Association of a specific Major Histocompatibility Complex class IIβ single nucleotide polymorphism with resistance to lactococcosis in rainbow trout, Oncorhynchus mykiss (Walbaum)

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Abstract

Major histocompatibility complex (MHC) loci encode glycoproteins that bind to foreign peptides and initiate immune responses through their interaction with T cells. MHC class II molecules are heterodimers consisting of α and β chains encoded by extremely variable genes; variation in exon 2 is responsible for the majority of observed polymorphisms, mostly concentrated in the codons specifying the peptide-binding region. *Lactococcus garvieae* is the causative agent of lactococcosis, a warm-water bacterial infection pathogenic for cultured freshwater and marine fish. It causes considerable economic losses, limiting the profitability and development of fish industries in general and the intensive production of rainbow trout, *Oncorhynchus mykiss* (Walbaum), in particular. The disease is currently controlled with vaccines and antibiotics; however, vaccines have short-term efficacy, and increasing concerns regarding antibiotic residues have called for alternative strategies. To explore the involvement of the MHC class II β-1 domain as a candidate gene for resistance to lactococcosis, we exposed 400 rainbow trout to naturally contaminated water. One single nucleotide polymorphism (SNP) and one haplotype were associated with resistance ($P < 0.01$). These results are promising for using MHC class IIβ as a molecular marker in breeding rainbow trout resistant to lactococcosis.

Introduction

Major histocompatibility complex (MHC) genes represent the most polymorphic genes known to date, with multiple loci and a considerable number of alleles at each given locus in mammals and teleost fish. These loci encode glycoproteins that bind to foreign peptides and thus initiate immune responses through their interaction with T cells mediated by T-cell receptors. In particular, MHC class II molecules present foreign peptides, phagocytosed and processed within the host cell, and initiate a type II response by CD4+ T cells. They are heterodimers consisting of α and β chains encoded by class IIα and class IIβ genes and are expressed on the cell surface of professional antigen-presenting cells (Elgert 1996).

Differently from other teleost fish, no more than one class-IIβ-chain-encoding locus is transcribed in rainbow trout, *Oncorhynchus mykiss* (Walbaum). The open reading frame encodes a 247-amino-acid-long polypeptide composed of two domains (β-1 and β-2), a connecting peptide, a transmembrane region and a cytoplasmic tail. The β-2 domain and the transmembrane region are conserved, especially the CD4-binding site located inside the β-2 domain. The β-1 domain is instead highly variable: only nine amino acid residues are identical among vertebrates and 17 among teleost fish (Glamann 1995).

Because of important role of MHC alleles in innate and adaptive immune responses, their association with diseases has been well investigated in mammals. However, much less is

known about involvement of MHC alleles in teleost fish. The role of MHC class IIβ alleles in modulating susceptibility/resistance has been reported for disease-causing bacteria such as *Aeromonas salmonicida* in brook charr, *Salvelinus fontinalis* (Mitchill) (Croisetière *et al*. 2008), and Atlantic salmon, *Salmo salar* (L.) (Langefors *et al*. 2001); *Vibrio anguillarum* in Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel) (Zhang *et al*. 2006; Xu *et al*. 2008); and *Edwardsiella tarda* in turbot*, Scophthalmus maximus* (L.) (Xu, Chen & Ding 2009). Some data have been reported also for viral diseases such as infectious haematopoietic necrosis (IHN) or infectious salmon anaemia (ISA) in salmonids (Palti *et al*. 2001; Kjøglum *et al*. 2006).

Lactococcus garvieae, a Gram-positive bacterium belonging to the *Streptococcaceae* family, is the causative agent of lactococcosis, warm-water bacteria pathogenic for cultured freshwater and marine fish at water temperatures above 15 °C. It causes considerable economic losses, limiting the profitability and development of fish industries in general and the intensive production of rainbow trout in particular (Eyngor *et al*. 2004). *Lactococcus garvieae* infection results in a hyperacute systemic disease characterized by high mortality rates of up to 50%, typical of capsulated strains (Ghittino *et al*. 1998; Vendrell *et al*. 2006).

