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# Multiple Antimicrobial Resistance of *Escherichia coli* Isolates from Nile Tilapia Sold in Wet Markets in Metro Manila and the Conjugative Transferability of the Drug Resistance

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**Multiple drug resistance (MDR) in *Escherichia coli* poses great risks to both human and animal health as it compromises the management of diseases caused by *E. coli* strains. *E. coli* was isolated from the gills of 35 *Oreochromis niloticus* or Nile tilapia procured from the Balintawak, Cubao and Libertad wet markets in Metro Manila. The isolates were studied for their antimicrobial susceptibility patterns using the standard disc diffusion method, and for the transferability of their resistance determinants. Results showed the highest percentage resistance of the isolated *E. coli* to ampicillin (77.14%) and trimethoprim-sulfamethoxazole (60%). These were followed by the resistance to tetracycline (45.71%), cephalothin (17.14%), gentamicin (11.43%), nalidixic acid (8.57%), and the least to ciprofloxacin (5.71%). On the other hand, all the isolates were found to be susceptible to the aminoglycoside amikacin, and were also found to be susceptible to the carbapenem imipenem, suggesting the absence of carbapenemase-producing strains. These were also found to be susceptible to the extended spectrum β-lactams aztreonam, cefotaxime, and ceftazidime signifying absence of extended spectrum β-lactamase (ESBL)-producing *E. coli*. Multiple drug resistances were found in 21 out of 35 isolates or 60%, while the remaining five or 14.28% were completely susceptible to all test antimicrobials. Moreover, seven of ten isolates completely transferred their resistance determinants to a drug-susceptible, res- *E. coli* J53-2 through conjugation. Three isolates transferred only the ampicillin resistance gene.**

Key Words: antimicrobial resistance, conjugation, drug resistance, *Escherichia coli*, Nile tilapia gills, *Oreochromis niloticus*

Abbreviations and Acronyms: AMP – ampicillin, AMI – amikacin, ATCC – American Type Culture Collection, ATM – aztreonam, BHIA – brain heart infusion agar, CAZ – ceftazidime, CDC – Centers for Disease Control and Prevention, CEP – cephalothin, CIP – ciprofloxacin, CLSI – Clinical Laboratory Standards Institute, COT – co-trimoxazole, CTX – cefotaxime, EMB – eosin methylene blue, ESBL – extended spectrum beta-lactamase, FAO – Food and Agriculture Organization of the United Nations, GM – gentamicin, IMI – imipenem, MAC – MacConkey agar, MAR – multiple antimicrobial resistance, MDR – multiple drug resistant, MHA – Mueller Hinton agar, NA – nalidixic acid, SXT – trimethoprim-sulfamethoxazole, TE – tetracycline, TSB – tryptic soy broth, WHO – World Health Organization

## INTRODUCTION

*Oreochromis niloticus* or Nile tilapia is the second most important group of farmed fish after carps, and is the most widely grown of the farmed fish worldwide (FAO 2018). Nile tilapia was introduced in the developing countries and cultured on a subsistence level to meet local protein needs. As production techniques improved and off-flavors were controlled, tilapia moved into the mainstream seafood markets of these countries (FAO 2018). However, the growing tilapia industry presents a considerable risk for microbial contamination of the fish during the production and postharvest processes, which is of critical concern to public health. Foodborne pathogens are major agents that cause worldwide morbidity and mortality that can result from the consumption of various foods including seafoods (Iwamoto et al. 2010). Diarrheal diseases are the most common illnesses resulting

from the consumption of contaminated food, affecting millions of people every year (World Health Organization 2017). Thus, food safety becomes a vital concern in public health that connects human health to food production (Mizan et al. 2015), and the need to control these microbial contaminants must be implemented. Nile tilapia may be exposed to various microbial contaminants such as Escherichia coli in the fish farms where they are cultured. At the same time, the fish sold in the market may also be exposed to the bacterium. Although the gills are covered with the operculum, contamination with *E. coli* is still possible due to infected handlers or contaminated ice used during storage (Lateef et al. 2004; Rocha et al. 2014). The bacterium is known to be exclusively fecal in origin, and is used as an indicator for the biological conditions of food and environment. A number of strains cause various diseases in humans (Carson et al. 2001). While no cases of diseases linked to *E. coli* in farmed fish like Nile tilapia have been reported, transmission of the bacterium presents a public health concern especially if the strains are drug resistant. These are selected for by the presence of antimicrobials in the environment, such as in the animal feeds used in the aquaculture industry. This may lead to the dissemination of resistance genes by mobile genetic elements to potentially pathogenic bacteria (Alexander et al. 2010; Berglund 2015).

