COMPARATIVE GENETIC DIVERSITY
AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF
Escherichia coli FROM SELECTED ANIMALS AND
ENVIRONMENTAL SOURCES IN SARAWAK,
MALAYSIA

Chong Yee Ling

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COMPARATIVE GENETIC DIVERSITY AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *Escherichia Coli* FROM SELECTED ANIMALS AND ENVIRONMENTAL SOURCES IN SARAWAK, MALAYSIA

Chong Yee Ling

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DECLARATION

I hereby declare that no portion of the work referred to this thesis has been submitted in support of an application for another degree or qualification to this or any other university or institution of higher learning.

__________________________

(Chong Yee Ling)

Date:
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ABSTRACT

A total of 312 *Escherichia coli* isolates were recovered from broiler and village chickens, bats, rodents, and swine sewage effluents, in Sarawak, based on their biochemical tests and morphological characteristics. A comparison of the Enterobacteriaceae species revealed that enteric bacteria and *E. coli* are common members of microflora in all the environmental samples tested except for bat samples. The total recovery rate of *E. coli* was only 5.5% from a total of 239 bat samples. More than 70% of the total isolates were detected as typical *E. coli* (sorbitol fermenter and possess GUD activity). Detection of *stx1*, *stx2*, *rfbE* and *fltC* genes using multiplex PCR method revealed that none of the 130 representative *E. coli* isolates was confirmed as STEC or *E. coli* O157:H7. The absence of pathogenic genes indicates that these animals and environmental sources are not important reservoirs of STEC or *E. coli* O157:H7 in Sarawak. The antimicrobial susceptibility characteristics of 172 *E. coli* isolates for antibiotic agents commonly used in the animal husbandry industry in Sarawak was studied. In general, the most frequently encountered form of resistance in all samples was resistance to tetracycline (43.6%) and sulphamethoxazole-trimethoprim (25.58%). The multiple-antibiotic-resistance (MAR) indices were highest for broiler chicken isolates (0.479) and lowest for bat isolates (0.013). All isolates from both broiler chicken and swine sewage effluent samples were multidrug-resistant *E. coli*. High MAR indices as well as prevalence of multiple-resistance patterns of isolates from broiler chickens and swine sewage effluents raise a concern on the dissemination of these bacteria to human via food-consumption. The *E. coli* isolates from wildlife had an average MAR index of only 0.172. Thus, wildlife does not present a high risk of spreading antibiotic-resistant *E. coli* to the environments. A total of 58 representative *E. coli* isolates were assessed to study the genetic profiles of these bacteria
using PFGE, RAPD-PCR, and ERIC-PCR genotyping methods. Among these methods, only RAPD-PCR generated dendrogram successfully grouped the *E. coli* isolates into their respective animal sample sources and clearly differentiate between food animal sources and wildlife sources. This study also provided the first approach to combine three different molecular subtyping methods in a single dendrogram for differentiating the animal sources of *E. coli* isolates. Dendrograms generated from RAPD-PCR and combined-RAPD-ERIC-PFGE fingerprinting patterns demonstrated that *E. coli* isolates sampled from environmental sources in Sarawak were non-randomly distributed but specific to an animal host which accounted for the genetic variation in the *E. coli* populations. Further study to trace any changes that may occurred in the genetic composition of the isolates during the transition from the chickens’ hosts to the external environments was done using 38 representative *E. coli* isolates from chicken samples. As village chickens were regarded as the samples from natural habitat, this study revealed that clonal relationships occurred between the *E. coli* isolates from two different habitats of village chicken samples. Broiler chickens were considered as control sample sources. Based on the genetic profiles, some changes in the genetic materials had occurred during the transition of the *E. coli* isolates from the broiler chickens to the external environments. This findings based on the fingerprinting profiles through genotyping methods would aid in the future study on the genetic diversity and epidemiological investigations of this bacterium.
