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Characterization of *Escherichia coli* isolated from cultured catfish by antibiotic resistance and RAPD analysis

[1,*]Samuel, L., [1]Marian, M. M., [1]Apun, K., [1]Lesley, M. B. and [2]Son, R.

[1]*Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia*
[2]*Centre of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

Abstract: Antibiotic susceptibility and genetic diversity of *E. coli* isolated from cultured catfish and their surrounding environment were determined. The levels of resistance of the *E. coli* isolates towards six different antibiotics tested differed considerably. Though the isolates displayed resistance towards some of the antibiotics tested, none of the isolates showed resistant towards norfloxacin, sulphametoxazole/trimethoprim and chloramphenicol. RAPD-PCR analysis using single primer and primers combination clustered the *E. coli* isolates into 3 and 5 groups, respectively. The results of this study suggest that the *E. coli* isolates from the catfish and their surrounding environment derived from a mixture of sensitive and resistant strains with diverse genetic contents. The use of the RAPD analysis is sufficiently discriminatory for the typing of the *E. coli* isolates.

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

Introduction

Escherichia coli has been known as one of the most common bacteria found in the intestinal tract of human and warm blooded animals (Levine, 1987). Their ability to survive outside the body for longer period of time makes them an ideal indicator organism to test food and environmental samples for fecal contamination (Geldrich, 1966; Levine 1987; Lihan *et al*., 1999). Though people generally understand *E. coli* as harmless intestinal flora, they are opportunistic and some of the strains have been identified as the serious causal agents of various illnesses (Levine, 1987). In our local setting, the health hazards associated with *E. coli* have become complicated by the fact that some of the causal agents have over the years, developed resistance against commonly used antibiotics (Son *et al*., 1997a; Son *et al*., 1998a; Son *et al*., 1999; Son *et al*., 2001; Wan *et al*., 2003).

Antibiotics are widely used in livestock, poultry and fish farming as therapeutic and prophylactic purposes. They are used to increase growth and feed efficiencies. They represent an extremely important tool in the efficient production of animal products such as eggs, milk and meat. Antibiotics used in both veterinary and human medicine include penicillins, cephalosporins, tetracyclines, chloramphenicols, amynoglycosides, spectinomycin, lincosamide, macrolides, nitrofurans, nitromidazoles, sulfonamides, trimethoprim, polymyxins, quinolones and many others (Michael, 2001). However, evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by over prescription of drugs in clinical settings and their heavy use as growth promoters for farm animals such as fish. When antimicrobial drugs are administered to food animals, they can thus promote the emergence of resistance in bacteria that may not be pathogenic to the animals, but are pathogenic to humans (Bates, 1994; Piddock, 1996).

It is important to implement monitoring systems to be aware of the emergence and spread of bacterial resistance to antimicrobial agents. There is limited data on the antibiotic resistance of bacteria in fish sampled directly from the farm in Malaysia although the antibiotic resistance of bacteria isolated from fish and shellfish samples purchased from wet markets has been studied (Son *et al*., 1997b; Son *et al*., 1998b; Zaiton *et al*., 2001). This study was undertaken to determine the incidence of antimicrobial resistance and genetic diversity among *E. coli* isolated from cultured catfish and its water environment.

Materials and Methods

Bacterial strains, cultivation and media

Seventeen strains of *E. coli* isolated from cultured catfish and water from four different aquaculture locations within Kuching, Sarawak were analyzed in this study (Table 1). These *E. coli* strains were isolated by direct plating of the fish intestine and water

*Corresponding author.
Email: *lsamuel@frst.unimas.my*
Tel: +608 2583014; Fax:+608 2583160

Table 1. Antibiotic resistance and RAPD patterns of *E. coli* isolated from catfish and their water environment

