UNIVERSITI PUTRA MALAYSIA

PLASMID PROFILING AND ANTIBIOTIC RESISTANCE OF ESCHERICHIA COLI AND ESCHERICHIA COLI 0157 STRAINS

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By

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UPM

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LIST OF ABBREVIATIONS

BCIG	5 bromo-4-chloro-3-indolxyl-ß-D-glucuronic
ccc	Covalently close circular
CO_2	Carbon dioxide
CT-SMAC	Cefizime tellurite sorbitol MacConkey agar
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EMBA	Eosin methylene blue agar
g	gram
HCl	Hydrocloric acid
IMViC	Indole methyl red Voges-Prokauer citrate
KH_2PO_4	Potassium phosphate
min	minute
MUG	4-methylumbelliferone glucuronide
ml	Milliliter
Na_2HPO_4	di-sodium phosphate
NaOH	Natrium hydroxide
%	Percentage
oc	Open circular
SDS	Sodium dodecyl sulphate
SMAC	Sorbitol MacConkey agar
TBE	Tris-Boric-EDTA
UV	Ultra violet
µl	Microliter
w/v	Weight per volume

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PLASMID PROFILING AND ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* AND *ESCHERICHIA COLI* O157 STRAINS

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Enterohaemorrhagic *Escherichia coli* O157:H7 has received considerable amount of attention because of its implication in sporadic outbreaks of hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. The incidence of *E. coli* O157:H7 have been reported in various parts of the world, however, its incidence in Malaysia is relatively unknown. From March to June 1996, 25 beef samples were obtained from 4 supermarkets in Selangor and Federal Territory, Malaysia. Twenty five g of beef samples were enriched in 225 ml of EC broth containing novobiocin (20 mg/l) for four hours at 37°C and were plated on MacConkey agar. Nineteen to fifty lactose fermenting colonies per sample (n=1091) were transferred onto Eosin Methylene Blue Agar, Sorbitol MacConkey Agar and Cefizime Tellurite Sorbitol MacConkey Agar. Sixty five of 112 metallic sheen and sorbitol negative colonies were positive when tested for O157 antigen using latex agglutination test kit of Oxoid and Serobact. Nineteen (76%) samples were positive

for *E. coli* O157:H7. The 65 strains of *E. coli* were tested for susceptibility against 20 antimicrobial agents and screened for plasmid DNA. All 65 isolates of O157 were resistant to four or more antimicrobial agent tested. All strains were resistant to bacitracin (100%), methicillin (100%) and vancomycin (100%). All strains were susceptible to cephalosporin, kanamycin, nalidixic acid and furazolidone. Fourty isolates contained plasmid ranging in sizes from 1.38 megadalton to 86.0 megadalton; and 29 possessed the 60.0 megadalton *E. coli* O157 serotype-specific plasmid (pO157) of EHEC. Two of eight selected *E. coli* O157 isolates were observed to transfer their resistant phenotypes at frequencies 2.7×10^{-7} to 3.0×10^{-6} per donor cells. The concomitant transfer of donor plasmids in two of the selected *E. coli* O157 isolated was also detected. These results may suggest that antibiotic resistance among the selected *E. coli* O157 isolates were encoded on conjugative R plasmids.

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PROFAIL PLASMID DAN KERENTANGAN ANTIBIOTIK *ESCHERICHIA COLI* DAN STRAIN-STRAIN *ESCHERICHIA COLI* O157

Oleh

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Perhatian telah diberikan ke atas Enterohemorragik *Escherichia coli* O157:H7 kerana ia menyebabkan implikasi yang jarang berlaku seperti hemorrhagik kolitis, hemolitik uremik sindrom dan thrombotik thrombositopenik purpura. Insiden yang disebabkan oleh *E. coli* O157 telah dilaporkan diseluruh dunia tetapi kejadian jangkitan yang berlaku di Malaysia belum pernah diketahui. Dari Mac sehingga Jun 1996, dua puluh lima sampel telah didapati dari 4 buah pasar-raya di Selangor dan Wilayah Persekutuan Malaysia. Dua puluh lima gram sampel daging telah dikayakan dalam 225 ml larutan EC yang mengandungi novobiocin (20 mg/l) selama 4 jam pada 37°C dan diplatkan ke atas agar MacConkey. Sembilan belas sehingga lima puluh koloni penapai laktosa per sampel (n=1091) telah dipindahkan di atas agar Eosin Metillin Biru, agar Sorbitol MacConkey dan agar Cefizim Telurit Sorbitol MacConkey. Enam puluh lima dari 112 kilauan metalik dan koloni-koloni sorbitol-negatif telah memberikan keputusan yang positif bila diujikan dengan kit

