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# *Pangasius* Hypophthalmus Viscera as A Potential Vector of Bacterial Cross-Contamination and Resistance of *Escherichia Coli* to Antibiotics

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**Abstract**

The viscera of *Pangasius* fish was studied to provide baseline information about the presence of antibiotic-resistant *E. coli* on it. This aimed to assess the possible resistance of bacterial pathogens to antibiotics and cross-contamination into the fish's muscles during processing, as well as to evaluate the effect of starvation on the microbial loads of *Pangasius* fish viscera. The resistance of *E.coli* was tested against 15 antimicrobial agents using the disk diffusion method. The findings revealed that starvation reduced microbial loads on the viscera compared to non-starvation *Pangasius*. LAB, coliforms, and *E. coli* count on viscera of non-starved *Pangasius* were 7.0±0.5, 5.5±0.9 and 5.4±1.0 log CFU $g^{-1}$, whereas those of the starved fish were 2.6±0.8, 3.8±0.4 and 3.1±0.3 log $CFUg^{-1}$, respectively. A total of 55 *E. coli* isolated from *Pangasius* viscera were tested for antimicrobial susceptibility as stated above. Surprisingly, 69.09% of *E. coli* isolates were multi-antibiotic resistant from three to fifteen antibiotics tested. A high level of resistance to ampicillin (63.64%), ceftazidime (69.09%), nalidixic acid (78.18%) was observed. More importantly, 9.09% of the *E. coli* isolates were resistant to all kinds of antibiotics tested. As *E. coli* is a potential vector for transfer of antibiotic resistance gene, causing cross-resistance with human enteric pathogens, there is a need for both the prudent use of these antimicrobial agents in aquaculture and stringent appropriate infection control in the processing chain in Vietnam.

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## Introduction

With the aquaculture industry's rapid development, the probability of fish serving as a vector for human enteropathogenic bacteria has received increased attention.[1] Enteric and other infectious diseases have been linked to the existence of certain bacterial species such as *Listeria*, *Aeromonas*, *Pseudomonas*, *Staphylococcus*, *Salmonella* and *Escherichia*, on the flesh of fish, indicating that they have the ability to cause human disease when consumed or handled.[2-7] Gram-negative bacteria such as *Aeromonas hydrophila*, *Citrobacter freundii*, *Escherichia coli*, Enterobacter aerogenes, *Vibrio* anguillarum, Klebsiella, and *Pseudomonas* and Gram-positive bacteria such as *Listeria*, Bacillus and *Staphylococcus* were present in abundance in the fish's intestine.[1,8] These bacteria exist with high levels in the different organs of the fish intestines (i.e. liver, kidney, intestine etc.) which may be a great source of cross-contamination during processing.

Antibiotic-resistant *E. coli* derived from animals with elevated human carriage rates should not be ignored, as according to evidence, they contribute to the burden of human microbial resistance.[9,10]

The fish farming industry is rapidly expanding, particularly in Asia (including in Vietnam).[11] Besides, Vietnam's main source of foreign exchange is fisheries exports, aquaculture production accounts for about 65% of Vietnam's total fisheries exports. *Pangasius* (catfish) is the fisheries sector's second-largest commodity (behind shrimp), accounting for about half of the total export value.[12] The use of antibiotics extensively in aquaculture has resulted in the selection of multi-resistant bacteria in the fish gut flora, which are then transferred to the human gut commensal flora when the fish are consumed. The wide spread use of anti-microbials such as oxolinic acid, tetracycline, florfenicol, and nitrofurantoin has resulted in the selection of resistance in fish pathogens.[11,13]

In view of these facts, this study was to establish a baseline understanding for the existence of antibiotic-resistant *E. coli* in starved and non-starved *Pangasius* viscera. This aimed to assess the possible resistance to antibiotics and cross-contamination of the bacterial pathogens into the muscles of the fish during processing, as well as to evaluate the effect of starving *Pangasius* fish to limit cross-contamination in the products at the processing companies in the Mekong Delta, Vietnam.

