Distribution and prevalence of chloramphenicol-resistance gene in *Escherichia coli* isolated from aquaculture and other environment

Ng, K. H., *Samuel, L., Kathleen, M. M., Leong, S. S. and Felecia, C.

Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

Abstract

In Malaysia, the aquaculture industry, particularly the production of freshwater aquaculture fish, is growing rapidly. Nevertheless, the illegal use of banned antimicrobial agents such as chloramphenicol in aquaculture has become a major concern in relation to the safety of consumers and also the development of drug-resistant strains in bacteria. Driven by those factors, the main intention of this study was to determine the prevalence and types of chloramphenicol-resistance genes in *E. coli* isolated from aquaculture and other environmental waters. The respective chloramphenicol-resistance genes in the isolates were detected by multiplex PCR with four sense primers C-1, C-2, C-3, C-4 and one antisense primer C-R for targeting cat I, cat II, cat III and cat IV genes, respectively. Out of 27 *E. coli* isolated, 19 were resistant to chloramphenicol. *Cat* I, *cat* II, *cat* III and *cat* IV genes were detected in 19, 13, 10, and 6 of the *E. coli* isolates, respectively. The results of this study revealed that chloramphenicol-resistance *E. coli* is present in aquaculture and environmental waters, in the study area. This finding suggested that although banned, there could be illegal usage of chloramphenicol antibiotic in local aquaculture. The bacteria in aquaculture may have spread to other environmental water through disposal of aquaculture waste water to other environments.

Introduction

Chloramphenicol is a broad spectrum antibiotic that is effective against both gram-positive and gram-negative bacteria (Sorensen *et al*., 2003). It is commonly used in several fields, especially in the medical field for therapeutic purposes. Chloramphenicol is inexpensive to produce, and it is usually utilize widely in the Third World for the treatment of a variety of gram-negative pathogens, such as *Salmonella*, *Vibrio* and *Rickettsia* (Davies and Webb, 1998). However, chloramphenicol has decreased rapidly as the drug of choice due to the chronic toxicity, in other words, depression of bone marrow function causing blood disorders such as aplastic anemia (Davies and Webb, 1998).

Apart from medical field, chloramphenicol is also used in aquaculture, in which chloramphenicol is used either as a chemotherapeutic agent to control diseases, or as a disinfectant to prevent diseases (Lu *et al.*, 2009). Chloramphenicol can be spread directly to the environment by filtering from uneaten feeds or from the waste products of aquatic animals (Cravedi *et al.*, 1987; Ervik *et al.*, 1994). A selective pressure in favour of antimicrobial-resistant bacteria could be established when antimicrobial residues are exposed to the environment (Lu *et al.*, 2009). The bacteria in environmental water could possibly develop antimicrobial-resistant due to the used of antimicrobial, such as chloramphenicol in aquaculture to control or to prevent diseases.

As an important antibiotic in the treatment of central nervous system infections and some epidemic diseases in humans and none food producing animals (Chinabut *et al.*, 2005), chloramphenicol has become one of the banned antibiotics in animal production in a number of Asian countries (Huys *et al*., 2007). Although it has been banned in the aquaculture producing countries of Asia and South East Asia (SEA), there is still reported case for the rejection of Asian aquaculture products by the European Union (EU) due to the detection of chloramphenicol residues in the importation of some Asian aquaculture products (Chinabut *et al*., 2005).

In fact, any presence of antibiotics in aquaculture products signifies their use on the farm, which may result in the development of antibiotic resistance and dissemination in the aquatic environment (Chinabut *et al*., 2005). Besides, the rising of the resistance in the farmed species may further cease the therapeutic value of the antibiotic to the farmer, whether it is used legally or illegally (Chinabut *et al*., 2005). Therefore, this study was undertaken to determine the prevalence and distribution of chloramphenicol resistance and the *cat* genes among *E. coli* from the aquaculture and other environmental waters.

