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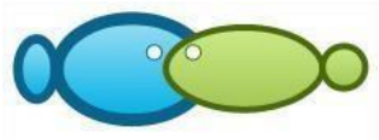

Bacterial resistance of *Escherichia coli* against antibiotics in *Clarias batrachus* digestion

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Abstract. Catfish (*Clarias batrachus*) is a freshwater fish with a high demand and an affordable price in Indonesia. However, in aquaculture, farmers usually use antibiotics for treatments, prophylaxis, growth improvement and feed efficiency. The excessive use of antibiotics can lead to bacterial resistance, especially for *Escherichia coli*, and can increase the risk of treatment failure in infected fish. This study aims to determine the resistance of *E. coli* found in the catfish digestion system to several antibiotics used in aquaculture. This study used a descriptive method with samples of 25 g from the digestive organs of *Clarias batrachus*. This study was conducted at the Education Laboratory of the Faculty of Fisheries and Marine Science, Surabaya, Universitas Airlangga, Indonesia. The main parameter observed was the inhibition zone produced by antibiotics, in the form of a clear zone. The determinations were made by a sensitivity test. *E. coli* bacteria obtained from the digestive organs of the catfish were isolated and several types of tests were conducted, *E. coli* assay, *E. coli* assertion, biochemical and antibiotic resistance tests. The resistance pattern of *E. coli* presents a resistance of 100% to tetracycline and ampicillin and 77.78% to chloramphenicol. The highest level of *E. coli* bacteria resistance was to tetracycline and ampicillin. However, gentamicin has the lowest resistance value and can be concluded that gentamicin still presents inhibitory abilities against pathogenic *E. coli* bacteria in catfish.
Key Words: ampicillin, aquaculture, catfish, fish disease, tetracycline.

Introduction. Catfish (*Clarias batrachus*) is a freshwater fish that has a high demand, affordable price and it is a good protein source (Debnath 2011; Ume et al 2016). The high demand attracts the development of catfish farming (Adewumi & Olaleye 2011). Farmers usually use antibiotics in catfish farming (Benbrook 2002; Chuah et al 2016). Antibiotics are administered for the treatment and prevention of diseases in catfish. Antibiotics are also used as growth accelerators and for maximizing feed efficiency (Samuel et al 2011).

The use of antibiotics as agents for healing disease is not always carried out correctly (Watts et al 2017). This can be triggered by an incorrect diagnosis of the disease or excessive use (Allocati et al 2013; Romero et al 2012; Watts et al 2017). Excessive use of antibiotics can lead to bacterial resistance and increase the risk of treatment failure in infected fish (Chuah et al 2016; Romero et al 2012). Ampicillin accounted for 28% of theurapetic antibiotics used in feed for aquaculture in Denmark, causing the resistance of *E. coli* and *Salmonella* to this antibiotic (Olesen et al 2004). In Malaysia, 17 *E. coli* strains were isolated from catfish and 7 of them had been shown to be positively resistant to tetracycline and ampicillin antibiotics (Samuel et al 2011).

E. coli is a gram negative bacterium found in the digestive tract of warm-blooded animals and humans (Kaper et al 2004). These bacteria are able to survive outside the host organism a long period of time (Allocati et al 2013). They are opportunist and several strains have been identified to cause serious diseases (Allocati et al 2013). *E. coli* can be easily disseminated in different ecosystems through the food chain and water (Ryu et al 2012). This bacterium belongs to the enterobacteriaceae group, which is commensal in nature but dangerous if it develops beyond its normal numbers in the digestive tract. The amount of *E. coli* from each gram of feces is 106-109 colonies (Susanto 2014). *E. coli* can grow with or without oxygen, even in nutrient-poor media, such as water, floors, and inorganic matter (Blackburn & McClure 2009). These

microorganisms are able to exchange genetic material with other bacterial species and it is possible that these microorganisms pass antibiotic resistance genes to transient pathogenic bacteria that cause disease in humans (Allocati et al 2013; Kaper et al 2004). Therefore, this study was conducted with the aim of determining the resistance of *E. coli* bacteria from catfish to several antibiotics that are often administered by farmers. It is expected to be used as preliminary data to deal with and overcome cases of commensal bacteria and pathogens that are resistant to antibiotics by knowing the resistance pattern of *E. coli* from catfish.

