The resistance of the synthetic population of Indonesian common carp (*Cyprinus carpio*) to Koi Herpesvirus (KHV)

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Abstract. One of the effects of a decrease in the genetic quality of common carp is a decrease in resistance to disease. Establishing a synthetic population, blended from 5 strains, namely Rajadanu, Majalaya, Sutisna, Wildan, and Sinyonya, is expected to increase the disease resistance in the Indonesian common carp population. This study aims to evaluate the resistance of this synthetic population to Koi Herpesvirus (KHV) disease. The three common carp strains, Majalaya carp, Subang carp, and Cangkringan carp, were challenged with a cohabitation approach compared to the synthetic population. The water temperature was adjusted to the optimum condition for KHV development, which ranged from 20 – 22 °C. The result showed that KHV infection started on day three and peaked on days 6 to 10 after cohabitation. At the end of the challenge test, the survival rate of the synthetic population was 62.0 %, significantly higher than other strains (P<0.05), which ranged from 20.0 to 26.7 % of the constituent populations. The results of this challenge test indicated that the synthetic population of common carp had better resistance to KHV infection than other strains of common carp cultured by farmers.

1 Introduction

Common carp (*Cyprinus carpio*) is one of the world’s most popular aquaculture commodities. In Indonesia, the production of cultivated carp has begun to decline since the emergence of a disease caused by the Koi Herpesvirus (KHV) in 2002 [1, 2, 3, 4]. Control of this disease has been carried out through several ways, i.e., culture management [5], increasing the body's immunity against diseases externally through the administration of immuno-stimulants [6, 7], and use of the SPF (Specific Pathogen Free) seed [8]. However, these efforts have not yet obtained maximum results. Until 2015, common carp farming had not fully recovered from the previous period [9].

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Apart from being triggered by pathogenic agents, the emergence of disease outbreaks is also influenced by the internal conditions of the fish and the cultivation environment [10]. The low quality of carp seeds and also supported by low environmental conditions will result in fish being susceptible to disease. Several studies related to the genetic quality of carp seeds in Indonesia are relatively low [11, 12, 13]. The low genetic quality of carp seeds is thought to be due to the uncontrolled hatchery system in the breeders. [12] reported that the most common carp farmers are small-scale hatcheries with a limited number of broodstock. The limited number of broodstock used results in a high inbreeding rate in the fish culture seed. This impacts decreasing the quality of seeds, making them susceptible to various diseases when growing up in culture media. These two problems, namely the outbreak of KHV disease and the low genetic quality of common carp seed, are the main problems in Indonesia’s carp farming activities.

The lack of success in overcoming carp diseases through externally enhancing immunity has spurred efforts to increase carp immunity internally through genetic quality improvement. Improving carp’s genetic quality, which aims to improve phenotypic performance, can be done through breeding programs, including selection methods. As an initial step in the selection program, it is necessary to establish a base population with a wide level of genetic variation [14, 15]. The best way to obtain this base population is to form a "synthetic or composite population" by utilizing several available germplasm [16, 17, 18, 19].

The establishment of a synthetic population of Indonesian common carp has been carried out at the Research Institute for Fish Breeding. [13] reported that this synthetic population has a higher genetic variation and better growth than its founder population and other populations. To evaluate the resistance of this synthetic population to disease infections, especially the KHV, it is necessary to carry out the challenge tests of the population before being used by the farmers [20]. This study aims to evaluate the resistance of the synthetic population of common carp to KHV infection. The result will be used to assess the potential of the synthetic population as the base population in selective breeding programs, especially in forming resistant carp to disease populations.

2 Material and methods

2.1 Tested fish

The tested fish is the synthetic population, blended from 5 strains: Rajadanu, Majalaya, Sutisna, Wildan, and Sinyonya. The samples were taken randomly from nursery ponds stage 2. The fish tested was ± two months old with a length of 7-9 cm and weight ranging from 10-15 g/individual fish. As the comparisons, seeds from three populations of other common carp strains cultured by farmers were used: Majalaya carp, Cangkringan carp, and Subang carp. Fish age and size of each population are equivalent to the synthetic population, using individuals per population. A mixed strains population of carp not infected with KHV was used as a negative control of the challenge test.

