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FULL PAPER

Prevalence of antibiotic resistance *Escherichia coli* isolated from *Pangasius* catfish (*Pangasius hypophthalmus*) fillet during freezing process at two factories in Mekong Delta Vietnam

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Abstract

Total of 261 samples of fish and environmental samples (i.e. wash water, swabs of hand/ gloves of workers, fish contact surfaces i.e. knives, cutting boards and working tables) were collected from two *Pangasius* processing factories (PPF1 and PPF2). A total of seventy-one (71) isolates of *Escherichia coli* were selected to study the prevalence of antibiotics resistance using disk agar diffusion method. Overall, it was determined that 61% (22/36) of PPF1 isolates were resistant except to colistin while 68.57% (24/35) of PPF2 isolates were resistant except kanamycin. High resistance was against ampicillin in both PPF1 and PPF2 isolates (47.22% and 42.86%), followed by cefotaxime (33.33% and 40%) respectively. Varying resistance response to all other tested antibiotics such as streptomycin, meropenem, tetracycline, sulfamethoxazole/trimethoprim and nalidixic acid was also observed among the *E. coli* isolates from both factories. About 50% of the multi-drug resistant (3-9 antibiotics) among PPF1 were observed whereas there were 45.83% multi-drug resistant (3-7 antibiotics) among PPF2 isolates. The result from this study reflected that there was a prevalence of multi-drug resistance of *E. coli* isolated during the processing of *Pangasius* at the studied factories. Therefore, there is a need for an effective risk management assessment models and management plans from stakeholders involved in the *Pangasius* value chain (i.e. farmers, processors and government) to ensure the food safety of production chain.

1. Introduction

Pangasius hypophthalmus is a key aquaculture species in Vietnam which accounts for more than 90% of what is sold on the international market (Little *et al.*, 2012). This industry has become an important source of employment and wealth generation in the Mekong Delta as the product is almost totally exported to over 100 countries, as frozen fillets making it one of the key success stories of Asian aquaculture (Phan *et al.*, 2009). To meet up with the booming exports of *Pangasius* fillets, there are currently almost 140 *Pangasius* catfish processing units operating with a cumulative capacity of nearly one million ton of processed *Pangasius* fillets (De Silva and Phuong, 2011). To ensure that safe and quality aquatic product is produced both for domestic and international markets, as well as the reduction in the number of food contamination, several quality regulations, standards and certifications, were put in place which all stakeholders in the industry must comply with. The processed *Pangasius* products had currently implemented Good Manufacturing and Hygienic Practices (GMP and GHP), Hazard Analysis Critical Control Point (HACCP), and/or other food quality or Food Safety Management Systems (FSMS) such as BRC, IFS, ISO 9001, ISO 14001, etc. (VASEP, 2020).

In aquaculture sectors, the intensive and semi-intensive practices employed to produce large stocks of fish result in the outbreak of diseases, and the use of antimicrobials has become a customary practice to control them (Santos and Ramos, 2018). So, over the years, antibiotics have also been used in animal husbandry and aquaculture for growth promotion, improvement of feed efficiency, prophylaxis, and treatment of infections in South Asian countries (Manage, 2018). It is highlighted that the lack of stringent control on the use of antimicrobial agents resulted in the emergence of antimicrobial resistant bacteria, representing a risk of dissemination of

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Antimicrobial Resistance (AMR) organisms and their genes to consumers (Manage, 2018). As such, antimicrobial resistance is now recognized as one of the most serious global threats to human health (Liu *et al.*, 2016). High use of antibiotics has been noticed among *Pangasius* farms and Vietnamese aquaculture generally (Rico *et al.*, 2013; Pham *et al.*, 2015; Long and Lua, 2017; Hedberg *et al.*, 2018). There was a rapid alert notification of ofloxacin categorized as an unauthorized substance in the exported *Pangasius* products to Switzerland recently (RASFF, 2019). On the other hand, antibiotics residue has also been reported in exported *Pangasius* products (Jansomboon *et al.*, 2018).