*Corresponding author.
Email: *lsamuel@frst.unimas.my*
Tel: +6082 583014; Fax: +6082 583160

Materials and Methods

Bacterial strains, cultivation and media

Ten strains of *E. coli* isolated from the environmental water samples from Damai Central and seventeen strains of *E. coli* isolated from the water of different aquaculture farms within Kuching, Sarawak were analyzed in this study. The *E. coli* strains were isolated by direct plating of the water or sediment 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.* The tests included indole-methyl red-Voges Proskauer-citrate (IMViC). All the confirmed isolates were stored on nutrient agar (NA) (Oxoid Ltd., England) slant and were cultivated on nutrient agar (NA) (Oxoid Ltd., England) plate under optimal incubation conditions to obtain a fresh overnight grown culture prior to use.

Antibiotic susceptibility testing

The antimicrobial susceptibility tests were performed essentially by the disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2007), with antibiotic containing discs (Oxoid Ltd., England). The antibiotic disc tested and its standard concentration used was chloramphenicol (C, 30 μg). Along with the tested organism, a reference culture (*Escherichia coli* ATCC 25922) was included during each antibiogram determination. The zone diameter for each antibiotic disc were translated in prefixed susceptibility (S), resistant (R) or intermediate (I) categories by referring to the criteria suggested by the Clinical and Laboratory Standards Institute (CLSI, 2007).

Crude DNA preparation

DNA from the isolates was extracted by boiled cell method as described by Soumet *et al.* (1994) with a slightly modifications. The *E. coli* isolate was inoculated into 5 ml of LB broth and grown for 24 hours with shaking at 120 rpm at 37°C. From the LB broth culture, 1.5 ml was spun at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 0.5 ml of sterile distilled water. The suspension was boiled for 10 minutes and followed by immediate cooling at -20°C for 5 minutes. After that, the suspension was spun at 10,000 rpm for 10 minutes. The supernatant (4 μl) was used for the multiplex PCR analysis.

Multiplex PCR analysis

Four sense primers C-1 (5'-GGTGATATGGGATAGTGTT-3'), C-2 (5'-GATTGACCTGAATACCTGGAA-3'), C-3 (5'-CCATACTCATCCGATATTGA-3'), C-4 (5'-CCGGTAAAGCGAAATTGTAT-3') and one antisense primer C-R (5'-CCATCACATACTGCATGATG-3') were used to analyze all the DNA samples (Yoo *et al.*, 2003). The multiplex PCR was performed in 25 μl volume containing 5 μl of 10X PCR buffer solution, 0.8 μl of 2 mM dNTP mix, 3 μl of 50 mM MgCl$_2$, 1 μl of each primer (C-1, C-2, C-3, C-4 and C-R), 0.3 μl of 5 units of *Taq* DNA polymerase (Promega, Madison, USA), 6.9 μl of sterile distilled water and 4 μl of DNA template. A negative-DNA control was included by adding 4 μl of sterile distilled water instead of template DNA in the reaction. Amplification was performed in a Bioer Little Genius thermal-cycler (Bioer, China) with a temperature program consisting of the initial denaturation at 95°C for 3 minutes, followed by 34 cycles of denaturation at 95°C for 1 minute, annealing for 1 minute at 55°C, polymerization at 72°C for 1 minute and final elongation at 72°C for 5 minutes. The amplification products were analyzed by electrophoresis in a 1.0% (w/v) agarose in 1.0 X TBE (0.1 M Tris, 0.1 M boric acid, 0.1 mM EDTA) at 80 V for 45 minutes. A 1kb DNA ladder (Promega, USA) was used as a DNA size marker. Gels were stained with ethidium bromide and the amplified fragments were visualized with UV transilluminator.

