Determination of withdrawal time, efficacy, and safety test of enrofloxacin in carp (Cyprinus carpio)

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Abstract. The use of antibiotic such as enrofloxacin for the treatment of fish diseases needs to be appropriately controlled so that the medicine function effectively and does not cause antibiotic residues above the maximum residues limit (MRL). The study aims to determine the duration of administration, efficacy, and safety of enrofloxacin in carp. The size of the carp used was 10-40 g, with a length of 8-12 cm. The methods used in this study were (a) withdrawal time test, (b) efficacy and safety test, and (c) histopathology analysis. The results of determining enrofloxacin withdrawal time for 0.5 MRL and 1 MRL were two days and eight days, respectively. The survival rates of carp in post-challenge with Aeromonas hydrophila for the positive control and efficacy groups were 86.7% and 88.9%, respectively, and were not significantly different. The results of histopathological observations on the liver, spleen, kidney, and muscle of the carp showed no significant pathological changes between the safety test dose treatment and the control treatment. Keywords: Aeromonas hydrophila, enrofloxacin, carp, withdrawal time

1 Introduction

Carp (Cyprinus carpio) is a type of consumption freshwater fish with a long shape and soft flesh. It has been kept since 475 BC in China, while carp were held around the 1920s in Indonesia. Carp found in Indonesia was a type of carp from China, Europe, Taiwan, and Japan. Until now, ten types of carp have been identified based on their morphological characteristics [1]. It is also used as an ornamental fish, which is quite popular.

The carp farmer has a problem with fish disease, which always occurs in fish culture, mainly caused by pathogenic bacteria. One of the bacterial diseases that often attacks fish is a red-sore disease, caused by the bacteria Aeromonas hydrophila, also known as “Motile Aeromonas Septicemia (MAS)” [2]. Aeromonas disease can cause up to 80% mortality in a short period (1-2 weeks) [3], which is detrimental to fish farmers. This disease occurs due to stressful conditions due to high fish density, poor feed (malnutrition), rough fish

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handling, poor water quality (e.g., low oxygen), and extreme fluctuations in water temperature.

Bacterial infection of *A. hydrophila* is acute, infects all freshwater fish, and can cause death up to 100% [4]. Many methods have been carried out to control *A. hydrophila* bacterial infection in fish by treatment and prevention. One way to control fish disease is by administering antibiotics. However, the problem that often occurs is fish diseases become more resistant to antibiotics used, caused by using antibiotics not in the correct dose and time [5].

Enrofloxacin is the type of antibiotic which often used in the treatment of fish diseases. This fluoroquinolone group was first used for veterinary medicine with its primary metabolite, ciprofloxacin [4]. Excessive and continuous use of enrofloxacin can cause antibiotic residues in fishery products and cause bacterial resistance to antibiotics. Therefore, antibiotics such as enrofloxacin for treating antibiotic fish diseases must be adequately controlled so that the medicine functions effectively and appropriately and does not cause antibiotic residues above the maximum residues limit (MRL).

Japan and the USA still tolerate certain antibacterials regarding the quality standards or Maximum Residue Limit (MRL) set. This antibacterial use policy needs to be regulated in such a way as to meet food safety requirements with residue content below the residue limit. It is necessary to carry out effective control through pharmacokinetic studies, including withdrawal time. Determination of withdrawal time must be carried out systematically and comply with scientific principles. With the withdrawal time data, antibiotic residues still contained in fish meat after treatment are expected to be likely below the Maximum Residue Limit (MRL) so that the product is safe for consumption and can be accepted in the global market. The study aims to determine the duration of administration, efficacy, and safety of enrofloxacin in carp.

### 2 Material and method

Materials used in this activity included carp with a length of 8-12 cm and a weight of 10-40 g per fish, enrofloxacin antibiotics, enrofloxacin ELISA kit, and *Aeromonas hydrophilla* isolate. The equipment needed includes 2 m³ fiber tank, ELISA reader, and water quality checker.

