

Use of Microarray Technology to Improve DNA Vaccines in Fish Aquaculture – The Rhabdoviral Model

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1. Introduction

Fish rhabdoviral infections, specially those caused by novirhabdoviruses, can be tackled with commercial DNA vaccines such as the one against infectious haematopoietic necrosis virus (IHNV) (Salonius et al., 2007). Nevertheless, rhabdoviral diseases continue to pose a considerable threat to aquaculture because a number of practical problems regarding vaccination remain unsolved (for instance, mass delivery methods for small fish and requirements for safer vectors). Furthermore, some fish rhabdoviruses appear to be spreading to wild-type species.

Theoretically, efficient DNA vaccines could be used for any fish pathogen, such as other viruses (nodaviruses and orthomyxoviruses, for instance), bacteria and parasites. However, in practice, many fish DNA vaccines do not perform satisfactorily in most other pathogens than novirhabdoviruses. Therefore, fish novirhabdoviral vaccines are suitable models in which to study why similar DNA vaccines have not been successfully developed for other viruses (Gomez-Casado et al., 2011; Kurath, 2008). Those studies include the use of microarrays.

To date, effective vaccines against fish rhabdoviruses have been achieved simply by using their glycoprotein G gene (Einer-Jensen et al., 2009; Kurath, 2008; Kurath et al., 2007; Lorenzen, 2000; Lorenzen et al., 2009; Lorenzen & LaPatra, 2005). The glycoprotein G of rhabdoviruses is a widely studied antigen in fish (Bearzotti et al., 1995; LaPatra et al., 1994; McAllister et al., 1974; Vestergaard-Jorgensen, 1972; Winton et al., 1988) and its crystal structure has recently been elucidated in a similar mammalian rhabdovirus (Roche et al., 2006; Roche et al., 2007).

Most of our present knowledge about the factors that affect DNA vaccination efficacy in fish (vaccine dosage, delivery route, water quality, host species/size, time to challenge, severity of challenge, viral strain, etc) derives from work on fish rhabdoviral models (Kurath, 2008). Thus, the first fish DNA vaccines against IHNV were reported in 1996 (Anderson et al., 1996a; Anderson et al., 1996b) and against viral haemorrhagic septicemia virus (VHSV) in 1998 (Lorenzen et al., 1998). In 2005, Vical-Aqua Health Ltd. of Canada (Novartis APEX-

IHN) received authorization to commercialize an IHNV DNA vaccine (Salonius et al., 2007). In support of their licensing, millions of salmon were vaccinated in British Columbia in 2004 and 2005. However, there are no reports on the efficacy of this vaccine against natural viral challenges (Kurath, 2008; Salonius et al., 2007).

When injected intramuscularly in each fish, plasmid-based G glycoprotein-coding rhabdoviral vaccines induce long-term (months) specific immunity, preceded by an early (4-8 days) non-specific protective response (Kim et al., 2000; Lorenzen, 2000; Lorenzen et al., 2002). Non-specific short-term protective immunity results from the induction of interferon-*mx* and related genes, while specific long-term protection may have this effect as a result of the induction of G glycoprotein gene-specific antibody or cellular responses (Kurath et al., 2007). However, most changes in gene expression that occur with resistance mechanisms in short-term and long-term immunity are not fully understood (Goetz & MacKenzie, 2008). Furthermore, more basic knowledge on mucosal immunity is required to move rhabdoviral DNA vaccines from the laboratory into the field, as existing vaccines still require either intramuscular injection in individual fish or stronger (adjuvanted) immune responses to facilitate mass delivery methods, such as those using oral- (delasHeras et al., 2010; Tian et al., 2008) or ultrasound-aided (Fernandez-Alonso et al., 2001) immunization. Studies using microarrays could greatly contribute to furthering this basic knowledge (Secombes, 2008).

Theoretically, for best performance an optimal vaccination should mimic viral infection steps such as entry and replication. For instance, since the entry of rhabdoviruses would be first detected by cellular membrane toll-like receptors (TLRs) through the G glycoprotein and their later cytoplasmic replication by endosomal TLRs through dsRNA intermediates, the question arises as to whether DNA vaccines should include not only the G glycoprotein gene but also dsRNA intermediates (ie.: RNA hairpins). Again, new data obtained from microarrays could shed some light on these possibilities.

As established by quantitative RT-qPCR before the advent of microarrays, 4 to 8 days after DNA vaccination by intramuscular injection, gene expression by fish haematopoietic organs showed an increase in interferon-inducible *mx* (Acosta et al., 2005; Boudinot et al., 1998; McLauchlan et al., 2003; Purcell et al., 2004; Robertsen, 2008; Tafalla et al., 2007), virally-induced genes (*Vig*) (Boudinot et al., 1999; Boudinot et al., 2001) and *mhc* and *tcr* genes (Takano et al., 2004).

In this context, the recent availability of fish microarrays (Martin et al., 2008), which allow the expression profiling of thousands of genes simultaneously, has provided new opportunities to further study fish immunological responses in several rhabdovirus/fish models.

Expressed sequence tag (EST)-based microarrays of the Japanese flounder, trout, salmon and zebrafish have been used in gene-discovery efforts. These studies included infections with IHNV (MacKenzie et al., 2008; Purcell et al., 2006a), VHSV (Byon et al., 2005; Byon et al., 2006; Encinas et al., 2010) and hirame rhabdovirus (HRV) (Yasuie et al., 2007) (Table 1). However, no studies have reported on the largest microarrays that have recently become available, such as the ~ 32 K cDNA of salmonids (von Schalburg et al., 2008) and the ~ 37 K 60-mer oligos of trout (Salem et al., 2008), most probably due to the complexity of the interpretation of the data.