Material and Method

Design and variables. This study was conducted at the Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, East Java, Indonesia, in April 2016. This study used descriptive methods. The main parameters observed in this study were the inhibition zone in the form of a clear zone in the sensitivity test. Measurements were carried out 4 times on different sides of the inhibition zone because the inhibition zone did not form a perfect circle. Growth barriers to *E. coli* bacteria can be seen from the presence or absence of the established resistance zone. The obstacle zone formed was a clear area, which was measured using calipers. Biochemical tests for *E. coli* were conducted by using IMVIC media. The data obtained were analyzed descriptively by presenting the results of the antibiotic resistance test of *E. coli* isolated from the digestive organs of catfish. Analyses were performed regarding the inhibitory power of bacterial growth. Bacterial growth observations and measurements were done on the diameter of the inhibition zone in the form of a clear zone around the antibiotic disk. The bacterial isolation process was carried out by transferring 10^{-1} sample dilutions into a 1 mL of Butterfield's phosphate buffer (BFP). Furthermore, 1 mL of solution was incubated for 24 hours at 35°C. Turbidity and gas formation were observed in the Durham tube. The process was continued by conducting several types of tests, consisting of *E. coli* estimation test, *E. coli* confirmation test, biochemical tests and antibiotic resistance test.

Materials. The material used consisted of samples from 15 catfish (*Clarias batrachus*) farmed in the Mojokerto region (East Java, Indonesia). Buttterfield's phosphate uffer (BFP), lauryl tryptose broth (LTB), EC broth, Levin's eosin methylene blue (L-EMB) agar, trypticase soy agar (TSA), biochemical test media - tryptone broth (TB), MRVP, Simmons citrate agar, Mueller-Hinton agar (MHA), physiological NaCl, glycerol, antibiotics and alcohol were used in this study. The antibiotics used were ampicillin, chloramphenicol, tetracycline, and gentamicin.

E. coli estimation test. The *E. coli* estimation test was performed by observing the turbidity and gas formation after the incubation period. The EC broth tube was marked as positive when turbidity occurred in the solution and there was gas formation in the Durham tube. *E. coli* bacteria ferment lactose, resulting in changes in turbidity and gas. Lactose contained in the EC broth medium is fermented by *E. coli* bacteria, thus the solution becomes foggy because of the gas produced. The test was conducted at a normal temperature of 35°C, which is the general temperature of warm-blooded animal intestines and the temperature of the normal habitat of *E. coli* bacteria (Hazar et al 2012).

E. coli confirmation test. The confirmation test for *E. coli* was performed by using the streak plate method, by scratching the inoculating needle containing bacteria on the agar surface. After an incubation period of 24 hours at 35°C, the formation of bacterial colonies was observed. In regard to the EC broth positive tubes, the samples were inoculated on L-EMB agar medium. *E. coli* bacteria formed metallic shiny colors. The colonies that were found in the L-EMB medium were sterilized using TSA medium.

Biochemical test. The biochemical test consisted of the Indole (I) production test, the Voges Proskauer (VP) test, the Methyl Red (MR) test and the Citrate test. In the Indole

production test, a positive reaction is indicated by the formation of a purple ring in the upper layer of the media and a negative reaction is indicated by the formation of a yellow ring. In the Voges Proskauer (VP) test, positive reactions are characterized by the formation of eosin pink to ruby red colors. In the Methyl Red (MR) test, a positive reaction is characterized by the formation of a red color and the negative reaction by the formation of a yellow color. In the Citrate test, a positive reaction is indicated by the apparent change in color from green to blue and the negative reaction is marked by no apparent color change in the media. Citrate test was done by inoculating 9 isolates in Simmon's citrate agar.

Antibiotic resistance test. Antibiotic resistance tests were performed using the disc diffusion method. From the antibiotic resistance tests, observations were made on the presence of inhibitory zones that formed around the antibiotic disk. The inhibition zone was measured using calipers to determine the strength of *E. coli* bacterial resistance to the antibiotics tested. Susceptible (s), intermediate (I) and resistant (T) bacteria were determined by the size of the diameter of the inhibition zone formed by the CSLI standard. Bacteria were grown in MHA media, using the streak plate method with cotton swabs. Four antibacterial disks were placed with equal distance among them. Antibiotics would diffuse into the media, thus inhibiting bacterial growth. The inhibition zone in the form of a clear zone formed was measured and compared to the standard to determine the nature of bacterial resistance. The antibiotics used were ampicillin, tetracycline, gentamicin and chloramphenicol. These four antibiotics are very easy to obtain and are found to have been resistant to pathogenic bacteria.

Results and Discussion. Biochemical test results of *E. coli* bacteria from the digestive organs of catfish for confirming the presence of *E. coli* bacteria can be seen in Table 1. The 15 colonies from petri presented a metallic green color with or without a black spot in the middle, thus 15 isolates of Escherichia coli bacteria were obtained.

