INVESTIGATION OF IMMUNOGLOBULIN HEAVY CHAIN ISOTYPES IN AN ANCESTRAL MUCOSAL IMMUNE MODEL

A Thesis

by

CHRISTINA C. DU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Laboratory Animal Medicine

Investigation of Immunoglobulin Heavy Chain Isotypes in an Ancestral Mucosal Immune Model

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Approved by:

Chair of Committee,	Michael F. Criscitiello
Committee Members,	Jane Welsh
	Sara Lawhon
	Vincent Gresham
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ABSTRACT

Investigation of Immunoglobulin Heavy Chain Isotypes in an Ancestral Mucosal Immune Model. (August 2011)

Christina C. Du, B.A.; B.S., University of California, Irvine; D.V.M., Ross University

Chair of Advisory Committee: Dr. Michael F. Criscitiello

The importance of gut associated lymphoid tissues has been extensively reported in higher vertebrates, but less is known in lower vertebrates. In mammals immunoglobulin (Ig)A is the primary Ig of mucosal immunity. But no IgA has been identified in cold-blooded animals. In higher vertebrates, antigen must stimulate the lymphoid tissues in the intestines to elicit an IgA response, and cytokines from CD4 positive helper T cells are required for B cell switch. It is not known if this is the case in lower vertebrates, or if T cell help evolved before or after class switch recombination between functional antibody isotypes. My study will fill in these gaps in our knowledge by comparing oral antigen inoculation relative to intraperitoneal antigen inoculation in frogs (*Xenopus* sp.). Oral immunization is a novel approach to eliciting immune responses in *Xenopus*. I propose that IgX will increase with oral inoculation compared to intraperitoneal injection. This would be the first demonstration of class switch upon oral immunization to a mucosal isotype in the first vertebrates that employs higher vertebrate Ig heavy chain switch mechanism, which would shed light on the most fundamental aspects of our humoral adaptive immune system.

Using a total Ig ELISA protocol, measuring total relative levels of IgM, there was no difference between the first three groups of orally immunized frogs compared to intraperitoneally immunized frogs. However, a response to serum IgX was seen in the first group. On the other hand, the refined Ag-specific ELISA protocol did present a significant increase in serum IgM response in frogs immunized systemically over orally immunized animals, but not an overall IgX response.

Phylogenetic analysis suggests that, contrary to initial reports, IgA evolved from IgX. With consideration of entire constant region and individual constant domain analyses as well as synteny and function, we suggest new hypotheses of vertebrate antibody evolution to be tested as immunogenetic coverage of more species continues to expand.

DEDICATION

I would like to dedicate this thesis to my father, James S. Du, who passed away a month before I started this residency program. Without you, none of this would have been possible. Thank you…

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Criscitiello, and my committee members, Dr. Welsh, Dr. Lawhon, Dr. Gresham, for their guidance and support throughout the course of this research.

I would like also like to thank Sara Mashoof, Jonathan Walker and Natalie J. Jacobs for their assistance in this project and Patricia Chen's assistance in the laboratory.

I would like to thank my family for their support in the pursuit of my veterinary career for over a decade. It's been a long journey and I thank you for your patience and understanding.

Thanks also go to my friends and colleagues and the department staff for making my time at the Comparative Medicine Program, Veterinary Pathobiology Department at Texas A&M University a memorable one.

This work was supported by the NIH through grants to MFC (AI56963) and a graduate student award to me from the College Of Veterinary Medicine, Texas A&M University.

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1. INTRODUCTION AND LITERATURE REVIEW

Xenopus laevis, from an evolutionary standpoint, is the choice model for many adaptive immune system studies. Aside from the fact that they can easily be manipulated during larval stages and have limited maternal and environmental influences during early development, *Xenopus* are the most primitive major model species that share a fundamentally similar immune system to humans (Du Pasquier et al. 1989). All vertebrates' first line of defense is the innate immune system, which is ready at birth. It involves barriers, anti-microbials, phagocytosis, inflammation, and complement. The innate system can be activated by foreign molecules with pathogen-associated molecular patterns (PAMPs). These are recognized by germline-encoded pattern recognition receptors (PRRs) on specific cells, which initiate a cascade of events, the end result being elimination of the pathogen. It is fast to respond and non-specific for any individual pathogen. On the other hand, the adaptive immune system of vertebrates is slow, with high molecular specificity and a memory component. It is executed by lymphocytes and requires induction by antigen presenting cells. The adaptive immune system provides the ability to recognize and mount responses to pathogens. This mechanism allows a small number of genes controlled by

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This thesis follows the style of Veterinary Immunology and Immunopathology.

somatic recombination activating gene (RAG) dependent rearrangement to generate a vast repertoire of different antigen receptors.

