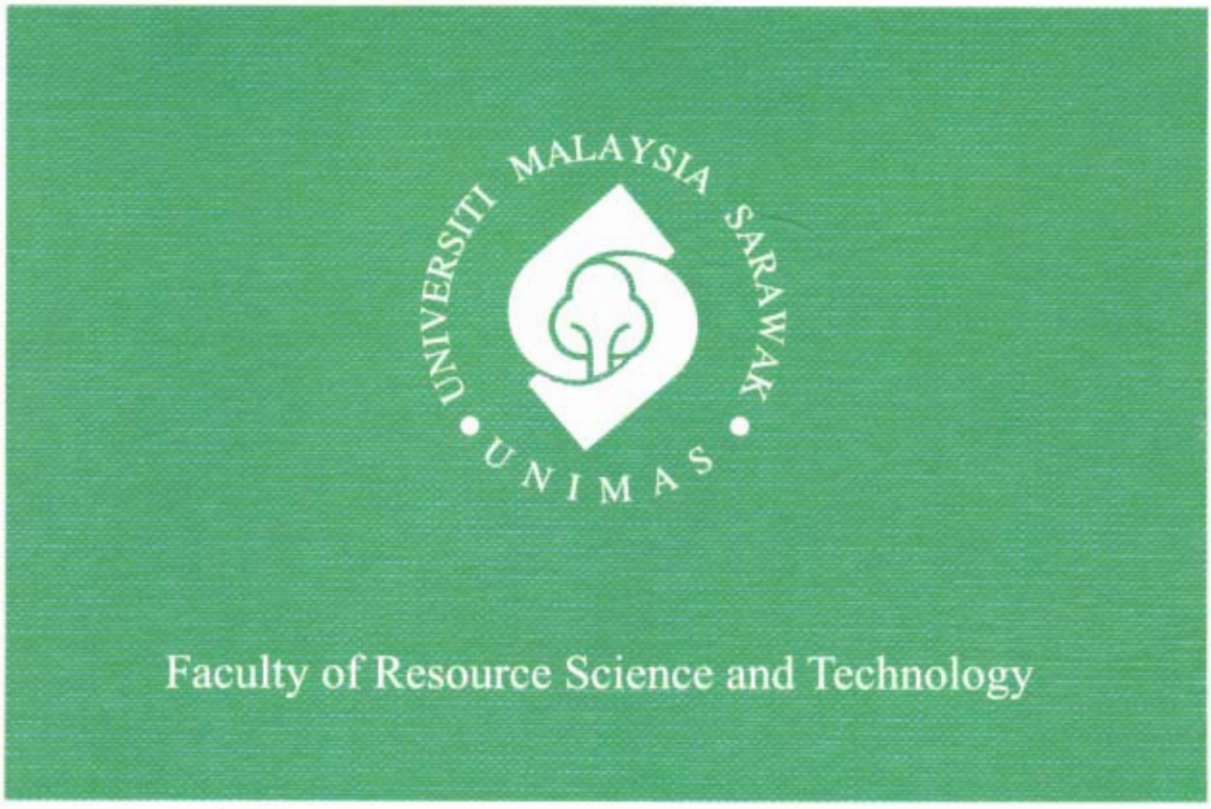

GENETIC DIVERSITY OF ANTIBIOTIC RESISTANT *ESCHERICHIA COLI* FROM CULTURED CATFISH (*CLARIAS BATRACHUS*) AND THE ENVIRONMENT

Marian Madonna Mobilik (18950)

Bachelor of Science with Honours
(Resource Biotechnology)
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Genetic Diversity of Antibiotic Resistant *Escherichia coli* from Cultured Catfish (*Clarias batrachus*) and the Environment

Marian Madonna Mobilik (18950)

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: Dr. Samuel Lihan
Co-Supervisor: Dr. Lesley Maurice Bilung

Resource Biotechnology Program
Department of Molecular Biology

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2010

I dedicate this work to my parents, Julius Mobilik and Mariana Norman and to my whole family who have given trust and have supported me in my education and to Eddy Jamilih for giving the strength and faith for me to believe in myself. I would not make it through if it was not because of friends who have dearly journeyed together.