Table 1

Biochemical tests for *Escherichia coli* bacteria from the digestive organs of catfish (*Clarias batrachus*) on several antibiotics

Isolate	*Biochemical Test Parameters*				*Identification Results*
	Indole	MR	VP	Citrate	
MJ 1	+	+	-	-	*E. coli*
MJ 2	+	+	-	-	*E. coli*
MJ 3	+	-	-	+	No *E. coli*
MJ 4	-	+	-	-	*E. coli*
MJ 5	+	+	-	+	No *E. coli*
MJ 6	-	+	-	-	*E. coli*
MJ 7	+	+	-	+	No *E. coli*
MJ 8	+	+	-	-	*E. coli*
MJ 9	-	+	-	-	*E. coli*
MJ 10	-	+	-	-	*E. coli*
MJ 11	+	-	-	+	No *E. coli*
MJ 12	+	+	-	+	No *E. coli*
MJ 13	+	+	-	-	*E. coli*
MJ 14	+	+	-	-	*E. coli*
MJ 15	+	+	-	+	No *E. coli*

Note: MJ – sample of digestive organs from catfish; MR - methyl red test; VP - Voges Proskauer test.

The results of the biochemical test analysis in Table 1 depicted that of the 15 isolates tested, 9 bacterial isolates were identified as positive were *E. coli* and 6 bacterial isolates were not *E. coli* bacteria. This shows that the digestive tract of the catfish contains *E. coli* bacteria, further tested in the antibiotic sensitivity test to determine the bacterial

resistance profile to antibiotics - tetracycline, ampicillin, chloramphenicol and gentamicin. The results of the sensitivity test for *E. coli* bacteria can be seen in Table 2.

Table 2

Escherichia coli sensitivity to antibiotics test

Antibiotics	*Disc Content*	*Isolate Count*		
		Susceptible	Intermediate	Resistant
TE	30 µg	0	0	9
AMP	10 µg	0	0	9
C	30 µg	1	1	7
CN	10 µg	7	0	2

Note: TE - tetracycline; AMP - ampicillin; C - chloramphenicol; CN - gentamicin.

The bacterial sensitivity test results for 9 *E. coli* bacterial isolates show that all 9 isolates are resistant to ampicillin and tetracycline. 7 isolates showed resistance to chloramphenicol, while only 2 isolates showed resistance to gentamicin.

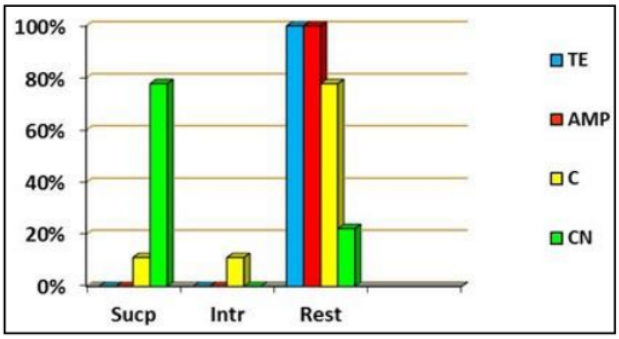

Figure 1. *Escherichia coli* resistance profile. Sucp – susceptible, Intr – intermediate; Rest – resistant; TE – tetracycline; AMP – ampicillin; C – chloramphenicol; CN - gentamicin.

Based on Figure 1, the highest resistance levels were found for tetracycline and ampicillin, while the lowest resistance value was noted for gentamicin. Gentamicin is still able to inhibit the growth of pathogenic *E. coli* bacteria. Chloramphenicol experienced a decrease in antibacterial properties, but still presented some antibacterial properties, the bacterial resistance being 77.77%. The resistance pattern of *E. coli* isolated from the digestive organs of catfish presented resistance to tetracycline and ampicillin by 100%.

According to the test results on the dilution of the LTB and EC broth tubes, the lactose medium, which was incubated for 24 to 48 hours, showed more than 10% gas and turbidity in the tube.

Petri MJ, 3, 5, 7, 11, 12 and 15 showed a growth of *E. coli* bacteria with a metallic green color, but they did not form a single colony. Meanwhile, in petri MJ 4, 6, 8, 10, 12 and 14, there was no metallic shiny green color formation found, but there was a single colony indicating that *E. coli* grows on this medium. Isolation of *E. coli* on L-EMB agar media did not form a single colony due to the presence of other bacteria that accumulated and were not completely separated.

From 15 samples of isolates that had gone through the Indole test, 9 isolates contained *E. coli* bacteria, while the other 6 showed negative results. This can be seen from the formation of a red ring on the upper part of the fermentation medium after the addition of Kovac's reagent. In a previous study, Indole test proved that the *E. coli* bacteria belonging to the faecal group could break down tryptophan (Percival & Williams 2014). The tryptophanase enzyme contained in the bacterium *E. coli* breaks tryptophan and ammonia (Hazar et al 2012).