Fish name	Genus species	Size , ~K	Type	Name	References
Flatfish					
Japanese flounder	<i>Paralichthys olivaceus</i>	1	cDNA	HRV-infected Leukocytes	(Aoki et al., 1999) (Nam et al., 2000)
European flounder	<i>Platichthys flesus</i>	3	cDNA	GENIPOL	(Diab et al., 2008)
Atlantic halibut	<i>Hippoglossus hippoglossus</i>	7	oligo	---	(Douglas et al., 2008)
Turbot	<i>Scophthalmus maximus</i>	3	oligo	Aeromonas-infected organs	(Pardo et al., 2009)
Salmonids					
Atlantic salmon	<i>Salmo salar</i>	16	cDNA	TRAITS-SGP	(Salem et al., 2008)
Atlantic salmon	<i>salmonids</i>	32	cDNA	GRASP	(von Schalburg et al., 2008) (Koop et al., 2008)
Rainbow trout	<i>Oncorhynchus mykiss</i>	37	oligo 60mer	RTGI	(Salem et al., 2008)
Other species					
Zebrafish	<i>Danio rerio</i>	40	oligo 60mer	----	Agilent (commercial)

GENIPOL, <http://www.pleuronectes.ca>. GRASP, <http://web.uvic.ca/grasp/microarray/array.html>. TIGR, <http://biocomp.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon>. TRAITS-SGP, <http://www.traitsdb.stir.ac.uk>. RTGI, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=r_trout. Zebrafish, http://www.ensembl.org/Danio_rerio/Info/Index and Sanger zebrafish project http://www.sanger.ac.uk/Projects/D_rerio/.

Table 1. Summary of rhabdoviral-sensitive fish species with microarrays in different stages of development.

In this review we focus on the data published on the use of microarrays for the identification of rhabdoviral-induced genes with properties that make them candidate adjuvants for the improvement of fish DNA vaccines.

2. Vertebrate viral infections, vaccination and adjuvants

Pathogen-associated molecular patterns (PAMPs) are sensed in higher vertebrates by pattern recognition receptors (PRRs). There are several PRR classes (retinoic acid-inducible gene-like helicases, nucleotide-binding oligomerization domain-like receptor, peptide recognition proteins, etc). The most studied PRRs belong to the family of toll like receptors (TLRs) (Manicassamy & Pulendran, 2009). When expressed at the cell (TLRs numbers 1,2,4,5,6,10,11) and at the endosomal (3,7,8,9) membranes, TLRs detect PAMPs outside and inside the cells, respectively. Most natural infections start through mucosal surfaces that contain dendritic cells (DCs) specialized in sensing PAMPs through their cell-specific TLRs-

enriched membranes (Iwasaki, 2007a; Iwasaki, 2007b; Thompson & Iwasaki, 2008). After recognition of their corresponding PAMP, TLRs generate TLR-mediated signals, these resulting in a complex signalling network whose integration by the host determines the final immune response (Manicassamy & Pulendran, 2009).

Since the most effective vaccinations are obtained after infections with live or attenuated pathogens, several PAMPs from a unique pathogen (such as external glycoproteins and internally synthesized dsRNA/glycoproteins in rhabdoviruses) simultaneously stimulate several TLRs. In contrast, dead recombinant protein subunits and antigenic genes contain fewer PAMPs than live/attenuated pathogens. Nevertheless, single PAMPs have also been used to immunize against live pathogens, mostly with the help of adjuvants to replace the missing PAMPs. Therefore, the purpose of vaccine adjuvants is to increase the immune responses of otherwise weak individual PAMPs.

Most adjuvants in mammals are believed to target professional antigen-presenting cells, such as tissue DCs (De Gregorio et al., 2009; Lambrecht et al., 2009). The expression patterns of pro-inflammatory genes such as cytokines, chemokines, MHC and co-stimulatory molecules are altered in adjuvant-targeted DCs (Figure 1). Subsequently, maturing DCs migrate to lymph nodes and activate naive CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells to produce antigen-specific antibodies, cytotoxic cells, antimicrobial peptides and regulatory cytokines (Craig et al., 2009; Longhi et al., 2009; Manicassamy & Pulendran, 2009; Secombes, 2008). DCs also process PAMPs into peptides for presentation onto major histocompatibility (*mhc*) molecules to T cell receptors (*tcr*). Thus DCs are crucial for both adjuvant effects and innate/adaptive immune responses (Figure 1).

Although most PAMP-derived vaccine adjuvants act through TLRs on mammalian DCs (Figure 1), other internal adjuvants, such as *hmgbl* released from lysed cells, exert their action through cell damage molecules (Lambrecht et al., 2009). Artificial TLR-independent adjuvants, such as those derived from particulate compounds administered together with mammalian vaccines (mineral salts, liposomes, microparticles, saponins, and emulsions) either increase antigen persistence or uptake by DCs. Traditionally, vaccine adjuvants have been empirically identified as enhancers of antibody responses to a co-administered antigen. However, new adjuvant candidates have also been found among molecules of the signalling cascades of DC activation. According to a recent review (Secombes, 2008), the molecules with potential capacity to act as fish vaccine adjuvants might be found among: i) cytokine/chemokine molecules; ii) co-stimulatory cluster differentiation (*cd*) antigen receptors; and iii) blocking molecules, which might inhibit negative regulators. Microarray analysis of rhabdoviral fish immunizations have identified some of these molecules, as it will be reviewed here.

3. Microarrays in the study of the flatfish/HRV/VHSV models

Traditional sequencing, annotation and estimation of frequencies of each rhabdovirally-induced transcript in flatfish, is one of the strategies designed to identify genes transcribed after rhabdoviral infections (pathogen-induced gene approach) (Aoki et al., 2011). Thus, the first attempts to identify HRV-induced genes were made in the Japanese flounder *Paralichthys olivaceus* by sequencing 300-596 expressed sequence tag (EST) clones from leukocytes 2-5 days after infection. The frequencies of each EST were estimated within a short 1 to 10 range (Aoki et al., 1999; Nam et al., 2000).