Sebanyak 312 pencilan Escherichia coli telah dipulihkan daripada ayam daging, ayam kampung, kelawar, rodensia, dan efluen kumbahan khinzir di Sarawak berdasarkan ujian biokimia dan ciri morfologi. Perbandigan di antara spesis Enterobacteriaceae menunjukkan bahawa bakteria enterik dan E. coli adalah mikroflora umum dalam semua sampel alam sekitar yang diperiksa kecuali sampel kelawar. Jumlah kadar pemulihan bagi E. coli adalah hanya sebanyak 5.5% daripada sejumlah 230 sampel kelawar. Lebih daripada 70% daripada jumlah pencilan telah dikesan sebagai E. coli tipikal (penapai sorbitol dan memiliki aktiviti GUD). Pengesanan gen-gen stx1, stx2, rfbE dan fliC₁₇ dengan menggunakan kaedah tindak balas berantai polimeras multipleks menunjukkan bahawa tiada di antara 130 pewakil pencilan E. coli yang disahkan sebagai STEC atau E. coli O157:H7. Ketidakhadiran gen berpatogen menunjukkan bahawa sumber haiwan dan alam sekitar adalah tidak penting sebagai takungan bagi STEC atau E. coli O157:H7 di Sarawak. Ciri-ciri rentan anti-mikrob untuk 172 pencilan-pencilan E. coli bagi agen antibiotik umum yang digunakan dalam industri perternakan haiwan di Sarawak telah dikaji. Secara umum, rintangan yang paling kerap ditemui dalam semua sampel adalah rintangan terhadap tetrasiklina (43.6%) dan sulphamethoxazole-trimethoprim (25.58%). Indeks multiple-antibiotic-resistance (MAR) adalah tertinggi bagi pencilan-pencilan ayam daging (0.4719) dan terendah bagi pencilan-pencilan kelawar (0.013). Semua pencilan E. coli bagi kedua-dua sampel ayam daging dan efluen kumbahan khinzir adalah E. coli jenis rintangan ubat berbilang. Indeks MAR yang tinggi berserta dengan corak tentangan berbilang yang tersebar luas bagi pencilan-pencilan daripada ayam daging dan efluen kumbahan khinzir meningkatkan kebimbangan ke atas kemungkinan penyebaran bagi bakteria ini kepada manusia melalui pemakanan. Purata
indeks MAR pencilan \textit{E. coli} hanya sekadar 0.172. Maka, hidupan liar tidak menunjukkan risiko tinggi dalam penyebaran \textit{E. coli} jenis rentangan antibiotik ke sekitar alam. Sejumlah 38 pencilan-pencilan \textit{E. coli} seterusnya telah diaksir untuk menguji profil genetik bagi bakteria ini dengan menggunakan kaedah PFGE, RAPD-PCR dan ERIC-PCR. Di antara kaedah-kaedah ini, hanya dendrogram yang dijanakan daripada RAPD-PCR berjaya mengumpulkan pencilan-pencilan \textit{E. coli} ke dalam sampel sumber haiwan masing-masing dan membezakan secara jelas antara sumber haiwan makanaan dengan hidupan liar. Kajian ini merupakan cubaan pertama kali untuk menggabungkan corak cap-jarian daripada RAPD-ERIC-PFGE dalam satu dendrogram untuk membezakan pencilan-pencilan \textit{E. coli} daripada sumber haiwannya. Dendrogram yang dijanakan daripada corak cap-jarian RAPD-PCR dan gabungan RAPD-ERIC-PFGE menunjukkan bahawa pencilan-pencilan \textit{E. coli} yang disampelkan daripada sumber alam sekitar di Sarawak adalah diagihkan secara tidak rambang tetapi spesifik kepada perumah haiwan yang bertanggungjawab dalam variasi gen di dalam populasi \textit{E. coli}. Kajian lanjutan untuk mengesan sama ada perubahan dalam komposisi gen boleh berlaku semasa peralihan daripada perumah ayam ke persekitaran luarinya telah dibuat dengan menggunakan 38 pencilan \textit{E. coli} daripada sampel ayam. Ayam ayam kampung adalah dianggap sebagai sampel daripada habitat semulajadi, keputusan daripada kajian ini menunjukkan bahawa hubungan klon wujud di antara pencilan \textit{E. coli} daripada sampel ayam kampung bagi dua habitat yang berbeza ini. Ayam daging dianggap sebagai sumber sampel kawalan. Melalui kajian ini, perubahan dalam profil genetik telah berlaku semasa peralihan pencilan \textit{E. coli} daripada ayam daging ke dalam persekitaran luaran. Hasil kajian berdasarkan profil cap-jarian melalui pelbagai kaedah genetik boleh membantu pada kajian masa depan dalam kelainan genetik dan peyelidikan epidemiologi bagi bakteria ini.
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LIST OF ABBREVIATIONS