Results and Discussion

The 27 *E. coli* isolates used in this study were isolated from aquaculture and environmental sources. Their susceptibility towards chloramphenicol were tested against commercially prepared chloramphenicol discs (Jorgensen and Ferraro, 2009). Among the 17 isolates of *E. coli* isolated from the aquaculture, 9 isolates displayed resistance to chloramphenicol (30 μg), whereas the rest of the 8 aquaculture isolates were susceptible towards chloramphenicol (Table 1). All 10 isolates of *E. coli* isolated from the environmental water were observed to be resistant against chloramphenicol.

The *E. coli* strains that displayed resistance to chloramphenicol showed the presence of *cat* (chloramphenicol acetyltransferase) genes (*cat* I, *cat* II, *cat* III and *cat* IV) as detected by multiplex PCR (Figure 1a and 1b). In this multiplex PCR, four sense primers opposed to variable regions and one antisense primer opposed to conserved region of different *cat* genes were used in a single reaction mixture to distinguish the type of *cat* gene by referring to the different sizes of the amplified products (Yoo *et al.*,

Table 1. Chloramphenicol resistance and types of *cat* genes among *E. coli* isolated from aquaculture and environmental water

E. coli strains	Source of samples	Locations of sampling sites within Kuching, Sarawak	Chloramphenicol resistance patterns	Multiplex PCR Patterns	Types of *cat* genes			
					Cat I	*Cat* II	*Cat* III	*Cat* IV
B5-1-S-4	Aquaculture	5th Mile	R	A1	-	✓	✓	-
B6-1-W-4	Aquaculture	6th Mile	S	-	-	-	-	-
B6-1-W-5	Aquaculture	6th Mile	R	A2	-	✓	✓	-
B6-1-W-6	Aquaculture	6th Mile	R	A3	✓	✓	✓	✓
B6-1-W-7	Aquaculture	6th Mile	S	-	-	-	-	-
B6-1-W-8	Aquaculture	6th Mile	S	-	-	-	-	-
B6-1-W-10	Aquaculture	6th Mile	R	A4	✓	✓	✓	✓
B6-1-F-1	Aquaculture	6th Mile	S	-	-	-	-	-
B6-1-F-2	Aquaculture	6th Mile	R	A5	-	✓	✓	-
B6-1-F-3	Aquaculture	6th Mile	R	A6	-	✓	✓	-
B7-2-S-BC4	Aquaculture	7th Mile	S	-	-	-	-	-
B7-2S-BC6	Aquaculture	7th Mile	R	A7	-	✓	✓	-
B7-1-F-2	Aquaculture	7th Mile	R	A8	✓	✓	✓	✓
SB-1-F-1	Aquaculture	Semariang Batu	S	-	-	-	-	-
SB-1-W-1	Aquaculture	Semariang Batu	S	-	-	-	-	-
SB-1-F-2	Aquaculture	Semariang Batu	S	-	-	-	-	-
SB-1-F-3	Aquaculture	Semariang Batu	R	A9	✓	✓	✓	✓
DDS-W-1-1	Environmental	Damai Central	R	E1	✓	✓	-	✓
DDS-W-1-2	Environmental	Damai Central	R	E2	✓	✓	-	✓
DDS-W-1-3	Environmental	Damai Central	R	E3	✓	✓	-	-
DDS-W-1-4	Environmental	Damai Central	R	E4	✓	✓	-	-
DDS-W-1-5	Environmental	Damai Central	R	E5	✓	✓	-	-
DDS-W-1-6	Environmental	Damai Central	R	E6	✓	✓	-	-
DDS-W-1-7	Environmental	Damai Central	R	E7	✓	✓	-	-
DDS-W-1-8	Environmental	Damai Central	R	E8	✓	✓	-	-
DDS-W-1-9	Environmental	Damai Central	R	E9	✓	✓	-	-
DDS-W-1-10	Environmental	Damai Central	R	E10	-	✓	✓	-