## Material and Methods

### Sampling

Thirty of *Pangasius* viscera samples including 14 samples of starved fish and 16 samples of non-starved fish were collected randomly from two *Pangasius* processing company in An Giang and Dong Thap provinces, Vietnam from November 2018 to July 2020.

Samples of the whole *Pangasius* fish weighing approximately 2-3 kg were put into sterile bags, sealed and transported to Can Tho University's Food Microbiology Laboratory in insulated ice boxes within 2-3 hours after collection for analyzing bacterial criteria.

### Microbial analysis

The whole of the viscera of raw *Pangasius* fish were taken and transferred aseptically to new sterile containers (Stomacher bags, France) via sterile scalpels and tweezers. Twenty-five grams of these viscera samples taken from different parts (e.g. intestine, liver, kidney, stomach etc.) were transferred aseptically to a stomacher bag using sterile scissors and tweezers. Maximum Recovery Diluent (MRD, Merck, Darmstadt, Germany) was used for a ten-fold dilution,then the samples mixture was homogenized for five minutes. Subsequently, a decimal dilution, using 1 mL of the samples was made in MRD of 9 mL. Total aerobic mesophilic counts (TMC) and total anaerobic mesophilic counts (TAMC) was counted by pour-plating 1 mLof appropriate sample dilutions on Plate Count Agar (PCA, Merck, Darmstadt, Germany), with an over-layer for TAMC, followed by incubation at 37oC for 48-72h. *E. coli* and Coliforms were counted by the spreading of 0.1 mL of appropriate sample dilutions on Coliform Agar Enhanced Selectivity (Coliform Agar ES, Merck, Darmstadt, Germany) and incubated at 37°C for 24h (blue to violet colonies were counted as *E. coli* and pink to red colonies were counted as Coliforms-including *E. coli* group). For the determination of mesophilic lactic acid bacteria (LAB), de Man Rogosa Sharpe agar media (MRS, Merck, Darmstadt, Germany) was used by pour-plating with an over-layer and

then incubation for 48-72 h at 37°C. After incubation, the bacterial colonies were counted manually and presented as logarithms (log CFU $g^{-1}$).

For *E. coli*, after counting on Coliform Agar ES, three to five colonies (or fewer if five were not available or showed confluent growth) of each observed morphology (color, margin, surface and shape) were selected and then isolated to collect the pure colonies and carry out biochemical tests.

The confirmation of *E. coli* colonies was done using five biochemical tests: Indole, Methyl red, Voges-Proskauer, Citrate and Kligler Iron Agar test (Merck, Darmstadt, Germany).[14] All the confirmed *E. coli* isolates were then stored under -80° C in order to perform antibiotic susceptibility testing.

**Antibiotic Resistance Test**

The disk diffusion method was used to test antibiotic susceptibility of the *E. coli* isolates using Mueller-Hinton agar plates (MHA, Merck, Darmstadt, Germany).[15] As a control sample, *E. coli* strain ATCC 25922 was used. Antimicrobial agents used[14] were: ampicillin10 µg (AMP), meropenem 10 µg (MER), gentamicin 10 µg (GEN), tetracycline 30 µg (TET), chloramphenicol 30 µg (CHL), ciprofloxacin 5 µg (CPR), fosfomycin 200 µg (FOS) (Abtek, United Kingdom), ceftazidime 30 µg (Cz), cefotaxime 30 µg (Ct), cefoxitin 30 µg (Cn), kanamycin 30 µg (Kn), streptomycin 10 µg (Sm), sulfamethoxazole/ trimethoprim 23.75/1.25 µg (Bt), nalidixic acid 30 µg (Ng) and colistin 10 µg (Co) (Nam Khoa, Vietnam). The *E. coli* isolates were labeled as susceptible, intermediate, and resistant to the antibiotics according to the zone diameter interpretative standards recommended by Clinical and Laboratory Standards Institute (2019).[15]

**Statistical Analysis**

Microsoft Excel version 2019 (Microsoft Office, U.S.A.) was used to compute and graph the data. The microbial counts were compared using analysis of variance with significance level of 5%via SPSS Statistics version 20.0 (SPSS Inc., Chicago, U.S.A.). The data were reported as the mean value ± standard deviation.