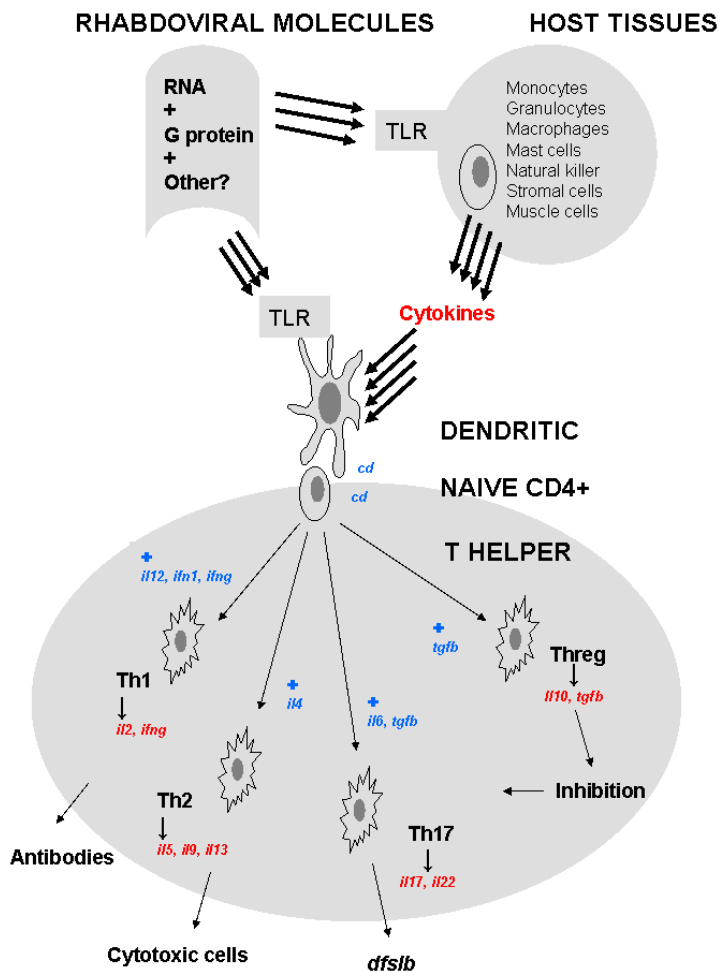


Fig. 1. Scheme of possible mechanisms of adjuvanticity. Modified from several published schemes (De Gregorio et al., 2009; Manicassamy & Pulendran, 2009; Secombes, 2008). External incoming or internally synthesized rhabdoviral molecules (dsRNA, glycoprotein G, other viral proteins, etc) activate dendritic cells (DCs). These recognize rhabdoviral molecules and are activated either through toll-like receptors (TLRs) or cytokines produced by other cells (monocytes, granulocytes, macrophages, mast cells, natural killer cells, stromal cells, muscle cells, etc). Each combination of rhabdoviral molecules induces simultaneous stimulation of DCs to induce the expression of secreted cytokines and costimulatory membrane *cds*. The induced membrane *cds*, together with other signals (blue), induce differentiation of *cd4+* cells to T helper cells (Th1, Th2, Th17 and/or Threg). Each differentiated Th cell produces a series of cytokines (red), which are required to make antibodies, cytotoxic lymphocytes, antimicrobial peptides and molecules involved in the regulation of other Ths. Theoretically, any of the up- or down-regulatory molecules that increase defensive responses could be candidate molecular adjuvants for vaccines.

The new mass-sequencing technologies, such as those offered by the Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA), Illumina Solexa (Illumina Inc., San Diego, CA, USA) or ABI SOLiD (Applied Biosystems, Foster City, CA, USA), could improve the pathogen-induced transcript frequency-estimation strategy. Thus the mass-sequencing technologies produce millions of sequences per run, facilitating significant statistical data for the quantitation of each sequence frequency. However correct annotation of such a mass of new sequences continues to be a problem (Goetz & MacKenzie, 2008). For instance, of 58 million cDNA sequences of ~ 100 bp from largemouth bass, only 31391 unique sequences could be annotated (Garcia-Reyero et al., 2008). Although, the recent production of longer sequence sizes (200–400 bp), will facilitate their annotation, comparison of transcripts from many samples by this ultra-high-throughput sequencing technology is still not economically feasible. Massive sequencing could be used as a first approach, while a more focused microarray developed with selected genes could then be used for quantification of larger numbers of samples (Goetz & MacKenzie, 2008).

Japanese flounder EST-derived cDNA microarrays were applied to *in vitro* kidney cell cultures 3–6 h after HRV infection (Kurobe et al., 2005). The number of expressed transcripts changed in 20.8 % of the genes after HRV infection. The 91 immune-related genes of the microarray were preliminarily categorized into 8 clusters on the basis of their known pattern of gene expression. After 3 h of HRV infection, several genes included in the chemotaxis, apoptosis, cell growth and antigen-presenting clusters were increased while the expression of some genes, including *mx*, decreased. Among the genes of unknown function that changed after HRV infection, 13 showed a similar response profile to that of the genes of known function mentioned above. This observation may be indicative of their association.

Improved versions of Japanese flounder EST-derived cDNA microarrays (779 spots containing 228 immune-related genes) were used for *in vivo* differential gene expression after intramuscular injection of DNA vaccines containing the G gene of VHSV (Byon et al., 2006) and/or HRV (Yasuike et al., 2007). The differential expression of their transcripts was studied in kidney tissue 1, 3, 7 and 21 days after vaccination. The greatest number of differentially expressed genes (Figure 2) was observed 3 days after injection (91.4 % were increased, of which 31 % were known genes). Genes with increased expression/transcription include those related to the non-specific immune responses, such as *tnf*, *il1r*, *ccr*, and *mx*, transcription factors, and even a few genes associated with the late specific antibody response, such as *cd20*. Many interferon-inducible genes including *mx* and interferon regulatory genes were the most strongly induced genes 3 and 7 days after injection. The expression of a number of unknown genes was also increased (Aoki et al., 2011). Among these, the *LB3(8)* gene increased a maximum of 56-fold 3 days after infection and then remained increased during one week (Byon et al., 2005).

Later versions of the Japanese flounder EST-derived cDNA microarrays of up to 1187 unique flounder ESTs (691 identified genes) were then used to compare the injection of recombinant G protein (non-protective) with the G gene (protective) (Byon et al., 2006). A number of IFN-related genes (including the unknown *LB3(8)*) and *mx* increased 7 days after injection, thereby confirming the observations made in previous studies using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) (Acosta et al., 2005; Robertsen, 2008). Further studies included differential gene expression in kidneys from Japanese flounder injected with the *HRV G* gene (protective) in comparison with the *N* gene

