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Zebrafish immunoglobulin IgD: unusual exon usage and quantitative expression profiles with IgM and IgZ/T heavy chain isotypes

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Abstract

The zebrafish is an emerging model for comparative immunology and biomedical research. In contrast to the five heavy chain isotype system of mice and human (IgD, IgM, IgA, IgG, IgE), zebrafish harbor gene segments for IgD, IgM, and novel heavy chain isotype called IgZ/T which appears restricted to bony fishes. The purpose of this study was to design and validate a suite of quantitative real time RT-PCR protocols to measure IgH expression in a vertebrate model which has considerable promise for modelling both pathogenic infection and chronic conditions leading to immune dysfunction. Specific primers were designed and following verification of their specificty, relative expression levels of IgD, IgM, and IgZ/T were measured in triplicate for zebrafish raised under standard laboratory conditions. During embryonic stages, low levels of each heavy chain isotype (IgH) were detected with each increasing steadily between 2 and 17 weeks post fertilization. Overall IgM>IgZ>IgD throughout zebrafish development with the copy number of IgM being several fold higher than that of IgD or IgZ/T. IgD exon usage was also characterized, as its extremely long size and presence of a stop codon in the second IgD exon in zebrafish, raised questions as to how this antibody might be expressed. Zebrafish IgD was found to be a chimeric immunoglobulin, with the third IgD exon spliced to the first IgM constant exon thereby circumventing the first and second IgD exons. Collectively, the qRT-PCR results represent the first comparative profile of IgD, IgM, IgZ/T expression over the lifespan of any fish species and the primers and assay parameters reported should prove useful in enabling researchers to rapidly quantify changes in IgH expression in zebrafish models of disease where altered IgH expression is manifested.

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Introduction

Because of their key role in a variety of diseases and immune responses, antibodies have been studied in many capacities and consequently represent some of the best-characterized genetic regions in traditional animal disease models (mice and humans). Data mining of the zebrafish genome has facilitated identification of the gene segments encoding antibodies in this animal model (Danilova *et al.* 2005, Hsu and Cristicitello 2006, Zimmerman *et al.* 2008). In contrast to mice and humans which harbor gene segments for five immunologlobulin heavy chain isotypes (IgD, IgM, IgA, IgG, IgE), equivalents of IgG, IgA, and IgE gene segments are not found in zebrafish. Surprisingly, a third heavy chain isotype referred to as IgZ/IgT was identified in both zebrafish (Danilova *et al.* 2005) and rainbow trout (Hansen *et al.* 2006). This IgZ/IgT isotype has also recently been found in stickleback (Gambon-Deza *et al.* 2010) and carp (Ryo *et al.* 2010) and appears to be a unique heavy chain isotype restricted to bony fish. To date, quantitative age-dependent expression of all three (IgD, IgM, IgZ) isotypes has yet to be elucidated in zebrafish, trout, or any other teleost species.

Changes in the relative proportion of IgH isotype expression are a hallmark of immune responses in mammals as the binding of antigen to a naïve B cell triggers the cell to proliferate and secrete IgM and IgD antibodies. As the immune response progresses, antigen stimulated B cells in mice and humans can further alter their expression patterns to IgA, IgG, or IgE through class switch recombination (CSR). It is important to note that neither CSR nor IgA, IgG, IgE isotypes have been found in bony fish despite the presence of the AID gene which is considered a key regulator for CSR in mammals (Saunders and Magor 2004). In humans, deficiencies in CSR have been found to be underlying features of several chronic pathological conditions correlating to elevated levels of IgM with a relative absence of IgA, IgG, and IgE (Levy *et al.* 1997, Notarangelo *et al.* 2006, Buckley 2008). Immunodeficiencies involving immunoglobulins have also been found to manifest conditions of recurrent respiratory and gastrointestinal infections, autoimmunity, and cancer predisposition in humans (Arason *et al.* 2010). Thus, it appears both isotype diversity and changes in quantitative expression of IgH are central to maintaining overall health. Although the genes encoding IgD, IgM, and IgZ/T have been identified in bony fish by database mining, the biological functions of these IgH isotypes are yet unknown (Ryo *et al.* 2010).