2.2 Disease resistance test

The disease resistance test for the synthetic population of common carp was conducted through the Koi Herpesvirus (KHV) challenge test. The experiment was conducted with a completely randomized design (CRD), with the genotype of common carp as the different factor. The experiment was conducted for 14 days with three replications for all treatments.
The cohabitation approach was used for the challenge test, based on the Indonesian Common Carp Breeding Protocol No. 03 [21]. The challenge test was conducted in the 60 x 30 x 40 cm aquarium. All aquarium was filled with 25 cm water depth, equivalent to 45 liters in each aquarium. The aquarium’s water temperature was adjusted to the optimal condition for KHV development, which ranged from 20 – 22 °C. The number of tested fish is 30 fish/aquarium, and the number of the KHV transmitter fish is three fish/aquarium (10% of the sampled fish). The KHV transmitter fish is the KHV-infected fish, intramuscularly injected with 0.5 ml 10^5 homogenate KHV filtrate (Figure 1 A). Before stocking the KHV transmitter fish, the tested fish were acclimated first in the aquarium for three days (Figure 1 B).

![Fig. 1. A: Injection of homogenate KHV filtrate to the KHV transmitter fish. B: Cohabitation rearing unit for KHV transmitter fish with tested fish in the aquarium. The red arrow showed the thermometer for the water temperature check.](image)

The parameters observed are the number of dead fish daily and the final survival rate of each population of common carp at the end of the experiment. The daily number of dead fish was analyzed and presented descriptively. All populations' final survival rate was analyzed with variance analysis (ANOVA), followed by Duncan’s Multiple Range Test.

### 2.3 The confirmation of KHV infection in tested fish

The presence of KHV infection was determined in the laboratory using the Indonesian Common Carp Breeding Protocol No. 03 [21]. According to the manufacturer's protocol, the genomic DNA from the sample's gill was extracted using DNAzol (DNAzol® Reagan Invitrogen). The PCR was carried out using the RTG beads kit (GE Healthcare). A KHV-specific primer with a fragment length of 290 bp was utilized for amplification [22]. Electrophoresis was carried out on 1.5% agarose gel in 1TBE buffer. For 40 minutes, electrophoresis was performed using a 100 V electric current. Following ethidium bromide staining, DNA was visualized using a UV gel doc trans-illuminator. The zymogram of the KHV molecules was analyzed descriptively.

### 3 Results and discussion

The disease resistance, especially to KHV, of the synthetic population and other populations of common carp strains was presented in Table 1. Confirmation results regarding KHV infection in tested fish are shown in Figure 2. The daily dead fish in each population during the challenge test period was described in Figure 3.

Table 1. The number of dead fish, survivors, and survival rate of common carp in challenge test with KHV disease for 14 days.
<table>
<thead>
<tr>
<th>Fish population</th>
<th>The number of dead fish (individual)</th>
<th>The number of survivors (individual)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control population</td>
<td>---</td>
<td>30,0±0,0(^a)</td>
<td>100,0±0,0(^a)</td>
</tr>
<tr>
<td>Synthetic population</td>
<td>11,4±1,8(^b)</td>
<td>18,6±1,8(^b)</td>
<td>62,0±6,1(^b)</td>
</tr>
<tr>
<td>Majalaya strain</td>
<td>22,0±4,6(^c)</td>
<td>8,0±4,6(^c)</td>
<td>26,7±15,3(^c)</td>
</tr>
<tr>
<td>Cangkringan strain</td>
<td>22,0±3,0(^c)</td>
<td>8,0±3,0(^c)</td>
<td>26,7±10,0(^c)</td>
</tr>
<tr>
<td>Subang strain</td>
<td>24,0±3,0(^c)</td>
<td>6,0±3,0(^c)</td>
<td>20,0±10,0(^c)</td>
</tr>
</tbody>
</table>

Note: The values followed by different superscripts in the same column showed significant differences (P<0.05).

The presence of fish mortality in both synthetic populations and other strains, as shown in Table 1, indicates that the KHV virus infected the fish and increased mortality. The absence of fish mortality in the negative control population (not infected with KHV) strengthens the indication that fish mortality in the tested population was caused by KHV infection. The negative control population has a survival rate of 100%, significantly different (P<0.05) from all the tested populations. KHV infection in tested fish was proven, as shown in the molecular confirmation result presented in Figure 2. The KHV transmitter fish and all tested fish were infected with KHV, which has a molecular length of around 290 base pairs [23, 24].

Fig. 2. Laboratory confirmation result for KHV infection in the tested fish. M: marker; 1: transmitter KHV fish; 2: control (+) filtrate KHV; 3: control (-) fish; 4-6: tested fish; bp: base pair.