#### 2.1 The efficacy and safety test

The efficacy and safety test design consisted of 4 groups (negative control group, positive control group, and treatment group, each with three replications, and the safety test group without replication). The description of each group is as follows:

a. The negative control groups
   - Carp were kept in a rearing tank without being injected with bacteria and without antibiotics.

b. The positive control groups
   - Carp were kept in a rearing tank and injected with bacteria and without antibiotics.

c. The efficacy test group
   - Carp were kept in a rearing tank, injected with bacteria, and given antibiotics as recommended dosage.

d. The safety test group
   - Carp were kept in a rearing tank, injected with bacteria, and given antibiotics twice the recommended dosage.

The isolate used *Aeromonas hydrophilla* with an injected dose of $0.5 \text{ ml } 10^5 \text{ CFU per fish}$, and antibiotics used enrofloxacin with a recommended dose $1 \text{ g}/100 \text{ g feed}$. 

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The test was carried out in 2 m³ fiber tank, with a 30 fish/tank. The test fish were acclimatized for three days, then the treatment was carried out according to the test design. For the efficacy test, the treatment group's treatment was carried out via feed one day after being injected with the bacteria. Mixing antibiotics in the feed using the surface binder method and then drying it. The feed is made every day (the antibiotic feed that will be given the next day is made the previous day). Antibiotic feeding was carried out three times a day. For the safety test, the way of making and the feeding time is the same as the efficacy test, with antibiotics being two times the recommended dose. One-way ANOVA analyzed the test samples (fish meat) were homogenized with a tissue grinder and analyzed in duplicate. Analysis was performed by ELISA method data analysis using exponential or logarithmic regression equations on upper values with 95% confidence intervals. The withdrawal is determined based on the most extended time equation in meat until it has the same value as the MRL (Maximum Residue Limit) or 50% from MRL (1 MRL = 100 μg/kg).

2.2 The withdrawal time test

There were two groups in the withdrawal test, control group (feed without antibiotics) and the treatment group (feed added with antibiotics). Each group has three replications. About 150 carp were reared in 2 m³ fiber tank. Fish were fed two times a day. The treatment group was provided a mixture of enrofloxacin antibiotics at 1 g/100 g of feed. Administration of enrofloxacin is carried out 3-5 days in a row.

Six fish samples were taken after the last treatment on days 0, 3, 6, 12, 24, 36, and 48 (day 0 is the last day of treatment), respectively. The test fish samples (fish meat) were homogenized with a tissue grinder and analyzed in duplicate. Analysis was performed by ELISA method data analysis using exponential or logarithmic regression equations on upper values with 95% confidence intervals. The withdrawal is determined based on the most extended time equation in meat until it has the same value as the MRL (Maximum Residue Limit) or 50% from MRL (1 MRL = 100 μg/kg).

2.3 Histopathology analysis

Histopathological data analysis was carried out on several organs of the carp, specifically the liver, spleen, kidney, and muscle. Based on the results of the diagnosis, further conclusions are drawn as follows:

a. Antibiotics are considered safe (positive safety test) if the antibiotics do not produce pathological changes that are pathognomonic when compared to the control group.

b. Antibiotics are considered unsafe (negative safety test) if the administration of antibiotics causes pathognomonic pathological changes.

2.4 Water quality analysis

Examination of water quality parameters such as DO (dissolved oxygen), pH (acidity), and temperature are measured twice daily. In contrast, nitrite parameters are carried out at the beginning of the test.

3 Results and Discussion

3.1 Determination of withdrawal time of enrofloxacin

The results of enrofloxacin concentrations in ordinary pellet feed, pellet feed mixed with enrofloxacin, and pre-treated carp meat were presented in Table 1 below.
Table 1. Concentration of enrofloxacin antibiotics in pellets and pre-treated carp meat.