ujian agglutinasi latek antigen O157 yang dibekalkan dari Oxoid dan Serobact. Sembilan belas (76%) sampel telah menunjukkan kehadiran positif *E. coli* O157. Ke enam puluh lima strain *E. coli* O157 telah diuji kesensitifannya terhadap 20 agen antimikrobial dan diskrinkan untuk DNA plasmid. Kesemua 65 asingan, rentang kepada empat atau lebih agen mikrobial yang diujikan. Selain dari itu kesemua enam puluh lima strain menunjukkan kerentangan terhadap bacitrasin, (100%), meticillin (100%) dan vankomisin (100%). Bagaimanapun, kesemua strain sensitif terhadap cephalosporin, kanamisin, acid nalidizik and furazolidon. Empat puluh asingan mengandungi saiz DNA plasmid yang berjulat dari 82.0 megadalton sehingga 1.38 megadalton; dan dua puluh sembilan memiliki 60.0 megadalton plasmid yang spesifik terhadap serotip (pO157) EHEC. Dua dari 12 asingan *E. coli* O157 telah ditunjukkan memindahkan penotif kerentangan pada frekuensi 2.7×10^{-7} to 3.0×10^{-6} per sel penderma. Pemindahan DNA plasmid juga didapati seiring terhadap kedua-dua *E. coli* O157. Keputusan tersebut mencadangkan bahawa kerentangan antibiotik di antara asingan *E. coli* O157 mungkin dienkodkan di atas plasmid R konjugatif.

CHAPTER 1

INTRODUCTION

Escherichia coli was first described by Theobald Escherich in 1885 (Sojka, 1965). He examined the faeces of new-born breast-feeding babies and found that they contained bacteria; he called this microorganism as *Bacterium coli commune* which is now accepted as *E. coli*. It was first considered to be a harmless saprophytes. However in 1889, Laraeke (Sojka, 1965) demonstrated that this coliform bacteria existed in cases of appendicitis with peritonitis, which suggested that it might be pathogenic.

The presence of *E. coli* in food is always associated with lack of cleanliness, inadequate processing, and more specifically indicating the occurrence of fecal contamination. Thus, the detection of *E. coli* can be used to asses the sanitary quality of food, as well as in water and is considered primarily as an indicator organism for the possible presence of pathogenic bacteria. *E. coli* also carries a functional system for lactose utilisation study which led to the formulation of the operon model for regulation of expression of prokaryotic genetic material (Glass, 1982).

This microorganism has been extensively studied, and it is known that *E. coli* commonly exist in the lower part of the intestine of most warm-blooded animals and recognised as opportunistic pathogen because of its continuous presence in gut. It

has ample opportunity to cause extraintestinal diseases of any type when local or general immunity has been compromised in favour of bacteria. Most common in man are urinary tract infections, of which majority are cause by *E. coli*. Other infections caused by *E. coli* are gall bladder, peritoneal cavity or wound infections. In rare instances it may cause meningitis, septicemia, endocarditis and arthritis in animal (Soltys, 1979). In 1982 two outbreaks of acute bloody diarrhea occurred in U. S. A (State of Oregon and Michigan) (Riley *et al.*, 1983) where *E. coli* was recognised as the pathogen. The new strain was identified as *E. coli* O157:H7 which is now associated with food-related outbreaks of an unusual gastrointestinal illness. The illness is generally quite severe and can cause three different syndromes; haemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura.