E. coli has been reported to be a candidate vehicle for transfer of antibiotic resistance gene (Van *et al.*, 2008). Multi-drug resistant bacteria was found on exported *Pangasius* fillets (Khan *et al.*, 2006; Boss *et al.*, 2016). It is evident that the prevalence of multi-drug resistance of *E. coli* on fresh *Pangasius* fish and pond water of cultured fish has also been reported (Sarter *et al.*, 2007; Nguyen *et al.*, 2016; Boss *et al.*, 2016; Long and Lua, 2017). The report of multi-drug resistance in *E. coli* that is rampant in, food sources, environment and human beings in Mekong Delta region is alarming (Van *et al.*, 2008; Dyar *et al.*, 2012; Nhung *et al.*, 2015).

With the booming export of *Pangasius* products from Vietnam, the prevalence of antibiotics resistant bacteria from farm levels or final fish products has been studied. Very little to no scientific information is available about the resistance prevalence in *E. coli* obtained from *Pangasius* processing factory (PPF) influenced by industrial processing. Therefore, the objective of the current research was to study the antibiotics resistance of *E. coli* isolated from *Pangasius* fish and environmental samples during processing from two processing factories located at Dong Thap and Vinh Long provinces of the Mekong Delta Region.

2. Materials and methods

2.1 Characteristics of Pangasius processing factories (PPF)

The processing factories evaluated in this study are located at Dong Thap (PPF1) and Vinh Long (PPF2) provinces of the Mekong Delta region, Vietnam. PPF1 has a production capacity of approximately 100 tons per day and between 800-900 people work in the factory. Food safety and/or quality management systems implemented include HACCP, BRC, GMP, SSOP, ISO 9001-2000, HALAL and SQF 2000. *Pangasius* products especially frozen fillets from this factory are being exported to South America including Brazil, Arab region, China, Hong Kong and Europe. PPF2, on the other hand, has a production capacity between 70-80 tons per day, with 500 employees working in the factory. Food safety and/or quality management systems implemented are HACCP and HALAL. The *Pangasius* frozen fillets from this factory are being exported to Spain, Poland, Germany, Malaysia, Indonesia and China.

2.2 Enumeration and isolation of E. coli

2.2.1 Sampling

Total of 261 samples (i.e. n = 144 from PPF1 and n = 117 from PPF2) of fish and environmental samples (i.e. wash water, swabs of hand/gloves of workers, fish contact surfaces i.e. knives, cutting boards and working tables) with 9 replicates per sample were collected at the different processing steps critical to the safety and quality of fillets such as raw material receiving, filleting, trimming, cooling and packaging as well as intervention steps of washing in water baths from two factories between January and August 2019 and subjected to microbiological analysis within 6 to 24 hrs of sampling.

2.2.2 Microbiological analysis

Approximately 25 g of fish samples were weighed and 225 mL of Maximum Recovery Diluent (MRD) was added to make first primary dilution, and a ten-fold serial dilution was done. For the water and swab samples, they were vortexed and a ten-fold serial dilution was done as well. Enumeration of *E. coli* was done by streaking 0.1 mL of inoculum of three consecutive dilutions taken from each sample on Coliform Agar ES (Enhanced Selectivity) (Merck Merck Darmstadt, Germany) and incubated for 18-24 hrs at 37°C. Colonies of blue color suspected as *E. coli* were picked and subsequently purified until pure *E. coli* isolates were obtained. Biochemical confirmation was done for the *E. coli* isolates using IMViC and KIA tests. *E. coli* isolates that tested positive were streaked on Tryptic Soy Agar (TSA, Merck Darmstadt, Germany) slant, incubated at 37°C for 18-24 hrs after which the purified *E. coli* were used for antimicrobial susceptibility tests.

2.3 Antimicrobial susceptibility testing

About seventy-one (PPF1, n = 36 and PPF2, n = 35) *E. coli* isolates were selected to be representative of the different samples and processing steps mentioned above. They were subjected to antimicrobial susceptibility test which was replicated twice using the disc agar diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2017). *E. coli* isolates were pre-cultured in Tryptic Soy Broth (TSB, Merck Darmstadt, Germany) and incubated at 37^{o}C for 18-24 hrs. The turbidity of the isolates was then adjusted to 0.5 McFarland (approximately 10^{8} CFU/mL). The isolates tested were spread on Mueller Hinton Agar (MHA,

