Prevalence and Determination of Chloramphenicol-resistant Gene in *Escerichia coli* Isolated From Aquacultural Environment

Mohamad Al Aamin bin Razali
(24007)

A final project report submitted in partial fulfilment of the requirement for the degree of Bachelor of Sciences with Honours.
(Resource Biotechnology)

Supervisor : Dr. Samuel Lihan

Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2012

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List of Abbreviations

EDTA	Ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
MRSA	Methicillin-resistant *Staphylococcus aureus*
CAT	Chloramphenicol acetyltransferases
PCR	Polymerase chain reaction
EMB	Eosin methylene blue
TBE	Tris-Borate/EDTA
mM	milimolar
UV	Ultra-violet

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ABSTRACT

Aquaculture nowadays seems as a promising sector, both in food production and economic intention. However, recently the public health hazards related to antimicrobial use in aquaculture were become a major concem regarding this sector; including the development and spread of antimicrobial resistant bacteria and resistance genes, and the occurrence of antimicrobial residues in products of aquaculture. Driven by above factor, the main intention of this study is to determine the prevalence of antiobiotic-resistance gene selected bacterial strains from aquaculture environment. *Escherichia coli* isolates from aquaculture origin were grown on selective media, EMB agar. These isolatess were screened for antibiotic resistance by antibiotic susceptibility test using chloramphenicol (30 μg) disk and the respective chloramphenicol-resistance gene in the isolates then were further detected by multiplex PCR, using primers C-1, C-2, C-3, C-4 and C-R for *cat* I, *cat* II, *cat* III, and *cat* IV genes respectively in a single reaction mixture. From all 19 isolates, 7 were resistance to chloramphenicol, and only 4 isolates indicates the presences of *cat* gene during agarose gel electrophoreses, which is *cat* II gene. The presence of *cat* gene in this study indicates the potential chloramphenicol antimicrobial resistance hazard to consumers and aqualculture workers. Further studied should be carried out to determine the origin and the resistance mechanism of *cat* gene in the studied isolates.

Keywords: resistance gene, aquaculture, multiplex PCR, *E. coli*, chloramphenicol

ABSTRAK

Akuakultur pada masa kini dilihat sebagai sektor yang memberangsangkan, sama ada dalam sektor pengeluaran makanan mahupun dengan tujuan ekonomi. Walaubagaimanapun, kebelakangan ini ancaman bahaya kesihatan awam yang berkaitan dengan penggunaan antimikrobial dalam akuakultur telah menjadi perhatian khusus, termasuk pembangunan dan penyebaran bakteria dan gen kalis antimikrobial, didorong terdapatnya sisa penggunaan antimikrobial yang dikesan dalam produk akuakultur. Didorong oleh faktor di atas, tujuan utama kajian ini dijalankan adalah untuk menentukan kadar kelaziman strain yang mengandungi gen kalis antiobiotik daripada bakteria yang diperolehi dari persekitaran akuakultur persekitaran. E. coli yang diperoleh daripada persekitaran akuakultur telah dibiakkan pada media selektif, iaitu agar EMB. Kesemua pencilan ini telah diskrin untuk menguji tahap kerintangan terhadap antibiotik melalui ujian kecenderungan antibiotik menggunakan disk chloramphenicol (30 μg) dan gen kalis chloramphenicol masing-masing kemudiannya dikesan oleh PCR multipleks, menggunakan primer C-1, C-2, C-3, C-4 dan C-R untuk gen cat I, cat II, cat III, dan cat IV. Daripada kesemua 19 pencilan bakteria, 7 daripadanya menunjukkan penentangan terhadap chloramphenicol, dan hanya 4 pencilan bakteria yang menunjukkan kehadiran gen cat melalui proses gel agarosa electrophoreses, iaitu gen cat II. Kehadiran gen cat dalam kajian ini menunjukkan bahawa bahaya rintangan bakteria terhadap chloramphenicol amat berpotensi kepada pengguna dan pekerja aqualkultur, disamping bahaya kesan sampingan chloramphenicol itu sendiri. Kajian yang lebih terperinci selanjutnya perlu dijalankan untuk menentukan origin dan mekanisme rintangan gen cat dalam pencilan yang dikaji.