Initially, infection due to *E. coli* O157:H7 was considered rare because of its lack of frequent isolation prior to 1982; however, subsequent experience has shown that the organism can be isolated with such an appreciable frequency that it is now not considered a rare serotype (Doyle, 1991). *E. coli* O157:H7 is now recognised as an important cause of foodborne disease, with outbreaks reported in U. S. A., Canada and United Kingdom (Doyle, 1991). Several additional outbreaks of *E. coli* O157:H7 infections have been reported in Mexico (Cravioto *et al*., 1990), China (Xu *et al*., 1990), Argentina (Lopez *et al*., 1989) and Belgium (Pierard *et al*., 1990). The illnesses caused by *E. coli* 0157:H7 appears to occur throughout much of the world (Doyle, 1991). However, no illness caused by *E. coli* O157:H7 has been reported yet in Malaysia.

Most outbreaks due to *E. coli* O157:H7 have been associated with eating undercooked ground beef or less frequently drinking raw milk. Surveys of retail raw meats and poultry revealed that *E. coli* O157:H7 was detected in 1.5 to 3.5% of ground beef, pork, poultry and lamb. Dairy cattle, especially young animals, have been identified as a reservoir (Doyle, 1991). Faith *et al.* (1996) reported that the range prevalence of *E. coli* O157:H7 strains in herds of dairy and beef cattle was 1.8% (19 of 1068 herds) to 16% (4 of 25 herds), respectively.

E. coli O157:H7 is typical of most *E. coli*, but does possess distinguishing characteristics. One distinguishing feature of *E. coli* O157:H7 is its lack of rapid (less than 48 hours) fermentation of sorbitol. This has being used as the basis of the most common screening procedure for this organism (Neill *et al.*, 1993). *E. coli* O157:H7 does not possess ß-glucuronidase activity and hence cannot hydrolyse 4-methylumbelliferone glucuronide (MUG) to a fluorogenic product; it is therefore MUG negative (Doyle and Schoeni, 1984). The organism has no unusual heat resistance; heating ground beef sufficiently to kill typical strains of Salmonellae will also kill *E. coli* O157:H7. The mechanism of pathogenicity has not been fully elucidated (Doyle, 1991), but clinical isolates produce one or more verotoxins which are believed to be important virulence factors.

Griffin *et al.* (1988) reported that almost all strains of *E. coli* O157:H7 are susceptible to ampicillin, trimethoprim-sulfamethoxazole, tetracycline and quinolones, although they are resistant to erythromycin, metronidazole and vancomycin. However, in the past few years, several reports had been published on the emergence of multiply antibiotic resistance *E. coli* O157:H7 (Swerdlow *et al.*1992; Kim *et al.*, 1994; and Farina *et al.*, 1996).

Since dairy cattle of young animals have been identified as a reservoir of *E. coli* O157:H7 and the contamination of retail meats may probably occur during the processing of meats at the retail level (Sekla *et al*., 1990), attempts are made to isolate *E. coli* O157:H7 from imported frozen beef.

Objectives

The objectives of this study are:

1. To isolate *E. coli* and *E. coli* O157:H7 from imported frozen beef and its biochemical tests.
2. To determine antibiotic susceptibility and plasmid profiles of the *E. coli* and *E. coli* O157:H7.
3. To carry out genetics transfer study on selected isolates. Such study would provide information on the possible correlation between the presence of plasmids and antibiotics resistance.

CHAPTER 2

LITERATURE REVIEW

Escherichia coli

Taxonomy

Escherichia coli is classified in the family *Enterobacteriaceae* and its taxonomic features includes Gram-negative, asporogenic, straight rods that may be peritrichously flagellated or nonmotile (Orskov, 1984). It coagulated milk, producing acid and gas. The ability to ferment certain carbohydrates, producing acid and gas was soon adapted as a basis for the differentiation of closely related enteric bacteria such as *Shigella* and *Salmonella.*

Five species of *Escherichia* now are recognised; *E. coli*, *E. hermanii*, *E. blattae*, *E. vulneris* and *E. fergusonii* (Edwards and Ewing, 1986). These were delineated by means of DNA relatedness studies by Brenner and Brenner *et al.* (Cited by Edwards and Ewing, 1986). *E. hermanii* was differentiated earlier and was called a biogroup of *E. coli* followed by *E. blattae*. The DNAs from all above mentioned species of *Escherichia* are related to those of *E. coli* (average, 84%) (Edwards and Ewing, 1986).

