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MOLECULAR CHARACTERISATION OF ESCHERICHIA COLI SEROGROUP 0 157:H7

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MOLECULAR CHARACTERISATION OF *ESCHERICHIA COLI* SEROGROUP O157:H7

By

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MOLECULAR CHARACTERISATION OF *ESCHERICHIA COLI* SEROGROUP O157:H7

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Chairman: Professor Dr. Gulam Rusul Rahmat Ali

Faculty: Food Science and Biotechnology

Fourteen *E. coli* O157:H7 beef isolates were characterised by using four specific epidemiological markers: combination of antibiogram and plasmid profiling, pulsed-field gel electrophoresis (PFGE) and arbitrary primed polymerase chain reaction (AP-PCR). These markers were assessed for their reliability, typability, rapidity and discriminatory power in differentiating beef *E. coli* O157:H7 strains from different locations, namely Bangsar, Kajang, Petaling Jaya and Serdang. The majority of the isolates were resistant to penicillin G (100%), vancomycin (100%), trimethoprim/ sulphametoxazole (100%), bacitracin (100%) and erythromycin (92.8%). Only 21.4% were resistant to carbenicillin. 14.3% and 7.1% were resistant to ampicillin and cephalotin, respectively.

Plasmid analysis revealed three basic plasmid patterns among *E. coli* O157:H7 strains, profile 1 characterised by plasmid DNA of 60 and 2.5 MDa, profile 2 characterised by plasmid of 60 MDa, and profile 3 characterised by the absence of any plasmid in the strains. Grouping according to combination of antibiogram and plasmid analysis indicated eight different groups as two strains with similar antibiotype could be distinguished into two different strains by their dissimilar plasmid profile. However, the reliability of antibiogram and plasmid analysis in typing *E. coli* O157:H7 can be questioned. Thus, other reliable methods such as PFGE and AP-PCR were then applied. In the present study, macrorestriction of genomic DNA of *E. coli* O157:H7 using *Xba*I, *Spe*I and *Hind*III and analysed by PFGE successfully grouped ten out of fourteen isolates into five groups and provided evidence of epidemiologically related strains between strains of different and same locations. However, AP-PCR using three short primers grouped the isolates into fourteen distinct groups and differentiates isolates that were not differentiated by PFGE. The overall analysis of the present study revealed AP-PCR as the most suitable method to differentiate *E. coli* O157:H7 because it was more discriminatory, less labor intensive and applicable to all isolates. Using this method, it was clearly shown that all fourteen *E. coli* O157:H7 existed as independent isolates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENCIRIAN MOLEKULAR *ESCHERICHIA COLI* SEROGROUP O157:H7

OLEH

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Pengerusi: Professor Dr. Gulam Rusul Rahmat Ali

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Empat belas isolat *E. coli* O157:H7 diciri menggunakan empat metod epidemiologi iaitu gabungan ujian ketahanan terhadap antibiotik dan analisis plasmid, PFGE dan AP-PCR. Marker diuji dari segi kemantapan, kebolehan pencirian, kepantasan dan nilai pemisahan untuk membezakan *E. coli* O157:H7 yang diisolat dari pelbagai tempat seperti Bangsar, Kajang, Petaling Jaya dan Serdang. Kebanyakan isolat adalah resistan kepada penicillin G (100%), vancomycin (100%), trimethoprim/ sulphametoxazole (100%), bacitracin (100%) dan erythromycin (92.8%). Hanya 21.4% resistan kepada carbenicillin. 14.3% dan 7.1% resistant kepada ampicillin dan cephalotin masing-masing. Analisis plasmid menunjukkan tiga corak asas di antara *E. coli* O157:H7, profil 1 diciri oleh kehadiran dua plasmid bersaiz saiz 60 MDa dan 2.5 MDa, profil 2