The MR and VP tests used the MR and VP liquid media in which the methyl red reagent was added in the MR test. In the MR test, there were 9 isolates which showed a positive reaction with the formation of a red liquid media surface. This can be interpreted that bacteria are able to produce acid as the final product of glucose fermentation. Besides, VP I and VP II reagents were added in VP test. There were also 9 isolates reacting positively in the VP test, which showed that the bacteria were able to produce acidic products from organic acids produced from the glucose metabolism.

The results of the citrate test did not indicate a change from the green color of the medium to blue and showed no growth of *E. coli* bacteria. It can be interpreted that *E. coli* did not use citrate as a carbon source. *E. coli* bacteria can use acetate as a carbon source (Hazar et al 2012). The largest inhibition zone was found in petri containing gentamicin and chloramphenicol. The greatest resistance was found for tetracycline and ampicillin. Ampicillin is a semisynthetic penicillin that is stable against acid or amidase, whereas it is not resistant to the β-lactamase enzyme (Olesen et al 2004). Ampicillin presents antibacterial activities against Gram positive bacteria and Gram negative bacteria and is a broad spectrum antibiotic. *E. coli* and *Proteus mirabilis* bacteria are sensitive Gram negative germs (MacInty et al 1991). Tetracycline has a broad antibacterial spectrum and it is effective against Gram positive and Gram negative germs, including the spectrum of penicillin, streptomycin and chloramphenicol (Chopra & Roberts 2001). Tetracycline is categorized as a bacteriostatic antibiotic (Sykes & Papich 2014). There are several bacteria that are sensitive to tetracycline, such as β-hemolytic *Streptococci*, non-hemolytic *Streptococci*, *Clostridia*, *Brucella* and *Haemophilus* bacteria. On the other hand, *E. coli, Pasteurella*, *Salmonella* and *Corynebacterium* are somewhat sensitive to tetracycline (Booth & McDonald 1988). Some germ species, especially hemolytic *Streptococci*, *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Bacteroides*, *Shigella* and *Staphylococcus aureus* are increasingly resistant to tetracycline.

Similar to tetracycline, chloramphenicol is also bacteriostatic (Smolin et al 2005). Chloramphenicol could strongly inhibit protein synthesis in microorganisms (Das & Patra 2017). Resistance to chloramphenicol could be mediated by the drug chemical inactivation and drug efflux (Bischoff et al 2002). Gentamicin is an antibiotic included in the aminoglycosides group (Krause et al 2016). Gentamicin is effective against various strains of Gram negative germs, including *Brucella* spp., *Calymmatobacterium granulomatis*, *Campylobacter*, *Citrobacter*, *Escherichia*, *Enterobacter, Klebsiella, Proteus*, *Providencia*, *Pseudomonas*, *Serratia*, *Vibrio* and *Yersinia* (Lee et al 2017).

Antibiotic resistance in this study was manifested against tetracycline, ampicillin and chloramphenicol. Usually, the use of tetracycline, in addition to causing resistance in *E. coli*, also increases resistance in *Streptococcus β Haemolyticus* (Samuel et al 2011). Antibiotic resistance from *E. coli* isolates obtained from the digestive organs of catfish could be an outcome of the use of supertetra drugs (Tetracycline + HCl 250 mg/capsule) that have been administered regularly by farmers in order to prevent and treat diseases (Beier et al 2004). Supertetra itself is a generic drug that contains the active substance of tetracycline. Thus, if we continue to use supertetra in the prevention and treatment of the diseases of catfish, it will have an impact on bacterial resistance. Irrational use of antibiotics will cause low effectiveness, followed by unnecessary toxicity and resistance acceleration (Maharani 2015; Sáenz et al 2004).

The location of a pond adjacent to a chicken or duck coop also affects the possibility of transferring bacterial genes into catfish ponds. At the time of maintenance, the catfish were not treated with ampicillin or chlorogenic acid, but the results show that there are very high resistance levels of *E. coli* to ampicillin and tetracycline. This can occur due to gene mutations in *E. coli* bacteria against recognizable antibiotics. Whereas, gentamicin antibiotics still had antibacterial properties, the resistance being low. This is possible because of the rare use of aminoglycoside antibiotics in catfish farming, as well as in the farming of poultry located near fish ponds. Catfish farmers rarely use gentamicin, because it is only used for the treatment of Gram-negative bacterial infections.

Conclusions. *Escherichia coli* bacteria isolated from the catfish digestive organs showed a high level of resistance against tetracycline, ampicillin and chloramphenicol. However, gentamicin still presents antibacterial properties against *Escherichia coli* bacteria. Thus, it can be concluded that gentamicin is still able to inhibit the growth of pathogenic *E. coli* bacteria in catfish farming.

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