Merck Darmstadt, Germany) and then placed on antibiotics disc before incubation at 37°C for 18-24 hrs. Fifteen antibiotics commercially available and frequently used in *Pangasius* aquaculture (ampicillin 10 µg, cefotaxime 30 µg, ceftazidime 30 µg, cefoxitin 30 µg, meropenem 10 µg, gentamicin 10 µg, kanamycin 30 µg, streptomycin 10 µg, tetracycline 30 µg, chloramphenicol 30 µg, sulfamethoxazole/trimethoprim 23.75/1.25 µg, nalidixic acid 30 µg, ciprofloxacin 5 µg, fosfomycin 200 µg and colistin 10 µg) (Abtek Ltd., United Kingdom and Nam Khoa., Ltd., Vietnam) were used for the test. Along with the tested isolates, a reference culture (*Escherichia coli* ATCC 25922) was used as control. The zone diameter for each antibiotic disc was measured and categorized as susceptible (S), resistant (R) or Intermediate (I) by referring to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2017).

2.4 Data analysis

The results of the microbial analysis of fish, the water, and swab samples were expressed as log CFU/g, log CFU/mL, and log CFU/100 cm^2, respectively. The results were reported as the mean value ± standard deviation. The results of the microbial contamination were statistically analysed using IBM SPSS statistics version 20.0. (SPSS Inc., Chicago, U.S.A.) ($\alpha = 0.05$). Prevalence of resistance of isolates to each antibiotic was calculated as the number of the isolates resistant to the antibiotics over the number of tested isolates and expressed in percentage using MS Excel 2013.

3. Results and discussion

3.1 Escherichia coli contamination during processing between PPF1 and PPF2

The trend of *E. coli* counts from fish and environmental samples along the processing steps in both PPF1 and PPF2 is shown in Figure 1 A-D. From PPF1, the highest count of *E. coli* was observed at the trimming (3.67 log CFU/g) and cooling steps (3.73 log CFU/g) for the fish samples compared to other steps ($p<0.05$). *E. coli* on packaged fish (2.07 log CFU/g) was not significantly different on the raw fish (1.28 log CFU/g) at PPF1 ($p>0.05$). From PPF2, *E. coli* counts on fish ranged from 1.0 to 2.0 log CFU/g among processing steps. For hands samples, the high *E. coli* counts on trimming, being 3.22 at PPF1 and 2.87 log CFU/100 cm^2 at PPF2. For food contact surfaces, there was a statistically significant difference ($p<0.05$) between *E.coli* at the skinning (3.58) and packaging (2.0 log CFU/100 cm^2) steps at PPF1 whereas there was no significant difference between *E.coli* at the skinning (1.78) and packaging (1.67 log CFU/100 cm^2) steps at PPF2 ($p>0.05$). For water samples, the highest *E. coli* was seen in bleeding (2.6 and 2.2 log CFU/ml) whereas the lowest *E. coli* was in glazing step (1.0 and 1.08 log CFU/ml) at PPF1 and PPF2, respectively ($p<0.05$). From this study, the fillets went through several handlers at the trimming step which increased the chances of contamination for the fillets. Also, during observation at the skinning step, several fillets passed through the machine surface where skinning is being done without intermittent cleaning of the surface during this operation, therefore, there is a greater possibility of contamination at this step as well. This shows that the contamination of *Pangasius* fillets with *E. coli* can occur at any point during production, and this might originate from the raw fish itself, food operators, environment and poor quality of wash water used for fish processing along the processing chain (Tong Thi *et al.*, 2013; Divyashree *et al.*, 2019). The increasing trend observed in the *E. coli* of sampled raw fish and fillets at the trimming step in this study is the same as observed by Tong Thi *et al.* (2014) with the counts from this study higher than those observed in their study. This might be because of the differences in sampled factories or some subtle differences in the processing steps of both factories. However, the count of *E. coli* conforms to the microbiological criteria for production of frozen Tra fish (*Pangasius hypophthalmus*) fillets (tolerance level for *E. coli* is 2 log CFU/g) according to (Ministry of Science and Technology of the Socialist Republic of Vietnam, 2010). The presence of *E. coli* on the packaged fillets is an indication that a contamination pathway exists between source of bacteria and the fillets across the processing chain. It is therefore recommended that improved hygiene practices among the food handlers as well as the cleaning efficiency of the contact surfaces of both factories should be implemented to reduce and/or prevent fillet contamination.