An important aspect of adaptive immunity is that an individual can be immunized against a specific antigen in a process called vaccination. It is generally long-term and protects when the same antigen is encountered a second time. The immune response will be different than the first time it was encountered. It will be faster because specific lymphocytes have already undergone clonal expansion and affinity maturation. When B cells and T cells are activated, some become memory cells. These memory cells form an expanded database of B and T lymphocytes. This process occurs when a random, diverse primary repertoire of lymphocyte specificities is created in the thymus and bone marrow. After removal of cells that recognize self, the remaining mature, naive cells populate the secondary lymphoid tissues. On binding specific antigen, the lymphocyte is activated to clonally expand and produce progeny specific to that antigen. Upon interaction with a previously encountered antigen, the specific memory cells are selected and activated. In this manner, the second and subsequent exposures to an antigen produce a stronger and faster immune response. This is "adaptive" because the body's immune system prepares itself for future challenges.

The adaptive humoral immune response is responsible for the production of antibodies that bathe the extracellular spaces and neutralize and flag extracellular pathogens and toxins for destruction. B cells, necessary for the production of antibodies, usually require helper T cells to help differentiate into antibody-secreting plasma cells. Antibodies can be either secreted or act as a surface receptor. Antibodies have three

basic functions: neutralization, opsonization and complement activation; to bind to molecules that elicit an immune response, to neutralize complete virus or bacterial cells and to signal to other cells to destroy the foreign pathogen. Antibodies have a similar basic structure comprising variable regions and a constant region and consist of di-sulfide linked light and heavy chains, made of constant and variable immunoglobulin superfamily domains. The antigen-binding region varies extensively between antibody molecules. The total repertoire of antibodies made by an individual allows any foreign structure to be recognized. The constant region does not carry this variation and is greatly conserved across evolution. The constant regions are important for 1) binding Fc receptors 2) binding complement and 3) delivering antibodies to specific locations such as colostrum, gut lumen or across the placenta. RAG-mediated V(D)J rearrangement increases diversity of the variable region, while class switch recombination (also known as isotype switching) maintains the variable region and switches the constant regions.

Five immunoglobulin isotypes have been identified in humans: IgM, IgD, IgG, IgE and IgA. The first antigen receptors expressed by B cells are IgM and IgD and can be expressed without class switch recombination. IgM largely functions in complement activation. Function for IgD was generally unknown until recent studies showed that it aides activation of basophils and eosinophils (Chen et al. 2009). IgG is the most abundant isotype in serum and (like IgM) is able to cross the placenta. IgE functions in parasitic and allergic response, and IgA, by far the most abundant isotype in the body, is the major mucosal immunoglobulin. Aside from functional differences, there are structural differences between the isotypes in their number of constant domains,

tendency to polymerize, and presence or absence of a hinge region. The immunoglobulin isotypes are encoded at the heavy chain locus by a cluster of immunoglobulin heavy-chain C-region genes. These heavy chain clusters are split into exons that correspond to an individual immunoglobulin domain. In mammals, the same assembled variable region may be expressed in IgG, IgA or IgE antibodies, after class switch from IgM. Switch of the constant region occurs through non-homologous DNA recombination and is guided by switch regions upstream of each gene. This process is mediated by several enzymes including AID (the activation induced cytidine deaminase), and create nicks in the DNA. The two switch regions are brought together and the coding regions and DNA between the regions are deleted. For example, the B cell with a heavy chain VDJ rearrangement that recognizes a helminth would now be able to make that specific V domain on IgE instead of IgM.

Cartilaginous fish are the oldest animals that have an adaptive immune system based upon rearranging immunoglobulin superfamily antigen receptors. They have the same genes encoding the RAG recombinase that lead to immunoglobulin and T cell receptor gene rearrangement and a polygenic, polymorphic major histocompatibility complex. Amphibians are the oldest vertebrates that share with mammals the capability of class switch recombination (Du Pasquier et al. 2000). Fellah et al., concluded that amphibians may have been the species in which diversification of Ig occurred which lead to various Ig isotypes in higher species (Fellah et al. 1993). Therefore, *Xenopus laevis* is the best choice for our research model to study the evolution of mucosal immune isotypes.

The major barrier breached by most pathogens is the mucosal surface, which comprises an enormous area to be protected. This includes the gastrointestinal tract, the respiratory tract, the urogenital tract, salivary glands, lactating glands and the conjunctiva of the eye. In mammals, secretory IgA is the predominate immunoglobulin of the mucosal surfaces and recognition of antigen in mucosal immune tissues causes B cell switch to this isotype. However, IgA has not been identified in cold-blooded vertebrates, and little has been studied of the evolution of our secretory mucosal immunoglobulin despite its importance in host defense.