In order to understand the complex molecular events involved in the initiation and progression of immunodeficiency disorders, and to develop conditions that modulate either infection or disease, animal models that attempt to mimic human pathology are often utilized. The zebrafish has been used for *in vivo* modeling of chronic and autoimmune disorders including neurological diseases (Guo 2004), muscular dystrophy (Bassett and Currie 2004), acute renal failure (Hentschel *et al.* 2005), diabetes (Kinkel *et al.* 2009), hematopoietic disease (Traver *et al.* 2004, Walters *et al.* 2010) and cancer (Patton and Zon 2005, Mione and Trede 2010). Both gram-positive bacteria *Mycobacterium* spp. (Hegedus *et al.* 2009, Tobin *et al.* 2010) and gram-negative *Aeromonas* spp. (Lam *et al.* 2004, Rodriguez *et al.* 2008) have also been associated with infectious disease in zebrafish. Given that the gene segments encoding immunoglobulin loci have now been described in zebrafish, expression of these genes during normal development and in response to pathogens can now be studied in detail. Moreover, understanding how different Ig isotypes contribute to immunity may prove valuable in establishing knockout models of immunodeficiency in the zebrafish model.

In this study, we designed and optimized quantitative real-time PCR (qRT-PCR) primers, reagent conditions, and cycle parameters to quantify immunoglobulin heavy chain (IgH) gene expression in the zebrafish model. These qRT-PCR protocols were used to generate

comparative analyses of IgD, IgM, and IgZ expression over a range of embryonic, juvenile, and adult stages in zebrafish. The specificity of the qRT-PCR protocols were verified using zebrafish rag$^{-/-}$ models for immune deficiency as a negative control. In addition, zebrafish IgD exon usage was characterized, as its extensive repetitive exon configurations, unlike that of mice and humans, raises questions as to how this large antibody might be expressed. Taken together, a novel chimeric splicing pattern in IgD was identified and the qRT-PCR results provide baseline profiles for immunoglobulin heavy chain isotype expression over a wide range of developmental stages in the zebrafish model.

Materials and Methods

Animals

Zebrafish (Tübingen strain) were obtained from the Zebrafish International Resource Center (ZIRC, Eugene, Oregon) and subsequent matings of these fish were utilized to generate fish at different developmental time points. To ensure developmental synchrony, all embryos, juvenile, and adult fish were raised at low densities according to standard laboratory conditions described by Westerfield (1994). A separate population of rag$^{-/-}$ zebrafish was generated by breeding rag$^{+/-}$ fish obtained from Artemis Pharmaceuticals (Colonge, Germany). Rag$^{-/-}$ zebrafish are deficient in V(D)J-rearrangement (Wienholds *et al.* 2002) and as such were used as negative controls in this study.

RNA isolation and cDNA synthesis

Total RNA from Tübingen embryos at 0, 2, 4, 6, 8, 10 days post fertilization (dpf) and 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14 weeks post fertilization (wpf) were isolated in triplicate for each age group using Trizol (Life Technologies) in accordance with the manufacturer's guidelines. In addition, total RNA was prepared from adult Tübingen fish at 4.3, 7.1, 9, 10.8, 11, 17 and 19 months of age and from rag$^{-/-}$ fish at 8 months of age. Due to vast differences in body size, approximately 50 embryos were used for 0–10 dpf isolations, 10–50 fish were pooled for weekly time points, and for adult stages three or more fish were pooled. For all samples, whole fish were snap frozen in liquid nitrogen, pulverized with a mortar and pestle, and samples maintained at −80°C until RNA was isolated. RNA concentrations were determined by OD$_{260}$ measurements, and 1.0 µg of RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Biorad) incorporating oligo-dT and random hexamer primers. In addition, cDNA was prepared using the protocol described above for a tissue panel (thymus, pronephros, mesonephros, spleen, heart, liver, brain, intestine, muscle, gills) pooled from 20 zebrafish at 8 months of age.