Note: R, resistance; S, susceptible; ✓, positive for *cat* gene; -, negative for *cat* gene.
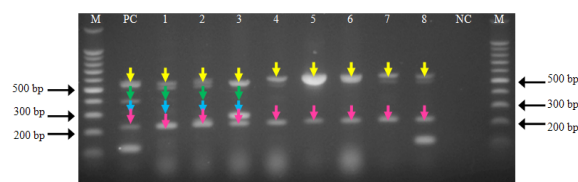

Figure 1a. Multiplex PCR of *E. coli* isolates from aquaculture electrophoresed on 1.0% (w/v) agarose gel. Lane M: 100 bp ladder; PC: positive control (A8); Lane 1: A3; Lane 2: A9; Lane 3: A4; Lane 4: A2; Lane 5: A1; Lane 6: A5; Lane 7: A7; Lane 8: A6; NC: negative control. (↓) *cat* II gene (567 bp); (↓) *cat* IV gene (451 bp); (↓) *cat* I gene (349 bp); (↓) *cat* III gene (275 bp).
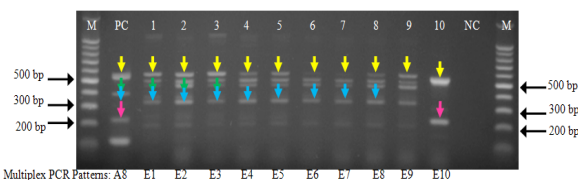

Figure 1b. Multiplex PCR of *E. coli* isolates from environmental water electrophoresed on 1.0% (w/v) agarose gel. Lane M: 100 bp ladder; PC: positive control (A8); Lane 1: E1; Lane 2: E2; Lane 3: E3; Lane 4: E4; Lane 5: E5; Lane 6: E6; Lane 7: E7; Lane 8: E8; Lane 9: E9; Lane 10: E10; NC: negative control. (↓) *cat* II gene (567 bp); (↓) *cat* IV gene (451 bp); (↓) *cat* I gene (349 bp); (↓) *cat* III gene (275 bp).

2003).

In this study, 19 isolates (10 aquaculture and 9 environment) of *E. coli* displayed resistant to chloramphenicol. Among these 19 isolates, 4 isolates were detected carrying all the four *cat* genes (cat I, *cat* II, *cat* III, and *cat* IV). This could be a new finding for the study related to *cat* genes, as Yoo *et al.* (2003) reported that there were no isolates used in their study carried more than two different types of *cat* genes. In fact, they reported that there has not been a report of the use of multiplex PCR with four sense primers and an antisense primer to discover and characterize four different types of *cat* genes carried in microorganisms.

In this study, the overall prevalence of chloramphenicol-resistant *E. coli* is 33.3%. The *E. coli* isolates shows the relatively high frequency of the *cat* II, followed by *cat* I and then *cat* III, whereas *cat* IV is the least common to be present in the *E. coli* isolates. According to Yoo *et al.* (2003) on the multidrug-resistant fish pathogens from Korea, *cat* II gene is the most prevalence and the multidrug-resistant fish pathogens are believed to carry restricted types of *cat* gene, which are *cat* II and *cat* IV. Nevertheless, Shaw (1983) reported that *cat* I and *cat* III genes are widely distributed, whereas *cat* II gene is less common in different Gram-negative and Gram-positive bacteria. The frequency of *cat* genes as reported by Shaw (1983) is different from this study and also the study by Yoo *et al.* (2003). Therefore, larger numbers of bacteria isolated from different geographical locations with different environments at different times can be further investigated in order to deal with the question on the different frequency and distribution of the *cat* genes (Yoo *et al.*, 2003).

In fact, *E. coli* isolates which are resistant to chloramphenicol still can be found from the samples of aquaculture and environmental water as shown in this study. Dang *et al.* (2008) reported that there are certain environmental and biological factors that might affect the alteration of resistance populations of the bacteria carrying the same antibiotic resistance genes at the same location but appear at different period of a year. The most probable reasons could be the dramatic decline of temperature, pH and dissolved oxygen (Dang *et al.*, 2008). In addition, the temporal and spatial shift of antibiotic-resistant bacteria populations could also be caused by the alterations of the source of the resistance determinants through river runoff and sewage discharge (Dang *et al.*, 2008). Chloramphenicol-resistant molecular determinants are varied with environments and geological locations (Dang *et al.*, 2008).