**Results and Discussion**

**Microbial Counts of the *Pangasius* Viscera**

Figure 1 shows microbial loads (i.e. total counts of TMC and TAMC), LAB, coliforms and *E. coli* of non-starved and starved *Pangasius* viscera. Specifically, TMC, TAMC, LAB, coliforms and *E. coli* on viscera of non-starved *Pangasius* were 7.9±0.3, 7.9±0.4, 7.0±0.5, 5.5±0.9 and 5.4±1.0 log CFU $g^{-1}$, respectively. TMC, TAMC, LAB, coliforms and *E. coli* on viscera of starved *Pangasius* were 6.7±1.2, 6.8±1.0, 2.6±0.8, 3.8±0.4 and 3.1±0.3 log CFU $g^{-1}$,respectively (Fig. 1). These results showed that no significant difference was found in the total anaerobic counts ($p = 0.179$) as well as the total aerobic counts ($p = 0.160$) on the viscera. There were significant differences found in LAB, coliforms and *E. coli* between non-starved and starved *Pangasius* viscera ($p = 0.018$, $p = 0.006$ and $p = 0.000$, respectively).

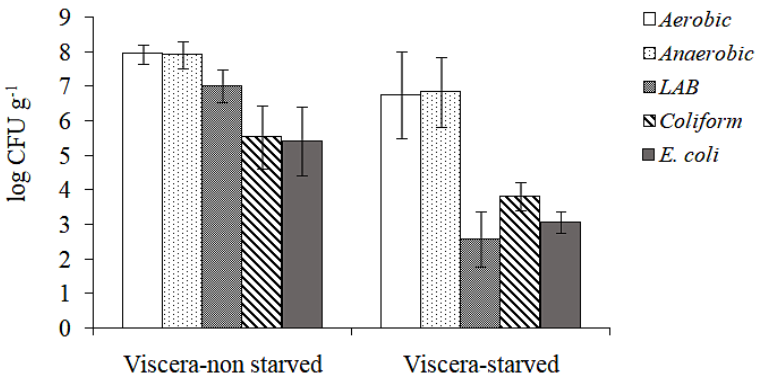


**Fig. 1: Total aerobic and anaerobicmesophilic counts (TMC and TAMC), lactic acid bacteria (LAB), coliforms, and E. coli of the Pangasius viscera**

The obtained results confirmed that starving *Pangasius* fish before slaughtering was effective at significantly reducing the intestinal microflora. The bacterial counts in digestive tract of fish can reach up to 8 log $CFUg^{-1}$. The number of microorganisms depended on various factors including the seasons, part of the digestive tracts of fish, and feeding types.[16] Depending on environmental conditions, the common microbiota in fish's gastro intestinal tract include *Vibrio*, *Aeromonas*, *Flavo bacterium*, *Plesiomonas*, *Pseudomonas*, *Enterobacteriaceae*, *Micrococcus*, *Acinetobacter*, *Clostridium*, *Fusarium* and, *Bacteroides* which may vary from species to species.[1,17] Diet (i.e. starvation) plays a major factor in forming gut microbiota and diverse bacterial species in the gut microbiota were extremely responsive to starvation.[18] In this study, the pathogens i.*e. coli forms* and *E. coli* enumerated on the viscera of starved *Pangasius* decreased significantly compared to the non-starved *Pangasius*. Therefore, starving of *Pangasius* fish before slaughtering (from two to three days) would be effective in partly limiting cross-contamination especially pathogenic and spoilage bacteria (i.e. LAB) for fish fillets during processing due to widely distributed microorganisms in the intestinal tracts.[19] The previous studies also reported that endogenous bacteria of intestinal tracts can contaminate at the filleting step due to viscera perforation.[4,20]