Molecular Cloning of Zebrafish IgD

Conventional PCR and the Expand Long Template PCR System (Roche) using cDNA from poly-A enriched pronepheros and mesonepheros samples were utilized to evaluate IgD exon utilization. PCR reactions were carried out using conditions optimized for a series of primers designed to amplify overlapping regions of zebrafish IgD. In all cases, primers spanned one or more introns to facilitate discrimination of genomic DNA from targeted cDNA. In addition, 3' and 5' RACE with IgD specific primers were carried out to obtain additional IgD sequences. Amplicons of appropriate sizes to candidate exons were purified from 2% agarose gels using a Qiaquick Gel Purification kit (QIAgen), cloned into pCR2.1-TOPO vectors (Invitrogen) and transformed into TOP10 cells (Invitrogen). Colonies were picked by blue/white screening, expanded, and plasmid DNA was purified using the QIAgen Miniprep Kit. EcoR1 restriction digests were performed to identify clones with inserts. Inserts were sequenced bi-directionally using either M13 forward and M13 reverse primers or gene specific internal primers.

Quantitative PCR

Primers were designed for IgD, IgM, IgZ/T and a control gene β-actin using Primer 5.0 (Premier Biosoft International, Palo Alto, CA). Pairs of optimized primers and cycling conditions for IgD, IgM, IgZ/T, and the β-actin gene are reported in Table 1. For each developmental time point, qRT-PCR reactions were performed in triplicate on a MyIQ (Biorad) instrument using the iQ SYBR Green Supermix (Biorad) in a reaction volume of 20 µl with a final concentration of 1X SYBR Green, 0.5µM of each primer and 1 µl of the first strand cDNA samples. Melting curves were performed from 60 to 95°C with reads every 0.2°C and 5 second holds between each to determine amplification specificity. In all cases, the amplifications were specific and no amplification was observed in the negative controls (water blanks, no primers, RNA without reverse transcription). Amplification was carried out for 40 cycles with fluorescence readings acquired at the annealing step of each cycle. For each PCR replicate, threshold cycle (Ct) values were determined separately. Amplification efficiencies (E) and correlation coefficient R^2 values were calculated based on the use of serial dilutions of control plasmids with IgD, IgM, IgZ/T, or β-actin inserts that we created in our lab (see footnote for availability). For each plate, appropriate blanks as controls (no-cDNA, no-reverse transcriptase, no-primers etc.) were used. Standard curves from the serial dilution plasmid series reactions were calculated using the Biorad MyIQ software package. β-actin measurements were used to normalize for variation in amount and quality of RNA between samples. For each time point, three replicates were carried out and for each replicate, PCR efficiencies (E) were between 85 and 115% with correlation coefficient R^2 values exceeding 0.98. Thus, expression values could be calculated as fold change in the target gene relative to the internal β-actin standard by using the following formula: fold change = $E^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ = (Ct target gene – Ct β-actin).

Results and Discussion

Identification of a novel IgD splicing pattern in Zebrafish

Exon usage of the extensive IgD gene sequence in zebrafish was revealed as illustrated in Figure 1. The genomic configuration of the zebrafish IgH locus (Danilova *et al.* 2005) is depicted in the upper portion of the figure showing an array of V_H gene segments upstream of tandem clusters of diversity (D), joining (J), and constant (C) gene regions. IgZ and IgM constant regions are both encoded by six exons whereas the IgD constant region is comprised of 17 IgD exons. To date, this constitutes the most extensive number of IgD exons found in any animal model. This exon assemblage includes an initial $C_\delta 1$ exon, four blocks of repeated (δ2-δ3-δ4) exons, exons $C_\delta 5$, $C_\delta 6$, $C_\delta 7$, and a single membrane exon (M). The first exon in the first block ($C_\delta 2.1$) carries a frameshift mutation (Danilova *et al.* 2005) which raised questions regarding IgD expression as the inclusion of $C_\delta 2.1$ during translation would result in a truncated IgD protein. As shown in Figure 1 we found that zebrafish bypass $C_\delta 2.1$ through splicing of the first IgM exon to $C_\delta 3.1$. This novel splicing pattern of IgD has yet to be observed in any other animal model.