Generally, the genus of *Escherichia* is composed of motile or non-motile bacteria that conform to the definitions of the family *ENTEROBACTERIACEAE* and the tribe *ESCHERICHIEAE*. Both acid and gas are formed from a wide variety of fermentable carbohydrate, but aerogenic biotypes occur; salicin is fermented by many cultures but inositol is not utilised and adonitol is utilised by members of only one species. Lactose often is fermented rapidly, some strains utilise it slowly, and some fail to ferment this substrate. Lysine and ornithine are decarboxylated by the majority of cultures, acid is formed from sodium mucate, and sodium acetate frequently is utilised as a sole source of carbon.

E. coli, the type species in this family was fully reviewed by Glass (1982) as a favourite organism for all types of microbiological studies. Prominent in any list of the advantages of research on bacteria must be the rapid growth and limited nutritional requirements of those microorganism.

Genome size (including chromosomal and extrachromosomal DNA) in *E. coli* strain varies from 2.3 X 10^9 to 3.0 X 10^9 daltons. The G + C content ranges from 49 to 52% (Selander *et al.*, 1987).

Habitat

Most strains of *E. coli* are harmless commensal members of the intestinal flora of mammals and, to an undetermined extent, birds in which some strain adhere to the intestinal mucous while others are only temporary transients in the lumen of the colon. *E. coli* is the major anaerobe of the large intestine, occurring in the

intestine, in normal densities of about 10^6 cells per g of colon contents. It is a minor component of the total intestinal flora, which consists largely of obligate anaerobes and in the aggregate reaches 10^{11} cells per g of colon contents (Selander *et al.*, 1987).

E. coli is also one of the first of the intestinal bacteria to colonise the newborn, generally being derived from the faeces of the mother in both humans and animals (Baltelheim *et al.*, 1974) and may be present in the cecum and lower intestine of mammals within hours or day after birth.

Growth in the intestine is much slower than in laboratory culture, the doubling time being estimated at about 1 day. While most strains of *E. coli* live as benign commensals, many perhaps all, are opportunistic pathogens of humans and other animals (Soltys, 1979). In 1982, two outbreaks of acute bloody diarrhea occurred in the state of Oregon and Michigan where *E. coli* (*E. coli* O157:H7) was first recognised as a pathogen (Riley *et al.,* 1983).

E. coli is also a prime agents in surgical and other nosocomial (hospital-acquired) infections (wound, secondary pneumonia, peritonitis) in compromised patients (Soltys, 1979). In addition *E. coli* is the major cause of neonatal septicemia, neonatal meningitis and urinary tract infection in humans and of a variety of invasive disease in mammals and birds (Sojka, 1965), including mastitis in cattle and sepsis in chicken (Sojka, 1965). It is also a leading cause of diarrheal disease in humans and other animals.

The intestine and tissues of warm blooded animals are the primary habitats of *E. coli*, secondary habitats are soil, sediment and water (Caugant, 1983). Under extreme conditions, such as around cattle feed lots, densities may reach fecal levels in the soil and 10^4 cells per ml in polluted water. In surface soils underlying manure, *E. coli* is reported to survive for several months, but normally it may live in soil or water for only a few days without dividing (Selander *et al.,* 1987). Human and food animals should perhaps also be regarded as a secondary environment of *E. coli*.

Isolation and Identification

E. coli can be easily isolated by growing the suspension samples on MacConkey or eosin methylene blue agar (EMBA). The colonies growing on MacConkey medium are rose-coloured owing to the action of acid produced during fermentation of lactose. However, most primary isolation of *E. coli* rely on EMBA. This medium is lactose-peptone agar containing eosin methylene blue. The colonies growing on this media will have a greenish metallic sheen due to the reflection of light. Therefore, *E. coli* can be distinguished from *Aerobacter aerogene* and *Candida albicans* which also have the ability to grow on the same media.