The emergence of antimicrobial resistance presents problems in disease management, which may result in longer duration of the disease period or in high mortality rates (WHO 2015). Treatment for *E. coli* infections has become complicated due to the resistance to most of the first-line antimicrobial agents (Sabate et al. 2008). Clinical isolates of *E. coli* have been reported to be commonly resistant to tetracyclines, quinolones, fluoroquinolones, cotrimoxazole, and the β-lactam antibiotics (Batard et al. 2016). Numerous studies show that some strains of *E. coli* are capable of cross-resistance among these antimicrobials (Strand et al. 2014). In addition, carbapenemase-producing and extended spectrum-β-lactamase (ESBL)-producing Enterobacteriaceae to which E. coli belongs, have been included in the Urgent Threat and Serious Threat categories of the Centers for Disease Control and Prevention (CDC) levels of concern for drug-resistant bacteria, respectively (CDC 2013).

Foodborne bacterial strains resistant to antibiotics pose health risks and favor the transfer to humans through the food chain (Sapkota et al. 2008; Ryu et al. 2012). Bacterial populations that are susceptible to antibiotics become resistant either through mutation or through horizontal transfer of resistance genes in mobile genetic elements from other strains, either distantly or closely related. This leads to the dissemination of the resistance genes to potentially pathogenic bacteria (Alexander et al. 2010; Berglund 2015). Among the different mechanisms of horizontal gene transfers, conjugation, particularly in the human intestine, is the most studied (Zechner et al. 2012; Arutyunov and Frost 2013).

In the Philippines, antimicrobial agents, which are related to those used for treatment of infectious diseases in man and animals, are still routinely used to promote the growth of food animals like tilapia fish (Jiao et al. 2007). The presence of multiple drug resistant *E. coli* in tilapia gills has not been studied in the Philippines, and surveillance of these microorganisms must be conducted. Gills may not be consumed for food, but the handler might get infected during the removal of the gills from the fish. In addition, *E. coli* present in the gills may contaminate the fish body parts such as muscles and intestines during meat processing, and eventually cause diseases when taken with the meat that is not thoroughly cooked. The study was thus conducted to determine the presence of *E. coli* in the gills of tilapia fish sold in selected wet markets in Metro Manila, and to determine their antimicrobial susceptibility patterns. The *E. coli* isolates were also studied to evaluate their ability to transfer their resistance

genes to the antimicrobial susceptible *E. coli* strain J53-2 through conjugation.

## MATERIALS AND METHODS

**Sample Collection and Sampling Area**

A total of 35 samples of Nile tilapia were procured from three different public wet markets in Metro Manila. Ten tilapia fish were purchased from each of the wet markets in Balintawak and Cubao in Quezon City, and 15 from the Libertad wet market in Pasay City in November 2016. The samples were wrapped in sterile plastic films or sterile zip-lock and were transported in an ice box to the Microbiology Laboratory of the De La Salle University-Manila for immediate bacteriological analysis.

**Isolation, Purification, and Identification of the *Escherichia coli* Species**

Sterile cotton swab was wiped against the gill filaments after lifting the operculum using a pair of flame-sterilized forceps. These swabs were streaked on MacConkey agar and eosin methylene blue agar plates and incubated at 37 °C for 24 h. A part of the gill filament was likewise aseptically removed and inoculated in lactose broth with phenol red and Durham tube, and incubated at 44.5 °C for 24 h. Bacterial colonies of suspected *E. coli* were selected from either of the agar plates. The inoculated lactose broth tubes that were positive for gas formation within 48 h were further processed to isolate *E. coli*. The isolates were checked for purity on brain heart infusion agar plates, Gram-stained, and studied for their reactions in indole, methyl red, Vogues Proskauer, and citrate tests.

**Antimicrobial Susceptibility Testing**

Susceptibility of the *E. coli* isolates to antimicrobials was determined using the standard disk agar diffusion method on Mueller-Hinton agar following the guidelines of the Clinical Laboratory Standard Institute (CLSI 2015). *Escherichia coli* ATCC 25922 was used as the quality control isolate. The turbidity of saline suspension of *E. coli* prepared from 18-h plated culture was adjusted to that of 0.5 McFarland turbidity standard, equivalent to $1.5 \times 10^8$ CFU $mL^{-1}$ (CLSI 2015). The susceptibility of the *E. coli* isolates was tested against the aminoglycoside family: amikacin (AMI; 30 μg) and gentamicin (GM; 10 μg); the carbapenem imipenem (IMP; 30 μg); the cephalosporins cephalothin (CEP; 30 μg), ceftazidime (CAZ; 30 μg) and cefotaxime (CTX; 30 μg); the fluoroquinolone ciprofloxacin (CIP; 5 μg); the monobactam aztreonam (ATM; 30 μg); the penicillin ampicillin (AMP; 10 μg); the quinolone nalidixic acid (NA; 30 μg); the sulfonamide sulfamethoxazole-trimethoprim or Co -trimoxazole (COT; 23.75/1.25 μg); and the tetracycline (TE; 30 μg). The diameters of the zones of inhibition were measured after 24 h of incubation at 35 °C and interpreted using the CLSI (2015) interpretative chart. Each test was done in triplicate.