diciri oleh kehadiran satu plasmid bersaiz 60 MDa dan profil 3 diciri oleh ketidakhadiran plasmid. Pengkelasan mengikut penggabungan antibiogram dan analisis plasmid menunjukkan lapan kumpulan bardasarkan pada pendapat yang mengatakan dua isolat yang mempunyai antibiotaip yang serupa boleh dibezakan dengan profil plasmid yang berlainan. Walaubagaimanapun, kebolehan antibiogram dan analisis plasmid untuk menciri *E. coli* O157:H7 boleh dipersoalkan. Jadi, metod lain yang lebih mantap seperti PFGE dan AP-PCR telah dicuba. Dalam kajian ini, pemotongan DNA genomik *E. coli* O157:H7 menggunakan enzim *Xba*I, *Spe*I dan *Hind*III, dan dianalisis menggunakan PFGE mengkelaskan sepuluh dari empatbelas isolat kepada lima kumpulan dan memberi bukti perkaitan epidemiologi antara isolat yang dipencil dari tempat yang sama atau pun berlainan. Dengan menggunakan AP-PCR dan tiga primer pendek, semua isolat berjaya dibezakan ke dalam empat belas kumpulan yang berlainan dan ia juga dapat memisahkan isolat yang tidak boleh dibezakan dengan PFGE. Secara keseluruhan, kajian ini mendapati bahawa AP-PCR merupakan metod yang paling sesuai untuk membezakan *E. coli* O157:H7 kerana ianya lebih cepat, mempunyai nilai pemisahan yg lebih tinggi, kurang memerlukan tenaga kerja dan boleh diaplikasikan keatas semua isolat. Dengan metod ini, amat jelas ditunjukkan bahawa empatbelas isolat yang dikaji tidak mempunyai perkaitan di antara sama satu lain.

CHAPTER I

GENERAL INTRODUCTION

The bacteria constituting the species *Escherichia coli* were first discovered by a German microbiologist, Theobald Escherich in 1885 and were commonly thought as normal flora of man and animals (Sojka, 1965). However, until late 1950's, certain strains were found to be capable of inducing disease, and *E. coli* were therefore regarded as a potential pathogen. These strains are classified into several groups based on their distinct clinical manifestations and virulence determinants, for example, the enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC) (Olsvik *et al.*, 1991).

In 1982, following two outbreaks of a distinctive bloody diarrheal syndrome and a sporadic case of bloody diarrhea, a new bacterial pathogen, *E. coli* O157:H7 was identified (Riley *et al.*, 1983). *E. coli* O157:H7 comprises a fourth group of *E. coli* associated with human diarrhea, i.e., enterohemorrhagic *E. coli* (EHEC). The first two outbreaks that occurred in Michigan and Oregon, involved a national fast-food chain distributing hamburgers. Since then, several additional outbreaks have been reported in other parts of United States (Ryan *et al.*, 1986,

Griffin *et al.*, 1988), Canada (Borczyk *et al.*, 1987) and with increased surveillance, *E. coli* O157:H7 outbreaks have also being reported in other parts of the world including Mexico (Cravioto *et al.*, 1990), China (Xu *et al.*, 1990), Argentina (Lopez *et al.*, 1989), Belgium (Pierrard *et al.*, 1990) and Malaysia (Son *et al.*, 1996).

As the incidence of by *E. coli* O157:H7 is in the increase in many parts of the world, specific surveillance of this pathogen is essential. For identifying the sources and monitoring the spread of *E. coli* O157:H7, a number of epidemiologic markers have been use, including antibiotic susceptibility pattern (antibiotype), plasmid profile, pulsed field gel electrophoresis (PFGE), biotype, phage typing and restriction analyses of chromosomal DNA by classical electrophoresis. The use of epidemiologic markers enables the bacterial stains to be typed and establish degree of relatedness between the strains.

As phenotypic systems have limitations in typing and stability, and most typing systems on their own have a low discriminatory ability, therefore, in the present study, we combined antibiotic susceptibility pattern and plasmid profile for subtyping *E. coli* O157:H7 beyond their serotype. Among genotypic analysis techniques, PFGE of macrorestriction fragments appears to be a highly sensitive method that may detect subtle genetic variations among phylogenetically and epidemiology related isolates of *E. coli* O157:H7.