Although the disease has been partially controlled using vaccines and antibiotics, current methods of prophylaxis and treatment are limited by decreasing vaccine effectiveness over time: only 3 months, increasing to six if the vaccine is respectively without or with adjuvant. This period is insufficient to cover the productive cycle of farmed fish, and the vaccine cannot be administered in immature fish due to their still incomplete immunocompetence (Ghittino *et al*. 2002). Antibiotic residues in fish are a further concern, potentially leading to environmental pollution and animal and human health problems due to the spread of antibiotic-resistant strains. The host range of *Lactococcus garvieae* is, in fact, not restricted to aquatic species. The agent has also been identified in cattle, water buffalo, poultry meat, swine, cat and dog. Its isolation in several human cases suggests that it could be considered as a potential zoonotic agent (Vendrell *et al*. 2006). Gene-assisted selection, which could enhance individual resistance to pathogens, might thus offer a promising solution to this problem.

To date, no data are available about the involvement of MHC class IIβ alleles in lactococcosis in trout. In this study, we analysed the variability of a fragment of exon 2 that encodes for the β-1 domain, comprising the peptide-binding region (PBR) of MHC class IIβ molecules, and investigated its role in resistance in rainbow trout naturally exposed to *Lactococcus garvieae*.

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Materials and methods

In July 2011, a total of 400 rainbow trout (323 females and 77 males) were exposed to water naturally contaminated by *Lactococcus garvieae*. The fish were located in a single farm in northern Italy (Western Po Basin). Inbreeding of the study population was avoided through the purchase of different rainbow trout batches. Water temperature was ranged from 19 to 21 °C. Feeding was composed of a standard daily diet. The average weight of fish was 500 g for females and 1400 g for males. The standard tank volume was 7.2 m^3 (1.5 × 4 × 1.2 m), with a continuous water flux (2 L/s).

Mortality was recorded daily in each tank, and the fish were observed for the appearance of clinical signs. Late October, because of decreasing temperature, was chosen as the end of the exposure period, and all the surviving fish were individually marked with a microchip. Fish found dead during the 3 months (August, September and October) after exposure and positive for *Lactococcus garvieae* were considered as cases.

For microbiological examinations, strains were isolated from the kidney after growth at 24 °C for 24 h on Columbia Agar Base (Microbiol) supplemented with 5% defibrinated

sheep blood. Bacterial colonies were then subjected to identification using classical phenotypical and biochemical methods (bile esculin agar and Slanetz–Bartley agar selective medium) and miniaturized systems of diagnostic API 20 STREP, API ZYM, API 50 CH (bioMérieux), consisting of galleries of different biochemical tests. In addition, a biomolecular technique based on species-specific PCR was used (Zlotkin *et al*. 1998) to differentiate *Lactococcus garvieae* with certainty from the very similar *Lactococcus lactis* subsp. *lactis*.

For genetic analysis, genomic DNA was extracted from adipose fin tissue with a PureLink™ Genomic DNA Mini Kit (Invitrogen).

A 257-base-pair fragment (including primers) of exon 2, coding for the polymorphic β-1 domain of the protein (from residue 33 to 112), was amplified using the primers B1RA and B1FA, as described by Miller (Miller, Withler & Beacham 1997).

PCR was carried out in a total volume of 25 µL containing 30–50 ng of genomic DNA using Platinum® qPCR Supermix-UDG (Invitrogen) and the primers reported above (300 nm each).

PCR conditions were the same as reported by Miller *et al*. (1997). The same primers were used for sequencing, using the BigDye 1.1 chemistry and the ABI3130 genetic analyser. Sequence alignment was carried out using Lasergene SeqMan software (DNASTAR).

As genotypic frequency in real populations can be predicted from the allelic frequencies only under the conditions of Hardy–Weinberg equilibrium, SNPs not fulfilling this assumption were not included in the association study and were not inserted in haplotypes definition; moreover, SNPs with low frequency (<0.05) were also not included in haplotypes definition. Haplotypes configuration for each individual was estimated using PHASE software (version 2.1) following the methodologies described by Stephens, Smith & Donnelly (2001). A permutation test was carried out using this software, simply tagging each sample as case or control (1 or 0) in the input file. As a first step in the genetic association study, Pearson's chi-squared test, considering Yate's correction, was carried out for each SNP both independently and as haplotype. SNPs significantly associated with the disease ($P < 0.05$) were used for further analysis.