The daily mortality of fish in the challenge test period of this study is presented in Figure 3. Figure 3 shows that the first mortality occurred on the third day in the Subang strain. The peak of the mortality of the tested fish happened between the 6\(^{th}\) to 10\(^{th}\) day. After that period, the daily mortality of the sampled fish decreased and was relatively low. Until the 14\(^{th}\) day of the challenge test, the Subang strain had the lowest survival rate, which was 20%, not significantly different (P>0.05) from the Majalaya and Cangkringan strains, which were 26.7%. The final survival rate for the synthetic population was 62%, significantly different (P<0.05) from the control negative population and the Majalaya, Cangkringan, and Subang strains.
The host's ability to control infection by limiting pathogen replication can be defined as disease resistance [25]. In practical terms, host resistance to viral and bacterial infections is frequently quantified as individual survival (and/or mortality) during an outbreak [20, 25]. Empirical data showed that KHV infection in cultivated common carp in Indonesia could cause mortality of up to 80-90% of the population. The first big epidemic occurred in cultured common carp in Subang Regency, West Java, at the end of April 2002. More than 450 metric tons of common carp cultivated in running water systems were lost economically. Within two weeks, the infection had expanded to West Java's regencies and the western section of Central Java [6, 26]. In laboratory challenge tests, the survival rates of the test fish varied from 0% [27], 5-7% [28], 13.3-36.7% [4], to 100% [29]. Several factors causing the varying mortality rates include the level of virus virulence, the amount or dose of the virus, the type or strain of common carp tested, and the age and size of the test fish [28]. In this research, the virulence level of KHV, the dose of virus applied, and the size and age of the fish were relatively the same among tested populations. The tests are carried out in the same laboratory simultaneously. Thus, the difference in the mortality rate of each carp population is strongly suspected to be due to the tested fish strains having different genotypes.

Based on both Table 1 and Figure 3, it can be seen that the synthetic population has the highest average survival rate compared to the other three strains. However, the survival rate of the synthetic population was only 62.0%. This result showed that 38% of the fish in the synthetic population died at the end of the challenge test. The Subang strain carp has the lowest resistance to KHV infection, with an average mortality rate of 24 of 30 fish or a survival equivalent of 20% of the population. The low survival rate of the three comparison fish strains in this challenging test is thought to be caused by the low genetic quality of carp. In general, the level of genetic variation of carp in Indonesia is relatively low due to the high rate of inbreeding in carp hatcheries [12].

Synthetic populations of Indonesian carp were formed to increase the genetic variation of that population. Therefore, this synthetic population was created for selection purposes, especially on growth characteristics [13]. The founder populations used are the dominant carp strains cultured by farmers in Indonesia, namely Majalaya, Rajadanu, Sutisna, Wildan, and Sinyonya. The synthetic population was further reported to have higher genetic variation than the founder populations. The high genetic variation in this synthetic population is suspected to significantly increase the body's resistance to disease infections, especially...
KHV. In general, [30, 31] explained that a population's genetic variation greatly influences that population's resistance to disease.

The results of further analysis using Duncan's multiple range test showed that the synthetic population had a final survival rate of 62%, significantly better than the three comparison strains, which ranged from 20-26.7%. The higher genetic variation in synthetic populations is thought to increase the population's resistance to KHV infection. This resulted in a markedly better synthetic population survival rate than other strains. As explained by [29], the level of heterozygosity is thought to influence the character of resistance to KHV disease infection in Majalaya carp. This is based on the research result by [32] related to the heterozygosity of the MHC I gene, particularly in the Cyca-DAB1 allele, which regulates the immune system in European common carp challenged with KHV.

Another study reported that genetic variation factors play a role in stress control systems in fish [33]. This is based on a study by [34] on the channel catfish (*Ictalurus punctatus*), which stated that as many as 61 genes were expressed in various ways in the brain in response to stress. The existence of high gene variation will increase the stress response in the fish so that it can deal with changes in environmental conditions. The same condition is thought to occur in fish challenged with a disease, one of the stress-causing factors in organisms. In this study, the level of genetic variation, especially the heterozygosity of the synthetic population, which was higher than the founder population [13], is thought to have played a major role in increasing the immune capacity of this population. The higher the immune level of a population, the better the stress response. This resulted in the population having higher resistance when challenged with KHV disease. Based on this result, this synthetic population of common carp has a high potential for being cultured by farmers or used as a base population for selection programs, especially for forming disease-resistant carp.

### 4 Conclusion

The synthetic population of common carp has a survival rate of 62%, significantly better than other carp populations. This result showed that the synthetic common carp population's establishment has succeeded in increasing carp populations' resistance to KHV infection. This synthetic population has a high potential for being cultured by farmers or used as a base population for selection programs.

### References