A
BLAST
BCIG
bp
C
cm
CDC
cfu
CT-SMAC
dNTPs
ELISA
EMB
ERIC-PCR
FDA
fruit bats
g
G
GUD
HC
HUS
IMS
insect bats
kb
LB
mEC+n
MEGA
MgCl₂
M
Mb
mg
MP
min
ml
mM
μm
μg
μl
mol
sec
NaCl
Na-EDTA
NCBI
NJ
PAUP

Adenine
Basic Local Alignment Search Tool
Chromogen 5-bromo-4-chloro-3-indolyl-β-D-glucuronide
Base pairs
Cytosine
Centimeter
Centers for Disease Control and Prevention
Colony forming unit
Cefixime-tellurite Sorbitol MacConkey agar
Deoxynucleotide triphosphates
Enterohemorrhagic Escherichia coli
Deoxynucleotide triphosphates
Enzyme-linked immunosorbent assay
Eosin methylene blue agar
Enterobacterial repetitive intergenic consensus sequence-based polymerase chain reaction
Food and Drug Administration
Megachiropterans
G
Guanine
B-glucuronidase activity
Hemorrhagic colitis
Hemolytic-uremic syndrome
Immunomagnetic separation
Microchiropterans
Kilobase pairs
Luria Bertani
Modified Escherichia coli medium with novobiocin
Molecular Evolutionary Genetics Analysis
Magnesium chloride
Molar or molarity
Megabase pairs
Milligram
Maximum parsimony method
Minute(s)
Milliliter
milliMolar
Micrometer
Microgram
Microliter
Mole
Second(s)
Sodium chloride
Sodium ethylenediamine tetra-acetic acid
National Center for Biotechnology Information
Neighbor joining method
Phylogenetic Analysis using Parsimony
<table>
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<th>Acronym</th>
<th>Term</th>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
<td></td>
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<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
<td></td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplification of polymorphic DNA</td>
<td></td>
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<tr>
<td>rpm</td>
<td>Revolution per minute</td>
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<tr>
<td>SMAC</td>
<td>Sorbitol MacConkey agar</td>
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<tr>
<td>SMAC-BCIG</td>
<td>Sorbitol MacConkey agar supplemented with chromogen 5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
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<tr>
<td>STEC</td>
<td>Shiga toxin-producing <em>Escherichia coli</em></td>
<td></td>
</tr>
<tr>
<td>Stx</td>
<td>Shiga toxin</td>
<td></td>
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<tr>
<td>T</td>
<td>Thymidine</td>
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<tr>
<td><em>Taq</em></td>
<td><em>Thermus aquaticus</em> DNA polymerase</td>
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<td>TBE</td>
<td>Tris-borate EDTA electrophoresis buffer</td>
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<tr>
<td>TE</td>
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<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) methylamine</td>
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</tr>
<tr>
<td>TTP</td>
<td>Thrombotic thrombocytopenic purpura</td>
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<td>USA</td>
<td>United States of America</td>
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<td>USFDA</td>
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CHAPTER 1

GENERAL INTRODUCTION

The bacterium *Escherichia coli* is a naturally occurrence and harmless commensal inhabitant of the intestinal tract of both humans and warm-blooded animals. Typical *E. coli* are sorbitor-fermenter and possess β-glucuronidase enzyme (Koneman *et al.*, 1997). The presence of *E. coli* is generally an indication for fecal contamination from environmental samples (Kuhnert *et al.*, 2000). Most studies concerning *E. coli* had been focused on clinical samples (Souza *et al.*, 1999) and little is known about the natural history of *E. coli* in populations of environmental sources (Wittam, 1998). Gordon and Cowling (2003) studied the distribution of *E. coli* in Australian vertebrates and suggested that host and ecology can influence the distribution of *E. coli* population in the environment. Based on the literature review, no other studies have been done to assess the occurrence of enteric flora and *E. coli* from animal and natural environmental sources in Sarawak, Malaysia. Thus, this study reports on the occurrence of this bacterium from chickens, bats, rodents, and swine sewage effluents in Sarawak.

