



## Human Behaviour and Responses Challenge towards Emergence of Infectious Diseases: *E.coli* clinical isolate

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### Abstract

Consumption of undercooked ground beef is the most common route of transmission of verotoxin-producing *E.coli*. It is estimated that non-O157 verotoxigenic *E.coli* (VTEC) can cause diarrhea. The sample was isolated from Universiti Malaya Medical Centre. All the isolates were identified using agarose gel electrophoresis method. This study aims to detect the verotoxin genes and detect the link or involvement of plasmids with these verotoxin genes. Therefore, this study will contribute to shed new light on resolving the significant and global problem of diarrheal disease caused by this particular pathogenic organism and help in improvising novel therapeutic approaches to improve human healthcare.

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### 1. Introduction

Non-O157 *E. coli* is commonly present in environment, food and food production animals. Problems associated with these emerging pathogens are increasing day by day (Bettelheim, 2000). Previously attention has been focused on infections caused by isolates of *E. coli* O157 serotypes. Infections with non-O157 *E. coli* are now increasingly recognized in many countries (Schmidt et al., 1999). Many non-O157:H7 isolates that are associated with outbreaks do not possess either *eae* or the pO157 plasmid (Bokete et al., 1993) indicating that there must be additional, as yet unknown, virulence factors that distinguish pathogenic from non-pathogenic non-O157 *E. coli* strains. The virulence profiles of most non-O157 *E. coli* are unknown (Schmidt et al., 1999). Therefore, characterization of non-O157 VTEC isolates is necessary for improving our knowledge of these organisms (Nielsen & Andersen, 2003).

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Plasmid is a small, covalently closed circular, double stranded extra chromosomal deoxyribonucleic acid (DNA) element. It is self-replicating independent DNA molecule. The plasmid contains the gene that is normally not essential for bacterial survival or growth. Plasmid commonly carries one or more genes that can confer the advantage to the bacterial cell harboring them. Plasmid may confer toxin production, drug resistance, etc. Plasmid profiling analysis sometime can be used for epidemiological analysis (Bratoeva et al., 1992). The size of the plasmid may vary from 1 kb to 250 kb. Different size of plasmids may be found in a single host bacterium, and not all bacteria may contain the plasmid. In the same bacterial cell, many copies of a single plasmid can be found which can be extracted in the laboratory using different methods. Many plasmid DNA extraction methods have been established to date. The presence of plasmids confers antibiotic resistance that plays a role in human behavior when they are under any antibiotic therapy.

## 2. Literature Review

In July 2015, the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) has announced and recalled approximately 26,975 pounds of tenderized steak and ground beef products issued by Denver's Lombardi Brothers Meats that may be contaminated with *E. coli* O157:H7. No illnesses are yet known to be associated with the meat, which was distributed to hotels, restaurants and institutional use customers in Colorado, New Mexico, Utah and Wyoming. This problem was discovered by Lombardi Brothers when the company's sampling program returned a positive *E. coli* test result. It then found that some products made from the same source were shipped into commerce (FSIS, 2015).

*E. coli* O157:H7 is a potentially deadly bacterium that can cause dehydration, bloody diarrhea and abdominal cramps 2-8 days (3-4 days on average) after exposure to the organism. While most people recover, some develop a type of kidney failure called hemolytic uremic syndrome (HUS). The condition can occur among persons of any age, but is most common in children younger than five and older adults. It is marked by easy bruising, pallor and decreased urine output. Persons who experience these symptoms should seek emergency medical care immediately (FSIS, 2015).

FSIS routinely conducts recall effectiveness checks to verify recalling firms notify their customers of the recall and that steps are taken to make certain that the product is no longer available to consumers. United State Department and Agriculture (USDA) advises all consumers to safely prepare their raw meat products, including fresh and frozen, and only consume beef products that have been cooked to a temperature of 145 degrees F for steaks and roasts and 160 degrees F for ground product (FSIS, 2015).