In addition, Schwarz *et al.* (2004) stated that chloramphenicol-resistant bacteria could still exist in the environments although the drug has no longer been used. This is because chloramphenicol resistance genes could be transferred between aquatic microorganisms without a high particular selective pressure (Yoo *et al.*, 2003). Meanwhile, the mechanism involved could be either the cross-resistance caused by cross-selection, or co-resistance caused by co-selection (Alonso *et al.*, 2001; Courvalin and Trieu-Cuot, 2001; Schwartz *et al.*, 2004). Apart from chloramphenicol acetyltransferases, Poole (2005) found that multidrug transporters could also contribute to chloramphenicol resistance. In addition, antibiotics resistance in the environments might be conferred as a result of exposure to heavy metals and other toxicants (Schwarz *et al.*, 2004; Baker-Austin *et al.*, 2006).

There are several reasons that make *E. coli* a suitable indicator organism to be tested against the

presence of *cat* genes in this study. The main reason is because the members of the genus Escherichia, particularly *E. coli*, could act as suitable indicators of chloramphenicol resistance in Southeast Asian aquaculture environments, since they were the only universal group of chloramphenicol resistance heterotrophs regardless of country, sample type or farm type (Huys *et al.*, 2007). Theoretically, antimicrobial resistance indicators are usually omnipresent in the environment, readily observed and having genetic flexibility that allow them to obtain mobile resistance elements (Huys *et al.*, 2007). In *E. coli*, the resistant to chloramphenicol is generally conferred by mobilizable genes encoding chloramphenicol acetyltransferases (Huys *et al.*, 2007). Besides, *E. coli* is believed as a probable reservoir of resistance genes, which is able to transfer resistance to other zoonotic or commensal organisms causing diseases in human or cattle (Linton, 1985; Winokur *et al.*, 2001; Hoyle *et al.*, 2005; Donaldson *et al.*, 2006).

In conclusion, there is a diverse type of *cat* gene among the *E. coli* isolated from aquaculture and other environment. The *cat* II gene shows a relatively high frequency, followed by *cat* I, *cat* III and *cat* IV. The present of chloramphenicol resistant *E. coli* with the *cat* genes could be due to the illegal use of the antibiotic in the aquaculture and the spread of antibiotic resistance bacteria into other environment as indicated by the resistance of *E. coli* from the environmental source.

Acknowledgements

References

Alonso, A., Sanchez, P. and Martinez, J. L. 2001. Environmental selection of antibiotic resistance genes. Environmental Microbiology 3: 1-9.

Baker-Austin, C., Wright, M. S., Stepanauskas, R. and McArthur, J. V. 2006. Co-selection of antibiotic and metal resistance. Trends in Microbiology 14: 176-182.

Chinabut, S., Somsiri, T., Bartie, K. L., Huys, G., Oanh, D. T. H., Giacomini, M., Bertone, S., Shariff, M., Yusoff, F. M., Swings, J. and Teale, A. 2005. Paper presented. The use of antimicrobials in Asian aquaculture: Aims of the EU-ASIARESIST project. Indonesia: World Aquaculture 2005.

Clinical and Laboratory Standards Institute (CLSI). 2007. Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement, M100-S17. Wayne, PA: CLSI.

Courvalin, P. and Trieu-Cuot, P. 2001. Minimizing potential resistance: The molecular view. Clinical Infectious Diseases 33: 138-146.

Cravedi, J. P., Choubert, G. and Delous, G. 1987. Digestibility of chloramphenicol, oxolinic acid and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. Aquaculture 60 (2): 133-141.