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Marian Madonna Mobilik

Kuching

5 May 2010

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LIST OF ABBREVIATIONS

CDC	-Centre for Disease Control and Prevention
DNA	-Deoxyribonucleic acid
E. coli	-*Escherichia coli*
EC	-*Escherichia coli*
EDTA	- Ethylenediaminetetraacetic acid
EHEC	-Enterohemorrhagic *E. coli*
EMBA	- Eosine Methylene Blue Agar
EPEC	-Enteropathogenic *E. coli*
ETEC	-Enterotoxigenic *E. coli*
EtOH	- Ethyl alcohol
IMViC test	-Indole, Methyl red, Voges-Proskauer, Citrate test
MHA	-Muellër Hinton Agar
NA	- Nutrient Agar
NCCLS	-National Committee for Clinical Laboratory Standards
OD	-optical density
PCR	-Polymerase Chain Reaction
RAPD-PCR	-Random Amplified Polymorphic DNA - PCR
TBE	- Tris/Borate/EDTA
TSA	- Typticase Soy Agar
TSB	- Tryptic Soy Broth
UV	-Ultraviolet
FAO	- Food and Agriculture Organization
UNO	- United Nations Organization

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Marian Madonna Mobilik
(18950)

Resource Biotechnology Programme
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Human gastrointestinal illness caused by *E. coli* has been known to occur due to the presence of shiga toxin and verotoxin gene carried by this bacteria. This food borne pathogen contaminates food through improper handling of food and also caused by poor practice of hygiene. However, the spread of this bacterium is reaching the point in which it inhabits in our food source, fish. Fish (catfish) grown in fish farms are treated with supplemented feeds to enhance its growth and to avoid the fishes from being infected by disease. However, the undigested feed pollutes the environment and caused the *E. coli* to acquire genetic alteration that may be harmful to human. Samples of fish (catfish), water and sediement were collected from fish farms around Kuching area and were tested through a series of biochemical test, antibiotic resistance test, and specific PCR and RAPD-PCR. There were 12 isolates resistant towards at least one type of antibiotic tested. The highest percentage of resistance is towards ampicilin (23.5%), while the lowest percentage of resistance is towards nitfurantoin (11.8%). No *E. coli* strains were resistant towards norfloxacin, sulphametoxazole / trimethroprim and chloramphenicol. The PCR analysis revealed that 4 and 6 *E. coli* strains possessed shiga toxin gene and verotoxin gene, respectively. The RAPD analysis showed that 9 RAPD fingerprinting profile were detected with the use of

primer GEN_1_50_09, one RAPD fingerprinting profile with the use of primer GEN_1_50_10 and 12 RAPD fingerprinting profiles with the use of combined primers (GEN_1_50_09 and GEN_1_50_10). This proves that the use of combined primers have higher power of discrimination. The results of this study suggested that the *E. coli* strains have multiple resistances towards commonly used antibiotics and these strains are genetically diverse. The occurrence of antibiotic resistance and shiga toxin and verotoxin possession among the *E. coli* may pose potential hazard to human health.

Keywords: *E. coli*, antibiotic resistance, RAPD-PCR

Pengagihan Genetik terhadap Ketahanan Antibiotik *Escherichia coli* daripada Kultur Ikan Keli (*Clarias batrachus*) dan Persekitarannya

Marian Madonna Mobilik
(18950)

Program Bioteknologi Sumber
Fakulti Sains dan Teknologi Sumber
Universiti Malaysia Sarawak

ABSTRAK

Penyakit berkaitan perut dan usus manusia yang disebabkan oleh *E. coli* adalah kerana ia memiliki gen untuk penghasilan toksin shiga dan toksin vero. Patogen yang disebarkan melalui makanan ini berlaku dengan cara pengendalian makanan yang salah dan tidak mengamalkan kebersihan semasa menyediakan makanan. Penyebaran bakteria ini kini diketahui mendiami salah satu punca makanan manusia iaitu ikan. Ikan keli yang dipelihara dan dikomersilkan di dalam kolam telah diberi makanan tambahan untuk mempercepatkan tumbesaran ikan dan meningkatkan ketahanan ikan tersebut terhadap serangan penyakit. Sisa makanan yang tidak dicerna mencemarkan habitat ikan tersebut dan menyebabkan *E. coli* dalam habitat itu mengalami perubahan gen yang membahayakan kepada manusia. Sampel ikan keli, air dan mendapan telah diambil daripada kawasan penternakan ikan keli di sekitar Kuching dan diuji melalui beberapa peringkat ujian biokimia, ujian daya tahan antibiotic dan PCR. Selain itu, sebanyak 12 pencilan *E. coli* menunjukkan daya tahan terhadap sekurang-kurangnya satu antibiotik. Peratus ketahan yang tertinggi adalah terhadap ampicilin (23.5%), manakala peratus ketahan yang paling rendah adalah terhadap nitrofurantoin (11.8%). Semua pencilan tidak mempunyai ketahan terhadap norfloxacin, sulphametoxazole / trimethroprim and chloramphenicol Sebanyak 4 dan 6 pencilan *E. coli* masing-masing didapati memiliki gen toksin