Non-O157 verotoxigenic *E. coli* (VTEC) are common typical *E. coli* and are commonly present in food and food production animals. Transmission is produced by consumption of contaminated food, by direct contact with animals or the environment and from person to person (Polifroni et al., 2014). However, consumption of raw/undercooked ground beef is the most common route of transmission of verotoxin-producing *E. coli* (Liorente et al., 2014). *E. coli* serotypes now known to be VTEC were present in humans and animals many years ago but have acquired the ability to produce VT and probably other virulence factors (Bettelheim, 2000). *E. coli* serogroups O26, O45, O103, O111, O121, and O145 are the leading cause of VTEC-associated infections in humans (Liao et al., 2014). Multidrug resistances in non O157 *E. coli* were also observed (Rajkhowa & Sarma, 2014). Research on the *E. coli* non-O157 serogroups is limited. In recent years increased attention has been focused on infections caused by non-O157 *E. coli* isolates as infections with non-O157 VTEC are now increasingly recognised in many countries (Ma et. al., 2014). It is estimated that non-O157 VTEC may cause diarrhoea at frequencies similar to those of other enteric bacterial pathogens, such as Salmonella and Shigella. The virulence mechanisms of most non-O157 *E. coli* are unknown (Schmidt et al., 1999, Joseph & Mohammad, 2012). Therefore, characterization of non-O157 VTEC isolates are essential for improving our knowledge about the virulence mechanism of these organisms (Misselwitz et al., 2003). To date, VTEC prevalence studies have focused primarily on *E. coli* O157: H7, because of its initial predominance in human clinical infection. Culture and molecular methods for the detection of VTEC have thus been developed and optimized for that serotype, with little attention to and resultant underestimation of the risks posed by non-O157 serogroups. However, non-O157 infections are of primary concern in several countries (Áine et al., 2011). VTEC produces two types of Verotoxin, VT1 and VT2. Both the antigenically distinct toxins VT1 and VT2 are encoded on two different lysogenic lambdoid bacteriophages (933J and 933W) inserted into the chromosome in O157 *E. coli* 933 (Nataro & Kaper, 1998). The T1-like bacteriophages were shown to lyse common phage types of verotoxin-producing *Escherichia coli* O157: H7 (VTEC O157: H7), but not non-O157 *E. coli* (Niu et al., 2014).

Our previous study (unpublished) suggests that the VT gene might be present in the plasmid, but the mechanism of its presence in the plasmid was not understood yet. How these verotoxin genes are transmitted to these bacteria? Is there any correlation between plasmid and verotoxin gene? Can verotoxin gene be located in the plasmid? In the light of this, we are prompted to study further. We are not aware of any studies of these verotoxin genes to be plasmid mediated. To the best of our knowledge, not enough studies have been carried out about the association of plasmids in verotoxin production. This prompted us to carry out this study that will help advance our knowledge about the implication of plasmid in verotoxin production among the clinical isolates of VTEC. On completion, this study aims to detect the verotoxin genes and detect the link or involvement of plasmids with these verotoxin genes. Therefore, we hope that from the findings, it can shed new light on resolving the significant and global problem of diarrheal disease caused by this particular pathogenic organism and help in improvising novel therapeutic approaches. Knowledge of plasmid related verotoxin genes will help in formulating novel therapeutic approaches.

### 3. Methodology

Sixty non-O157 *E. coli* clinical isolates were used in this study. These were obtained from Universiti Malaya Medical Centre, Kuala Lumpur. Supercoil DNA was used to provide reference plasmids of known molecular weight that was purchased from Promega, USA.

Perfectprep Plasmid Mini preparation kit from Eppendorf, Germany, was used to extract Plasmid DNA. The exact procedure was followed according to manufacturer's instructions, and no further modification was done. This isolation technique gave plasmid DNA bands in patterns, and this technique was found to be stable, i.e., reproducible recovery of all plasmid DNA was observed from all the *E. coli* isolates. Firstly, the plasmids of all the 60 *E. coli* isolates were extracted. The experiment was repeated few times for the confirmation of the findings. After purification, the plasmids were analyzed to estimate the approximate size by using the graphical method of Aij and Borst (1972).