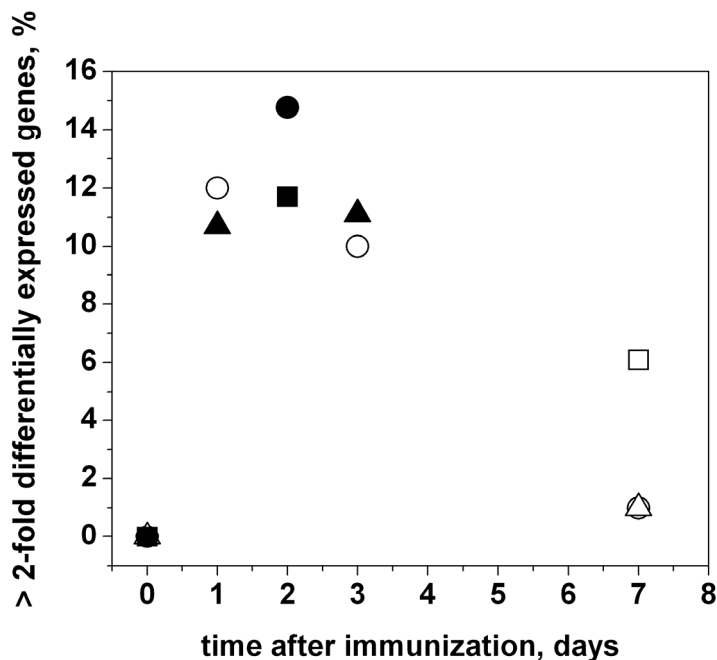


Fig. 2. Number of differentially expressed genes after rhabdoviral immunization. Genes with increase expression were defined as those genes with more than 2-fold increase in expression. ip, intraperitoneal injection. im, intramuscular injection. imm, immersion. Flounder, Japanese flounder, *Paralichthys olivaceus*. Trout, rainbow trout, *Oncorhynchus mykiss*. Zebrafish, *Danio rerio*. *, number of unique sequences or features and type of microarray (cDNA or oligo DNA). HRV, hirame rhabdovirus. IHNV, infectious haematopoietic necrosis virus. VHSV, viral haemorrhagic septicemia virus. ▲, infection-by-injection of trout with the IHNV G gene and expression on head kidney with p<0.01 (MacKenzie et al., 2008). ●, Infection-by-immersion of zebrafish with VHSV and expression on fins (Encinas et al., 2010). ■, VHSV infection-by-immersion of zebrafish and expression in internal organs (head kidney, liver and spleen) (Encinas et al., 2010). □, Injection of trout with the IHNV G gene and expression in muscle tissue with p>0.01 (Purcell et al., 2006b). △, injection of Japanese flounder with the HRV G gene and expression in head kidney (Yasuike et al., 2007). ○, Injection of Japanese flounder with the VHSV G gene and expression in head kidney (Byon et al., 2006).

(non protective). Results confirmed that the IFN-inducible genes, *LB3(8)* and *mx*, were also increased 7 days after vaccination but only when the G gene was used (Yasuike et al., 2007). Furthermore, it was shown that the *LB3(8)* gene has an homologous domain to that of a mammal IFN-inducible protein. Thus, this gene is an example of how new genes involved in rhabdoviral immunization can be discovered by the microarray approach.

However, in all the series of experiments on flounder commented above, only transcripts from pooled organs from 3-5 fish were compared. Biological replicates were not reported and therefore statistical biological variation could not be estimated. Furthermore, the number of genes in the microarrays were relatively small and their collected data has not

been deposited in any known banks to allow for independent or comparative analysis. Nevertheless, two main conclusions can be drawn from these experiments. Firstly, the largest number of differentially expressed kidney genes after fish rhabdoviral (VHSV or HRV) immunization-by-injection occurs 2-3 days after vaccination (Figure 2) and, secondly, IFN-induced gene responses are stimulated after 3-7 days (Byon et al., 2005; Byon et al., 2006; Kurobe et al., 2005).

In these earliest experimentations, microarrays based on cDNAs (100-500-mer) rather than oligos (60-70-mer) were used. Because one of the greatest concerns with cDNA arrays is cross-hybridization between similar genes or between repeated elements of different genes as a result of the pseudotetraploidy of many fish, the use of oligo microarrays would increase specificity (von Schalburg et al., 2008). However, in contrast to cDNA microarrays, oligo microarrays have a poorer performance when used for other related species. The current tendency appears to favour the use of the former. Thus, by using oligo microarrays, the printing layouts, total number of sequences and number of sequence replicates can be modified to meet any formats. Furthermore, oligo microarrays do not require maintenance of collections of bacterial clones coding for cDNAs. In addition, oligonucleotides can be selected and used in a range of various formats suitable for each experimental design. Improved sensitivity, increased dynamic range, lower variance and fewer outliers have also been demonstrated when using oligo rather than cDNA microarrays. Correlation between cDNA and oligo microarray results has been demonstrated, although some discrepancies have also been reported (Salem et al., 2008). High density oligo microarrays have been developed in other fish such as salmonids (von Schalburg et al., 2008), rainbow trout (Dios et al., 2008; Salem et al., 2008) and zebrafish (Cameron et al., 2005) (Table 1).

4. Microarrays in the study of the salmonid/IHNV/VHSV models

Large-scale genomic projects for salmon have been initiated by groups in Canada, the USA, the UK, Norway and France. As a result there are many physical and genetic maps, large collections of ESTs and a growing number of genomic sequences and derived microarrays. Thus three projects have developed salmonid microarrays. The first salmonid 16K cDNA microarray appeared in 2004. This array was developed by the Genomic Research on Atlantic Salmon Project (GRASP) (von Schalburg et al., 2005a) and led to the most recent 32K cDNA (von Schalburg et al., 2008) and the first 5K oligo DNA of 70-mer (Koop et al., 2008) microarrays. The high sequence similarity (~ 86 %) between salmonids (9 genera and 68 species) indicates that cDNA microarrays may be suitable for studies involving any member of this fish family. Transcriptome Analysis of Important Traits of Salmon (TRAITS) and the Norwegian Salmon Genome Project (SGP) also developed a 16K cDNA microarray (<http://www.abdn.sfire/salmon>) based on two independent collections of their bacterial clones kept in ARK, Genomics Facility at Roslin Institute, UK and at SGP Genetics Laboratory at the University of Oslo, respectively. The TRAITS-SGP cDNA array was obtained from ESTs from 15 tissues (pathogen-induced libraries, trait-specific subtractive EST, starvation-induced libraries, diet-response libraries, smoltification-response libraries and well-known genes). This array was conceived as a preliminary tool to develop an oligo microarray for routine health monitoring of Atlantic salmon. The first results found some artefactual expression patterns caused by cross-hybridization of similar transcripts and underlined the greater relevance of biological over technical replicates (Taggart et al., 2008).