E. coli strains	Sources	Locations of sampling farms within Kuching, Sarawak	Antibiotic resistance patterns	MARI	RAPD-PCR Patterns with primer	
					Gen 15009	Gen 15009 + 15010
EC1	Water	Semariang Batu	[i]-	0.000	P1	U1
EC2	Water	Semariang Batu	Amp, Te	0.333	P1	U2
EC3	Water	Semariang Batu	Amp, Te	0.333	P2	U3
EC4	Water	Semariang Batu	Amp	0.167	P3	U4
EC5	Water	Semariang Batu	-	0.000	P4	U5
EC6	Water	Semariang Batu	-	0.000	P4	U5
EC7	Water	5th Mile	Te	0.167	P5	U6
EC8	Fish	7th Mile	-	0.000	P5	U7
EC9	Fish	Semariang Batu	F	0.167	P3	U8
EC10	Fish	Semariang Batu	Amp	0.167	P3	U8
EC11	Fish	Semariang Batu	Amp	0.167	P6	U9
EC12	Water	6th Mile	Amp, Te	0.333	P7	U10
EC13	Water	6th Mile	F	0.167	P8	U11
EC14	Fish	6th Mile	-	0.000	P8	U12
EC15	Fish	6th Mile	-	0.000	P8	U13
EC16	Fish	6th Mile	-	0.000	P9	U11
EC17	Fish	6th Mile	-	0.000	P8	U13

Note: [i]susceptible to all the antibiotics tested

samples on eosine methylene blue agar (EMBA). The *E. coli* colonies showing metallic greenish sheen on EMBA after the plating were selected and subjected to the standard biochemical tests for the identification of *E. coli*. Among the tests included the Gram staining, morphology observation under microscope, indol-methyl red-Voges Proskauer-citrate (IMViC), lysine decarboxylase tests and TSI reactions. All the confirmed isolates were stored on trypticase soya agar (TSA) (Oxoid Ltd., England) slant and were cultivated on TSA (Oxoid Ltd., England) plate under optimal incubation conditions to obtain a fresh overnight grown culture prior to use.

Antibiotics and susceptibility testing

The antimicrobial susceptibility tests were performed essentially by the disc diffusion method as described by National Committee for Clinical Laboratory Standards (NCCLS, 1997), with antibiotic containing discs (Oxoid Ltd., England). The antibiotics tested and their standard concentration used were ampicillin (Amp, 10 µg), nitrofurantoin (F, 300 µg), norfloxacin (Nor, 10 µg), tetracycline (Te, 30 µg), sulphametoxazole / trimethoprim (SXT, 25 µg) and chloramphenicol (C, 30 µg). Along with the tested organism, a reference culture (*Escherichia coli* ATCC 25922) was included during each series of antibiogram determinations. The zone diameter for each antibiotic disc were translated in prefixed susceptible (S), resistant (R) or Intermediate (I) categories by referring to the criteria recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) and the BSAC (BSAC, 2001).

Multiple antibiotic resistance index (MARI)

The multiple antibiotic resistance index (MARI) of the isolates is defined as a/b where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed (Krumperman, 1983).

Crude DNA preparation

DNA from the isolates was extracted by boiled cell method as described by Abdulamir *et al*. (Abdulamir *et al*., 2010). A colony was picked from the TSA and inoculated into 5 ml of LB broth and grown for 24 h with shaking at 120 rpm at 37°C. From the LB broth culture, 1 ml was spun at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of sterile distilled water. The suspension was boiled for 10 min and placed on ice immediately for 10 min followed by centrifugation at 10,000 rpm for 5 min. The supernatant (5 µl) was used for the RAPD-PCR analysis.