Descriptions of media and reagents used for *E. coli* and other *Enterobacteriaceae* can be found in several common laboratory manuals, for example Edwards and Ewing (1986). To identify the presumptive *E. coli*, they (Edwards and Ewing, 1986) suggested a list of biochemical test as shown in **Table 1.**

Table 1 : The biochemical reactions of *Escherichia coli.*

Test or substrate	*E. coli* Sign	%+ (%+)	Test or substrate	*E. coli* Sign	%+ (%+)
Hydrogen sulphide (TSI agar)	-	0[a]	Rhamnose	d	83.5 (3.4)
Urease	-	0[a]	Malonate	-	0
Indole	+	96.3	Mucate	+	91.6
Methyl red	+	99.9	Christensen's citrate	d	18.2 (22.6)
Voges-Proskauer	-	0	Jordan's D-tartrate	+	97.6
Citrate (Simmon's)	-	0.2 (0.3)	Pectate	-	0
KCN	-	0	Sodium acetate	+ or (+)	83.8 (9.7)
Motility	+ or (+)	62.1	Ammonium salts glucose agar	+	94.5 (1.7)
Gelatin (22°C)	-	0	Sodium alginate	-	0
Lysin decarboxylase	d	80.6 (1.5)	Lipase		
Arginine dihydrolase	d	16.3 (39.1)	Corn oil	-	0
Ornithine decarboxylase	d	57.8 (8)	Tributyrin	-	0
Phenylalanine deaminase	-	0	Maltose	+	90.6 (2.4)
Glucose			Xylose	d	82.8 (6.6)
acid	+	100	Trehalose	+	98.2 (1.8)
gas	+	92	Cellobiose	-	1 (3)
Lactose	+	91.6 (4.2)	Glycerol	+ or (+)	89 (8.3)
Sucrose	d	53.7 (5.5)	Alpha-methylglucoside	-	0
Mannitol	+	97.5	Erythritol	-	0
Dulcitol	d	49.3 (18)	Esculin	d	30.9 (19.7)
Salicin	d	36 (12.3)	Beta-galactosidase	+	>96
Adonitol	-	1	Nitrate to nitrite	+	99.8 (0.2)
Inositol	-	0.9 (0.2)	Oxidation-fermentation	F	100
Sorbitol	d	80.3 (1)	Oxidase	-	0
Arabinose	+	99.3 (0.5)	DNase (22°C)	-	0
Raffinose	d	49.4 (2.1)	Yellow pigment	-	< 0.1

Key

+	90% or more positive within 1 or 2 days.
(+)	positive reaction after 3 or more days (decarboxylase tests: 3 or 4 days).
-	no reaction (90% or more) in 30 days.
+ or -	most cultures positive; some strains negative.
- or +	most strains negative; some cultures positive.
+ or (+)	most reactions occur within 1 or 2 days; some are delayed.
d	different reactions, +., (+), -.
F	Fermentative.
TSI	Triple sugar iron.
a	An occasional strain may produce abundant hydrogen sulfide and an occasional culture may hydrolyse urea. Both of these reactions are mediated by (different plasmid). The urease reactions are weak and usually are not apparent until after 24 hours of incubation.

(Source: Edwards and Ewing, 1986).

Serological Classification

Serological classification of *E. coli* is based on three kinds of antigens that occur in *E. coli*. The serotyping scheme was developed by Khaufman in 1947 (Doyle and Padhye, 1989) and divided into three categories:

a. Thermostable somatic "O" antigens closely resembling those of the *Salmonella*.

b. H or flagella antigens.

c. K or surface antigens.

The O antigen, which is based on the antigenicity of O-specific polysaccharide of the cells outer membrane lipopolysaccharides, is a thermo stable (stable at 100°C for 2 hours) surface antigen found in all smooth (S forms) *Enterobacteriaceae* (Doyle and Padhye, 1989). The O antigen forms the basis for classifying *E. coli* into serogroups, of which there are more than 170 (Orskov, 1984). Within each serogroup, are one or more serotypes that are based on the H antigen. For example O126:H27 and O126:H$^-$ represent two serotypes of *E. coli* within the same serogroup. H antigen are heat-labile flagella antigens compose of protein.

Three varieties of K antigen have been described: the heat-labile L antigen; the heat-stable A antigen which is associated with capsule formation and the B antigen which is partially altered by heat (Soltys, 1979). **Table 2** gives further information about K antigen.