By using all the tentative consensus sequences available at the Rainbow Trout Gene Index (RTGI) data base, a 37K oligo microarray was constructed (Salem et al., 2008), which is available at Agilent (design number 16271, deposited on the GEO with the GPL6018 number). The new rainbow trout (*Oncorhynchus mykiss*) high-density, oligonucleotide microarray was developed using 37394 specific 60-mer oligonucleotide probes assembled from 244984 ESTs from 12 tissues (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb%4r_trout). The specificity of each probe was checked for possible non-specific mRNA cross-hybridization by comparing all individual probes with all rainbow trout transcriptome sequences. Approximately 91 % of the sequences used for this microarray matched a previously annotated sequence in the GenBank.

Few attempts have been made to use these microarrays to study the rhabdoviral immunization of salmonids. In homozygous trout, the 16 K cDNA GRASP microarray was used to profile 7-day muscle transcripts after intramuscular injection of the IHNV G gene (Purcell et al., 2006a; von Schalburg et al., 2005b). After immunization, *irf3*, *mx*, *vig1*, and *vig8* transcripts were increased (Purcell et al., 2006a). Genes associated with antigen-presenting cells, lymphocytes, leukocytes, inflammation, antigen presentation, and interferon pathways were also augmented. The increased levels of transcripts associated with type I IFN pathways in systemic organs (gill, spleen and kidney) were corroborated by RT-qPCR. These observations confirmed that, when intramuscularly injected, the host-expressed viral G gene induces a systemic non-specific type 1 IFN innate immune response.

Using a 1.8K cDNA salmonid microarray, comparison of infection-by-injection with IHNV and attenuated IHNV in rainbow trout after 1 and 3 days showed an IHNV-dependent change in differential transcription in kidney towards adaptive immunity genes (MacKenzie et al., 2008). Thus, the rapid spread of the IHNV infection inhibited *tnfa*, *mhc1*, and several other gene markers while favouring *mhc2* and *ig* responses. The molecular mechanism for the development of late (months) specific cytotoxic T or B cell-mediated humoral responses has not been addressed by means of microarrays (Kurath, 2008; Kurath et al., 2006).

More recently, trout families with low (32% survival following challenge) and high susceptibility to VHSV (18% survival following challenge) were infected with VHSV by bath exposure and transcriptional data from internal organs were analyzed with the 16K GRASP microarray from day 3 post-challenge (Jorgensen et al., 2011). In total, 939 genes were differentially expressed between infected and non-infected fish. The genes increased in infected fish belonged to the following categories: stress and defence response, NFkappaB signal transduction, response to non-self, antigen processing and presentation, and proteasome complexes. Most were also increased among the 642 differentially expressed genes in the low-susceptibility trout family but not among the 556 differentially expressed genes in the high-susceptibility family. These results suggest that the innate immune system of internal organs plays a crucial role in eliciting an effective immune response to VHSV infection in rainbow trout (Jorgensen et al., 2011).

5. Microarrays in the study of the VHSV/zebrafish model

The zebrafish *Danio rerio* is one of the most suitable models in which to carry out microarray studies because, compared to other fish, its genome sequence is one of the most advanced. Furthermore, ~ 40 K annotated quantitative polymerase chain reaction (qPCR) arrays and

annotated oligo microarrays are available. In addition, large-scale experimentation with zebrafish is easier than with other fish models and zebrafish are susceptible to several viruses, most of these belonging to the fish rhabdoviral family (Sullivan & Kim, 2008). Of these, VHSV (Novoa et al., 2006) was chosen in a recent study using microarrays (Encinas et al., 2010) over IHNV (LaPatra et al., 2000), snake-head rhabdovirus (SHRV) (Phelan et al., 2005) and spring viremia of carp (SVC) (Sanders et al., 2003), because only in the VHSV/zebrafish model have infection-by-immersion (the natural route of infection) and successful vaccination been described (Novoa et al., 2006).

Damage and epithelial cell death immediately after VHSV infection in the surface portals of entry of these viruses, such as the fins (Harmache et al., 2006), should alert surrounding cells to promote epithelial cell division to replace dead cells, recruit inflammatory cells to the infection site, and send signals to internal immune organs. However, viral-induced signals to inhibit the most relevant host responses have also been detected. Detection of natural early responses may contribute to identifying vaccine adjuvants. Thus, the expression of the 636 immune-related transcripts that were increased after VHSV infection, as estimated by hybridization to oligo microarrays (confirmed by RT-qPCR arrays), was higher in fins than in organs. In contrast, the number of decreased transcripts was higher in organs than in fins (Figure 3). Therefore, an upregulated response of immune-related genes was greatest in fin tissues, while a downregulated response was most detected in the internal organ responses. The latter might be targets of viral inhibitory signals early after infection (Encinas et al., 2010). These results showed that 2 days after infection-by-immersion, VHSV had not yet caused an strong response from zebrafish internal organs, which contrasts with reports in other fish at later times after infection-by-injection (such as *ifn1*, *mx*, *il1b*, *tnfa*, etc) (Acosta et al., 2006; Samuel, 2001; Tafalla et al., 2007; Tafalla et al., 2005) or infection-by-immersion (Jorgensen et al., 2011; Zhang et al., 2009).

The zebrafish are refractory to rhabdoviral infection-by-immersion at high temperatures or without acclimation to low temperatures with IHNV (LaPatra et al., 2000), VHSV (Novoa et al., 2006) or SVC (Sanders et al., 2003). Therefore, a temperature-dependent response mechanism(s) that inhibits rhabdoviral infection and spread may occur. While these preliminary findings shed some light on the earliest effects of VHSV infection at the molecular level, some of the new immune-related genes identified might be suitable candidate adjuvants for fish vaccines (Rajcani et al., 2005; Secombes, 2008).

6. Comparative microarray study of fish/rhabdoviral models

To best detect innate immune responses, early times after rhabdoviral infection should be studied. Thus, according to the data obtained from flatfish, salmonid and zebrafish studies, the maximal number of >2-fold differentially expressed genes in microarrays was detected 2-3 days after rhabdoviral infection (Aoki et al., 1999; Byon et al., 2005; Byon et al., 2006; Kurobe et al., 2005; MacKenzie et al., 2008; Nam et al., 2000; Purcell et al., 2006a; von Schalburg et al., 2005b; Yasuike et al., 2007) (Figure 2).