Kata kunci: gen kalis mikrobial, akuakultur, PCR multiplex, E. coli, chloramphenicol

1.0 Introduction

Aquaculture is the production of aquatic organisms in an artificial system. This system has been in practice for over several centuries in some Asian countries. In recent years; our country also saw the aquaculture as a promising sector, both in food production and economic intention. However, public health hazards related to antimicrobial use in aquaculture were become a major concern regarding this sector; including the development and spread of antimicrobial resistant bacteria and resistance genes, and the occurrence of antimicrobial residues in products of aquaculture (FAO/OIE/WHO, 2006). This resistance can disseminate through the environment and can be transmitted to a variety of bacterial species, including bacteria that can infect humans as such as bacteria that present in aquaculture settings may be transmitted to humans who come in contact with this ecosystem (Shariff, 2006). Some antimicrobial residues also were recognized as causal of disease, e.g., the chloramphenicol that is use in veterinary was suspected carcinogenic to human. In the World Aquaculture Society meeting in 2005, following task should be done, which is developing standard operating procedures for the sampling of chloramphenicol resistant bacteria from diverse geographical and aquaculture backgrounds in Malaysia, Thailand and Vietnam, collecting descriptive and taxonomic data for each antibiotic-resistant bacteria isolate and enter into a project database, assessing the antibiotic susceptibility of each bacterial isolate, evaluating the genetic relationships among the strain collection by applying genotypic techniques (Chinabut *et al.*, 2005).

E. coli encompass an enormous population of bacteria that exhibit a very high degree of both genetic and phenotypic diversity. Genome sequencing of many isolates of *E. coli* and related bacteria shows that a taxonomic reclassification would be desirable, but this has

not been done, largely due to its medical importance (Krieg and Holt, 1984). Furthermore, *E. coli* remain one of the most diverse bacterial species: only 20% of the genome is common to all strains (Lukjancenko, Wassenaar and Ussery, 2010). Different strains of *E. coli* are often host-specific, making it possible to determine the source of fecal contamination in environmental samples (Feng, Weagant and Grant, 2002). These differences may result in changes to the physiology or life cycle of the bacterium, which is a strain may gain pathogenic capacity, the ability to utilize a different carbon source, the ability to survive in a particular ecological niche or the ability to resist antimicrobial agents.

Chloramphenicol found to be effective against typhus in 1948 and became the first antibiotic to undergo large-scale production (NTP, 2000). However, due to resistance and safety concerns; it is no longer a first-line agent for any indication in developed nations, although it is sometimes used specifically for remote cases. The microbial chloramphenicol resistance is mainly caused by drug inactivation mediated by chloramphenicol acetyltransferases (CAT) enzyme encoded by chloramphenicol-resistant genes, *cat,* of the microbes.

Due to these various concerns, including the urge to investigate the existence of antimicrobial residues in local aquaculture, the Faculty of Resource Science and Technology (FRST) of Universiti Malaysia Sarawak (UNIMAS) took the initiative to work out research on chloramphenicol-resistant gene from *E. coli* isolated from aquaculture environment. Thus, the objective of this study is to investigate the chloramphenicol resistance profile and to determine the prevalence of chloramphenicol-resistance gene in the *E. coli* isolated from local aquacultures.

2.0 Literature Review

2.1 Chloramphenicol

Chloramphenicol was first isolated from *Streptomyces venezuelae* in 1947 by David Gottlieb. It is bacteriostatic; functions by inhibiting bacterial protein synthesis thus has a very broad spectrum of activity: it is active against Gram-positive bacteria (including most strains of MRSA), Gram-negative bacteria and anaerobes (Neu and Gootz, 1996). The original indication of chloramphenicol was in the treatment of typhoid, but now, almost-universal presence of multiple drug-resistant *Salmonella typhi* has meant it is seldom used for this indication except when the organism is known to be sensitive. Chloramphenicol may be used as a second-line agent in the treatment of tetracycline-resistant cholera. There is sufficient evidence of carcinogenicity of chloramphenicol from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer. Numerous case reports have shown leukemia to occur following chloramphenicol-induced aplastic anemia (IARC, 1990).