RAPD-PCR analysis

Two arbitrary primers, GEN15009 (5'-AGA AGC GAT G-3') and GEN15010 (5'-CCA TTT ACG C-3') were used to analyze all the DNA samples (Lihan *et al*., 1999). The RAPD-PCR fingerprinting assay was performed in a 25 µl volume containing 2.5 µl of 10X PCR buffer solution, 0.5 µl of 10 mM dNTPs, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 25 pmol primer, 0.5 µl of 5 units of *Taq* DNA polymerase (Promega, Madison, USA), 14.0 µl of sterile distilled water and 5 µl of 30-40 ng DNA template. A negative-DNA control was included by adding 5 µl of sterile distilled water instead of template DNA in the reaction and a

positive control (*E. coli* ATCC 25922) DNA was also included. Amplification was performed in a personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 1 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 36°C and polymerization at 72°C for 2 min. Final elongation at 72°C for 5 min was included. The amplification products were analyzed by electrophoresis in a 1.0% agarose in 1.0 X TBE (0.1 M Tris, 0.1 M boric acid, 0.1 mM EDTA) at 90 V for 40 min. Gels were stained with ethidium bromide and the amplified fragments were visualized with UV transilluminator (Syngene, USA). The 1kb DNA ladder (Promega, USA) was used as a DNA size marker.

DNA fragment analysis

The fingerprint profiles obtained from the RAPD-PCR were analyzed using the RAPDistance package version 1.04. The banding pattern obtained from the gel was scored in the binary data format for each of the primers used. The scoring was made based on the presence or absence of the band. The score '0' indicates the absence of the band whereas '1' indicates the presence of the particular band. The distance calculation of similarity band profiles based on Dice formulation (Nei and Li, 1979) was generated based on the data recorded. From the calculated Dice formulation, a dendrogram of neighbor joining tree (NJTREE) was produced through the Tdraw clustering algorithm program.

Results and Discussion

E. coli is generally found in the lower intestinal of warm blooded animals and most of the strains do not affect the health of their host. While most *E. coli* strains do not cause human disease, earlier studies shows that some strains possess virulence factor (Koitabashi *et al*., 2006). In this study, seventeen isolates of *E. coli* were isolated from the catfish and pond water samples. Of the 17 *E. coli* isolates characterized, all (100%) of the isolates displayed sensitivity towards norfloxacin (10 µg), sulphametoxazole/trimethroprim (25 µg) and chloramphenicol (30 µg). On the other hand, resistance was observed against ampicilin (35.3%), tetracycline (23.5%) and nitrofurantoin (11.8%). In general, the more often an antimicrobial agent is used in an environment, the higher will be the frequency of resistant microorganism in that environment. The incidence of antibiotic resistant strains in catfish and the pond water tested in this study is an important concern of antibiotic usage either in the aquaculture or other agricultural activities in the surrounding environment. Multiple antibiotic resistances has been reported in fish pathogens and bacteria from aquaculture environments, often associated with the use of some drugs (Mc Phearson *et al*., 1991; Kerry *et al*., 1995; Schmidt *et al*., 2000). High incidence of multiple resistance patterns in bacterial isolates from catfish farm has also been reported elsewhere (Sarter *et al*., 2007). Son *et al*. (2003) found that high incidence of multiple antimicrobial resistance in some *Aeromonas* species isolated from retail fish.

In this study, samples of the intestinal part of the catfish were analyzed. When coming in contact with the inside of the fish intestines during the process of removing out the intestines, fish meat can become contaminated with the intestinal flora which may be antibiotic resistant. Antibiotics used as feed additives play an important role in the development of antibiotic resistance in normal flora bacteria (Kerry *et al*., 1995). However, other factors like stress from temperature, overcrowding, and management also contribute to the occurrence of antibiotic resistance in normal bacteria flora of fish. The consequences of the selection of antibiotic resistance bacteria include an increased risk of resistant pathogens transferred to humans by direct contact with the fish or through the consumption of contaminated fish or water, thus may pose public health risk (Piddock, 1996).

MAR indexing has been used as an indicator to identify high-risk contamination potentially hazardous to humans. In this study, the multiple antibiotic index (MARI) of the *E. coli* isolates ranged from 0.000 to 0.333 in which 82.4% of the isolates possess MARI of 1.677 and below, and the remainder 17.6% exhibit MARI of 0.333 (Table 1). This indicate that the samples are likely represents two different sources of contamination which are low risk (MARI < 0.2) and high risk (MARI > 0.2) sources (Krumperman, 1983).