Comparative expression of IgH isotypes

Quantitative results of IgD, IgM, and IgZ expression for juvenile zebrafish are presented as a log of standard copy number after normalization to B-actin in Figure 2. These results suggest that IgD is expressed in low quantities during embryonic and juvenile stages and increases during development. Overall the expression pattern of IgM>IgZ>IgD is prevalent throughout zebrafish development with the copy number of IgM being several fold higher than that of IgD in adults (Table 2). Notably, IgH expression was absent in rag$^{-/-}$ fish thus validating the quantitative real time PCR parameters developed to measure IgH expression in this study. These findings also confirm the applicability of utilizing rag$^{-/-}$ fish as suitable negative controls in experiments aimed at measuring differences in IgH expression.

In conclusion, the results presented in this study demonstrate, that IgM, IgD, and IgZ/T are co-expressed over a wide range of developmental stages in the zebrafish. IgM exceeded IgD and IgZ expression at the vast majority of points indicating a possible heighted role for the IgM isotype throughout embryonic, juvenile, and adult stages of development. In addition, the expression of all three isotypes during early embryonic stages before the apparent up regulation or rag1 and rag2 (Zapata *et al.* 2006) suggests maternal IgH mRNA may be present and operative in zebrafish embryos. Overall, with this study a suite of qRT-PCR parameters were discerned and optimized for measuring levels of IgH gene expression in the zebrafish which are reproducible, specific, and highly sensitive. In addition, the results presented reveal a novel chimeric splice variant for IgD and provide a baseline measure for comparative age-dependent IgM, IgD, and IgZ/T expression in zebrafish raised under standard laboratory conditions. Further experiments such as gene knockout studies and disease challenge experiments will be necessary to understand the biological function of the different heavy chain isotypes in this emerging immunological model for biomedical research.