#### *Agarose gel electrophoresis*

Plasmid DNA was resolved by electrophoresis in submerged horizontal agarose slab gel (0.7%) in Tris-Acetate buffer (TAE) (pH 8.3). The agarose (Sigma Chemical Co., USA) was dissolved by boiling in 1X Tris-Acetate (TAE) buffer (pH 8.3) followed by cooling at 50°C. Ethidium bromide (0.5 µg/ml) was added before casting. A 25 µl aliquot of extracted plasmid DNA was mixed with 5-10 µl gel loading buffer. The DNA-dye mixture was then loaded into the well. The tank was filled with 1X Tris-Acetate (TAE) buffer (pH 8.3) to completely submerge the gel. Electrophoresis was supplied by a power pack (Model Vokam 400, Shandon Co. Ltd, England). Electrophoresis was carried out from the cathode (-) to anode (+) at a constant voltage at room temperature. The voltage used was in the range of 70 -90. Electrophoresis was stopped when the tracking dye (loading buffer) was 5-10 mm from the anode end of the gel. The DNA-ethidium bromide complex was visualised using UV transilluminator (Model TFX, Vilber Lourmat, France). Gloves were worn at all times when handling agarose slab gels containing ethidium bromide. Photography was carried out with a Polaroid camera and Polaroid 665 black and white film. Exposure time was between 30-90 seconds. Digital camera was also used for photography and downloaded the image directly to the computer. The brightness and contrast of the image were adjusted before printing.

### 4. Findings

Plasmids were detected in 42 isolates with the Plasmid Occurrence Rate (POR) of 70%. These 42 isolates appear to harbor 1 or more plasmids with the maximum of 4 plasmids. Eighteen *E. coli* isolates (30%) did not carry any plasmids. The overall sizes of the plasmid DNA range from the lowest 1.6 kb to the highest 7.2 kb. (Figure 1)

The plasmid profile analysis revealed the presence of a 7.2-kb plasmid DNA harbored by 5 non-O157 *E. coli* isolates. This result is in agreement with Wan Himratul (1999) findings where different strains were used. This 7.2-kb plasmid from non-O157 was previously shown to be associated with VT1 production. DNA hybridization studies showed that, this 7.2 kb plasmid to be hybridized against VT1 probe (Wan Himratul, 1999).

Five of the *E. coli* isolates harbored a 6.6 kb plasmid. This size plasmid was also reported by Wan Himratul, (1999), and a plasmid of similar size was also reported by Scaletsky et al., (1995). Scaletsky et al., (1995) reported the 6.6 kb plasmid to be very significant in its pathogenesis. Bacteria containing this plasmid synthesize a protein of 32 kDa (pI 4.93) which seemed to be required for cell invasion. They isolated this 6.6 kb plasmid from an O111: H- EPEC that is capable of conferring to an avirulent, non-adherent *E. coli* k12 strain (DK1) the capacity to invade epithelial cells. Similar studies are required to observe the pathogenesis of the 6.6 kb plasmid harbored by the *E. coli* isolates in this study.

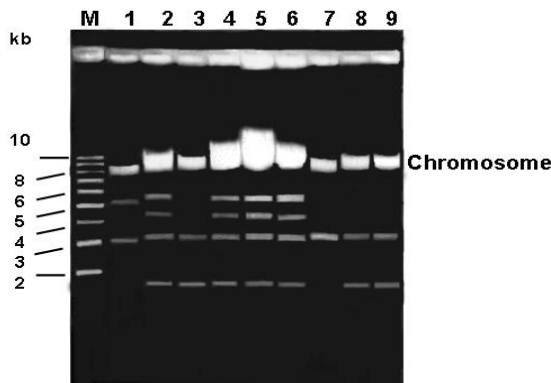


Fig. 1. Agarose (0.7%) gel electrophoresis of plasmid DNA extracted from non-O157 *E. coli* isolates and supercoil (M) as a standard marker. This is a representative picture of all the organisms with plasmids.