shiga dan gen toksin vero. Kaedah profil dan amplifikasi DNA polimorfik secara rawak (RAPD-PCR) telah digunakan untuk menganalisis perbezaan genetik 17 pencilan *E. coli* yang dipencilkan daripada kolam ternakan ikan keli. Dalam kalangan pencilan tersebut, terdapat 9 profil RAPD daripada pencetus GEN_1_50_09, 1 profil RAPD daripada pencetus GEN_1_50_10 dan 12 profil RAPD daripada gabungan dua pencetus (GEN_1_50_09 dan GEN_1_50_10). Ini menunjukkan bahawa penggunaan gabungan pencetus ternyata lebih sensitif. Hasil kajian ini mencadangkan bahawa pencilan *E. coli* mempunyai ketahanan terhadap beberapa antibiotik yang biasanya digunakan dalam bidang perubatan dan pencilan ini juga mempunyai kepelbagaian genetik. Ketahanan terhadap antibiotik dan kehadiran gen toksin shiga dan toksin vero di dalam pencilan *E. coli* menunjukkan bahawa ia mempunyai potensi unutk membahayakan kesihatan manusia.

Kata kunci: *E. coli*, ketahanan antibiotik, RAPD-PCR

CHAPTER 1

1.0 INTRODUCTION

Escherichia coli (*E. coli*) is a gram-negative, non-spore-forming, rod-shaped bacterium. It is also a facultative anaerobe that can be found in the large intestines of most warm-blooded animals (Leclerc *et. al.*, 2001). Although most strain are harmless, others can cause serious food poisoning, urinary tract infection, respiratory illness, and pneumonia (Weinstein *et. al.*, 1988). Food borne pathogen increases the risk of human consuming harmful toxins. The resident strain that lives in the human intestine is not dangerous but perturbs if there is an enteric infection (Nataro and Kaper, 1998). What differentiates the resident strains from other of its serotype is the virulence characteristic. Study shows that strains that causes food poisoning and other illness produces toxin that has an irreversible cytopathic effect on cultured vero cells (Grant, 2004). Antibiotics are widely used in aquaculture for therapeutic purposes and as prophylactic agents. However, the use of antibiotics will cause environmental organism, endogenous bacterial population of farmed species and pathogens to have transfer of resistance which jeopardizes human and livestock (FAO, 2006). It was recognized that the transfer of resistant microorganism from animals to human is through food chain (European Food Safety Authority, 2008). Therefore, if microorganism that infects human is readily resistant to the present medication, new drug must be discovered to overcome it (Sorum and Sunde, 2001).

1.1 OBJECTIVES

The objective of this study is to isolate *E. coli* from the intestine of catfish (*Clarias batrachus*), the water and sediment of the production pond and to characterize the isolates by antimicrobial susceptibility testing, Shiga toxin detection, and genetic diversity analysis.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Aquaculture

According to De Silva and Anderson (1995), the FAO of the UNO explains aquaculture as the farming of aquatic organism that involves certain processes, including the usage of growth hormone to increase production. The rate of production of freshwater fish increased since the introduction of aquaculture. This is supported by De Silva and Anderson in 1995 in their research that reported the quantity of freshwater fish produced from 1985 to 1989 have increased by 15.8% per annum compared to only 5.9% in 1985.