2.2 Chloramphenicol-resistant

The resistance to chloramphenicol is familiar in bacterial strains of veterinary origin and the basic of resistance's mechanism is due to the production of chloramphenicol acetyltransferases (CAT) enzyme, encoded by chloramphenicol bacterial plasmids (Gaffney, Foster & Shaw, 1978; Shaw, 1984; Shaw & Leslie, 1989). In accord to Roberts *et al.*,(1982), there are several types of *cat* genes with different nucleotide sequences, and the resulting CAT enzymes have different structures. In earlier study by Zaidenzaig and Shaw (1976) using enzymologic, biochemical and immunological methods, have discovered that

the CATs produced by the R plasmid in Gram-negative bacteria were classified into enzyme types I, II, and III. In later study discovery, CATs produced by R plasmids in Gram-negative bacteria are encoded by 4 types of resistance genes, *cat* I, *cat* II, *cat* III and *cat* IV (Aoki, 2000).

2.3 Aquaculture in Malaysia

After 1997 world financial crisis, Malaysia made a revised on its agricultural food production to ensure the sector's contribution to the national economy and its global competitiveness. Due to this, increase in production and contribution was set to be from aquaculture sector which is currently seem as not fully utilized. In local market, the main commodities singled out from aquaculture food production are; marine shrimp, marine fish, fresh water fish, cockle and sea weed. This is because it is a relatively cheap source of animal protein and due to that average Malaysian consumes more fish than other animal protein. Ibrahim *et al.* (2010) concluded that beside brackish water fish, freshwater fish are also the main aquaculture products in Malaysia especially for the red tilapia fish (*Oreochromis* sp.), patin (*Pangasius* sp.) and keli (*Clarius* sp.). Currently, the main production system for marine fish is still floating net-cages while the pond production is given a less consideration. Despite the volume it can produce, pond production may not be suitable for high value fish species which demand water of higher salinity, while the pond located inland. The government allocated RM252 million to develop the Integrated Aquaculture Zone in Batang Ai and Tanjung Manis in Sarawak, as well as Pitas, Sungai Telaga and Sungai Padas in Sabah, during the tabling of the 2011 budget, and such investment surely need a wise management. Due to this, the issues on production

sustainability, employment of improve technology, concept of eco-friendly, food safety regulations should be taken seriously.

2.4 Antibiotic in aquaculture

Antibiotic in a broader sense is a chemotherapeutics agent that capable of inhibit or abolishes the growth of microorganisms such as bacteria, fungi and protozoa (Kummerer, 2009). Antibiotic has been used extensively in human, veterinary medicine, agriculture and aquaculture business and the use has steadily increased especially in the developing countries (Kumar *et al.,* 2009). Nowadays, antimicrobial resistance is a growing public health threat and has been designated by the WHO as an emerging public health problem (Chai *et al.,* 2008). According to Roque *et al.* (2001), the most common way in Mexico to resolve the Vibriosis problem is by the use of feed plus antibiotics in shrimp's aquaculture freshwater farms or directly applied to the water. In Malaysia, antibiotic and other chemotherapeutics agents and also pesticides were commonly used in fish farms either as a feed additives or immersion baths to achieve either prophylaxis or therapy, also as a common practices to avoid the overgrowth of herbal plants and fish diseases beside promoting the fast growth of the fish (Majusha *et al.*, 2005 and Ibrahim *et al.,* 2010). Ibrahim *et al.* (2010) also reported that the chemical residue from the antibiotics or pesticides used at the farming activity can be accumulated in fish and could cause a chronic health effects to consumers and potentially to cause certain organ or system malfunction such as cancer, nerve problems and immunological problems in human.

2.5 Polymerase Chain Reaction of targeted gene

The choice of target genes and the design of oligonucleotide primers are critical elements in determining the sensitivity of PCR. However, in accord to He *et al.* (1994), even when the same gene is selected as a target, PCR with different primer sets shows a 100 to 1 000-fold sensitivity difference between primer sets. Therefore, the sequence of primers is important in the sensitivity and specificity of PCR. The sensitivity of PCR is also dependent on the target gene selected, because copy numbers of genes might be different. Due to this, if only the sensitivity of PCR is considered, reverse transcription-PCR is another selection method due to the multiple copy numbers of mRNAs per bacterium. However, the practical value of reverse transcription-PCR in diagnosis is limited due to the short life span and the fragility of bacterial mRNAs. In PCR of target gene, a frequently encountered problem in amplification of the sequences is the appearance of smaller bands in the product spectrum (Don *et al.*, 1991). This is usually interpreted to be due to mispriming by one or both of the oligonucleotide amplimers to the target template.