Dang, H., Ren, J., Song, L., Sun, S. and An, L. 2008. Dominant chloramphenicol-resistant bacteria and resistance genes in coastal marine waters of Jiaozhou Bay, China. World Journal of Microbiology and Biotechnology 24: 209-217.

Davies, J. and Webb, V. 1998. Antibiotic resistance in bacteria. In Krause R. M. (Ed.). Biomedical research reports: Emerging infections p. 239-273. New York: Academic Press.

Donaldson, S. C., Straley, B. A., Hegde, N. V., Sawant, A. A., DebRoy, C. and Jayarao, B. M. 2006. Molecular epidemiology of ceftiofur-resistant *Escherichia coli* isolates from dairy calves. Applied Environmental Epidemiology 72: 3940-3948.

Ervik, A., Thorsen, B., Eriksen, V., Lunestad, B. T. and Samuelsen, O. B. 1994. Impact of administering antibacterial agents on wild fish and blue mussels *Mytilus edulis* in the vicinity of fish farms. Diseases of Aquatic Organisms 18 (1): 45-51.

Hoyle, D. V., Yates, C. M., Chase-Topping, M. E., Turner, E. J., Davies, S. E., Low, J. C., Gunn, G. J., Woolhouse, M. E. and Amyes, S. G. 2005. Molecular epidemiology of antimicrobial-resistant commensal *Escherichia coli* strains in a cohort of newborn calves. Applied and Environmental Microbiology 71: 6680-6688.

Huys, G., Bartie, K., Cnockaert, M., Oanh, D. T. H., Phuong, N. T., Somsiri, T., Chinabut, S., Yusoff, F. M., Shariff, M., Giacomini, M., Teale, A. and Swings, J. 2007. Biodiversity of chloramphenicol-resistant mesophilic heterotrophs from Southeast Asian aquaculture environments. Research in Microbiology 158 (3): 228-235.

Jorgensen, J. H. and Ferraro, M. J. 2009. Antimicrobial susceptibility Testing: A review of general principles and contemporary practices. Medical Microbiology 49: 1749-1755.

Linton, A. H. 1985. Antibiotic resistance in bacteria associated with animals and their importance to man. Journal of Antimicrobial Chemotherapy 15: 385-386.

Lu, X. W., Dang, Z. and Yang, C. 2009. Preliminary investigation of chloramphenicol in fish, water and sediment from freshwater aquaculture pond. International Journal of Environmental Science and Technology 6 (4): 597-604.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. Journal of Antimicrobial Chemotherapy 56: 20-51.

Schwarz, S., Kehrenberg, C., Doublet, B. and Cloeckaert, A. 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiology

Reviews 28: 519-542.

Shaw, W. V. 1983. Chloramphenicol acetyltransferase: Enzymology and molecular biology. Critical Reviews in Biochemistry 14: 1-46.

Sorensen, L. K., Elbaek, T. H. and Hansen, H. 2003. Determination of chloramphenicol in bovine milk by liquid chromatography/tandem mass spectrometry. Journal of Association of Official Analytical Chemists International 86 (4): 703–706.

Soumet, C., Ermel, G., Fach, P. and Colin, P. 1994. Evaluation of different DNA extraction procedures for the detection of *Salmonella* from chicken products by polymerase chain reaction. Letter in Applied Microbiology 19: 294-298.

Winokur, P. L., Vonstein, D. L., Hoffman, L. J., Uhlenhopp, I. D. and Doern, G. V. 2001. Evidence for transfer of CMY-2 AmpC b-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates form food animals and humans. Antimicrobial Agents and Chemotherapy 45: 2716-2722.

Yoo, M. H., Huh, M. D., Kim, E. H., Lee, H. H. and Jeong, H. D. 2003. Characterization of chloramphenicol acetyltransferase gene by multiplex polymerase chain reaction in multidrug-resistant strains isolated from aquatic environments. Aquaculture 217: 11-21.