Recently, a polymerase chain reaction (PCR)-based method of genotypic analyses that is sensitive and yet efficient for typing pathogenic microbe has become very popular because of its convenience and simplicity. Arbitrarily primed-PCR has been reported to be useful for tracing route of infection and also for understanding the spread of pathogen, especially O157:H7 serotype. In the present study, we attempt to type strains of *E. coli* O157:H7 isolated from different samples and locations according to three methods, namely the combination of plasmid profile and antibiotype, PFGE and arbitrarily primed (AP)-PCR. The study here is motivated by the view that *E. coli* O157:H7, though members of one clone, can often be distinguished by methods that have been clarified by previous workers.

Objectives of the Study

This study is carried out to determine the relatedness of a group of 14 *E. coli* O157:H7 beef isolates using molecular techniques. The isolates are from Bangsar, Kajang, Serdang and Petaling Jaya. Three methods are used; combination of plasmid profiling and antibiotic susceptiblity pattern, macrorestriction digestion pattern by PFGE and arbitrarily primed polymerase chain reaction (AP-PCR) to detect polymorphisms in their genome. The results from each method are then discussed in order to determine which is the most discriminative for the purpose of strain differentiation.

CHAPTER II

LITERATURE REVIEW

Microbiology of *Escherichia coli* O157:H7

The strains of *E. coli* O157:H7 that were isolated during the outbreak in the Northwest Pacific, United States of America are typical of most *E. coli*, with some exceptions. *E. coli* serotype O157:H7 possess biochemical markers, growth and survival characteristics that are significantly different from those of other *E. coli* strains.

Biochemical Characteristics.

The inability to ferment sorbitol and the absence of β-glucuronidase activity are believed to be a specific phenotypic feature of *E. coli* O157:H7. More than 90% of *E. coli* isolates of human origin ferment sorbitol within 24 hours; however, *E. coli* O157:H7 does not (Johnson *et al.*, 1983; Ratnam *et al.*, 1988). Furthermore, other workers reported that *E. coli* O157:H7 failed to ferment sorbitol as late as seven days (Wells *et al.*, 1983). Workers exploited this biochemical feature and developed a modified MacConkey agar containing D-sorbitol (SMAC) instead of

lactose to detect *E. coli* O157:H7 (March and Ratnam, 1986; Farmer and Davis, 1985). The strain is similar to other *E. coli* on the lactose-containing agar such as Eosin Methylene Blue Agar (EMBA) making it very difficult to differentiate between them. However, on SMAC agar *E. coli* O157:H7 could be recognised as colorless colonies showing that they failed to ferment sorbitol. Ratnam *et al.* (1988) described supplementary biochemical markers, such as lysine and ornithine decarboxylation to increase the specificity of the sorbitol-only screened *E. coli* O157:H7. The inclusion of these tests reduced the number of organism to be serotyped and improved the specificity of the biochemical screen to 33.6% (Haldene *et al.*, 1986).

In addition, *E. coli* O157:H7 strains are different from other *E. coli* strains, as they do not posses β-glucuronidase activity. More than 90% of *E. coli* produce the enzyme β-glucuronidase, which is the basis for a rapid fluorogenic assay for *E. coli* (Feng and Hartman, 1982). The indicator, 4-methyl-umbelliferone glucuronide (MUG) used in this assay is hydrolysed by β-glucuronidase enzyme possess by normal *E. coli* and converted into 4-methylumbelliferone that fluoresces under radiation with ultra-violet (UV) light (366 nm). In contrast to most *E. coli* strains, *E. coli* O157:H7 is not capable of producing β-glucuronidase. When irradiated with long wave UV light, no fluorescence is formed, hence *E. coli* O157:H7 could be differentiated from other *E. coli*.