Survival of each individual was calculated from the date of introduction into water until the date of death or the last date of follow-up. Follow-up was defined as an observation over a period of time of an individual, group or initially defined population whose relevant characteristics were assessed to observe changes in health status or health-related variables (Last 1988).

Univariate analysis was carried out based on the Kaplan–Meier survival function and by comparing the survival differences for each significantly associated SNP using the log-rank test.

Finally, SNPs significantly associated with survival in the univariate model were entered in a Cox proportional hazards regression model. The proportional hazard assumption was tested to compare the hazard among individuals and to verify the time dependence of the risk factor. The hazard ratios were used to estimate the effect of SNPs on survival after adjusting for the potential confounding effect of covariates. As weight and sex were positively correlated, we decided to use only the sex of the animals as a covariate. For each hazard ratio (HR), a 95% confidence interval (95% CI) was calculated. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using Stata Statistical Software, release 10.0 (Stata Corp.).

For homology modelling, SWISS-MODEL (Arnold *et al*. 2006) based on crystal structure from humans as template (PDB id: 3lqz, B chain) was used. The model was then analysed with ProtScale (ExPASy Bioinformatics Resource Portal) to obtain a protein hydrophobicity plot according to the Kyte and Doolittle method based on a scale of positive scores attributed to hydrophobic amino acid by any amino acid (Gasteiger *et al*. 2005).

Subsequently, DeepView/Swiss-Pdb Viewer software (ExPASy Bioinformatics Resource Portal) was used to introduce amino acid changes to the model and to visualize the alterations in protein structure.

Results

In all, 183 fish died during the observation period. Mortality was strongly influenced by the unusual seasonal change, with alternating warm periods and short periods of decreasing water temperature. Ten dead trout were excluded from the study because they were negative for *Lactococcus garvieae* isolation (eight negative and two positive for *Yersinia ruckeri*), while 22 trout, positive for *Lactococcus garvieae,* were excluded because of incomplete genotyping. In all, 217 fish survived (four fish were excluded due to incomplete genotyping). Fish positive for *Lactococcus garvieae* were macroscopically characterized by ocular abnormalities (corneal clouding and exophthalmos), intracranial oedema, diffuse haemorrhage, congestion of internal organs, enteritis and darkening of skin.

Analysis of the MHC class II β-1 domain showed that 37 of 257 sites were polymorphic. The SNPs and the relative amino acid changes are reported in Table 1. Frequency and Hardy–Weinberg equilibrium were calculated for each SNP (Table 1).

Table 1. Single nucleotide polymorphisms (SNPs) (numbering with the leader peptide included), amino acid changes, relative frequencies and *P*-values for association. NA, not applicable because Hardy–Weinberg (H–W) equilibrium not fulfilled

SNPs	Amino acid	*P*-value H-W	Frequency	Cases		Controls
				Trout carrying mutation	Frequency	Trout carrying mutation
126 a>g	42 I>M	>0.05	0.32	72	0.31	118
130 t>c	44 F>H	**<0.05**	0.06	18	0.07	23
131 t>a	44 F>Y	**<0.05**	0.43	92	0.44	143
132 t>g	44 F>L	**<0.05**	0.10	21	0.09	41
135 a>t	45 I>I	**<0.05**	0.07	11	0.05	32
136 g>a	46 D>N	**<0.05**	0.07	11	0.05	32
137 a>c	46 D>A	**<0.05**	0.07	11	0.05	32
140 c>t	47 S>F	>0.05	0.06	6	0.02	38
143 a>t	48 Y>F	>0.05	0.16	37	0.15	68
145 g>t	49 V>F	>0.05	0.16	36	0.14	68
154 a>c	52 K>Q	>0.05	0.26	60	0.25	105
155 a>t	52 K>M	>0.05	**0.02**	5	0.02	11
158 t>c	53 V>A	**<0.05**	0.37	80	0.38	128
163 t>c-	55 Y>D 55 Y>H	**<0.05**	f (c) 0.16 f (g) 0.30	(c) 47 (g) 63	f (c) 0.19 f (g) 0.26	(c) 57 (g) 119