In humans, the thymus is required for T cell maturation and the spleen is the main peripheral organ where T and B cells migrate to sample antigen and become activated (Hsu 1998). At mucosal surfaces, the resident lymphoid tissues comprise the mucosal associated lymphoid tisssues (MALT). MALT is further broken down into bronchial-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT), as well as others. GALT include the tonsils, adenoids, appendix and Peyer's patches in the distal ileum. Other animals have similarly organized lymphoid organs in the intestines, such as those observed in the rabbit appendix (Becker and Knight 1990), the Peyer's patch in sheep (Reynaud et al. 1995) and the bursa of Fabricius in avian species (Cooper et al. 1966). In humans, the effector site of intestinal immune responses is the lamina propria, consisting of a heterogenous group of lymphoid and myeloid cells concentrated in germinal centers. These cells, including lymphocytes, macrophages, dendritic cells, neutrophils and mast cells, are responsible for production of a vast array of cytokines that aid in IgM to IgA isotype switching, nurture IgA B cell differentiation,

and secrete factors that aid in the transport of IgA from the lamina propria to the lumen (Malik et al. 2010). In the gut-associated lymphoid organs, naïve B cells must be stimulated by activated T cells. This in turn promotes B cell proliferation and maturation and leads to clonal expansion of memory B cells and antibody producing plasma cells. *Xenopus laevis*, the oldest vertebrate that shares the mammalian capability of immunoglobulin class switch recombination, apparently does this without germinal center formation required for B cell maturation (Du Pasquier et al. 2000). *Xenopus*, similarly to humans, do have thymus as a primary T lymphoid organ and a secondary peripheral organ in the spleen, but unlike mammals, they lack lymph nodes, germinal centers and Peyer's patches.

Other heavy chain isotypes have been described, besides our five, from other vertebrate groups. IgM has been well studied across species and is widely expressed throughout most immune tissues. IgM is conserved across vertebrates from shark to man. The identification of IgD in *Xenopus* (Ohta and Flajnik 2006), IgW in lungfish (Ota et al. 2003) and IgW in shark (Harding et al. 1990) has also linked mammalian IgD to an ancient origin in cartilaginous fish. IgY is identified in amphibians, reptiles and birds. In fact, the expression and function of IgY in amphibians has been shown to be similar to mammalian IgG and IgE, and phylogenetically has been shown to be the ancestor of these mammalian isotypes. In fact, the expression and function of IgY in *Xenopus* is similar to mammalian IgG and IgE and shows similarity to rabbit and ox IgG extracellular domains (Mussmann et al. 1996). The constant domains of IgF are very similar to IgY, indicating that they arose from a duplication event (Zhao et al. 2006).

IgX is similar to IgM, with four constant domains and forming polymers, but is not associated with the secretory J chain, yet is expressed by plasma cells found in the lamina propria of the gut epithelium (Mussmann et al. 1996). As in the case of IgA, polymerization with J chain is required for transport through epithelia. Expression of IgM and IgX are thymus independent and IgY is thymus dependent (Fellah et al. 1993) as determined by studies using intraperitoneal immunization. Recent literature has identified IgZ/IgT in teleost fish (Danilova et al. 2005) as a mucosal-associated lymphoid tissue immunoglobulin and it was suggested that this may be the last distinct isotype to be discovered (Flajnik 2005). Although, no J chain has been associated, IgT has been found to be a polymer in gut mucosa and a secretory component has been identified. IgT clearly shows homology to IgM, but no relationship to other isotypes has been found, suggesting that it arose after bony fish diverged from other vertebrates. Thus we have a very good idea of where four of the five antibody classes of placental mammals came from. IgM and IgD are ancient from the dawn of the system, IgG and IgE came from amphibian IgY. The natural history of IgA has not been as clear. An article by Mussmann in 1996 reported up to 60% of the B cells identified in the gut of the frog *Xenopus laevis* were IgX secretors, however, these were hardly identifiable in liver, spleen (Mussmann et al. 1996) or serum (Hsu 1998). Mussman also reported that systemic immunization in *Xenopus* elicited an IgM and IgY response, but not an IgX response. This mucosal localization of frog IgX, coupled with early reports of sequence similarity between IgM and IgX, suggested that IgX might be the functional analog, but not the ortholog of IgA (Hsu et al. 1985).