3.2 Antibiotic resistance

The comparison of the prevalence of resistance against 15 antibiotics is shown in Figure 2. Overall, it was determined that 61% (22/36) of PPF 1 isolates were resistant to at least one antibiotics except to colistin. A high level of resistance was against ampicillin (44.33%), followed by cefotaxime (33.33%) and streptomycin (22.22%). Furthermore, resistance response in the range of 11.01 to 16.67% was observed among the isolates to meropenem, tetracycline, sulfamethoxazole/trimethoprim and nalidixic acid while the rest of the tested antimicrobial agents was <10% except for colistin. A similar trend was also seen among PPF2 isolates, 68.57% (24/35) isolates were resistant to at least one antibiotics except for kanamycin. The highest resistance was to ampicillin (42.86%) and cefotaxime (40.00%).

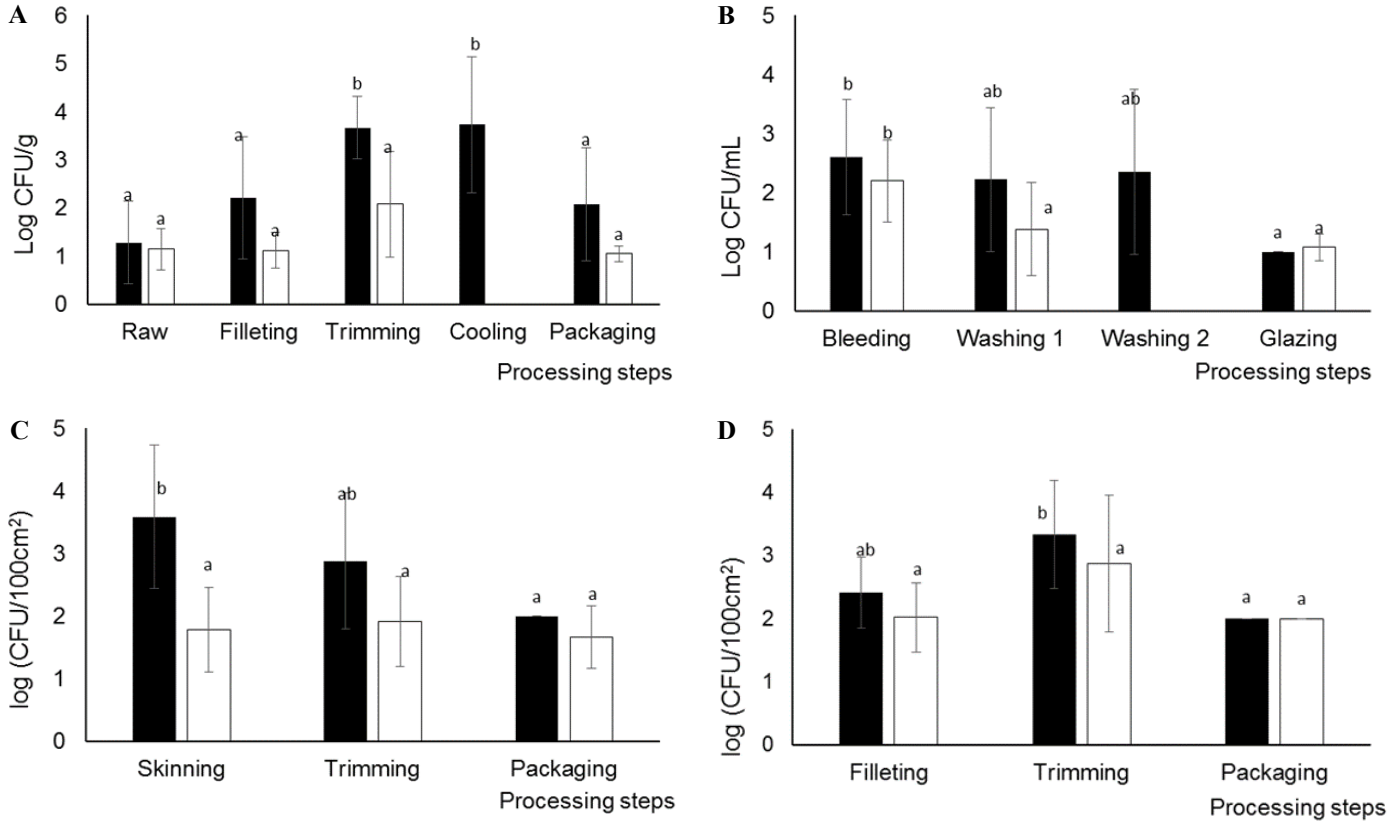

Figure 1. Trend of *E. coli* on fish (A), water (B), food contact surface (C) and hands (D) samples from PPF 1 (■) and PPF 2 (□) along the processing steps. Value with a different letter between processing steps in the same company sampled shows statistical significance ($p < 0.05$).

Resistance to sulfamethoxazole/trimethoprim and nalidixic acid was 20.00% and 22.86%, respectively while resistance to streptomycin, tetracycline and chloramphenicol was in the range of 11.00% - 17.00%.

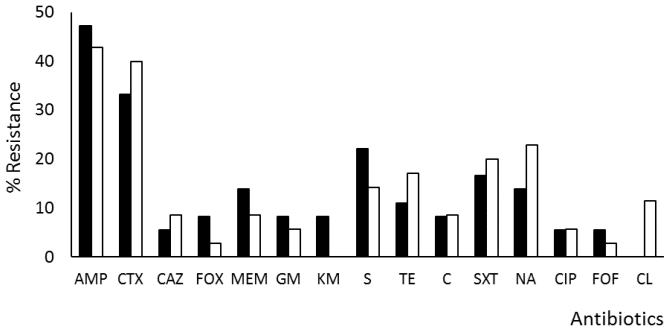

Figure 2. Resistance to antibiotics (%) among *E. coli* isolates from PPF 1 (■) and PPF 2 (□). AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, MEM: Meropenem, GM: Gentamicin, KM: Kanamycin, S: Streptomycin, TE: Tetracycline, C: Chloramphenicol, SXT: Sulfamethoxazole/trimethoprim, NA: Nalidixic acid, CIP: Ciprofloxacin, FOF: Fosfomycin, CL: Colistin

