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Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism

Krzysztof Ł. Rakus [a,*], Geert F. Wiegertjes [b], Mikołaj Adamek [a,c], Andrzej K. Siwicki [d], Agnieszka Lepa [d], Ilgiz Irnazarow [a]

[a] Polish Academy of Sciences, Institute of Ichthyobiology & Aquaculture in Gołysz, Zaborze, ul. Kalinowa 2, 43-520 Chybie, Poland
[b] Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands
[c] Department of Ichthyobiology and Fisheries, University of Agriculture, 30-199 Kraków-Mydlniki, Poland
[d] Department of Fish Pathology and Immunology, The Stanisław Sakowicz Inland Fisheries Institute in Olsztyn, Żabieniec, ul. Główna 58, 05-500 Piaseczno, Poland

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ABSTRACT

The role of MH class II *B* (*Cyca-DAB1*-like) genes in resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 (CyHV-3), also known as koi herpesvirus (KHV) was analysed. The material consisted of 934 fish from six carp crosses. Fish were challenged with CyHV-3 at an age of 7 and 10 months. During challenge experiments the peak of mortality caused by CyHV-3 was observed at days 8–12 p.i. and the overall cumulative mortality reached 79.9%. Among six *Cyca-DAB1*-like genotypes, revealed by PCR-RF-SSCP analysis, one genotype (E) was found associated with higher resistance to CyHV-3. Three other genotypes (B, H and J) could be linked to higher susceptibility to CyHV-3. Analysis of the alleles that compose the *Cyca-DAB1*-like genotypes linked one particular allele (*Cyca-DAB1*05*) to significantly increased, and two alleles (*Cyca-DAB1*02* and *Cyca-DAB1*06*) to significantly decreased resistance to CyHV-3. Our data indicate that MH class II *B* genes could be used as potential genetic markers in breeding of common carp for resistance to this virus.

1. Introduction

Within the last decade, mass mortality of farmed carp and ornamental domesticated varieties (koi), which have a viral aetiology, has been observed in numerous fish farms in Europe, Israel, USA, Japan, China, Taiwan, Thailand, Indonesia, Malaysia, and South Africa [1–8]. Outbreaks of disease also have been observed among wild common carp populations in the United Kingdom, Japan and several states in the USA [5,9]. The disease-causing virus has been identified as Cyprinid herpesvirus-3 (CyHV-3) [10] also known as Koi Herpesvirus (KHV) [2] and Carp Interstitial Nephritis and Gill Necrosis Virus (CNGV) [11]. CyHV-3 is a member of a new family *Alloherpesviridae* comprising piscine and amphibian herpes viruses, as proposed by McGeoch et al. [12]. Currently, the losses of farmed carp and koi carp populations caused by CyHV-3 have a very negative impact on production of carp over the whole world.

Several studies have been carried out to establish a strategy for controlling the disease of carp caused by the CyHV-3 virus. Even though there is a vaccine based on live attenuated CyHV-3 ('KoVax') available in Israel, legislation in the European Union does not allow for the use of such a vaccine. Furthermore, vaccination is an effective approach only in intensive systems and/or high market value species (koi), while traditional carp aquaculture is carried out in semi-intensive pond culture conditions and does not lead to high market value fish. Alternatively, genetic selection for improved carp lines and, to some extent, mass selection can be used to improve aquacultured fish species. For example, it has been shown that hybrid crosses of common carp were less sensitive to diseases than their parental purebred strains [13,14]. Also, Kirpichnikov et al. [15] developed a disease resistant breed of Krasnodar carp by the use of mass selection of fish for resistance to dropsy, a very serious infectious disease, but this long-term work included breeding for up to 8–9 generations. Recently, Shapira et al. [16] described differential resistance to CyHV-3 among selected common carp strains and crossbreds, which suggest that resistance of carp to this virus might be affected by genetic factors.

* Corresponding author. Polish Academy of Sciences, Institute of Ichthyobiology & Aquaculture in Gołysz, Zaborze, ul. Kalinowa 2, 43-520 Chybie, Poland.
E-mail address: krzysztof.rakus@fish.edu.pl (K.Ł. Rakus).

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Genes of major histocompatibility complex (MHC) are candidate genes for disease resistance. MHC genes encode for cell-surface receptors (class I and class II molecules) capable of binding short peptides for presentation to T cell receptors. MHC class II molecules present mostly exogenous derived peptides to $CD4^+$ T helper cells [17]. In addition, they can also present viral peptides derived from degradation in endosomal/lysosomal compartments to $CD4^+$ T helper cells. This stimulates the production of interferon gamma that can stimulate the production of virus-specific antibodies [18,19]. Although only a minor fraction of these antibodies have direct antiviral activity (referred as neutralizing antibodies), the vast majority of virus-specific antibodies (referred as non-neutralizing antibodies) have been shown to help to control virus infections by activating the complement system, facilitating phagocytosis and promoting antibody-dependent cellular cytotoxicity [19]. Actually, since the final stage of the virion assembly process of herpesviruses, occurs in endosomal compartments, viral peptides can enter the MHC class II pathway very efficiently and be presented to $CD4^+$ T cells [20–23]. In humans and mouse several reports suggest that $CD4^+$ T cells are essential in the control of herpesvirus infection [23,24]. Studies of mouse herpesvirus MHV-68 revealed that $CD4^+$ T cells could control an infection *in vivo*, independent of $CD8^+$ T cells and B cells [25]. It has been also shown that efficient presentation of Epstein Barr virus glycoproteins by MHC class II molecules can result in direct killing of EBV-infected B cells by $CD4^+$ T cells [24,26].