2.7 Multiplex Polymerase Chain Reaction

Multiplex PCR is defined as the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture, to produce amplicons of varying sizes that are specific to different DNA sequences. Since 1988, PCR multiplexing has been applied in diverse areas, regarding the DNA testing (Chamberlain *et al.*, 1988). Furthermore, the huge availability of genetic information due to the publishing of the sequence of the human genome makes the demand for multiplex PCR even greater in recent years. As an example, more than 1.4 million single nucleotide

polymorphisms (SNPs) have been identified in the human genome (Venter, 2001). By targeting multiple genes at once (single reaction mixture), additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform. Basically, annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes should be different enough to form significant bands when visualized by gel electrophoresis.

3.0 Materials and Methods

3.1 Preparation of working stock culture

In this study, 19 samples of *E. coli* were obtained from glycerol stock cultured; isolated from aquaculture environment by FRST, UNIMAS in the previous study. Firstly, one loopful of inoculums from the glycerol stock was streaked onto EMB agar to obtain the single colony of *E. coli* strain. Next, after incubation for approximately 48 hours, due to the selective behavior of EMB agar, metallic green-sheen colony were formed indicates the growth of *E. coli* colony. Subsequently, a loop of inoculums were obtained from a single colony and were streaked onto slant agar, then were stored in refrigerator at -20°C for storage. This procedure was applied to all of the 19 isolates.

3.2 Antibiotic susceptibility test

This method was carried out acording to the laboratory standards guideline by CLSI (2009). Prior to this method, bacterial inoculums from the slant stock culture were allowed to grow in LB broth for overnight. After the LB broth with the bacterial culture turned cloudy, then it were spread throughout the MHA using the sterile cotton swab. All of the *E. coli* isolates were tested against chloramphenicol antibiotic, by using a commercially prepared, 30 µg antibiotic disks (Oxoid, England), placed on the center of each inoculated MHA surface. The plates were incubated for 18 to 20 hours at 27°C temperature prior to determination of results. The zones of growth inhibition around each of the antibiotic disks (diameter) were measured to the nearest millimeter.

3.3 Boiling-Centrifugation DNA Extraction Method

The DNA extraction procedure was carried out according to Soumet *et al.* (1994), with few modifications. Briefly, the single colony from each isolates was inoculated into LB broth and was allowed to grow in the incubator. Then, 1500 µL of the culture were transferred into eppendorf tubes and were centrifuged at 10, 000 rpm for 5 min, and this procedure was repeated twice, to maximize the bacterial numbers in the pellet. Soon, supernatant were discarded and the pellet were resuspended in 500 µL of ddH_2O, the sample were heated in boiling water (approximately 100°C) for 10 minutes. Next, the bacterial solutions were cooled in ice for 5 minutes, and were centrifuged at 10, 000 rpm for 10 minutes. Subsequently, the supernatants were carefully collected into a new eppendorf tubes as a DNA samples. Finally, these DNA samples were stored in the freezer at -20°C.

3.4 Multiplex PCR amplification

The method for multiplex polymerase chain reaction was conducted according to Yoo *et al.* (2003) with slightly modification by referring to method by Dang *et al.* (2008). Four sense primers that were used in this PCR were specifically designed: C-1, C-2, C-3, and C-4, which were complementary to the specific regions of the respective *cat* I, *cat* II, *cat* III, and *cat* IV genes. One antisense primer, C-R that was derived from the conserved nucleotide sequence of the four different types of cat gene sequences, was used in this PCR (Table 1). These PCR amplification were carried out in a 25 µL reaction mixture containing the 4 µL of extracted bacterial DNA, 3 µL of $MgCl_2$, 0.8 µL of dNTPs, 1 µL of 1 µM for each primer, 0.3 µL of *Taq* DNA polymerase. The PCR amplification consisted of pre-

denaturing at 94°C, 35 cycles of denaturing, annealing, elongation processes, and lastly single final extension step at 72°C (Table 2).