<table>
<thead>
<tr>
<th>No.</th>
<th>Samples</th>
<th>Enrofloxacin residue</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ordinary pellet fish</td>
<td>1.04</td>
<td>μg/kg</td>
</tr>
<tr>
<td>2.</td>
<td>Pellet feed mixed with enrofloxacin</td>
<td>918.29</td>
<td>μg/kg</td>
</tr>
<tr>
<td>3.</td>
<td>Pre-treated carp meat</td>
<td>5.48</td>
<td>μg/kg</td>
</tr>
</tbody>
</table>

Enrofloxacin concentrations in fish pellets before and after mixing with antibiotics were 1.04 μg/kg and 918.29 μg/kg. This indicates that the fish pellets used in this activity did not significantly contribute to enrofloxacin. However, this is presumably due to the mixing process of antibiotics and pellets using water as an adhesive, then sprayed and stirred manually by hand and dried. The occurrence of homogenization is very relative to this technique. The expected assumption is that antibiotics can be mixed evenly in the pellet. However, this suit is done.

The carp used contained enrofloxacin with a concentration of 5.48 μg/kg. This could happen because there was an unknown track record of fish rearing from the fish farmer. The carp hatchery process carried out by farmers probably used a mixture of enrofloxacin antibiotics in the previous production process. In addition, weak supervision of the circulation of pellets containing antibiotics is also a factor in the occurrence of antibiotic contamination in fish fry. Other efforts that must be made are the application of Good Fish Hatchery Methods (CPIB) by fish farmers, including using vaccines to prevent fish diseases and controlling the use of antibiotics in hatchery activities so that the resulting fish seed products may not contain antibiotics.

The results of enrofloxacin concentrations in the control group and treatment group carp were presented in Table 2 below.

Table 2. The concentration of enrofloxacin antibiotics in the control group and treatment group carp.

<table>
<thead>
<tr>
<th>Days</th>
<th>Enrofloxacin residues (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
</tr>
<tr>
<td>0</td>
<td>1.88 ± 2.65</td>
</tr>
<tr>
<td>3</td>
<td>8.98 ± 6.66</td>
</tr>
<tr>
<td>6</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>24</td>
<td>1.15 ± 1.60</td>
</tr>
</tbody>
</table>

Table 2 shows that the concentration of enrofloxacin in control and treatment groups of carp meat decreased with the observation time. The reduction of enrofloxacin concentrations in control carp was relatively stable compared to carp treated with antibiotics. This could happen because, in average physiological processes, fish will experience natural detoxification of toxic substances, which could still be tolerated by each type and size of fish so that fluctuations in enrofloxacin concentrations are more stable. Meanwhile, carp that were given feed containing antibiotics showed a relatively constant decrease until the end of the observation.

The results of enrofloxacin antibiotics withdrawal time data processing on carp meat are presented in Figure 1 below.
Based on the intersection of the line equation between WT = 1 MRL, specifically the equation $y = 2$ and the upper log line equation for enrofloxacin concentration $Y = -0.0495X + 2.0691$, the withdrawal time (WT) of enrofloxacin antibiotics can be determined in Table 3 as follows:

**Table 3. Determination of antibiotics withdrawal time for enrofloxacin.**

<table>
<thead>
<tr>
<th>Description</th>
<th>Enrofloxacin concentrations (µg/kg)</th>
<th>Log (y)</th>
<th>Days (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT = 1 MRL</td>
<td>100.00</td>
<td>2.00</td>
<td>1.4 ± 2</td>
</tr>
<tr>
<td>WT = 0.5 MRL</td>
<td>50.00</td>
<td>1.70</td>
<td>7.46 ± 8</td>
</tr>
</tbody>
</table>

Based on the fish antibiotics withdrawal time testing procedure, the withdrawal time was determined based on the intersection of the linear regression lines from the upper limit of 95% confidence tolerance and the limit of WT = 1 MRL. Thus, the withdrawal time for enrofloxacin antibiotics at a dose of 1 g/100 g feed/day is two days. If the withdrawal time is determined based on the intersection of the lines between the linear regression of the upper limit of 95% confidence tolerance and the limit of WT = 0.5 MRL. In that case, the antibiotics withdrawal time is eight days. The results of enrofloxacin withdrawal time in carp for 1 MRL and 0.5 MRL were two days and eight days. That was presumably because each type and size of fish had a different ability to metabolize the antibiotics it absorbs.