2.2 *E. coli*

E. coli is a commensal organism that lives in mammals and it is usually isolated from the mammalian feces or large intestine (Russell *et. al.*, 2000). At some degree, it produces vitamin K for the host that makes it a symbiont (Ingledew and Poole, 1984). Certain strains are harmless but other strain may cause fever in infants and food poisoning in adults (Koch, 1995). Hence, this made *E. coli* a pathogen but an opportunistic one (Koch, 1995). Other than that, *E. coli* is a suitable model organism because it can multiply rapidly and its multiplication can be controlled in culture plates (Kaper, 2005). Diarrhoeagenic *E. coli* pose a worldwide threat to human health. The detection of enterotoxigenic strains of this bacterium usually requires biochemical or immunological testing of isolated strains, which are obtained by cultivation from food or environmental samples. Since most of the genes encoding *E. coli* enterotoxins are located on plasmids and selective enrichment steps may lead to plasmid loss, such detection strategies can yield false negative results (Dreyfus *et al.* 1983; Candrian *et* al. 1991a). A protocol based on

PCR that allows the detection of enterotoxigenic *E. coli* with the genetic potential for production of heat-labile toxin type I (LTI) in minced meat has been described by Wernars *et al.* (1991a). However, this procedure requires several cultivation steps and may detect only the LT1 allele, which was originally found in an *E. coli* of porcine origin. In contrast, PCR test that specifically recognizes DNA sequences of the malB operon of *E. coli* (Candrian *et al.* 1991b) was used to develop a PCR-based analysis method without cultivation steps for surface water and soft cheese. The protocol was applied to a second PCR test specific for both known alleles of LTI enterotoxigenic *E. coli* (Furrer *et al.* 1990) to determine the presence of such strains in soft cheese samples containing high levels of *E. coli*.

2.3 Antibiotic Resistant

Resistance of bacteria towards antibiotics is caused by prolong use of the antibiotics for a certain period of time which causes the bacteria to acquire resistance through natural selection or by evolutionary stress when antibiotics are given to a particular population. Many bacteria transfer the resistance capability via horizontal gene transfer: conjugation, transformation and transduction. Previous study on antibiotic resistance shows that genes that carry gene for resistance are usually found in the plasmid (circular DNA molecule that is separated from genomic DNA). Some bacteria carries resistance towards several different antibiotics which are termed bacteria with multiple resistance. This is one of the basic factors that small organisms like bacteria and virus do to continue to exist and multiply. The rate of evolution in bacteria is very fast which is one of the reasons a person can be infected with flu several times a year: same virus but different strain. It is a global issue in which emergence of antibiotic resistance not only

contribute to public health risk and increase in treatment cost but worst, it results in morbidity and motility due to treatment failure.

There are three methods for antibiotic susceptibility test: diffusion, dilution as well as diffusion and dilution method. For the purpose of this study, Kirby-Bauer disk diffusion method was used in the antibiotic susceptibility test. In this method, small disk containing a known amount of antibiotic is placed on an agar plate. After incubation, zone of inhibition can be seen depending on the level of resistance towards the antibiotics. The zone of inhibition is a clear area around the disc which the bacteria are incapable to grow. Each agar plate contains a single type of bacteria and is tested using six different types of antibiotic discs. The type of antibiotic used in this study was selected based on the possibility of antibiotic used in fish farming.

E. coli is one of the bacterium that exists naturally in the large intestine of human and other animals which facilitates in suppressing the growth of other harmful bacteria and also aids in the synthesis of some vitamins. The presence of *E. coli* in food indicates that the food is contaminated with other harmful bacteria such as *Salmonella* and Hepititis A virus. Alteration of gene in *E. coli* causes these bacteria to acquire similar toxin produced by *Shigella dysenteriea* known as Verotoxin and Shiga toxin that causes damage in the lining of the intestine which results in diarrhea and urinary tract infection.

Feeds given to fishes may be technologically derived from chemical or other harmful substances that are used in order to have cheap feeding cost. The feeds that were used contained anti nutrients that could affect not only the health of fishes but also the health of consumers and the

environment (Francis *et. al.*, 2001) by having the natural microflora in fish intestine to be resistant towards antibiotic that is being used in the feeds (Sorum and Sunde, 2001). When human consumes the fishes and experience diarrhea (due to the excessive amount of *E. coli*), the current antibiotic will not be able to kill the pathogenic *E. coli* because it is already resistant to it (Martinez, 2009). As a result, sick people cannot be cured by means of medication and new drugs or antibiotic will have to be generated.