Table 1: The primers used in The Multiplex PCR analysis

Primer	Orientation	Base sequence
C-1	Sense	5'-GGTGATATGGGATAGTGTT-3'
C-2	Sense	5'-GATTGACCTGAATACCTGGAA-3'
C-3	Sense	5'-CCATACTCATCCGATATTGA-3'
C-4	Sense	5'-CCGGTAAAGCGAAATTGTAT-3'
C-R	Antisense	5'-CCATCACATACTGCATGATG-3'

Table 2: The PCR programme

Process	Temperature (°C)	Time	cycles
Pre-denaturation	94	4 minutes	1
Denaturation	94	1 minutes	30
Annealing	58	1 minutes	
Elongation	72	45 minutes	
Final extension	72	10 minutes	1

Agarose Gel Electrophoreses

About 5 µL of each amplification products were electrophoresed on 1% agarose gels in 1x TBE buffer, 100 V for 40 minutes. In addition, 5 µL of 100 bp molecular ladder was used as DNA marker on the gel. The gel was pre-stained with 1 µL of Ethidium bromide, and the photograph of electrophoresed gel on UV light viewer was taken using a digital camera.

4. Results

4.1. Phenotypic and prevalence determination of chloramphenicol resistance in *E. coli* isolates

In all of the 19 tested isolates, 7 isolates shows the acceptable limits of inhibitory region diameter which is in range of 12 to 17 mm (CLSI, 2009) for antibiotic resistance determination. These isolates were B7-1-F-2, B7-1-F-3, B7-2-S-BC6, B6-1-F-3, B6-1-W-6, B6-1-W-10, and B5-1-F-4, subsequently. The results of inhibitory region to the respected *E. coli* isolates are shown in the **Table 3**.

Table 3: Susceptibility test result of *E. coli* isolates towards chloramphenicol

Isolates	Diameter of Inhibitory Region (mm)	Susceptibility status
B5-1-S-4	0	Resistant
B5-1-W-5	24	Susceptible
B6-1-W-4	24	Susceptible
B6-1-W-5	18	Susceptible
B6-1-W-6	17	Resistant
B6-1-W-7	26	Susceptible
B6-1-W-8	20	Susceptible
B6-1-W-10	10	Resistant
B6-1-F-1	25	Susceptible
B6-1-F-2	20	Susceptible
B6-1-F-3	15	Resistant
B7-2-S-BC4	21	Susceptible

Isolates	Diameter of Inhibitory Region (mm)	Susceptibility status
B7-2-S-BC6	12	Resistant
B7-1-F-2	14	Resistant
B7-1-F-3	12	Resistant
SB-1-F-1	20	Susceptible
SB-1-W-1	25	Susceptible
SB-1-F-2	21	Susceptible
SB-1-F-3	21	Susceptible

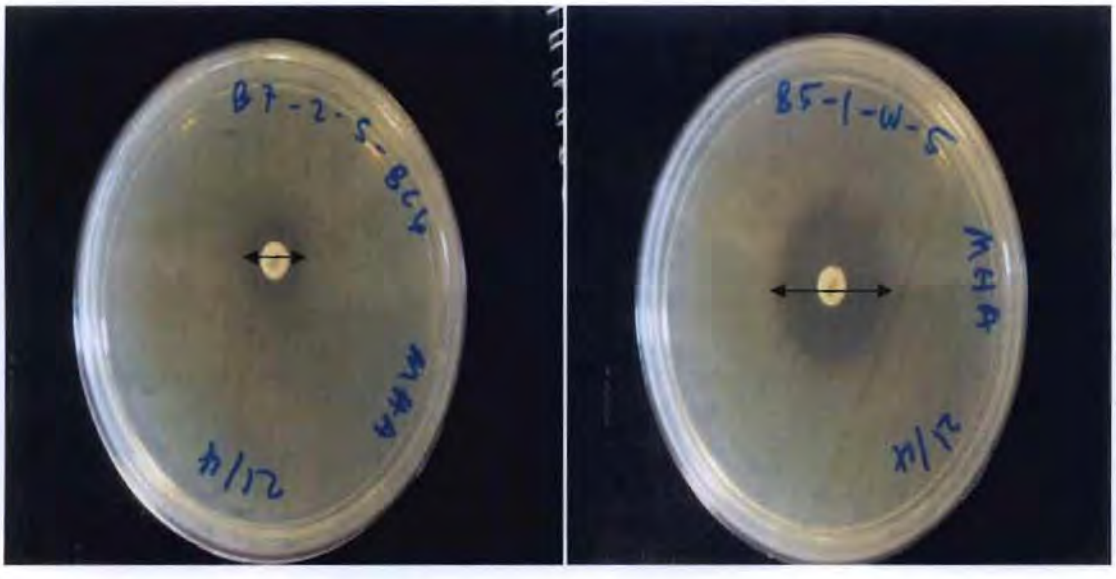

Figure 1: Antibiotic susceptibility test. The comparison of chloramphenicol-resistant isolates (left) and chloramphenicol-susceptible isolates (right), based on the diameter of visible inhibitory ('halo') region circulated around the antibiotic disk.