**Multiple Antimicrobial Resistance (MAR) Index**

The multiple antimicrobial resistance (MAR) indices were determined for the *E. coli* isolates from the gills of Nile tilapia following the method of Adzitey et al. (2012), and the following formula was employed:

> MAR index = (Number of resistance to antibiotics per isolate) / (total number of antimicrobials tested)

Isolates classified as intermediate on the basis of the inhibition zone were considered as sensitive for the MAR index (Singh et al. 2010).

**Conjugation Assay**

*E. coli* isolates from the Nile tilapia gills found to be susceptible to rifampicin, but resistant to some of the test antimicrobials were tested for the conjugative transferability of their resistance. On the other hand, rifampicin-resistant *E. coli* J53-2, which is susceptible to other antimicrobials, was used as the recipient in the conjugation assay. This was a kind gift from Dr. Jesus Silva-Sanchez of the Instituto Nacional de Salud Publica, Mexico.

Conjugation assay was conducted following the method of Jiao et al. (2007). The donor and the recipient strains were separately grown in tryptic soy broth (TSB) at 37 °C for 16–18 h, then sub-cultured in TSB for 4 h at 37 °C to bring them to the log phase of growth. The turbidity of the cultures was adjusted to that of 0.5 McFarland. Two hundred (200) µL of the recipient and 20 µL of the donor strain were mixed and spread plated on Mueller Hinton agar (MHA) plate using a flame-sterilized bent glass rod. The plates were incubated at 37 °C for 6 h to allow conjugation. The conjugation mixtures in the plates were harvested by adding 2 mL of sterile distilled water, and the cultures were scraped off from the plate using a bent glass rod. One hundred (100) µL of each mixture were inoculated into different selective plates of Mueller Hinton containing rifampicin (200 µg $mL^{-1}$) and tetracycline (16 µg $mL^{-1}$); rifampicin (200 µg $mL^{-1}$) and ampicillin (32 µg $mL^{-1}$); and rifampicin (200 µg $mL^{-1}$) and co-trimoxazole (4 µg $mL^{-1}$). The suspected trans-conjugants growing in the selective plates were then tested for their antimicrobial susceptibility patterns using the disc diffusion method to confirm the transfer.

## RESULTS AND DISCUSSION

### Antimicrobial Susceptibility of *E. coli* Isolates

Thirty-five (35) *E. coli* isolates from Nile tilapia fishes sold in three (3) wet markets in Metro Manila were tested for their susceptibility to 12 antibiotics prescribed by CLSI. Figure 1 shows the frequency of isolates with resistance to the antimicrobial agents. High resistance percentage was to ampicillin (77.14%), trimethoprim-sulfamethoxazole (60%), and tetracycline (45.71%). This was followed by resistance to cephalothin (17.14%), gentamicin (11.43%), nalidixic acid (8.57%), and ciprofloxacin (5.71%). Figure 2 shows samples of the test plates.

All isolates were susceptible to amikacin. At the same time, all isolates were susceptible to the extended spectrum β-lactams aztreonam, cefotaxime, ceftazidime, indicating the absence of ESBL-producing strains, while the susceptibility of all isolates to the carbapenem imipenem suggests the absence of carbapenemase-producing *E. coli* strains.

### Multiple Antimicrobial Resistance Index (MAR) of *E. coli* Isolates

Results of the antimicrobial susceptibility testing showed that 16 isolates (45.71%) were resistant to antimicrobials belonging to at least three categories (Table 1) and are thus considered multiple drug resistant strains (Magiorakos et al. 2012). As with Magiorakos et al.'s category of