The MHC contains some of the most polymorphic genes known to date and each MHC molecule has the ability to bind and present different groups of peptides. A single change in an amino acid within the peptide-binding site of an MHC molecule can directly affect the ability of the MHC molecule to bind a particular peptide. As a result, variations in the peptide-binding site can result in higher resistance or susceptibility to disease. There are a number of reports that described evidence for associations between MH polymorphism and disease resistance in several, but mostly salmonid, fish species [27–33]. We recently optimized a simple, but highly sensitive technique based on single strand conformation polymorphism (SSCP) that allows us to screen large numbers of individuals for MH class II *B* polymorphism [34]. We are in the process of optimizing this technique for MH class I polymorphism. In common carp, there are two paralogous groups of MH class II *B* genes; *Cyca-DAB1*-like genes, and *Cyca-DAB3*-like genes. Previous studies have indicated a ubiquitous presence and high polymorphism of *Cyca-DAB1*-like, but not *Cyca-DAB3*-like, genes [35]. For this reason we decided to focus on the *Cyca-DAB1*-like genes. Association between particular MH class II *B* (*Cyca-DAB1*-like) genotypes and higher resistance to CyHV-3 would allow us to use MH genes in future selection for increased resistance to disease in common carp. Such genetic selection would present a sustainable approach to disease control in semi-intensive carp pond farming.

This study has the following objectives: (*i*) to estimate the genetic differences in resistance to CyHV-3 infection between different common carp crosses; and (*ii*) to determine the association between major histocompatibility (MH) class II *B* genes (*Cyca-DAB1*-like) polymorphism and ability of carp to resist infection with CyHV-3.

2. Materials and methods

2.1. Fish

European common carp (*Cyprinus carpio carpio* L.) used in this study included 12 reciprocal crosses obtained at the Institute of Ichthyobiology & Aquaculture in Gołysz (Poland) by diallelic cross of four carp lines: Polish 'K' and 'R6', Hungarian 'R7' and French 'F' (Table 1). From each line one female and one male were randomly taken for propagation. All matings were performed on the same day. Eggs of one female from each line were divided into three equal portions and each portion fertilized with milt of one male from the appropriate carp line (Table 1). Separate incubation and hatching were used for 12 reciprocal crosses. At the swim-up stage, fish from each of reciprocal cross were moved into a system with UV treated recirculating water and bio-filters, and were grown in separate tanks (500 fish per tank) in triplicate. Total capacity of the system was 3 m^3 and the temperature of water was maintained at 21 ± 2 °C. Daily feeding with carp commercial pellets (Bestfeed) at 3% body weight per day was applied. Before challenge experiments, carp ($n = 934$) from the 12 reciprocal crosses were randomly sampled, marked by fin clipping, mixed and transported to the Laboratory of Fish Pathology and Immunology IFI, Żabieniec (Poland). Fish were allowed to acclimatize to the new recirculating system for a period of 14 days. No mortalities were observed during the acclimatization period.

2.2. Virus cultivation

Koi fin cell (KFC) cultures, kindly supplied by Professor Moshe Kotler from the Department of Pathology, Hebrew University-Hadassah Medical School in Jerusalem (Israel) were used for virus propagation. Cells were grown in culture medium containing 60% Dulbecco's modified Eagle's medium, 20% Leibovitz (L15) medium, 10% fetal bovine serum (Sigma), 10% tryptose phosphate (Difco) and supplemented with 1% HEPES and antibiotics to form of monolayer over a period of 10–14 days in a 22 °C incubator supplemented with 5% CO_2. The monolayer cultures were trypsinized and transferred into new flasks with fresh medium. The KFC cultures were inoculated with supernatants prepared from gills and kidney of CyHV-3 infected fish. The infected KFC cultures were incubated at 22 °C for 10 days and examined for cytopathic effect (CPE). The virus was released into the culture medium during the appearance of a CPE, but a significant amount remained associated with the cell. When CPE was observed in the KFC cultures, CyHV-3 viral identification was confirmed by PCR. Purification of virus from culture medium was done according to protocol [11]. Virus concentrations were estimated by $TCID_{50}$ using the method of Reed and Meunch [36] as presented for fish herpesvirus by Hedrick et al. [2]. Identification of CyHV-3 from gills and kidneys of experimental infected carps was confirmed by PCR.

2.3. Challenge test

Two challenge experiments were performed at different time points: when fish were 7 months and 10 months of age. Fish of both ages are considered immunology mature and were considered comparable groups. For both challenges fish were kept in three 500 L tanks. Each tank (approximately 240 fish) consisted of 12 reciprocal crosses, represented by 20 individuals per cross (Table 1). Tanks were supplied with aerated and filtrated water at 22 °C. Two

Table 1
Experimental design. For each challenge experiment (fish of 8 and 10 months old) fish from the 12 reciprocal crosses were mixed together in one tank, in duplicate, and infected by i.p. injection. The third tank was the control.

♀	♂			
	K	R6	R7	F
K	–	K × R6	K × R7	K × F
R6	R6 × K	–	R6 × R7	R6 × F
R7	R7 × K	R7 × R6	–	R7 × F
F	F × K	F × R6	F × R7	–