**Antibiotic Resistance**

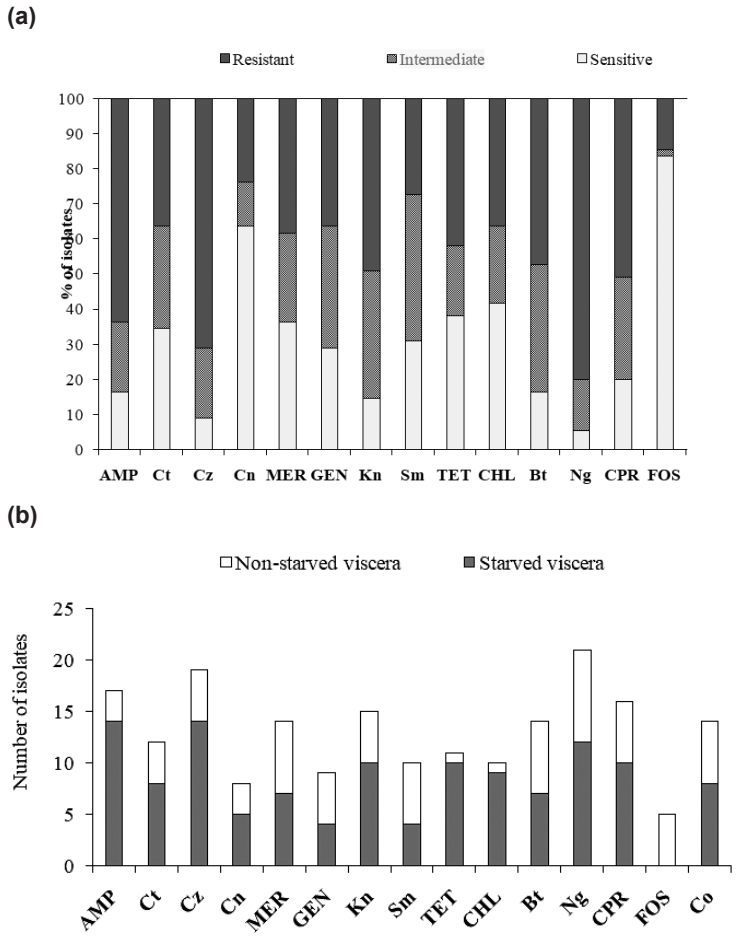


**Fig. 2: Profile of antibiotic resistance of 55 isolates of E. colifrom Pangasius viscera (a). The number and percentage of antibiotic resistance of theisolates from non-starved and starved Pangasius viscera (b) (AMP: ampicillin, Ct: cefotaxime, Cz: ceftazidime, Cn: cefoxitin, MER: meropenem, GEN: gentamicin, Kn: kanamycin, Sm: streptomycin, TET: tetracycline, CHL: chloramphenicol, Bt: sulfamethoxazole/ trimethoprim, Ng: nalidixic acid, CPR: ciprofloxacin, FOS: fosfomycin, Co: colistin).**

Fig. 2-a showed the high level of ampicillin resistance in *E. coli*i solates (63.64%), ceftazidime (69.09%) and nalidixic acid (78.18%), followed by sulfamethoxazole/trimethoprim, ciprofloxacin and kanamycin (45.5-49.1%); and chloramphenicol, cefotaxime, meropenem, tetracycline and colistin (36.4-41.8%). In contrast, the isolates were mostly susceptible to fosfomycin (83.64%). Fig. 2-b showed the prevalence of antibiotic-resistant *E. coli* isolated from the viscera of non-starved and starved *Pangasius*. The incidence rate of resistant isolates obtained from the viscera of non-starved *Pangasius* (47.8-71.2%) was seemingly higher compared to that from the starved *Pangasius* (16.7-52.2%). There were 38/55 (69.09%) isolates of multiple resistance *E. coli* (resistance to three or more kinds of antibiotics) among the samples. Surprisingly, 5/55 (9.09%) isolates were resistant to 15 antibiotics tested (Fig. 3).