### 3.2 The efficacy test

The parameters observed in the efficacy test were mortality and survival rate (SR) data. The number of fish used in each tank was 30 fish. Data on the number of mortal fish during antibiotics administration after fish were exposed to *Aeromonas hydrophila* were listed in Table 4.

The efficacy test results showed that there were 78 remaining survival fish in the positive control tanks, while in the efficacy group, there were 80 fish. The average percentage of survival fish in the positive control group and the efficacy group were 86.7% and 88.9%, respectively. The rate of survival fish in the positive control group was lower than in the efficacy group. This showed that the administration of enrofloxacin in an efficacy test was proven to control diseases caused by *Aeromonas hydrophila*, although there was no significant difference.
Table 4. Carp mortality and survival data on efficacy test of enrofloxacin post-challenge with *Aeromonas hydrophilla*.

<table>
<thead>
<tr>
<th>Days</th>
<th>NC1</th>
<th>NC2</th>
<th>NC3</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Mortality** 0 0 0 2 7 3 3 5 2  
**Survival** 30 30 30 28 23 27 27 25 28  
**% Survival Rate** 100 100 100 93.33 76.67 90.00 90.00 83.33 93.33  
**Mean % Survival Rate** 100 86.7 88.9  

NC = negative control, PC = positive control, E = efficacy

Based on statistical tests with a 95% confidence interval, it was shown that there was no significant difference between the positive control group and the efficacy group in Table 5 (p-value > 0.05). This indicates that the administration of enrofloxacin antibiotics did not affect the treatment of diseases caused by *Aeromonas hydrophilla*. The recommended dosage of enrofloxacin in this study may not be effective in controlling *A. hydrophila* infection and should be re-evaluated. Otherwise, an injected dose of *A. hydrophila* in carp was under quorum sensing levels of virulence so that carp in the positive group could recover from infection and efficacy group.

Table 5. One-way ANOVA of survival data of positive control and efficacy groups

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>0,67</td>
<td>1</td>
<td>0,67</td>
<td>0,14</td>
<td>0,72</td>
<td>7,71</td>
</tr>
<tr>
<td>Within Groups</td>
<td>18,67</td>
<td>4</td>
<td>4,67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19,33</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3 The safety test

Figures 2, 3, 4 and 5 show the safety test results for enrofloxacin antibiotics in carp. Initial histopathological changes in the control and safety groups showed similar changes in the liver, kidney, spleen, and muscle.

3.3.1 Liver

This organ has several significant changes, such as inflammation, degeneration, congestion, necrosis, oedema, and the melano-macrophage center (MMC) (Figure 2). At the start of testing, both in the control and safety groups, congestion and hepatitis predominate.
Meanwhile, degeneration and necrosis were more common in the early control group. At the end of the test, the fish with congestion and hepatitis in the control group were reduced more in the safety group. While changes in necrosis and degeneration in both groups increased, there was no difference.

![Liver Organ](image)

**Fig. 2.** Liver histopathological changes in all groups at the initial and final of the test.

### 3.3.2 Kidney

In the safety test, the kidneys changed, such as inflammation, necrosis, congestion, protein accumulation, melano-macrophage center (MMC), and vacuolization (Figure 3). At the beginning of the test, inflammation, protein accumulation, and congestion occurred a lot in both the control and safety groups with more safety composition. However, at the end of the test, the inflammation in the control group was reduced, but the congestion and protein accumulation increased a lot. In contrast, inflammation was not found, and congestion was greatly reduced. In both groups, at the end of the test, there was a higher amount of necrosis at the end of safety. At the same time, the amount of protein accumulation tends to increase in both groups.
3.3.3 Spleen