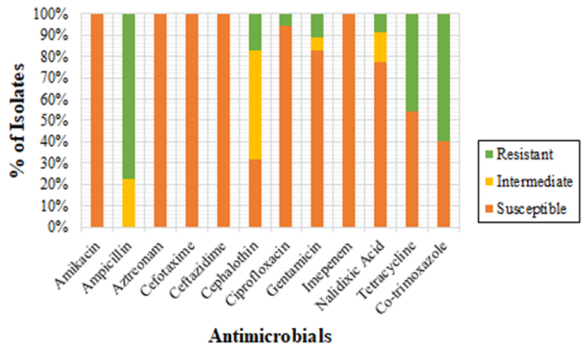


**Fig. 1.** Frequency of resistance to antimicrobial agents among *Escherichia coli* isolates from Tilapia gills.

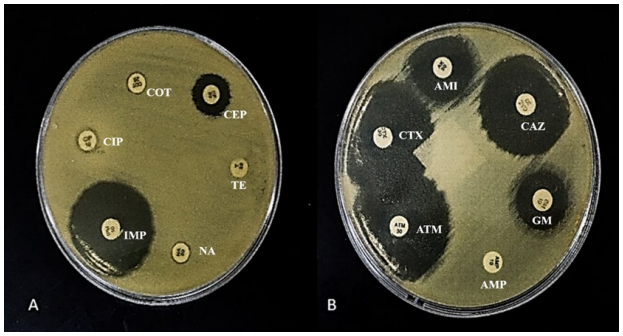


**Fig. 2.** (A) Mueller Hinton agar (MHA) plates showing multiple antibiotic resistance of *Escherichia coli,* (B) MHA plate showing the susceptibility of *E. coli* to several antibiotics: AMI – Amikacin, AMP – Ampicillin, ATM – Aztreonam, CAZ – Ceftazidime, CEP – Cephalothin, CIP – Ciprofloxacin, COT – Cotrimoxazole, CTX – Cefotaxime, GM – Gentamicin, IMP – Imipenem, NA – Nalidixic Acid, TE – Tetracycline.

MDR, 16 isolates (45.71%) have high MAR indices above 0.2 (Krumperman 1983; Titilawo et al. 2015), while 14 isolates (40%) have MAR index below 0.2; however, five isolates were susceptible to all antibiotics as shown in Table 2. These findings suggest that the MDR strains very likely originated from an environment where several antibiotics were present, such as fishpond waters where antibiotic-supplemented animal feeds were used, which are considered to be of high risk source (Singh et al. 2010).

In addition, Table 1 shows that seven (7) resistance patterns were observed among the *E. coli* isolates. High frequency of isolates was found with the resistance pattern of AMP-T-COT (40%), while a remarkable 5 antibiotic resistance patterns (T-NA-CIP-COT-AMP) were found in 2 (5.7%) isolates.

High prevalence of multidrug resistance in the isolates indicates a serious need for broad-based, local antimicrobial resistance surveillance and planning of effective interventions to reduce multidrug resistance in such potential pathogens (Osundiya et al. 2013). The use of antimicrobials in aquaculture must be regulated. The inappropriate use of antibiotics in medicine, agriculture, and aquaculture has been linked to the emergence of resistant bacteria in these settings (Economou and Gousia 2015).

The impact of antibiotic usage extends further, as antibiotic residues, resistant bacteria, and genetic resistance elements subsequently spread to adjacent environments (von Wintersdorff et al. 2016). In highly-developed countries, studies have shown the presence of high residues of tetracyclines and quinolones in tilapia and other aquaculture products. Resistance to tetracycline in one or more species of bacteria was reported for the same aquaculture species (Tusevljak et al. 2012). Results of the current study showed that 45.71% of the isolates were resistant to tetracycline, suggesting high use of the antibiotic in the environment to which the tilapia samples were exposed.

**Table 1.** Antimicrobial resistance patterns of *Escherichia coli* isolated from Tilapia gills.

| Resistance Pattern | No. of Isolates |
|---|---|
| AMP, TE, COT | 14 |
| COT, AMP | 1 |
| NA, GM | 1 |
| TE, NA, GM, CIP, COT, AMP | 2 |
| AMP | 6 |
| CEP, AMP | 3 |
| COT | 3 |
| Susceptible to all anti-microbials | 5 |
| **TOTAL** | **35** |

AMP – Ampicillin CIP – Ciprofloxacin GM – Gentamicin
CEP – Cephalothin COT – Cotrimoxazole
NA – Nalidixic Acid TE – Tetracycline

**Transferability of Drug Resistance in the *E. coli* Isolates**

The transferability of the drug resistance determinants among the *E. coli* isolates was determined through conjugation in a solid medium where *E. coli* J53-2 served as the recipient. Ten (10) *E. coli* isolates that were susceptible to rifampicin but resistant to at least two antimicrobial agents were conjugated with *E. coli* J53-2, which was resistant to rifampicin but susceptible to the other antimicrobials. Seven (7) donors completely transferred their resistance determinants (Table 3), converting the susceptible recipient to a multiple resistant strain with AMP-TE-COT resistance, while three (3) isolates transferred ampicillin resistance only. Figure 3 shows sample plates of the *E. coli* J532 trans-conjugants that were converted to resistance.