The most common plasmid found was a 4 kb plasmid, which was harbored by 14 *E. coli* isolates. Wan Himratul, (1999) also showed a similar finding in Malaysia when tested different isolates, where 16 diarrheagenic *E. coli* were found to harbor a 4.6-kb plasmid. Another common plasmid found was 5 kb plasmid. The significance of these plasmids is yet to be known. Gyles et al. (1974) found plasmids coding for heat stable enterotoxins was in the range of 3.2-12 kb. It suggests that any of this plasmid might be involved in producing heat stable enterotoxin but requires further study to confirm its association with the toxin production.

Eight *E. coli* isolates harbored two plasmids of 3.2 kb and 1.6 kb. One possible explanation for this 3.2 kb plasmid, is that may be a dimer of 1.6 kb plasmid, in which there is only one plasmid DNA that is present in two possible forms namely super helical of covalently closed circular (CCC) and relaxed form or open circular (OC). Two *E. coli* isolates were found to harbor only 3.2 kb plasmid without harboring 1.6 kb. Wan Himratul (1999) also showed that these two plasmids were harbored by two different *E. coli* isolates. These findings indicate that these two 3.2 kb and 1.6 kb plasmids may be of different origin and unrelated, not a dimer. But the significance of these two plasmids is yet to be reported.

Four *E. coli* isolates were observed to have identical plasmid profiles carrying four different size plasmids of 5, 4, 3.2 and 1.6 kb. Another 4 *E. coli* isolates also showed to harbor identical plasmid profiles carrying 3 different size plasmids of 7.2, 6.6 and 5 kb. Many other isolates also showed identical plasmid profile among them. Identical plasmid profiles have been reported when the isolates are in same serogroups (Fernandes et al., 1992). Fernandes et al., (1992) reported the usefulness of plasmid profile analysis to differentiate strains of *E. coli*. They showed no common plasmid profiles among strains of distinct serotypes. However, they grouped most of the strains within a few major profiles. They also reported that plasmid profile analysis is very useful to differentiate strains within specific *E. coli* serotypes. All the *E. coli* isolates in this study need to be further serotyped to provide a finer plasmid profiling analysis as to differentiate the strains within the specific serotype. This will also lead to a better understanding of the plasmid profiles among the respective serotypes. There are several common small plasmids of similar sizes among many of the *E. coli* isolates, but the significance of these plasmids is yet to be determined.

No plasmid bands were detected in 18 (30%) of the *E. coli* isolates. This finding also agrees with the findings of Wan Himratul (1999) where 22% different strains of non-O157 *E. coli* isolates were shown to be plasmidless. Plasmid occurrence rate is normally significant for the epidemiological studies where a large number of isolates is tested. Plasmids are known to be associated with different virulence property of the bacterial isolates. In this study, POR is significant for the basis of further studies and each plasmid needs to be characterized to detect their role in pathogenesis (if any).

## Conclusion

POR in this study was found to be 70%. POR is usually more significant for epidemiological studies where a large number of isolates are tested. The presence of plasmid indicates more antibiotic resistance that can influence the human behaviour during their treatment. This study will contribute to shed new light on resolving the significant and global problem of diarrheal disease caused by this particular pathogenic organism and help in improvising novel therapeutic approaches to improve human healthcare.

Further studies are required to confirm the contribution of plasmid in producing verotoxin. A Large number of isolates is also needed to be tested to use this POR as an epidemiological tool for non-O157 E. coli isolates. The further studies will improve the knowledge of plasmids in relation to verotoxin production and also to use plasmids as epidemiological tool.

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