During the enrofloxacin safety test, the spleen organ changed, such as splenitis, vacuolization, MMC, congestion, and protein accumulation. MMC and splenic congestion were present in the early control and safety groups. Inflammation of the spleen was only found in the initial control group. At the end of the test, changes in the spleen organ in the final control group varied. Splenitis, vacuolization, MMC, congestion, and protein accumulation were found in small amounts. While the final safety group only observed MMC for all individuals.
3.3.4 Muscle

In the muscle organs at the initial test, the initial control group occurred necrosis and myositis. Whereas the initial safety group only occurred inflammation. At the final of the trial, the changes in the initial control group were significant. The amount of necrosis decreased, but inflammation increased dramatically, coupled with degeneration and parasitic infestation. In the final safety group, there was a small amount of necrosis and inflammation, which did not change much compared to the beginning of the test.

![Muscle Organ Diagram](image)

**Fig. 5.** Muscle histopathological changes in all groups at the initial and final of the test.

In the safety test for enrofloxacin, both in the liver and kidneys, at the final of the trial, the safety group showed a decrease in individuals with inflammation and congestion. Changes such as necrosis and liver degeneration did not differ much from the final control. Kidney necrosis and protein accumulation were found in both groups at the final test. Still, the number of fish with these conditions was higher in the final safety group than in the final control.

The histopathological changes in the final safety group in the spleen looked better than in the control group. Melano-macrophage center (MMC) was observed in all safety groups. While in muscle, both the control and safety groups had histopathological changes. However, at the final of the test, necrosis and myositis were observed in the final safety group. necrosis and myositis were observed. However, this condition was similar to the control group at the last initial of the test.

Enrofloxacin is a quinolone antibiotic that is widely used in livestock and fisheries. Enrofloxacin given orally will enter the blood through intestinal absorption and then go to various tissues. Enrofloxacin will be metabolized in the liver and excreted by the kidneys [6,7]. Enrofloxacin will be eliminated in the kidney and passed through glomerular filtration and tubular secretion [8]. The highest residual concentrations of enrofloxacin in salmon were found in the skin, liver, back fat, kidneys, gills, muscles, and spine [9,10]. The distribution of enrofloxacin in tissues depends on the free drug concentration, which is known to depend on the free drug concentration and the strength of the binding [7].

In the liver, it was known that enrofloxacin could trigger apoptosis in fish liver cells [11]. Whereas in a study using sturgeon fish (*Acipenser baerii*), 24 hours after administration of enrofloxacin for 3-5 days caused liver organ changes in the form of...
apoptosis, atrophy, and blood cell infiltration (although this did not occur in all groups). In addition, administration of enrofloxacin at low doses (20 mg/kg) was considered not to cause liver damage. However, repeated administration of enrofloxacin at medium (40 mg/kg) and high (80 mg/kg) doses can trigger liver tissue damage [6]. In the enrofloxacin test, necrosis was reduced compared to the initial condition, but this condition was not much different from the control. The change that looked better in the final survival group was a decrease in the number of fish that experienced inflammation and congestion.

Administration of enrofloxacin induced pyknosis and changes in the shape of sturgeon kidney tubules [6]. However, this condition was only found in one group. At the same time, the other groups did not experience significant changes compared to the control. Pyknosis is one of the characteristics of necrotic changes. In the enrofloxacin test, necrosis was more numerous in the final safety group. Enrofloxacin will form crystals in the kidneys as the dose increases and could damage the nephrons [12]. Kidney damage by administration of enrofloxacin could occur if continuous administration at medium and high doses. This appears to be related to high levels of enrofloxacin in the kidneys [6]. However, the picture of inflammation and congestion in the final safety group was better than in the final control group.