**Table 2.** Multiple Antimicrobial Resistance (MAR) Index of *Escherichia coli* isolated from Tilapia gills.

| MAR Index | No. of Isolates |
|---|---|
| 0.0 | 5 |
| 0.1 | 9 |
| 0.2 | 5 |
| 0.3 | 14 |
| 0.4 | 0 |
| 0.5 | 2 |

**Table 3.** Resistance transferred to *E. coli* J53-2 from *Escherichia coli* isolated from Tilapia gills.

| Resistance Phenotype of Donor *E. coli* from Tilapia | Resistance Determinants Transferred to *E. coli* J53-2 | No. of Isolates |
|---|---|---|
| AMP, TE, COT | AMP, TE, COT | 7 |
| AMP | AMP | 3 |

AMP – Ampicillin, COT – Cotrimoxazole, TE – Tetracycline

Multiple drug resistance in bacteria is most commonly associated with the presence of plasmids which carry multiple resistance genes (Sandhu et al. 2016). The multiple drug resistance to different classes of antimicrobials among the isolates in the study suggests these to be plasmid mediated. The plasmid nature was confirmed by the transferability of the resistance determinants through conjugation. Thus, the results showed that the drug resistance in the isolates may be transferred not only vertically, but horizontally as well, from one bacterium to another. The transfer of plasmids in bacteria has led to the worldwide spread of numerous antimicrobial resistance genes encoding resistance to beta-lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, and

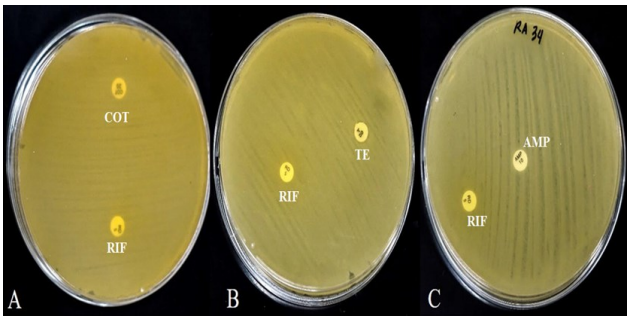


**Fig. 3.** Mueller Hinton agar plates showing resistance of trans-conjugant *E. coli* J532 to (A) RIF – Rifampicin and COT – Cotrimoxazole, (B) RIF – Rifampicin and TE –Tetracycline, and (C) RIF – Rifampicin and AMP – Ampicillin.

many other drug classes (Huddleston 2014). Several studies have reported spread of plasmids harboring carbapenem resistance (Carattoli 2013) and the recent discovery of plasmid-encoded colistin resistance in China (Liu et al. 2015), which has now already been identified in multiple continents (Arcilla et al. 2016) and may cause Enterobacteriaceae to truly become pan-drug resistant.

Moreover, multiple resistance genes are often co-localized on the same plasmid, which allows for the relatively easy spread of multidrug resistance. Once transmitted to humans, there is the possibility that the resistance genes in these microorganisms can be transmitted to more pathogenic bacteria that would compromise the treatment of serious infectious diseases (Jiao et al. 2007). In this regard, the impact of antimicrobials incorporated in animal feeds in selecting for resistant strains to survive and further be disseminated in the environment cannot be overemphasized. These resistant strains may enter the food chain due to undercooked livestock, improper handling and other contaminated produce, which may in turn cross-contaminate other food products because of common unsafe handling practices (Smith et al. 2007).

Results showed the *E. coli* isolates to be resistant to multiple antimicrobials belonging to different categories, suggesting that these bacteria were selected for in an environment where several antimicrobials were used. At the same time, conjugation results indicated that the multiple resistances in most of the isolates studied were carried in the same genetic element, as shown by their co-transfer. Findings of this study imply that the regulation of the use of antimicrobials in both agricultural and pharmaceuticals should be strictly implemented to eliminate the positive selective pressure for drug resistance strains, especially

those with transferable resistance genes. Moreover, the recovery of *E. coli* from the gills of tilapia fish indicates the presence of fecal contamination among the tilapia fishes sold in the wet markets, which suggests the possibility that other intestinal microbial pathogens that may be transferred through the fecal-oral route may be present. Fish that are improperly handled, undercooked or consumed raw may contribute to the spread of intestinal pathogens in the community. Thus, the results of the present study demand necessary actions to institute the proper use of antimicrobials in agriculture, and the proper handling of food for public safety.

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