Table 2 shows a list of some of the differentially transcribed immune-related genes detected using microarrays after rhabdoviral immunization, independently of immunization mechanism, fish species, rhabdoviruses and organs. Among the gene list, *ifn* and *irf*-related genes were expected to be present; however, their presence was scarce. As with many other

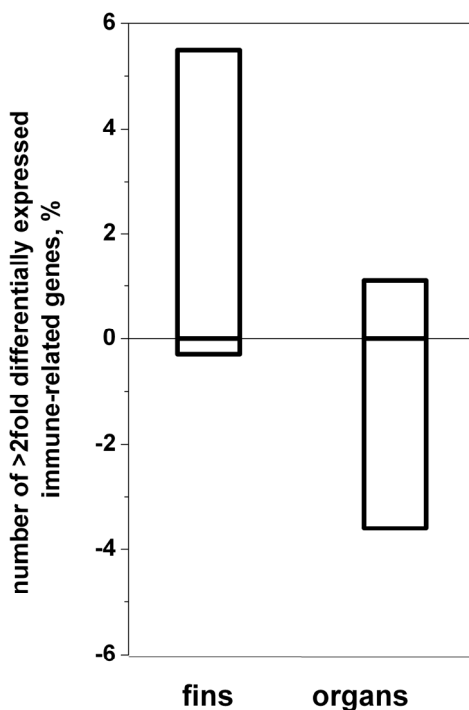


Fig. 3. Comparison of the number of differentially expressed genes 2 days after VHSV infection in zebrafish fins and organs (modified from Encinas et al., 2010). Groups of 10 zebrafish (*Danio rerio*) were infected with VHSV. To perform the hybridization to oligo microarrays, 636 selected immune-related sequences from the 44K microarray (Appligene) were used to analyze mRNA levels. Normalization was made with the ribosomal phosphoprotein p0 (*rplp0*) gene. Folds were calculated by the following formula: mRNA levels in VHSV-infected zebrafish / mRNA levels in non-infected zebrafish. The total number of genes increasing (+) or decreasing (-) expression > 2-fold and $p < 0.05$ was represented as the percentage of the total number of immune-related genes assayed ($n = 4$). Percentages were calculated by the formula, $100 \times \text{the number of differentially expressed genes} / \text{total number of immune-related genes assayed}$ (Encinas et al., 2010).

viruses and host species, an increase in *ifn1* expression is one of the first responses to the injection of any DNA vaccine (Acosta et al., 2006; Samuel, 2001) and to rhabdoviral infections (Samuel, 2001; Theofilopoulos et al., 2005).

Transcripts encoding several forms of *il17* were detected as differentially expressed only in one of the studies using microarrays (Encinas et al., 2010). *Il17* is produced by T helper 17 (Th17) cells (Figure 1) and acts together with *il22* on epithelial cells (Trifari et al., 2009) and other types of skin cells to trigger *il1b* and *tnf*. These responses induce neutrophil/macrophage recruitment in epithelial surfaces (Qiu et al., 2009), stimulate keratinocytes (Nogralles et al., 2008) and increase the production of antimicrobial peptides such as hepcidin (*hamp1*) and defensin β like-2 (*defbl2*). Both *hamp1* and *defbl2* were found

Gene classes	Genes	References
Interleukins & Receptors	<i>il17</i>	(Encinas et al., 2010)
	<i>il1r</i>	(Kurobe et al., 2005)
	<i>il8r</i>	(Nam et al., 2000)
	<i>il1b</i>	(Encinas et al., 2010)
Interferon - related molecules	<i>irf1</i>	(Kurobe et al., 2005)
	<i>mx</i>	(Yasuike et al., 2007)
	<i>isg15, 56</i>	(Yasuike et al., 2007)
	<i>iip56</i>	(Byon et al., 2006)
	<i>iip54</i>	(Aoki et al., 1999)
	<i>ifn3</i>	(Encinas et al., 2010)
Major Histocompatibility Complex	<i>mhc1</i>	(Aoki et al., 1999; Byon et al., 2006; Encinas et al., 2010; MacKenzie et al., 2008)
	<i>mhc2</i>	(Aoki et al., 1999; Byon et al., 2006; Kurobe et al., 2005; MacKenzie et al., 2008; Nam et al., 2000)
Antimicrobial peptides	<i>hamp1</i>	(Aoki et al., 2011; Encinas et al., 2010)
	<i>defbl2</i>	(Encinas et al., 2010)
Chemokines & receptors	<i>ccr</i>	(Byon et al., 2006; Kurobe et al., 2005)
Complement components	<i>c3</i>	(Byon et al., 2006; Encinas et al., 2010)
	<i>cfb/c2b, crpp, c3b, bfb, cfhp, clu, c6, c8a, c8g, c9, c1q</i>	(Encinas et al., 2010)
	<i>c3ar</i>	(MacKenzie et al., 2008)
High Mobility proteins	<i>hmgb</i>	(Aoki et al., 1999; Encinas et al., 2010; MacKenzie et al., 2008; Nam et al., 2000)
G proteins	<i>gnb</i>	(Byon et al., 2006; Encinas et al., 2010)
TNF-related molecules	<i>tnf</i>	(Byon et al., 2006; Encinas et al., 2010; Kurobe et al., 2005)
	<i>tnfr</i>	(MacKenzie et al., 2008)
	<i>tnfr1</i>	(Nam et al., 2000)
	<i>tnfr2-traf</i>	(Kurobe et al., 2005; Nam et al., 2000)
Toll-like receptors	<i>tlr2</i>	(Kurobe et al., 2005)
	<i>tlr5</i>	(Encinas et al., 2010)
	<i>tlr7</i>	(Encinas et al., 2010)
	<i>tlr9</i>	(Encinas et al., 2010)
Immunoglobulin chain domains	<i>igh</i>	(Byon et al., 2006; Encinas et al., 2010)
	<i>ighz</i>	(Encinas et al., 2010)
	<i>igl</i>	(Aoki et al., 1999; MacKenzie et al., 2008)
	<i>sid4</i>	(Encinas et al., 2010)

Table 2. Some of the fish immune-related genes differentially transcribed in microarray studies after rhabdoviral immunization. Independently of fish species, rhabdovirus, organ and time after immunization, annotated genes with a differential expression >2 fold were searched in the original papers and some of the most common were ordered on the basis of gene classes and listed in the table.

to be differentially expressed in some studies (Table 2) (Liang et al., 2006; Yu & Gaffen, 2008).