g						
166 a>g	56 I>V	**<0.05**	0.09	24	0.11	35
194 a>t	65 Y>F	**<0.05**	0.42	80	0.39	144
210 a>g	70 E>E	>0.05	0.16	39	0.16	66
211 c>t	71 H>Y	**<0.05**	0.05	16	0.07	20
217 g>c	73 V>L	**<0.05**	0.40	85	0.41	129
235 t>c	79 W>R	>0.05	**0.03**	8	0.03	11
236 g>t	79 W>L	>0.05	**0.04**	15	0.06	14
253 a>t	85 I>F	>0.05	0.07	14	0.05	38
255 c>g	85 I>M	>0.05	0.09	25	0.08	37
272 c>t-g	91 A>V 91 A>G	**<0.05**	f (t) 0.16 f (g) 0.22	(t) 39 (g) 55	f (t) 0.15 f (g) 0.25	(t) 65 (g) 86
274 c>g	92 Q>E	**<0.05**	0.61	117	0.61	179
283 a>c	95 S>R	**<0.05**	0.63	123	0.63	164
286 t>g	96 Y>D	**<0.05**	0.20	42	0.19	75
287 a>t	96 Y>V	**<0.05**	0.60	109	0.57	177
295 c>a	99 H>N	**<0.05**	0.05	18	0.08	15
296 a>c	99 H>P	>0.05	0.28	62	0.27	107
301 g>a	101 A>T	**<0.05**	**0.04**	14	0.06	13
305 a>c	102 D>A	>0.05	0.33	82	0.37	109
307 a>c	103 I>L	**<0.05**	0.33	66	0.28	124
308 t>a	103 I>N	>0.05	0.16	46	0.19	56
309 c>t	103 I>I	**<0.05**	**0.04**	18	0.07	6
310 g>c-t	104 D>H 104 D>Y	**<0.05**	f (c) 0.54 f (t) 0.19	(c) 109 (t) 41	f (c) 0.55 f (t) 0.17	(c) 169 (t) 74
311 a>c	104 D>A	**<0.05**	**0.04**	19	0.08	4

Only three SNPs of 37 were associated with disease after Pearson's chi-squared calculation: 140 c>t (47 S>F), 253 a>t (85 I>F) and 305 a>c (102 D>A) (Table 1). The

association of SNP 253 was lost when genotype rather than allele was considered (data not shown).

In all, 37 haplotypes were configured considering 11 SNPs. Only nine, with frequencies >0.05, were included in the association study (Table 2). Only haplotype 25, composed of the three mutated sites 140t (47F), 143t (48F) and 145t (49F) (Fig. 1), was associated with resistance to lactococcosis, as shown by the *P*-value reported in Table 3. Significance remained also when the analysis was carried out considering genotype (*P* = 0.00). The permutation test was statistically significant (*P* = 0.01), indicating diversity not due to chance between the two subpopulations.

Table 2. Haplotypes chosen for the association study (frequency >0.05). 0 for wild type and 1 for mutated single nucleotide polymorphism (SNP)

Haplotype number	Freq	SNP 126	SNP 140	SNP 143	SNP 145	SNP 154	SNP 210	SNP 253	SNP 255	SNP 296	SNP 305
4	0.07	0	0	0	0	0	0	0	0	0	1
5	0.12	0	0	0	0	0	0	0	0	1	0
8	0.07	0	0	0	0	0	0	1	1	0	0
11	0.08	0	0	0	0	0	1	0	0	1	0
13	0.07	0	0	0	0	1	0	0	0	0	0
20	0.08	0	0	1	1	0	0	0	0	0	0
25	0.06	0	1	1	1	0	0	0	0	0	0
26	0.06	1	0	0	0	0	0	0	0	0	0
27	0.06	1	0	0	0	0	0	0	0	1	0
32	0.14	1	0	0	0	1	0	0	0	0	1