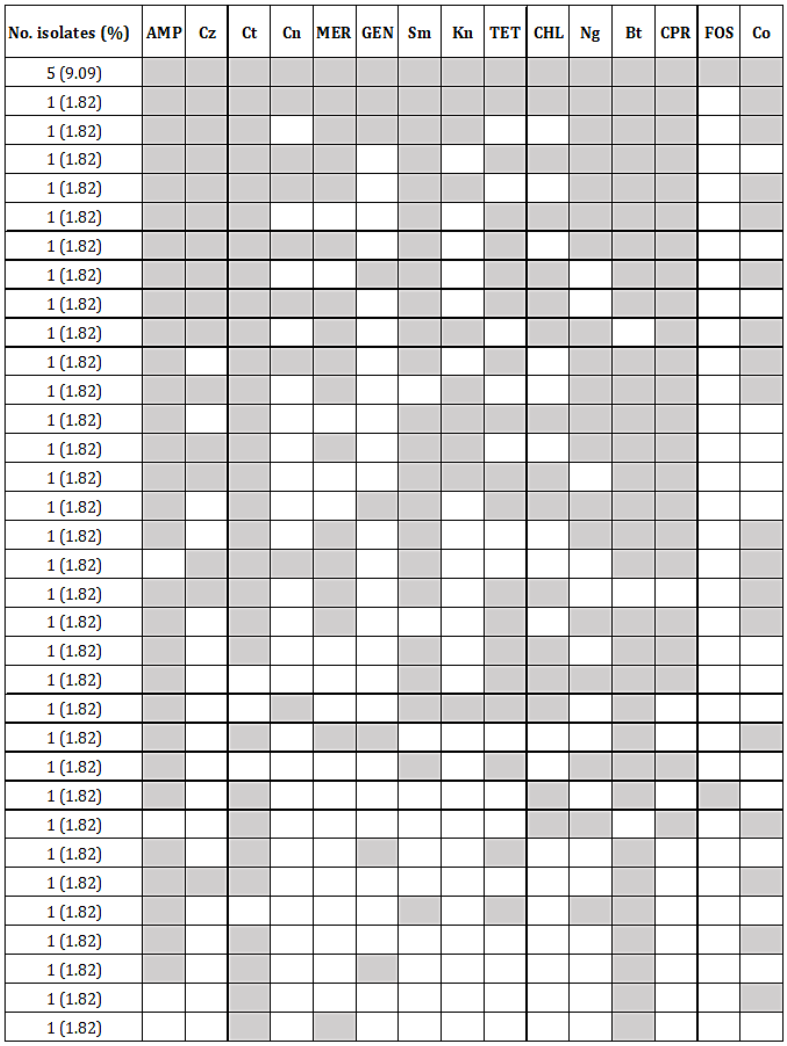


**Fig. 3: Multi-antibiotic resistance of 55 E. coli isolates from the viscera of Pangasius fish. The first column showed number of isolates resistant to multi-antibiotic and the percentage was calculated by the number of isolates resistant to 55 E. coli isolates in the bracket. The isolates that were resistant ▭ and not resistant ▭ to 15 antibiotics tested (AMP: ampicillin, Ct: cefotaxime, Cz: ceftazidime, Cn: cefoxitin, MER: meropenem, GEN: gentamicin, Kn: kanamycin, Sm: streptomycin, TET: tetracycline, CHL: chloramphenicol, Bt: sulfamethoxazole/trimethoprim, Ng: nalidixic acid, CPR: ciprofloxacin, FOS: fosfomycin, Co: colistin).**