Differentially upregulated transcripts of *il12* were detected in a zebrafish fin study (Encinas et al., 2010). In that context, *il12* is crucial because it has been widely described as a vaccine adjuvant in mammals (Bliss et al., 1996; Chong et al., 2007; Hirao et al., 2008; Stevceva et al., 2006), specifically increasing protective mucosal immunity (Arulanandam et al., 1999; Wright et al., 2008) to viral infection (Hancock et al., 2000; Jacobson et al., 2006; Skeen et al., 1996; Zheng et al., 2005). However, *il12* has not been tested in fish.

Co-stimulatory cell membrane cluster differentiation antigens (*cd*) molecules responsible for the antigen-presenting cell interactions with T cells were not differentially expressed, except those belonging to *mhc1* and *mhc2* molecules (Table 2). Nevertheless, the use of *cds* as vaccine adjuvants has been described for the *cd154* gene in zebrafish (Gong et al., 2009); the reasoning being that co-expression of *cds* with antigen in the same cell might accelerate specific immune responses. Thus, specific antibody responses obtained using *cd154* and the pMCV1.4 plasmid coding for the G gene of VHSV (Ruiz et al., 2008) were increased 3-4-fold with respect to the plasmid alone (Gong et al., 2009).

Transcripts of *c3* and *c3a* were differentially expressed in salmonid and flatfish internal organs after immunization-by-injection while many more complement components (*cfb/c2b*, *crpp*, *bfb*, *cflp*, *clu* and *c6* and *c8a*) were found in zebrafish fins after infection-by-immersion (Encinas et al., 2010). The use of *c3* derivatives (*c3a*, *c3d*, *c4a* and *c5a*) (Green et al., 2002; Ross et al., 2001; Sunyer et al., 2005; Villiers et al., 1999a; Villiers et al., 1999b) as vaccine adjuvants has been reported in mammals but not in fish. A possible relationship between *c3* trout genetic polymorphism and VHSV resistance (not confirmed by genetic evidence) (Slieëndrecht et al., 1993; Slieëndrecht et al., 1995; Slieëndrecht et al., 1996) may require further physiological studies.

TLRs, immunoglobulin chains, TNF -related molecules, and high mobility proteins were also found amongst the differentially expressed genes in several studies using microarrays and thus might deserve some consideration as adjuvant candidates (Table 2).

7. Future research

Microarray and mass sequencing technologies have opened up new avenues to analyze gene expression profiles. The data obtained by these technologies might facilitate the discovery of new immune-related genes (immunogenomics), clarify the molecular mechanisms of immunity and identify new candidates for vaccine adjuvants. Nevertheless, some problems remain in the application of these technologies to the amelioration of fish rhabdoviral vaccines. For instance there is a need to improve comparison of the data obtained from different models, to complete present gene annotations, to confirm transcriptional data with protein data and to develop mathematical models to facilitate interpretation of the abundant data.

In addition, the number of immune-related genes on zebrafish, trout, salmon, human and mice microarrays (Table 3), shows that more fish immune-related genes might have to be included in future microarray designs. The number of immune-related genes are still much lower in other cultured fish species (turbot, sea bream, sea bass, etc).

Immune-related key words	Zebrafish		Trout	Salmonids		Human	Mouse
	AFYz	AGIz	AGIt	GRAs	TRAs	AFYh U133v2.0	AFYm 430v2.0
	14K	45K	37K	36K	36K	47K	39K
interferon	19	53	62	92	42	103	91
chemokine	14	36	29	56	10	104	100
interleukin	8	57	62	49	40	187	142
cytokine	13	43	28	49	13	87	68
defensin	1	3	2	0	1	29	32
antiviral	0	2	1	0	0	9	5
LPS	0	0	0	0	2	2	7
histocompatibility	13	18	13	59	3	76	74
MHC	2	16	70	375	433	10	4
viral	40	73	29	16	8	171	96
Mx	0	1	0	0	1	0	0
complement	28	79	168	88	53	129	102
immunoglobulin	14	53	116	54	46	294	156
Toll	3	22	5	17	12	39	31
TNF	15	22	7	13	3	7	38
macrophage	5	11	22	25	25	34	36
lymphocyte	2	15	22	14	8	49	52
neutrophil	2	4	0	4	7	11	7
leukocyte	6	15	12	18	4	50	21
cytotoxic	5	5	14	3	4	30	22
natural killer	3	0	3	2	5	19	8
T cell	13	64	63	59	14	88	112
B cell	20	42	29	36	11	102	97
dendritic	0	3	15	0	3	0	0
TOTAL	226	637	772	1029	748	1630	1301

Table 3. Estimation of the numbers of immune-related genes in fish microarrays compared to human and mouse. Microarrays vary in the number of probes per gene, and gene nomenclatures. Many fish genes might be duplicated variants (due to pseudotetraploid genomes or transposon variations) and arrays may use different genes and/or cDNA or oligos per gene. All these facts make comparison of microarray platform gene contents difficult. The use of immune-related key words to preliminarily compare the relative abundance of the genes might serve for a first estimation. The future should bring about the use of a common languages such as gene abbreviations following the HUGO Gene Nomenclature Committee for human orthologues (<http://www.genenames.org>) and/or UniGene entries (<http://www.ncbi.nlm.nih.gov/unigene>). Genes should be also grouped by functional categories such as by using gene ontology (GO annotation for the immune system <http://www.geneontology.org/GO.immunology.shtml>) or the clusters of orthologous genes (COG, <http://www.ncbi.nlm.nih.gov/COG>).