Growth and Survival Properties

Doyle and Shoeni (1984) showed that *E. coli* O157:H7 can survive well up to 9 months at -20°C and at -80°C in ground beef. They also observed another important characteristic of *E. coli* O157:H7 i.e., they grew poorly at 44-44.5°C, which is the temperature generally used for the isolation of *E. coli* O157:H7. Hence, traditional procedures for detecting *E. coli* in foods would not likely detect *E. coli* O157:H7. Temperature range of 16.4 to 42.5°C and 48 hours incubation has been suggested by Raghukeer and Matches (1990) for detecting *E. coli* O157:H7 in foods.

E. coli O157:H7 has no unusual heat resistance. Most of the organisms will be killed if the food was pasteurised or heated to some extent. D' Aoust *et al.* (1988) observed that more than 10^4 *E. coli* O157:H7 per ml were killed following pasteurisation of milk (72°C, 16.2s). In addition, Kotula *et al.* (1977) observed ground beef patties that were undercooked 'well done' (4 minutes per side at 149°F griddle temperature) showed a reduction in the coliform count from four logs to less than 1/gram. Coliform count in retail raw ground beef are viable, but may be expected to be 1000/gram or more (Restaino and Lyon, 1987). This means that high initial coliform counts that results in a rare hamburger are likely to permit the survival of coliforms after cooking.

Outbreaks of *E. coli* O157:H7 caused by drinking apple cider indicates that *E. coli* O157:H7 is resistant to acidic pH and distinguished O157:H7 serotype from other *E. coli* (Miller and Kaspar, 1994). *E. coli* O157:H7 can grow at pH levels ranging from 4.0 to 9.0 (Glass *et al.*, 1992) which is not significantly different from the pH range of 4.4 to 9.0 reported for other *E. coli*. However, the ability of *E. coli* O157:H7 to survive at a pH of less than 4.0 was reported by Miller and Kaspar (1994). Their study revealed that *E. coli* O157:H7 can survive in unpasteurised apple cider at pH 3.6 to 4.0 for as long as 31 days at 8°C. Similarly, Zhao and Doyle (1994) observed that *E. coli* O157:H7, when initially present at 6.5 x 10^3 CFU/g, can survive in mayonnaise (pH 3.6 to 3.9) at 20°C for 21 days and at 5°C for 55 days.

Pathogenicity

The pathogenesis of infections with *E. coli* O157:H7 and other enterohemorrhagic *E. coli* is not completely understood. *E. coli* O157:H7 do not elaborate the heat-labile and heat-stable enterotoxins that are produced by enterotoxigenic *E. coli* and not enteroinvasive, as judged by both Sereny test and the absence of invasion into epithelial cells in tissue culture. However, *E. coli* O157:H7 do produce high levels of cytotoxin, variously termed as verotoxin or Shiga-like toxin. In addition to toxin, adherence to mucosal surfaces in the gastrointestinal tract is an

important primary step that results in bacterial colonisation of the intestine necessary for delivery of elaborated toxins to enterocytes.

Verocytotoxins

Verocytotoxin-producing *E. coli* (VTEC) were first described by Konowalchuk (1977), who identified a cytotoxin active on cultured Vero cells (African green monkey kidney cells) and produced by certain strains of *E. coli*; it was termed Vero cytotoxin (VT). VT is clearly indistinguishable, biologically and immunogically from the heat stabile (ST) and heat-labile (LT) enterotoxins of *E. coli,* and reported to be closely related to Shiga toxin which is produced by strains of *Shigella* dysentriae type I. Hence, these toxins were designated as Shiga-like toxins. Both Shiga and Shiga-like toxin share the same receptor, and have similar structure and modes of action (Robinson *et al.*, 1980). The gene coding for Shiga-like toxin I is very similar to that of Shiga toxin, while the gene for Shiga-like toxin II shows 58 percent overall homology with that for Shiga-like toxin.

Shiga-like toxins from *E. coli* O157:H7 were first reported in 1983 when O'Brien and co-workers found that isolates from two outbreaks in the United States produced high levels of a cell-associated cytotoxin for HeLa and Vero cells. On further analysis, it is clear that this organism produces two kind of cytotoxins, one of which can be neutralised by anti-

Shiga toxin. The neutralisable cytotoxin was designated as Shiga-like toxin I (SLT-I), and the other Shiga-like toxin II (SLT-II). If both toxins are produced by the same strains, SLT-I predominates in cell lysates while SLT-II is more active toxin in culture filtrate. The two Shiga-like toxins are antigenically distinct and differ in their biological effects: Shiga-like toxin II is less toxic to Vero cells, but more toxic for mice, and causes hemorrhagic colitis in the adult rabbit, while Shiga-like toxin I does not (Evans *et al.*, 1977).