The differences observed in the resistance response might be as a result of different antibiotics commonly used in catfish ponds (Hong *et al.*, 2018), sources of raw *Pangasius* fish produced (Tong Thi *et al.*, 2013), origins of contamination with *E. coli* and seasonal periods of sampling (Le Nguyen *et al.*, 2008) i.e. PPF1 sampling in the dry season (January 2019) versus PPF2 sampling in the rainy season (August 2019). This may be the first study of antibiotics resistance of *E. coli* isolated from processing factories during the processing chain of *Pangasius* product; however, high resistance of *E. coli* to ampicillin has been reported in previous studies from cultured catfish and pond water in Vietnam (Sarter *et al.*, 2007; Divyashree *et al.*, 2019; Shivakumaraswamy *et al.*, 2019). Divyashree *et al.* (2019) reported maximum resistance of *E. coli* isolates obtained from effluents of two fish processing factories in Mangalore, India to ampicillin, tetracycline, and cefotaxime. According to Heuer *et al.* (2009), a wide range of antibiotic classes used extensively in aquaculture and human medicine lead to the incidence of resistant *E. coli* to higher generation antimicrobials. The present study clearly demonstrated that ampicillin and cefotaxime resistant - *E. coli* was present in *Pangasius* processing factories. These antibiotics (ampicillin, beta-lactam penicillin and cefotaxime as a 3rd generation cephalosporin) represent a group of antibiotics used in treating serious infections caused by *E. coli* and *Salmonella* (Nguyen *et al.*, 2016). Resistance to meropenem was also observed among the *E. coli* isolates from both PPF1 (13.89%) and PPF2 (8.57%) isolates. Meropenem is a carbapenem which is one of the most important groups of antimicrobials considered as the last line of drugs for treatment of severe infection (Murugan *et al.*, 2019). Likewise,

resistance to streptomycin, sulfamethoxazole/ trimethoprim and nalidixic acid among *E. coli* isolates from this study is also of importance both in treating bacterial diseases in *Pangasius* aquaculture and also in human medicine. The implication of these results is that such antimicrobial resistant bacteria may be transmitted through the human gut thereby entering the food cycle when undercooked fish is consumed.