Table 3. Haplotype distribution of cases and controls and relative *P*-values

Haplotype	Cases		Controls
	Trout carrying haplotype	Frequency	Trout carrying haplotype
4	21	0.09	26
5	29	0.13	45
8	14	0.05	35
11	19	0.07	39
13	12	0.05	32
20	26	0.10	30
25	5	0.02	38
26	17	0.07	23
27	13	0.04	34
32	34	0.13	63

Figure 1. Amino acid sequence alignment between peptide derived from Glamann's continuous sequence of *Oncorhynchus mykiss* (1995) and peptide derived from haplotype 25, associated with resistance. The solid black line indicates the presence of the same residues as compared to Glamann's peptide; the beginning of the line represents the starting point of the sequence analysed in this study.

By the end of the follow-up period, 38% events (deaths) had occurred. Overall survival estimates were based on the Kaplan–Meier survival function, with statistical significance assessed using the log-rank test. The Kaplan–Meier survival curve for SNP 140 (Fig. 2) was associated with a log-rank test, *P*-value equal to 0.0002.

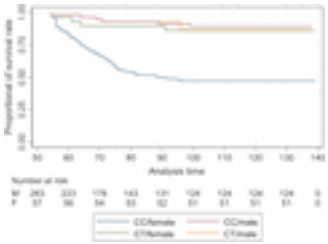

Figure 2. Kaplan–Meier curve for overall survival among trout, by single nucleotide polymorphism (SNP) 140 genotype stratified by sex; after adjustment for sex, the wild-type genotype was associated with shorter survival among individuals, as compared to animals carrying the mutation.

In the univariate proportional hazards model, the trout heterozygous for the minor allele 140t were at a lower risk of mortality as compared to individuals with the wild-type genotype (HR 0.24, 95% CI 0.106–0.54). This association remained significant after adjustment for non-proportional hazard by stratifying for sex: the adjusted HRs for survival associated with the heterozygous genotype and with sex were 0.28 (95% CI 0.12–0.63).

As the Kaplan–Meier curve in the male group was apparently inconsistent with SNP 140, the interaction between sex and genotype was tested: it was not significant (HR = 6.07, 95% CI 0.92–39.94), whereas the hazard ratio for SNP 140 was 0.20 (95% CI 0.07–0.54).

Trout carrying mutation 305c demonstrated a shorter survival time: the mutation was significantly associated with susceptibility to the disease (HR 2.16, 95% CI 1.41–3.30). The association was also significant after adjustment for non-proportional hazard by stratifying for sex (HR 2.10, 95% CI 1.38–3.22).

Finally, a multivariate proportional hazard model was fitted, considering SNP 140, SNP 305 and sex as covariates: consistently, the mutated 140 and 305 genotypes remained statistically significant predictors of survival. The adjusted HRs for survival associated with the mutations were 0.30 (95% CI 0.13–0.68) and 1.87 (95% CI 1.216–2.86), respectively.

Protein hydrophobicity plots, comparing the structures of the wild-type protein (Fig. 3a) and of the mutated protein derived from haplotype 25 (Fig. 3c), showed an increase in peak height in the area involved by amino acid residue changes. Moreover, a change in residue polarity was observable in the 3D model, considering the typing of the protein based on the chemical features of the amino acids (Fig. 3b,d).

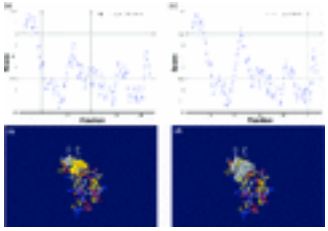

Figure 3. (a) and (c): Hydrophobicity plots of wild-type and mutated protein. The *x*-axis represents the amino acids' position in the peptide, and the *y*-axis represents the hydrophobicity score for each single residue. (b) and (d): Amino acid typing of the 3D homology model of wild-type and mutated protein. Non-polar residues (grey), polar residues (yellow), acidic groups (red), and basic groups (blue).