Phage conversion is responsible for controlling the production of several important bacterial toxins, including diphtheria toxin, streptococcal erythrogenic toxin, botulinum toxin and staphylococcal enterotoxin (Betley *et al.*, 1986). Recently, the production of Shiga-like toxin was shown to be determined by specific phages in selected strains of both EPEC and EHEC (O157) serotype isolated from humans (Smith *et al.*, 1983) which indirectly suggests that *E. coli* O157:H7 may have acquired these toxins through phage mediated transfer (Strockbine *et al.*, 1986).

Adherence and Attachment

Another property that makes *E. coli* O157:H7 virulent is its ability to adhere to intestinal cells. The mechanism of bacterial attachment to the intestinal mucosal cell remains controversial because there is no direct

evidence from human cases to asses the nature of intestinal colonisation by *E. coli* O157:H7. However, experiments to observe the phenomenon have been conducted in animal and cell cultures.

Studies in gnotobiotic piglets and cell cultures showed no evidence of the extensive invasion and intracellular multiplication as seen with the invasive bacteria such as Shigella (Tzipori *et al.*, 1986). *E. coli* O157:H7 produce a distinctive microscopic lesion characterised by intimate attachment of the bacteria to the apical intestinal mucosal cell and localised destruction of the microvilli (Tzipori *et al.*, 1988; Tzipori *et al.*, 1989). The bacteria also exhibit localised adherence to cells in culture, with dense concentration of actin microfilaments in a cup-like structure in the cytoplasm beneath the attached bacterium (Knutton *et al.*, 1989). This attaching-efficacing lesion resembles that produced by enteropathogenic *E. coli* strains in piglets and cell culture (Knutton *et al.*, 1989; Tzipori *et al.*, 1989).

The mechanism may indicate an initial contact followed by more intimate attachment, such as has been described by enteropathogenic *E. coli* (Knutton *et al.*, 1989). The initial attachment may be mediated by the 60 MDa plasmid while the intimate attachment may be chromosomally mediated (Toth *et al.*, 1990). Most *E. coli* O157:H7 carry a 60 MDa plasmid . There are several different opinions on the role of the plasmid. Karch *et al.* (1987) determined that the plasmid was required for

expression of a fimbrial adhesin and adherence to Henle 407 intestinal cells. The strains that were cured of the plasmid failed to express fimbriae and lost the ability to adhere to intestinal cells. Recently, Toth *et al.* (1990) determined that the 60 MDa plasmid appear to modify the eucaryotic cell adherence of *E. coli* O157:H7 and that adherence was conferred to an *E. coli* transformant.

However, Junkin and Doyle (1989) revealed that adherence to Henle 407 cells by *E. coli* O157:H7 strain 932 was not dependent on the 60 MDa plasmid mentioned. They showed that the test strain 932 adheres to the human small intestine cell line INT407 at an average level of 7.1 bacteria per INT407 cell without the strain harboring the plasmid.

Complications of *E. coli* O157:H7 Infections

Since the occurrence of the outbreaks in 1982, continued epidemiologic, clinical, and laboratory investigations clearly established *E. coli* O157:H7 to be an important etiologic agent of hemorrhagic colitis. Typical hemorrhagic colitis can be distinguished clinically from bloody diarrhea or dysentery seen in shigellosis, *Campylobacter* spp. and enteroinvasive *E. coli* enteritis, amoebiasis, or other enteric illnesses such as narcrotising enterocolitis or pseudomembraneous colitis by the lack of prominent fever (Riley, 1987). The illness caused by *E. coli* O157:H7 resolves in most patients with no sequel. Early outbreak studies in which