_2$. A thermostable polymerase enzyme, such as *Thermus aquaticus* (*Taq*) polymerase, is also added to the solution as well as the source of base nucleotides, which is dNTP. The PCR, then, runs for 25-30 cycles to produce around $10^6$-$10^8$ fold of targeted sequences. A cycle consists of three stages, 30 s denaturation at 95 °C, 30 s re-annealing at 55 °C, and 1 min elongation at 72 °C (Tamani-Shacoori et al, 1996). Slight temperature and time differences may be found in other methods during the amplification, depending on the optimum temperature of polymerase enzymes (Candrian et al, 1991; Blomen et al, 1993; Yano et al, 2007).

**Agarose electrophoresis**

**Electrophoresis.** Electrophoresis at 80 V for 1.5 h on 1.5% agarose gels in 40 mM Tris-acetate (pH 8) and 1 mM $Na_2EDTA$ buffer is applied to analyse the aliquots from the PCR step (Tamani-Shacoori et al, 1996). The gels, then, are stained in 0.5 μg $mL^{-1}$ solution of ethidium bromide for 15 min before photographed on a UV trans-illuminator. The basic principle of electrophoresis is the DNA sequences are moving from one end of the gels to the other as the electrical current is applied. The marks that indicate the same distance with positive controls is interpreted as the same DNA sequence (Wolcott, 1992; Candrian et al, 1991).

**Southern blot.** The electrophoresis result of DNA is transferred to a membrane of nylon, in which, then, pre-hybridized (Tamani-Shacoori et al, 1996). DNA probes are hybridized in the subsequent step. There are various probes can be used, such as *mal*B probe or digoxigenin UTP (Candrian et al, 1991). In the following step, the membrane is washed with SSC-01% SDS to reduce stringency (Tamani-Shacoori et al, 1996). NitroBlue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate solution is applied to aid visualisation after end-labelled with an alkaline phosphatase conjugate probe (Tamani-Shacoori et al, 1996; Candrian et al, 1991). The southern blot technique enables a clearer result of electrophoresis compared with the conventional method.

## SURVEILLANCE

In an isolated outbreak, tight surveillance can be carried out by visiting locations in a defined period. For example, there was an outbreak of ETEC in Africa, observed by Putnam et al (2004). In order to get a correct data, Putnam et al (2004) established a fortnightly visit in a small village for three periods of time during 1995 and 2000. The

recording included vomiting and diarrhoea as the symptoms as well as food and water samples in correlation with the accidents. However, a stringent observation can only be carried out in such targeted area, and it is not applicable in a larger region.

Since enterotoxin *E. coli* is causing mild to severe diarrhoea, there is a problem of gathering correct statistical data. In many countries, the number of outbreaks is often under reporting. Therefore, Adam and Moss (2000) suggested that a system of notification and reporting should be established. Currently, there are four possible levels of gathering information in relation with the outbreak which are:

1. Notification without further confirmation of the microbial involved;
2. Reporting only a confirmed case of foodborne disease, enclosed with full laboratory test;
3. Reporting all cases of gastrointestinal illness, regardless some of them were not caused by foodborne pathogen; or
4. Reporting cases in relation with salmonellosis, which has similar symptoms to ETEC outbreak.

With regard to local conditions of East Kalimantan, whereas the potential of foodborne illnesses is high, we need to establish a good reporting system of outbreak surveillance. The reporting should include healthcare units to report any incidence related to foodborne disease. Other action that likely can be established in a short time is encouraging people to report stomach illness, that probably caused by food ingestion.

## Reducing the number of outbreaks

Recently, there are two types of food business, catering and manufacturing. Catering is producing food for smaller audience within shorter period of shelf life, as the assumption of serving ready to eat food. The food manufacturers produce massive products to be sold in the markets. Both of the businesses are potential sources of food poisoning problems, including enterotoxigenic *Escherichia coli*. Pimbley and Patel (1998) strongly encourage food producers to adopt HACCP in the manufacturing lines to reduce the risk of toxin producing bacteria. Toxin testing should be conducted regularly to ensure the quality of the final product as there are many rapid test kits can be obtained to do so. However, the current system of monitoring still cannot guarantee the safety of the product.

Pennington (1998) suggested having end to end control through food chain productions from farms and livestock to the point of consumption. The general concepts of reducing the number of outbreaks are summarized as:

1. The awareness of potential prevalence and sources of Escherichia coli;
2. The need to apply good manufacturing practices, including sanitary standard procedure (SSOP), HACCP, good personal hygiene, and storage handling;
3. good documentation and record keeping to be able the back tracking process;
4. To increase worker's awareness; and
5. To educate young generation about the importance and how to recognize and avoid the potential causing of foodborne illness.

# FUTURE CHALLENGE

### *Antimicrobial susceptibility*

Some strains of *Escherichia coli* are not susceptible to some types of antibiotic. The failures of antimicrobial treatments are relatively high according to Putnam et al (2004). During their extensive research between 1995 and 2000, the decreasing susceptibility against the group of ampicillin, trimethoprim-sulphamethoxazole, and tetracycline is as high as 60%. Therefore, the ability of these bacteria to survive and causing illness is becoming a major concern in the future.

# CONCLUSION

Enterotoxin *Escherichia coli* outbreak remains to be a potential threat for food safety. The outbreak can be occurred in any place with a devastating effect, sometimes leading to death toll. Escherichia coli toxins are very similar to Shigella-like toxins. Therefore to distinguish the differences of organism require time and labour-consuming tests. Molecular identification becomes a recommended test to produce, since it has high sensitivity, high reliability with a lesser time and resources required. PCR technique is a major molecular detection that should be skilled by local microbiologists.

As a final recommendation, especially with regard to local condition of East Kalimantan, the local authority should expert in this molecular technique to cope with current threat in protecting people from foodborne diseases. Other recommendation to local authority is to establish a good surveillance to food security as an indicator of increasing wealthiness in local economy.

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