Discussion

To our knowledge, this is the first study to investigate the role of the MHC class IIβ gene in resistance/susceptibility to lactococcosis in rainbow trout. Our findings are in line with previously published data on the variability of the β-1 domain in trout. There are, in fact, small regions of the sequence, and consequently of the peptide, that are extremely conserved: the most conserved peptide motif in teleost fish is VGYT (valine, glycine, tyrosine, threonine, from residue 66 to 69, numbering with the leader peptide included). This motif is conserved in humans too, where it is defined by other residues (RAVT; arginine, alanine, valine, threonine) and it seems to contribute to the dimer interface, according to the crystal structure of the HLA-DR molecule.

Another strongly conserved motif in trout is (arginine, phenylalanine, asparagine, serine [RFNS]) from residue 57 to 60. The same positions in mammals seem to be involved in cell-to-cell interaction with the T-cell subset molecule CD4 (Glamann 1995).

In contrast, wide variability in the amino terminal half of the β-1 domain, especially in positions 55 and 56, has been reported (Glamann 1995). In agreement with these data, we found a trimorphism at nucleotide 163, encoding for two different amino acids (D/H) at residue 55.

The association study showed that mutation 140t was significantly associated with resistance to the disease, with a longer survival time between individuals carrying the mutation. Also related to resistance was haplotype 25: it is characterized by mutations at sites 140, 143 and 145, all coding for phenylalanine at positions 47, 48, 49, thus causing the appearance of a polyphenylalanine site formed by the three mutated residues and a fourth phenylalanine, which derives from the wild type at residue 50. And given that in haplotype 25, there is also the resistance-associated mutation 140t, it could be debated whether this single mutation only or rather its combination with the other mutations in the haplotype is responsible for resistance to the disease.

In this study, mutation 140t was not found in any other haplotype, except for haplotype 37, in which 140t was again associated with mutations at sites 143 and 145, along with a mutation at site 126. Unfortunately, haplotype 37 was too rare to search for any association.

On the other hand, mutations 143t and 145t were not significant when considered singularly ($P = 0.50$ and $P = 0.43$, respectively). Moreover, they also formed haplotype 20, which differs from haplotype 25 in the absence of 140t and in not being associated with the disease ($P = 0.20$), thus showing that these two mutations are insufficient to confer resistance.

In summary, while phenylalanine at residue 47 seems to be crucial for determining resistance to the disease, it cannot be excluded that the contiguous positions 48 and 49 (and maybe 50) can enhance this property.

Phenylalanine is characterized by an aromatic side chain common in polyproline-binding sites. Many surface proteins in pathogenic streptococci and staphylococci have been shown to include a proline-rich region: in particular, the conformation of the proline-rich region of the streptococcal β protein, which is a protein typical of group B streptococci (GBS) also known as *Streptococcus agalactiae*, has been shown to be exposed on the bacterial surface and to adopt the conformation of a PPII helix (with three residues per turn) (Areschoug *et al*. 2002). This structure is characteristic of the peptides bound by MHC class II molecules (Jardetzky *et al*. 1996). Considering that *Streptococcus agalactiae* and *Lactococcus garvieae* are members of the same family and that they are, together with *Streptococcus parauberis* and *Streptococcus iniae,* warm-water pathogens, a similar surface protein structure could be presumed also for *Lactococcus garvieae*. This could thus explain the association with resistance found when phenylalanine is present in amino

acid changes.
The hydrophobicity plot and typing of the 3D homology model of the protein derived from haplotype 25 clearly show an increase in the hydrophobic and non-polar residues. These features are related to a change in the mutated protein conformation, because such residues tend to be buried in the protein structure.
The gain of resistance could thus be attributed to a differential peptide presentation related to an alteration of peptide-binding sites or to changes in the conformation of the complex MHC/peptide or to changes in the interaction between MHC and T-cell receptor (TCR).
Further studies are ongoing in which genetically resistant rainbow trout have been challenged with LD_{50} and LD_{70} of *Lactococcus garvieae* in controlled conditions to determine the maintenance and the level of resistance.
Even if based on a single study and on a limited number of fish, the obtained results are promising and if the protective role of 140t will be confirmed, breeding for selection could constitute an alternative or complementary approach to the traditional methods of lactococcosis control.

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