Although usually harmless, certain pathogenic strains of *E. coli* have been implied as the causative agents in numerous human diseases (Bekal *et al.*, 2003). Among these, Shiga toxin-producing *E. coli* (STEC) is often identified as the etiologic agents of urinary tract infections, enteric and diarrhea diseases, and neonatal meningitis (Nataro and Kaper,
1998). STEC has also been reported to be involved in a variety of invasive diseases in mammals and birds, including mastitis in cattle and sepsis in chickens. The ability of STEC to cause serious diseases in humans is related to their principal virulence trait, the production of Shiga toxin I and II, Stx1 and Stx2 (Griffin and Tauxe, 1991), which inhibits protein synthesis of host cells, thus leading to cell death (O’Brien et al., 1992). Transmission of STEC to humans from animal reservoirs typically occurs by fecal contamination of food or water, direct or indirect contact with animals, or by person-to-person contact (Willshaw et al., 2001). Cattle are generally considered to be the principal reservoir of STEC as well as E. coli O157 (Bettelheim et al., 2003). Other animals such as sheep, deer, horses, dogs, and birds are recognized as a major risk factor.

The predominant STEC serotype that is consistently associated with human infection and death is O157:H7 (Gansheroff and O’Brien, 2000; Grauke et al., 2002). STEC emerged as human pathogens in the USA for the first time in the early 1980s during large-scale outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) caused by E. coli O157:H7. Ever since, this strain has been epidemiologically and clinically important worldwide. In addition, a wide portion of serotypes other than O157 STEC such as 026:H11, 048:H21, 0103:H2, 0111:NM, and 0145:NM (Panutdaporna et al., 2004), are also an emerging threat to human and animal health. Thus, this study attempted to isolate STEC or E. coli O157:H7 strains from selected animal and environmental source samples in Sarawak.
In recent years, there has been increasing concern of the side effect of the use of antibiotics which contribute to the possible emergence, selection and dissemination of resistant Enterobacteriaceae, especially \textit{E. coli} strains to antimicrobial agents used in animal husbandry and for human prescription (Witte, 1998; van de Bogaard and Stobberingh, 2000). A wide spectrum of \textit{E. coli} antimicrobial susceptibility patterns was reported and \textit{E. coli} strains from humans, chickens, and dairy cows have higher resistance indices than strains obtained from wild animals (Dombek \textit{et al.}, 2000). The development of antibiotic resistance in human pathogens has been closely associated with the use of antibiotics for therapy, diseases prevention, growth promotion, and control of diseases in the animals in modern production systems (Philips \textit{et al.}, 2004). These conditions assist the spread and persistence of antimicrobial resistant pathogens, including \textit{E. coli} O157:H7 which poses a public health threat, due to the higher risk of treatment failures in human (Zhao \textit{et al.}, 2001). Thus, to safeguard public health, the selection and spreading of resistant bacteria from animals and other environment sources should be examined. There is a need to identify the antibiotic resistance profile of \textit{E. coli} isolates and to relate them with their source samples, as well as to investigate whether wild environmental sources are more susceptible to antibiotics agents when compared to domestic samples. Therefore in the present study, the antimicrobial characteristics of \textit{E. coli} from these samples were assayed.

Studies concerning \textit{E. coli} have formed the empirical basis of our understanding of the genetic profiles of bacterial populations. Assumption is made where the genetic variation of \textit{E. coli} strains from environmental sources might be possible due to
geographical isolation or animal host-specific (Gordon, 2001). Numerous studies have suggested that horizontal DNA transfer, inversions, translocations, and point mutations could have caused some of the genotypic variation in *E. coli* strains (Nikolich et al., 1994; Netherwood et al., 1999). Therefore, the genetic diversity and correlation of *E. coli* strains from environmental sources in Sarawak should be studied. Investigation should be carried out to determine whether the genetic diversities of *E. coli* isolates are randomly distributed or are more assigned by animal host. Knowledge of the genotypic diversity of *E. coli* strains from wildlife samples is lacking (Souza et al., 1999). Thus, this study assessed the genetic diversity of *E. coli* from bat and rodent samples and compared with chicken and swine samples. The study on the genetic profiles of this bacterium in their animal host as well as their external environments is also of importance. This is to understand whether clonal relationships of the bacterium do occur between these two distinct habitats in the transmission from the host to the external environments. If they share similar genetic composition, the samples from animal’s secondary habitats (such as feces and soils in the vicinity) could be used to represent the animal host when doing source-tracking of fecal contamination from river water or other environmental sources.

In recent years, the application of PCR-based techniques such as enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), pulsed-field gel electrophoresis (PFGE), and random amplification of polymorphic DNA-PCR (RAPD-PCR) have been increasingly used in the detection and characterization of *E. coli* genotypic profiles (Tenover et al., 1995). ERIC-PCR had been used for genetic characterization of *E. coli* populations from host sources of fecal pollution (McLellan et al., 2003; Panutdaporna et