Antibiotics still remain the mainstay for treating bacterial infections and alternatives to the use of the antimicrobial agents, such as active and passive immunoprophylaxis, non-specific stimulation of the immune system, use of probiotics, or competitive exclusion cannot effectively replace antimicrobial chemotherapy, they may usually represent additional preventive measures rather than, real alternatives. Vaccines may be used for controlling bacteria that have high incidence of antibiotic resistance. In addition to developing new antibiotics it may be possible to reinforce the effect of currently used antibiotics.

The genetic relatedness among the *E. coli* strains in this study was distinguished by performing RAPD

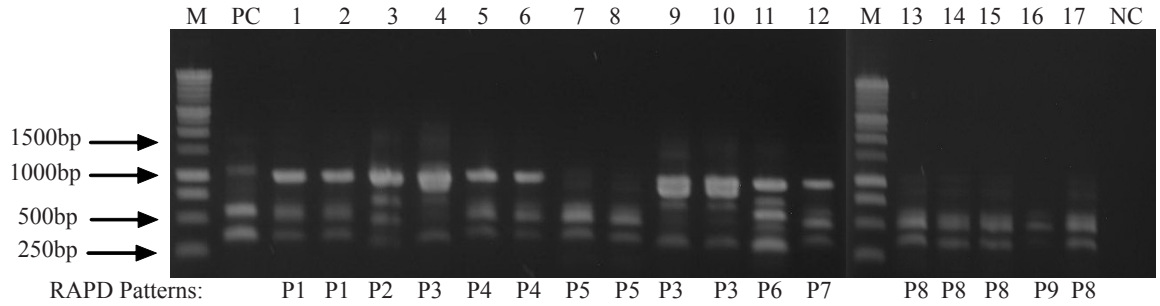

Figure 1a. RAPD-PCR fingerprinting of *E. coli* isolates obtained with primer GEN15009 (5'-AGA AGC GAT G-3') electrophoresed on 1.0% agarose gel. Lane M: 1kbp DNA ladder; lane PC: positive control; NC: negative control; lane 1-17: EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9, EC10, EC11 and EC12, EC13, EC14, EC15, EC16, EC17.

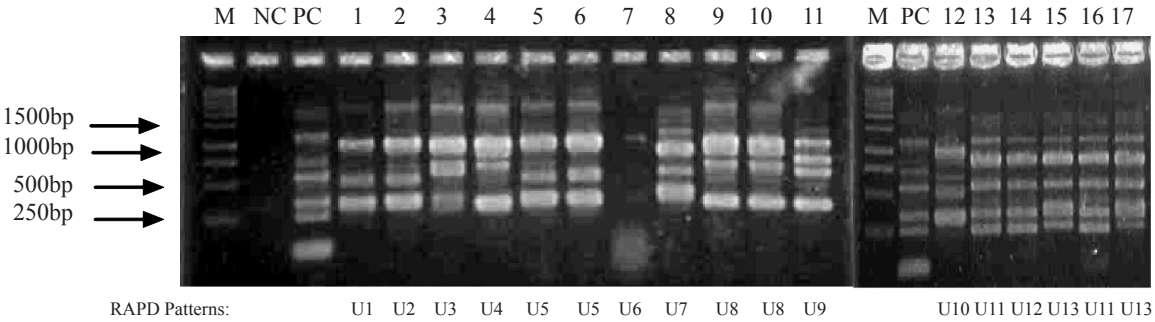

Figure 1b. RAPD-PCR fingerprinting of *E. coli* isolates obtained with primer combination of GEN15009 and GEN15010 electrophoresed on 1.0% agarose gel. Lane M: 1 kbp DNA ladder; lane PC: positive control; NC: negative control; lane 1-17: EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9, EC10, EC11 and EC12, EC13, EC14, EC15, EC16, EC17.