AFYz (AFFYMETRIX, zebrafish)	14K, ~ 15 oligos 25-mer/gen (Santa Clara, CA,USA).
AGIz (AGILENT, zebrafish vs2)	45K, 1 oligo 60-mer/gen (Palo Alto,CA, USA)
AGIt (AGILENT, trout)	37K, 1 oligo 60-mer/gen
GRAs (GRASP, salmonids)	36K, 1 cDNA/gen
TRAs (TRAITS-SGP, salmonids)	36K, 1 cDNA/gen
AFYh (AFFYMETRIX, human)	47K, U133plus v2.0 ~ 11 oligos 25-mer /gen
AFYm (AFFYMETRIX, mouse)	39K, 430 v2.0 11 oligos 25-mer/gen

The use of microarray technology stems from the availability of genome, mRNA and EST (<http://www.ncbi.nlm.nih.gov/dbEST>) sequences to build representative annotated (gene-identified sequences) microarrays. For most commercial fish species, there is a lack of information on the annotated genome or known mRNA sequences and thus most microarrays used for these species mostly apply EST sequences. However, correct and complete annotation continues to be a bottleneck.

At present, it is quite difficult to compare data from distinct microarrays, even between salmonid microarrays such as GRASP, TRAITS-SGP and RTGI. We consider that reanalysis of the data deposited in data banks, for instance by the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo>), to identify similar or identical genes should be undertaken.

For the most studied fish species, such as zebrafish, trout and salmon, identified mRNA sequences present in current GenBanks can alternatively be used to design focused microarrays enriched in some gene classes. For instance, the zebrafish and trout microarrays that are currently available omit a number of immune-related genes, the number of which vary depending on each microarray. A possible alternative to this problem in fish species for which abundant annotated mRNA sequences are held in GenBanks is to search for keyword-selected sequences to build up the corresponding microarrays.

One example of using some of the most obvious keywords corresponding to immune-related genes for the trout *O. mykiss* and the zebrafish *D. rerio* is shown in Table 4. Thus, this table shows that the number of some immune-related genes extracted from GenBank data are 2-24-fold higher than their corresponding numbers in the microarrays of trout and zebrafish commercially available. Furthermore, trout and zebrafish 2-4K microarrays designed on unique sequences selected from GenBank immune-related genes (using ~50 keywords in GenBanks) are enriched an average of 2-3-fold in immune-related sequences with respect to the more general 37-44K commercial alternatives (data not shown). The design of smaller, focused (ie: immune-related) microarrays based on existing GenBank sequences could contribute to making the experiments less expensive and their results easier to interpret.

Although the advent of mass sequencing technologies might soon change this scenario, correct annotation will still require a considerable research effort for most fish species.

Care must be taken not to over interpret differential transcript gene expression. Thus, some rhabdoviral-induced changes might involve protein cleavages (complement *c3*) and/or protein post-translation modifications (*hmgbl*) in which transcriptional control may not be essential. Although in most reports some of the microarray results were confirmed using RT-qPCR, true confirmation would require estimation of its corresponding protein levels by parallel proteomic studies. Thus, although the differential expression of some fin proteins

Gene names	Zebrafish		Trout	
	*Available microarrays	GenBank	**Available microarrays	GenBank
Interleukin	88	355	47	97
Chemokine	93	367	24	84
Interferon	89	299	48	107
Toll	41	107	8	37
Immunoglobulin	96	2207	98	1234
MHC	13	320	56	411
Vig	0	0	6	14

* vs 3 of 44K oligo microarray of zebrafish (Agilent's ID 26437)

** 37K oligo microarray of trout (Agilent's ID 16271) (Salem et al., 2008)

MHC, major histocompatibility complex

Vig, VHSV important genes

Table 4. Comparison of some immune-related genes found in commercially available microarrays with those obtained from GenBank sequences. GenBank at <http://www.ncbi.nlm.nih.gov/nucleotide>

(transferrin, hemopexin, annexin, ATP binding, alpha actin, and kinesin) show a parallel variation with their transcript levels, in most of them, the changes in the differential expression of proteins do not correlate with their corresponding transcript changes (Encinas et al., 2010). This observation suggests that regulation of their expression is not at the transcriptional level, at least in that study. Although correlation of gene and protein expression has been found in some plants (Gallardo et al., 2007; Joosen et al., 2007), most studies found no correlation, including a recent report on individual *E. coli* cells (Taniguchi et al., 2010). Correlation values comparing gene/protein expression levels in several systems are consistently very low (Hack, 2004), suggesting that mRNA levels are poor indicators of the expression of their corresponding protein. Therefore, the study of mRNA levels is justified only when protein levels cannot be detected by the proteomic approach because of their low concentrations or short lives.

Finally, mathematical modelling of microarray data may shed light on gene changes and be useful for testing new hypotheses. From the first symposium held on 2003 (Petrovsky et al., 2003), some progress has been reported on the use of mathematical modelling for early response genes (Lawrence et al., 2007), whole immune responses (Ahmed & Hashish, 2006; Kalita et al., 2006), immunity to infectious diseases, including microarray data (Morel et al., 2006) and future perspectives (Li et al., 2009; Ta'asan & Gandlin, 2009). Mathematical modelling is expected to develop further since there are few other alternatives available to interpret the massive amount of information generated by microarrays.

8. Conclusions

Novirhabdoviruses are among the few fish viral diseases for which efficacious DNA vaccines are available; however, they continue to affect aquacultured fish worldwide.

Despite DNA vaccines being commercial in Canada, the actual method of delivery by fish-to-fish intramuscular injection and safety concerns are the major bottle necks to wide

acceptance of DNA vaccination. In addition, a complete understanding of the molecular events induced after rhabdoviral fish infection and immunization may contribute to improving DNA vaccines not only for rhabdoviroses but also for other fish infections for which there are no current remedies.

Knowledge about infection, vaccination and adjuvant mechanisms in mammal models, together with high throughput genomic techniques, such as hybridization to microarrays (cDNA or oligo, wide or focused) and new massive sequencing technologies (largely unexplored in fish), offer the opportunity to gather a considerable amount of new transcriptional data in fish models.

Indeed, microarrays have already been used to quantify fish gene expression as well as to discover new genes involved in defense in several fish rhabdovirus models, such as flatfish, salmonid (salmon and trout) and zebrafish.

Genes that show increased transcription after infection (hypothetically signalling internal organs to react against the viral invasion) and also genes whose transcription is inhibited (possibly due to viral shut-off of critical host defences) might help researchers in their quest to identify new adjuvant candidates for fish vaccines.

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