2.5 PCR

PCR technology has been applied to detect a wide range of micro-organisms in a variety of situations and although it does not appear to be feasible to apply the technology directly with some types of sample, it does offer the possibility to reduce the time taken to obtain a result (Fricker and Fricker, 1994). There has been considerable interest in the application of PCR technology to the detection of bacteria (Bej *et. al.*, 1991), protozoan cysts and viruses in water. This is particularly true for detection of pathogens in water where the procedures are often laborious, time-consuming and incentives. However, the water industry relies to a large extent on the use of indicator organism to monitor water quality and much of the microbiology effort required is concentrated in this area. In particular, *E. coli* and the coliform group are used to indicate faecal and a lack of system integrity.

2.6 RAPD-PCR

One way to determine the similarities or differences in strains that are being isolated in a study is to use RAPD-PCR. RAPD markers are a modification of PCR contrived in the late 1980's (Wiliams *et al.*, 1990). The technique is one of the best available DNA-based tools for scoring

variations between cultivars within species (Lakshmikumaran and Bhatia, 1998). This is done by using arbitrary primers to detect regions in the genomic DNA that are complimentary to it. Hence, the genetic diversity can be identified to in a certain population. Identifying the genetic diversity is an important key in analyzing specific bacteria because one type of bacteria may have a variety number of strain. These different strains, although the same type of bacteria, have different characteristics. One strain can be beneficial to other strains or may be more hazardous. The factor that differ strains of the same type of bacteria may be as simple as a sequence of gene that codes for protein or the genetic make up of the plasmid in which a strain carries. For example, different *E. coli* strain has different degree of pathogenicity to humans and other animals. Some of it may even be beneficial to human such as the *E. coli* strain that inhabits the large intestine of humans and some mammals but there is also strain that is hazardous to human health. Thus, RAPD-PCR is a good molecular typing for distinguishing genomic diversity and this has been frequently used in bacterial study to discriminate bacteria into genus, species and strain. Some arbitrary primers can distinguish different strains effectively; others may not even present any bands. Hence, optimization is required in order to get result that can be analyzed.

This can be done by using the randomly designed primer to detect Usage of certain primers will result in the formation of a variety of bands that can tell the similarities or differences in several bacterial strain. One probable disadvantage, however, is the degree of reproducibility of these markers which can sometimes be low (e.g., Muralidharan and Wakeland, 1993; Ellsworth *et al.*, 1993; Skroch and Nienhuis, 1995). This is due to the RAPD banding patterns which is highly sensitive to reaction conditions. A slight change in PCR conditions will result in a different band result. Other than that, one may face difficulty in exactly replicating reaction conditions across laboratories, where different brands of thermocyclers may be used.

Despite of all of the disadvantages, RAPD-PCR result can be optimized by adjusting the PCR conditions. The process require much cheaper cost compared to other method, it requires minimal labour and does not involve hazardous chemicals.

2.7 Catfish (*Clarias batrachus*)

Clarias batrachus or also known as the walking catfish is widely distributed in Asia and it lives in muddy and marshy waters (Garg *et. al.*, 1995). Some people regard this walking catfish as a delicacy but others regards this as invaders to aquaculture farms that feed on culture fish. This fish has been described as a benthic, nocturnal, tactile omnivore that consumes detritus and opportunistically forages on large aquatic insects, tadpoles, and fish (Smithsonian Marine Station, 2007). It is reared locally in Malaysia for people to cook as a local delicacy.

2.8 Public Health

The pathogenic strains of *E. coli* can cause distinct disease syndrome such as diarrheal diseases, wound infection, meningitis, septicemia, atherosclerosis, hemolytic uremic syndrome and immunological diseases such as reactive and rheumatoid arthritis (Olsvik *et al.*, 1991). The ETEC strains produce one or more of toxins from the heat-labile and the heat-stable enterotoxin families (Levine *et al.*, 1985). These strains possess specific adhesion fimbria for intestinal attachment and colonization (Olsvik *et al.*, 1991). Some EPEC strain produces one or more of the cytotoxins, but adheres also to intestinal cells interfering with the electrolyte transport system (Olsvik *et al.*, 1991). The EHEC strains have been identified and shown to produce one or more of the cytotoxins (vero-cytotoxins, shiga-like toxins) (Olsvik *et al.*, 1991).