tests. Figure 1a and 1b shows the RAPD-PCR gel patterns of the *E. coli* strains obtained with primer GEN15009 and combination of primer GEN15009 and GEN15010, respectively. The number of DNA bands produced for a given primer ranged from 1 to 5 with molecular sizes ranging from 200 bp - 1500 bp. The possible number of RAPD patterns was estimated by the changes of one or more clear bands or band size. There were 9 RAPD patterns (P1-P9) observed using GEN15009 primer (Figure 1a). The RAPD-PCR analysis using combination of primers GEN15009 and GEN15010 allowed all the strains of *E. coli* to be differentiated into 13 different genome types, U1 to U13 (Figure 1b). This indicate that by using combination of primers, the *E. coli* strains can be differentiated into more groups compared to when using these primers individually. Hence, combined primers provide increased sensitivity towards genetic variations. Some of the *E. coli* strains have indistinguishable RAPD fingerprinting profiles which suggest that they may be closely related or have no genetic differences. Certain isolates did not produce band in this analysis mainly because there were no sequence in the bacterial DNA which is complementary to the sequence of the primer.

The dendrograms generated clearly established the genetic relatedness of the *E. coli* strains, the information that may be useful in epidemiological and population studies. There was prove of the repeated isolation of similar strains (strains assigned to the same vertical bar) and the isolation of genetically different strains (strains assigned to a single horizontal bar) among the *E. coli*. These results further reveals that while there is the potential for a single strain to be become dominant and get better adaptation within a population, there is also the potential for different contaminating strains of diverse genetic contents to present within the same population. The useful criteria for superior genotyping methods are rapidity, ease of performance, reproducibility and the ability to differentiate between closely related strains. The results of this study demonstrated that the RAPD-PCR analysis of the *E. coli* strains fulfills these criteria. This low-cost but powerful and reliable means of typing individual strains can be used either on its own or in conjunction with other established methods; because the results obtained with a single typing system cannot always be relied on during epidemiological investigations. The dendrogram generated from the RAPD-PCR analysis clustered the isolates into 3 groups by using a single primer (Figure not shown) and 5 groups by using primers combination (Figure 2).

In conclusion, the presence of *E. coli* were known to be a major cause of infections in human and animals as it signified a potential threat to farmers, consumers and other forms of livestock through potential transfer of resistance to human and animal pathogens. The difference in the RAPD-PCR and the antibiotic resistant profiles suggest that different

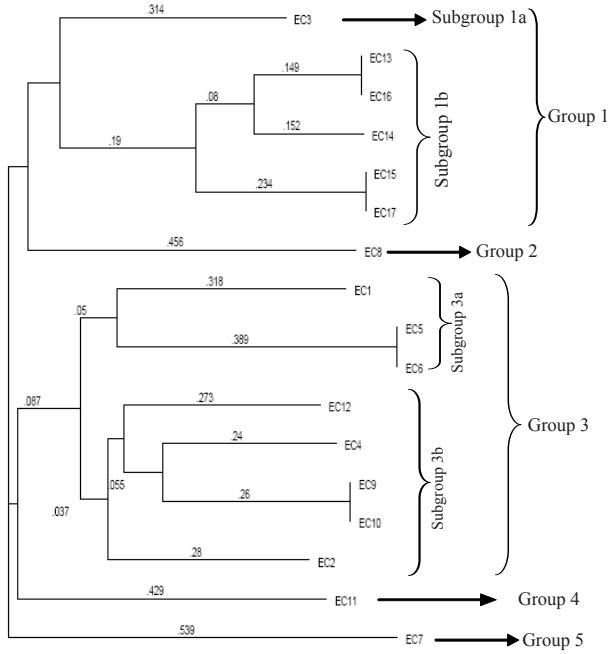

Figure 2. Dendrogram generated from the RAPD-PCR fingerprinting among 17 isolates of the *E. coli* with combination of primers GEN15009 and GEN15010.

strains of *E. coli* are circulating within the catfish environment and inhabiting the catfish in the study area.

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