Table 1a and 1b shows the antibiotic resistance pattern of individual *E. coli* from PPF1 and PPF2. The resistant *E. coli* isolates from PPF1 possess 16 distinctive resistance patterns (Table 1a). 50.00% (11/22) of the isolates from PPF1 were resistant to two or fewer antibiotics; whereas the remaining 50.00% of the isolates were multi-drug resistant (resistance 3-9 antibiotics). These multi-drug resistant isolates represent fish (i.e. raw fish, filleting and packaging), water (i.e. bleeding, first and second washing steps) and hand/gloves (i.e. filleting and trimming steps) (Table 1a). For isolates from PPF2, there were 19 distinctive patterns (Table 1b). 54.17% of the isolates were resistant to two or fewer antibiotics whereas 45.83% are multi-drug resistant (3-7 antibiotics) (Table 1b). The results from both factories showed possible sources of contamination of *Pangasius* products with multi-drug resistant bacteria along the processing chain. Multiple antibiotics resistance in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes each encoding a single antibiotic resistance phenotype (Daini *et al.*, 2008). For the raw fish, microbial flora present on fish is a representative of the aquaculture environment as well as the feed (Salgado-Miranda *et al.*, 2010). Even though the fish were starved while awaiting processing, they may still be kept in the fishing boat with the catch nets holding fish possibly inside the Mekong River close to the processing factories, and multi-drug resistant bacteria may be abundant in water bodies (Holt *et al.*, 2011). It is suggested that the processors should not only focus on reducing spoilage and pathogenic bacteria from the fillets but also put in high consideration the multi-drug resistant bacteria as they are present in almost every stage of processing and production chain. Some studies reported the prevalence of multi-drug resistant *E. coli* from cultured *Pangasius* catfish, other cultured fish species as well as wild fish species in Mekong Delta area

Table 1a. Antibiotic resistance pattern of *E. coli* isolates from PPF1 (n = 36)

No.	Resistance pattern	Number of antibiotics	Code	Processing step	Isolate source	%
1	NA	1	E33FT2	Trimming	Fish	
	NA	1	E12HF2	Filleting	Hands	
	NA	1	E12HF3	Filleting	Hand	
2	AMP	1	E22FP	Packaging	Fish	
	AMP	1	E22FF1	Filleting	Fish	
	AMP	1	E22FF2	Filleting	Fish	50.00[a]
	AMP	1	E12HT1	Trimming	Hand	
3	MEM	1	E11FF2	Filleting	Fish	
4	AMP-CTX	2	E22WB2	Bleeding	Water	
	AMP-CTX	2	E13FT2	Trimming	Fish	
5	CAZ-CTX	2	E21FT4	Trimming	Fish	
6	AMP-CTX-SXT	3	E33FR1	Raw fish	Fish	
7	AMP-CTX-MEM	3	E33W13	Washing 1	Water	
8	AMP-CTX-CAZ	3	E12FP2	Packaging	Fish	
9	AMP-S-TE-SXT	4	E31HF2	Filleting	Hand	
10	AMP-S-CTX-FOX	4	E13HT2	Trimming	Hand	
11	AMP-S-CTX-SXT-GM	5	E33FF1	Filleting	Fish	50.00[b]
12	AMP-S-CTX-SXT-TE	5	E31HF1	Filleting	Hand	
13	AMP-S-CTX-GM-C-NA-SXT-CIP	8	E33FR4	Raw fish	Fish	
14	AMP-S-CTX-FOX-MEM-KM-TE-FOF	8	E12WB2	Bleeding	Water	
15	AMP-S-MEM-KM-C-GM-NA-SXT-CIP	9	E32FR2	Raw fish	Fish	
16	AMP-S-MEM-KM-C-CTX-FOX-TE-FOF	9	E11W22	Washing 2	Water	

*total number of resistant isolates (22/36), [a]number of isolates resistant to two or less antibiotics over total number of resistant isolates, [b]number of isolates resistant to three or more antibiotics over total number of resistant isolates. AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, MEM: Meropenem, GM: Gentamicin, KM: Kanamycin, S: Streptomycin, TE: Tetracycline, C: Chloramphenicol, SXT: Sulfamethoxazole/trimethoprim, NA: Nalidixic acid, CIP: Ciprofloxacin, FOF: Fosfomycin, CL: Colistin