Acknowledgments

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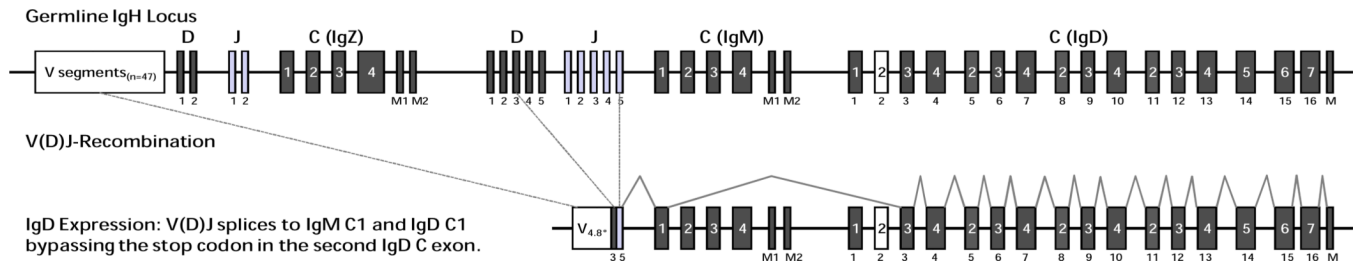
Figure 1. Zebrafish IgD is a chimeric immunoglobulin with a novel splicing pattern
The upper portion of diagram shows genomic configuration of the zebrafish IgH locus (Danilova *et al.* 2005). Given all V, D, J, and C gene segments are in the same transcriptional orientation, V(D)J rearrangement to the second cluster results in the deletion of the IgZ constant region (shown). The lower portion of the diagram depicts the exon usage of IgD as deduced by cloning IgD fragments from cDNA. V(D)J splicing to the first exon of the IgM constant region (Cµ1) revealed zebrafish IgD to be a chimeric immunoglobulin. In addition, Cµ1 splicing to the third IgD exon ($C_\delta 3.1$) bypasses the first two IgD exons, one of which contains a frameshift mutation ($C_\delta 2.1$). The presence of the frameshift mutation had raised questions as to how or if zebrafish IgD might be expressed as its inclusion would result in a truncated IgD protein. Here we show that IgD is expressed and that functional in-frame transcripts are produced by a non-standard splicing pattern which bypasses both $C_\delta 1.1$ and $C_\delta 2.1$. The bypass of the first and second IgD exons has yet to be found in any other animal model.
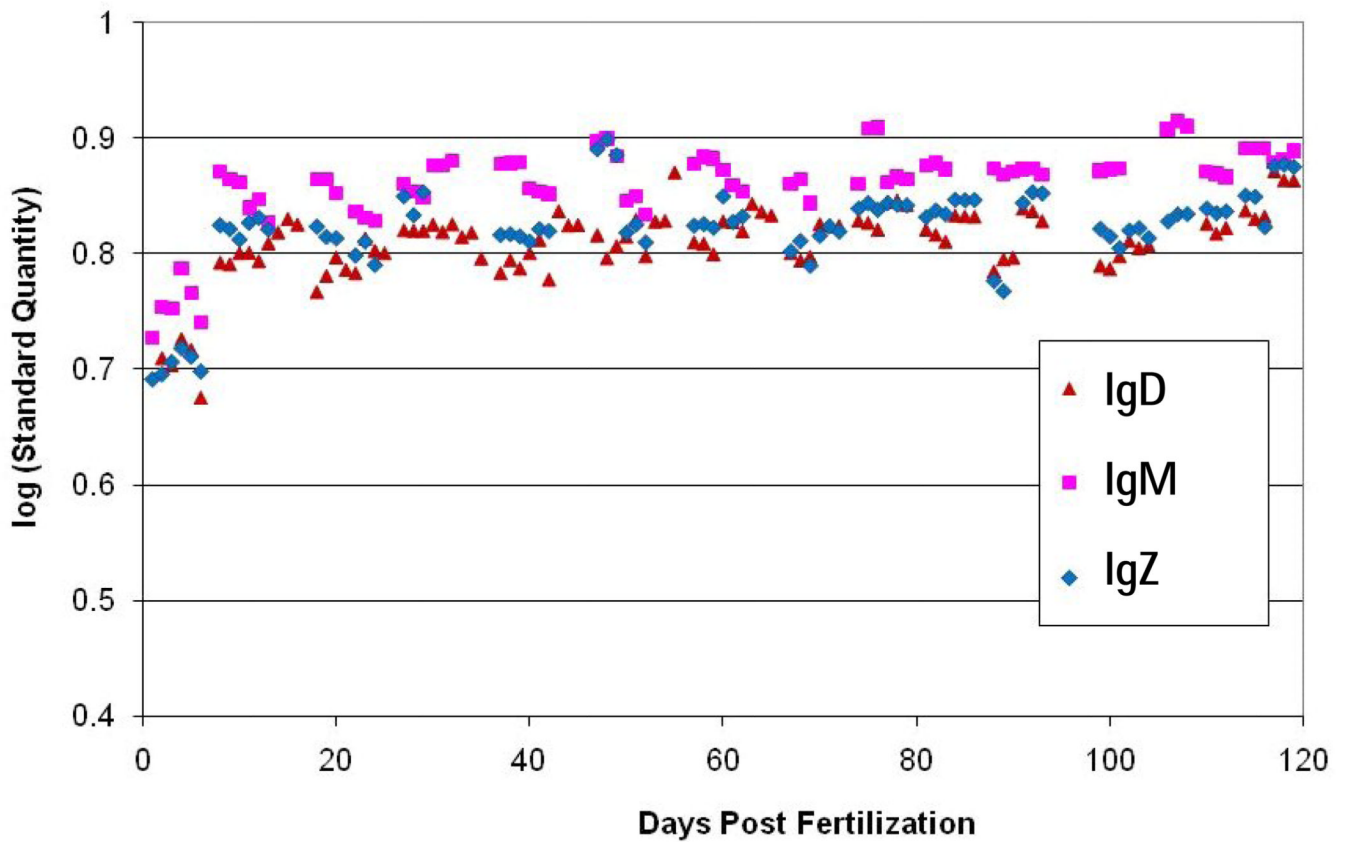
Figure 2. Quantitative expression profiles of IgD, IgM, and IgZ isotypes from zebrafish aged 2–129 days post fertilization