Enrofloxacin in the spleen induced congestion [12]. The final safety group, in the enrofloxacin spleen test, looked better, with only changes observed in the Melanomacrophage center (MMC). In fish, MMC will scavenge pathogens that spread through the circulation. This formation will participate in the adaptive immune response. MMC in histology is a parameter of the presence of an immune response in fish [13]. In this test, the inflammation that occurred in the safety groups at the beginning and the end showed no difference. This means that muscle inflammation is not caused by enrofloxacin administration. Inflammation of the muscles due to administration of enrofloxacin could occur if the administration was carried out by intramuscular injection [14]. Inflammation of muscles could also be generated by pathogens such as bacteria [15], parasites [16], fungi [17], exposure to heavy metals [18], and administration of nanoparticles [19].

Thus, based on the analysis of the results of the enrofloxacin antibiotic safety test results, it was concluded that the administration of antibiotics showed relatively safe histopathological changes compared to the control group. Changes in the safety group have improved or were not much different from the control group.

### 3.4 Water quality measurement

The pH values of the water in the rearing medium for control and efficacy carp for four weeks was between 7.3-7.7, which means that they were still within normal limits with a range of values between 6-9. Figure 6 shows an overview of the pH value for four weeks.

The carp rearing medium's water temperature in the control and efficacy withdrawal time test carp ranged between 27.45°C-28.58°C and 27.55°C-28.53°C. The temperature was still in good condition for fish rearing media, which is between 25°C-30°C. Figure 7 presents a graphical image of fluctuations in water temperature for carp rearing media.

Dissolved oxygen (DO) parameter values of control and efficacy rearing media of carp ranged from 5.5 – 6.1 mg/l (Figure 8). The dissolved oxygen obtained in maintenance was still at the appropriate value. Fish were kept in reasonable condition because of a flowing water system (flow trough) that carries dissolved oxygen, so carp also get an adequate supply of oxygen carp.

The thing that should be done was to flow water with a larger or heavier discharge so that it can have the effect of increasing the value of dissolved oxygen in the water. In addition, good feed management can control the accumulation of dissolved organic matter in it.
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![pH parameter](image1)

**Fig. 6.** The range of pH values of water in the carp rearing medium for 4 weeks.

![Temperature parameter](image2)

**Fig. 7.** The water temperature values range in the carp rearing medium for 4 weeks.

The nitrite parameter (NO₂⁻N) measured in this test activity was carried out at the beginning of the test fish rearing. The measurement results can be seen in Table 6 below.

**Table 6.** Data of nitrite value of water parameter in the carp rearing medium.

<table>
<thead>
<tr>
<th>Tank</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitrite (mg/L)</td>
<td>0.8939</td>
<td>0.8690</td>
<td>0.8935</td>
<td>0.4983</td>
<td>0.4770</td>
<td>0.4881</td>
</tr>
<tr>
<td>Mean</td>
<td>0.8855 ± 0.014</td>
<td></td>
<td></td>
<td>0.4878 ± 0.011</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The measured value of the nitrite parameter was above the threshold, which was >0.06 mg/l. This occurred presumably because the test fish reared had experienced acclimation for seven days (a week). Hence, the accumulation of uneaten feed residue and the results of carp metabolism generated nitrite content exceeded the threshold value. However, because the maintenance of carp uses a running water system, fluctuations in the increase in nitrite values could be controlled, and the fish did not have illness or death. The water condition of the rearing medium is generally feasible for the maintenance and testing of carp.

![Dissolve oxygen parameter](image)

**Fig. 8.** The range of dissolved oxygen values of water in the carp rearing medium for 4 weeks

### 4 Conclusions

Determining enrofloxacin withdrawal time for 0.5 MRL and 1 MRL were two days and eight days, respectively. The survival rates of carp in post-challenge with *Aeromonas hydrophila* for the positive control and efficacy group were 86.7% and 88.9%, respectively, and were not significantly different. The results of histopathological observations on the liver, spleen, kidney, and muscle of the carp showed no significant pathological changes between the safety test dose treatment and the control treatment. The value of water quality parameters for carp rearing media for four weeks offers a range still feasible for fish rearing activities in ponds.

### References

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Fig. 8. The range of dissolved oxygen values of water in the carp rearing medium for 4 weeks

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