A study of Salako *et al.* [21] reported that 61-69% of *E. coli* isolated from *Pangasius* freezing process at two factories in Mekong Delta Vietnam were resistant to ampicillin (43-47%), followed by cefotaxime (33-40%); and, prevalence of multi-drug resistance of the isolated *E. coli* was also reflected. It may explain that the accumulation of antibiotics in the water, environment, and ponds resulted in the occurrence of antibiotic resistant bacteria isolated from *Pangasius* fish during farming.Generally, starving of *Pangasius* fish before slaughtering would be effective in partly limiting the cross-contamination of fish fillet with pathogenic, spoilage, and antibiotic resistant bacteria during processing.

There was a connection between antibiotic use and the development of antibiotic resistance in bacterial pathogens.[22] It was recorded that there were more than 70 notifications of frozen *Pangasius* products contaminated with antibiotic substances i.e. chloramphenicol, ciprofloxacin, enrofloxacin, neomycin, etc.[23] which showed an evident of uncontrolling well the use of antibiotics during *Pangasius* fish farming. On the other hand, the prevalence of multi-antibiotic resistance of bacteria in pond water of cultured fish and fresh *Pangasius* fish has also been reported.[24,25] Jiang *et al.*[11] also reported that high levels of resistance to ampicillin, florfenicol, tetracycline and co-trimoxazole were found in 218 *E. coli* isolates recovered from farmed fish gut. In addition, some studies have shown that antibiotic use in animal husbandry can contribute significantly to the selection and spread of antibiotic-resistant bacteria in the environment.[26,27] Furthermore, aqua cultural products are sometimes at risk of antibiotic-resistant bacteria through the food chain and from handlers.[28,29] In contrast, a number of studies have mentioned that antibiotic over use did not always result in an increase in resistance because of a complex relationship between the use of antibiotics and the occurrence of antibiotic-resistant bacteria,[30,31] antibiotic-resistant bacteria might be abundant in the environment even when the corresponding antibiotics are not available and in the presence of co-selection by other antibiotics, horizontal gene transfer of resistant genes can play a key role in their dissemination in the environment.[32,33]

The emergence of *E. coli* isolates with multiple antibiotic-resistant pheno types (more detail in Fig. 3), involving co-resistance to four or more different antibiotic families has been previously mentioned and is regarded a serious health concern.[34,35] As *E. coli* is a potential vector for antibiotic resistance gene transfer, the possibility of horizontal transfer to human pathogens may occur.[22] In the present study, the origin of antibiotic resistance bacteria can be determined from viscera of *Pangasius* where they can contaminate into *Pangasius* fillets during processing. Hence, it should be taken into account that the cross-contamination of bacteria, especially antibiotic resistant bacteria can be limited during processing steps if the bursting of gut is avoided during processing i.e. gutting and filleting. This study, as far as we know, is one of the first report highlighting the microbial loads and the incidence of antibiotic-resistant *E. coli* isolates derived from the viscera of *Pangasius*. Intervention to control antimicrobial resistant bacteria during farming and processing is necessary in the follow-up studies.

## Conclusions

Generally, lower counts of lactic acid bacteria, coliform and *E. coli* in viscera of starved *Pangasius* compared to that of non-starved *Pangasius* was observed. The results of *E. coli* isolated from the *Pangasius* viscera showed that there were 69.09% of the isolates which were multi-antibiotic resistant. It was determined that there was a high level of resistance to ampicillin, ceftazidime and nalidixic acid. A total of 9.09% of isolates were resistant to fifteen antibiotics tested.There is a need for both the prudent use of these antimicrobial agents in aquaculture and stringent appropriate infection control in *Pangasius* processing chain in industry. Besides, treatments to control effectively antimicrobial resistant bacteria are also suggested for study in subsequent research.

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**Conflict of Interest**

The authors declares no conflict of interest.

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