Real time PCR results show an expression pattern of IgM>IgZ>IgD throughout early zebrafish development. The collective contribution of membrane and secreted forms of IgM, IgD, and IgZ are demonstrated as amplicons spanned internal exons thus amplifying both membrane and secreted forms. Results are presented as mean values of the log (relative copy number) after normalization to B-actin. Each sample was run in triplicate with a dilution series of plasmid cDNA to generate standard curves and appropriate non-template controls. Melting curves also indicated single specific melting peaks thus demonstrating that the protocols developed and optimized have amplification specificity for the targeted heavy chain isotype.

Table 1

Optimized quantitative real-time PCR primers and reaction conditions to measure IgD, IgM, IgZ/T, and β-actin gene expression in zebrafish.

Targeted transcripts	Primers	Primer Location[*]	Amplicon Size	Conditions
IgD FWD	5'-GACACATTAGCCCATCAGCA-3'	BX510335; 43011-42992	156 bp	95°C 3 min; 30 cycles: (95°C 10 sec; 58°C 10 sec; 72°C 3 sec)
IgD RVS	5'-CTGGAGAGCAGCAAAAGGAT-3'	BX510335; 39712-39731		
IgM FWD	5'-GAAGCCTCCAATTCTGTTGG-3'	AY643751; 258-277	147 bp	95°C 3 min; 30 cycles: (95°C 10 sec; 56°C 10 sec; 72°C 5 sec)
IgM RVS	5'-CCGGGCTAAACACATGAAG-3'	AY643751; 404-386		
IgZ/T FWD	5'-GAACCAAACTCAGGGTTGGA-3'	AY643750; 300-281	152 bp	95°C 3 min; 30 cycles: (95°C 10 sec; 56°C 10 sec; 72°C 2 sec)
IgZ/T RVS	5'-CACCCAGCATTCTACAGCAA-3'	AY643750; 149-168		
β-actin FWD	5'-CCGTGACATCAAGGAGAAGCT-3'	AF057040; 680-700	201 bp	95°C 3 min; 30 cycles: (95°C 10 sec; 58°C 10 sec; 72°C 10 sec)
β-actin RVS	5'-TCGTGGATACCGCAAGATTCC-3'	AF057040; 880-860		

[*] Position of primers in the nucleotide sequence of corresponding GenBank Accession numbers.

Table 2

Quantitative and comparative expression of IgM, IgD, and IgZ/T heavy chain isotypes in adult zebrafish of various ages.

Developmental Stage (months post fertilization)	IgM	IgD	IgZ/T
4.3	2.15E+03	5.25E+02	3.17E+01
7.1	3.83E+04	6.44E+01	3.29E+02
9.0	3.09E+03	1.02E+01	7.23E+01
10.8	4.68E+03	3.11E+02	4.46E+01
11.0	3.05E+02	1.56E+00	2.19E+01
17	7.16E+02	2.05E+00	3.37E+01
19	3.24E+02	1.23E+00	3.32E+01

Data are reported as fold increase of each isotype over negligible baseline expression of rag$^{-/-}$ zebrafish after normalization to B-actin. The Mann Whitney U test revealed significant differences between comparisons of IgM to IgD ($p = 0.006$) and IgM to IgZ/T ($p = 0.004$) across developmental stages. No significant differences were found between comparisons of IgD to IgZ/T expression at the time points measured. Collectively the results show IgM expression to be several fold higher than IgD or IgZ/T in zebrafish raised in standard laboratory conditions.