The prevalence values were defined by percentage, by using the followed formula:

$$\text{Prevalence (\%)} = \frac{\text{the number of occurrence of antibiotic-resistant activity at a given time}}{\text{the number of the total tested samples}} \times 100$$

In this case;

$$\frac{\text{7 of chloramphenicol-resistant isolates}}{\text{19 of total } \textit{E. coli} \text{ isolates}} \times 100$$

Thus, the prevalence value of chloramphenicol-resistant *E. coli* in this study is 36.84 %.

4.2. Molecular detection of chloramphenicol-resistant gene, *cat*

The presence of chloramphenicol acetyltransferase gene, *cat,* were carried out by electrophoresed the PCR products of *E. coli* isolates on 1x agarose gel. Despite 7 isolates positive of chloramphenicol resistance, 4 of them showed the DNA band on position slightly above 500 bp by referring to 100 bp molecular ladder, thus signify the presence of *cat II* gene (Dang *et al.,* 2008). These isolates were B7-2-S-BC6, B7-1-F-3, B5-1-F-4 and B7-1-F-2. The results of the agarose gel electrophoresis of the multiplex PCR products are shown in **Figure 2.**

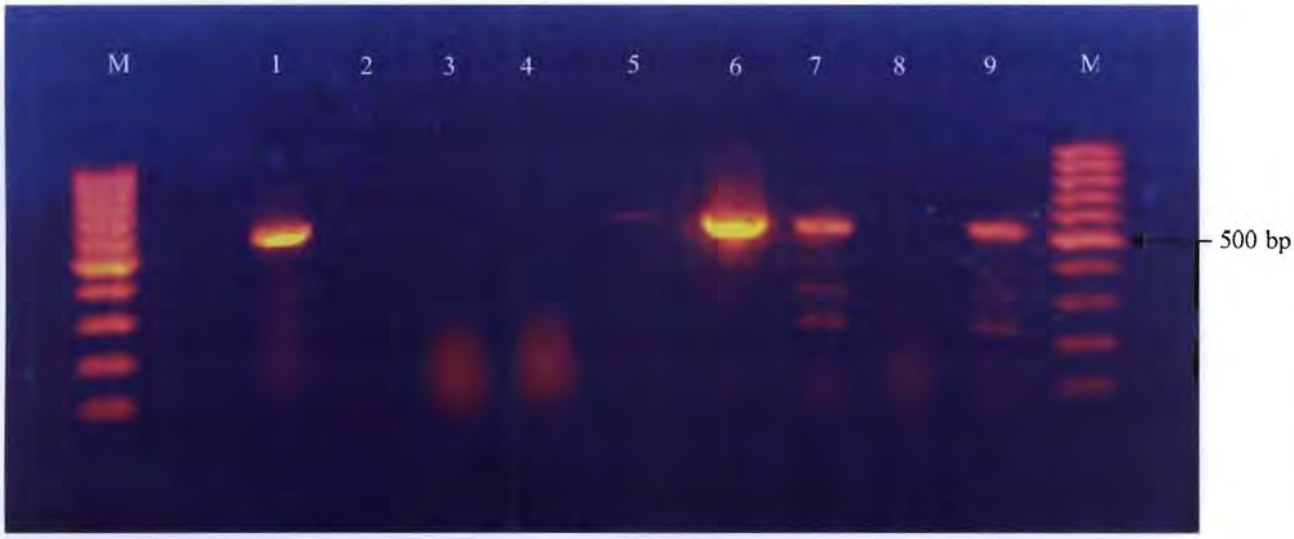

Figure 2: Agarose gel electrophoresis of multiplex PCR products. Lane M, 100 bp marker; lane 1, positive control; lane 2, negative control; lane 3, B7-2-S-BC6, lane 4; B6-1-F3; lane 5, B7-1-F-3; lane 6, B5-1-F-4; lane 7, B7-1-F-2; lane 8, B6-1-W-10; lane 9, B6-1-W-6. The clear band can be seen on 4 lanes at between 500 bp and 600 bp marker, indicating the presence of cat II gene, which is 567 bp in size (Dang *et al.*, 2008).