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Table 1b. Antibiotic resistance pattern of *E. coli* isolates from PPF2 (n = 35)

No.	Resistance pattern	Number of antibiotics	Code	Processing step	Isolate source	%
1	CAZ	1	E11FT1	Trimming	Fish	54.17[a]
	CAZ	1	E33FT1	Trimming	Fish	
	CAZ	1	E33W11	Washing 1	Water	
2	SXT	1	E12WB1	Bleeding	Water	
	SXT	1	E22FR1	Raw fish	Fish	
3	AMP	1	E21FRI	Raw fish	Fish	
	AMP	1	E22W11	Washing 1	Water	
4	FOF	1	E13WB2	Bleeding	Water	
5	NA	1	E33FF3	Filleting	Fish	
6	CTX-S	2	E33HT1	Trimming	Hand	
7	AMP-CAZ	2	E32FR2	Raw fish	Fish	
8	CAZ-FOX	2	E32WB1	Bleeding	Water	
9	GM-NA	2	E32WB2	Bleeding	Water	
10	AMP-S-SXT	3	E32FR1	Raw fish	Fish	45.83[b]
11	S-CAZ-CTX	3	E33FF2	Filleting	Fish	
12	AMP-CAZ-SXT-CL	4	E22FR4	Raw fish	Fish	
13	AMP-CAZ-S-TE	4	E22SS1	Skinning	Surface	
14	AMP-CAZ-MEM-C	4	E22WB1	Bleeding	Water	
15	AMP-TE-C-SXT	4	E31FR1	Raw fish	Fish	
16	AMP-CL-CAZ-TE-NA	5	E33HT2	Trimming	Hand	
	AMP-CL-CAZ-TE-NA	5	E33SS2	Skinning	Surface	
17	AMP-CL-CAZ-CTX-MEM	5	E31SS2	Skinning	Surface	
18	AMP-TE-SXT-GM-C-NA-CIP	7	E21W11	Washing 1	Water	
19	AMP-TE-SXT-CTX-CAZ-MEM-S	7	E33WB2	Bleeding	Water	

*total number of resistant isolates (24/35), [a]number of isolates resistant to two or less antibiotics over total number of resistant isolates, [b]number of isolates resistant to three or more antibiotics over total number of resistant isolates. AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, MEM: Meropenem, GM: Gentamicin, KM: Kanamycin, S: Streptomycin, TE: Tetracycline, C: Chloramphenicol, SXT: Sulfamethoxazole/trimethoprim, NA: Nalidixic acid, CIP: Ciprofloxacin, FOF: Fosfomycin, CL: Colistin

of Vietnam (Sarter *et al.*, 2007; Hon *et al.*, 2016). These multi-drug resistant *E. coli* was not only limited to cultured fish but also prevalent in raw food items, food distribution systems and healthy human which also carried resistance and virulence genes (Van *et al.*, 2008; Dyar *et al.*, 2012; Rasheed *et al.*, 2014; Done *et al.*, 2015; Danielle Mroz, 2018). The emergence of *E. coli* with multiple antibiotics resistant phenotypes, including co-resistance to four or more unrelated families of antibiotics was therefore considered a serious health concern (Sarter *et al.*, 2007). It is highly recommended that antibiotic resistant *E. coli* should be controlled as it was not only present in *Pangasius* catfish farms and environment but also prevalent in processing factories and among food operators. These findings are from two *Pangasius* processing factories and it can serve as background information for more studies to be done on several other *Pangasius* processing factories in the Mekong Delta region.

4. Conclusion

It can be concluded that even though both factories studied had implemented food safety and quality management systems such as HACCP, antibiotic resistant and multi-drug resistant *E. coli* were still found across the *Pangasius* processing chain. This showed that the current food safety management systems need to be reviewed to take into consideration multi-drug resistant bacteria. In addition, microbiological criteria required for *Pangasius* products destined for local and international markets should include multi-drug resistant bacteria to prevent the product from being a source of transfer of resistant genes both locally and internationally.

Conflict of interest

The authors declare no conflict of interest.

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