5.0 Discussion

The frequency of resistance to chloramphenicol among isolated *E. coli* strains in this research were determined, thus, signify the availability of chloramphenicol in the local aquaculture environment, probably to the misuse of this relatively cheap drug by local farmer or aquaculturist, despite its ban for use in food-producing animals in the European Union (EU) and in many other countries, including the USA, Canada, Australia, Japan, and China. Other than directly use to the aquaculture, the antibiotic residues from animal farm entering the aquacultural environment may also result in establishment of selective pressure, in favour of antimicrobial-resistant bacteria. This is the reason *E. coli* were choose as the biological marker for chloramphenicol contamination, because *E. coli* are host-specific, making it possible to determine the source of fecal contamination in environmental samples (Feng, Weagant and Grant, 2002), thus determine the origin of chloramphenicol usage. As being mentioned by Hatha and Lakshmanaperumalsamy (1995) and Miller (1998), even the residues of antibiotics in sediment slurries pose a potential risk to public health or may increase the occurrence of antibiotic-resistant bacteria in the aquatic environment. One of available option to decrease the dissemination of genes for antibiotic resistance is to release the selective pressure for these resistance elements, by means of legislatively prohibiting usage of cheap antibiotics as additives to animal and poultry feed.

In this study as shown by the results obtained, the *E. coli* isolates shows the relatively high frequency of the *cat* II gene in studied local aquacultural sites, which is the main factor of the resistance toward the chloramphenicol. However, 3 isolates (B7-2-S-BC6, B6-1-F3, B6-1-W-10) which were resistance to chloramphenicol as determined by antibiotic susceptibility test, not indicates any significant band on the electrophoresed agarose gel.

This phenomenon might be explained by relating all available recognized factors of chloramphenicol-resistance behavior of the microbes. In the scientific recognition, it is understood that chloramphenicol resistance could be genetically related to two major mechanisms which is, antibiotic inactivation by CAT enzymes produced from groups of *cat* genes, including the gene that has being studied in the research (*cat* I, *cat* II, *cat* III *and cat* IV) and secondly, by alteration in permeability to antibiotics through an efflux mechanism mediated by *cmlA* gene product (Gaffney *et al.* (1978), Burns *et al.* (1986), Bissonnette *et al.* (1991). By referring to these available literatures, it is plausible to infer that the isolates B7-2-S-BC6, B6-1-F3, B6-1-W-10, were actually categorized in the second type of major mechanism, thus not yield the expected results preferably because of different genetic content related to the chloramphenicol-resistance due to different mechanism of resistance. Although the previous findings that the enzymatic chloramphenicol resistance mechanism is more frequent mechanism whereas the efflux resistance gene, *cmlA*, is a less frequent mechanism for chloramphenicol resistance (Burns *et al.* (1986), Bissonnette *et al.* (1991), White *et al.* (2000), Cloeckaert *et al.* (2001), George and Hall (2002), and Al-Agamy (2006)), the chance of non-enzymatic chloramphenicol-resistance occurrence is acceptably possible.

The other available explanation that can be inferred to the phenomenon is related to the position of the studied gene (i.e. *cat* I, *cat* II, *cat* III *and cat* IV) in the genetic makeup of the microbes itself. It is scientifically assured that the bacterial genetic content are resides in two possible places, the chromosomal DNA or plasmid DNA. However, in this study, the DNA extraction method used is optimum for extracting the chromosomal DNA, in spite of plasmid DNA. In 1996, researchers in Japan identified a novel plasmid-encoded gene (*pp-*

flo) from *Photobacterium piscicida* that encoded resistance to both chloramphenicol and florphenicol (White *et al.*, 2000). In addition, the conjugation experiments by Al-Agamy (2006) showed that the most of chloramphenicol-resistance genes were encoded on conjugative plasmids, showing that inferring the position of studied *cat* gene to the plasmid are scientifically permitted.