My hypothesis is that frog IgX, a proposed functional analog of our IgA isotype, will be specifically produced against oral immunization and is in fact an ortholog of our IgA. I believe it shares a common ancestor with our IgA in early tetrapod. I propose that IgX will increase with oral inoculation compared to intraperitoneal injection and that IgM will be increased in systemically challenged animals compared to orally challenged animals. Oral immunization is a novel approach to eliciting immune response in *Xenopus* and this methodology will have to be developed. This would be the first demonstration of class switch upon oral immunization to a mucosal isotype in the first vertebrates that employ our Ig heavy chain switch mechanism. We do not know if T cell help evolved before or after class switch recombination. Results would shed light on the most fundamental aspects of our humoral adaptive immune system. My study will fill in these significant gaps in our knowledge by **inoculating frogs orally** and comparing this route to intraperitoneally immunized frogs. This work will be complemented by phylogenetic analysis of IgX and IgA in light of recent immunoglobulin findings in diverse tetrapods.

2. MATERIALS AND METHODS

2.1. Development of Frog Experimental Techniques

Xenopus laevis were initially purchased from *Xenopus* Express (Brooksville, Florida). Animals were housed in two XenoPlus (Tecniplast) recirculating systems. Tanks are constructed of polycarbonate with two water delivery pipes nested within each other. This system allows reduction of cleaning operations and minimizes overflow if debris clogs the outer pipe. A sleeve with smaller holes fits over the outer pipe for tadpoles. The system is equipped with a touchpad that provides control of buffering, temperature and water change operations. Visual and auditory alarm warnings help alert the user to parameters that have been exceeded. The water treatment unit is equipped with prefiltration, biological filtration, fine mechanical filtration, chemical filtration and UV light disinfection. Juvenile and adult frogs are provided a pellet diet, while tadpoles are fed a powdered yeast diet. Room conditions are maintained at a 12:12 hour light:dark cycle and ambient temperature of 23ºC. All subsequent generations were bred in house using chorionic gonadotropin hormone (Sigma). Each frog used in an experiment was identified with an identification chip (AVID) inserted intraperitoneally at the time of immunization.

Previous literature reported euthanasia dosage in adult frogs weighing 100-125 g (Torreilles et al. 2009). The average size of animal used in this immunization study was 20-40 g. Using tricaine methane sulfonate (MS-222), a range of concentrations was tested to sedate the frogs quickly, yet provide a wide safety margin against any suffering

before euthanasia. Since MS-222 is acidic, the solution was buffered with phosphate buffered saline (PBS, 80 g of NaCl, 2.0 g of KCl, 27.2 g of $Na_2HPO_4 \bullet 7H_2O$, distilled H_2O to 1L) prior to immersion of animals. Each frog was weighed prior to submersion. One frog was immersed to test each dose (500 mg/L, 1 g/L, 2 g/L and 5g/L) and observed for movement and righting reflex at 5 minutes and 10 minutes post-immersion. All animals were recovered in a separate recovery tank without complications. The results of this study also assisted in determination of euthanasia concentrations. All frogs euthanized in MS-222 were confirmed dead with a secondary method; pithing or decapitation.

Blood collection techniques from *Xenopus* have not been recently published. Cardiac puncture, toe clipping and venous cut down are currently used methods, but for small 20-40 g frogs these methods are unsatisfactory for providing consistent and reliable samples. To exsanguinate, frogs were heavily sedated in MS-222 and opened along the ventrum to expose the heart. A small incision was made at the base of the heart and using a 1 ml syringe, blood was collected as it pooled. Frogs were pithed after exsanguinations to verify euthanasia according to protocol Criscitiello #2008-033.

2.2. Gavage Technique

Mice and rats are routinely gavaged using feeding needles made specific for drug or vehicle delivery (Table 1, adapted from Braintree Scientific). Using this same technique as a model, a frog was euthanized in 5g/L of MS-222 and dissected to observe all major organs. A feeding needle was inserted into the mouth and observed to pass

into the stomach. We tested the technique with stainless steel and plastic feeding needles of various diameters and lengths (Figure 1). During actual immunizations, the frogs were sedated in 2 g/L of MS-222. The bulb of the feeding needle was placed at the joint of the upper and lower jaw. Slight pressure was applied to open the mouth and the needle directed towards the center into the stomach. The full gavage needle was inserted up to the hub for proper delivery into the stomach of a 20-40 g frog (Figure 2). Recovery of each sedated frog was uncomplicated within 30-60 minutes in a separate recovery tank with regular XenoPlus system water.

Table 1: Standard Feeding Needle Size Recommendations.

Laboratory Animal		Recommended Standard Sizes			
Species	Wgt. range in grams	Gauge	Length	Ball Dia.	Shape
Mice	to 14 gms	24	1"	1 1/4 mm	Straight
	15-20 gms	22	1", 1 1/2"	1 1/4 mm	Straight
	20-25 gms	20	1", 1 1/2",3"	2 1/4 mm	Straight, Curved
	25-30 gms	18	1", 1 1/2",2"	2 1/4 mm	Straight, Curved
	30-35 gms	18	2", 3"	2 1/4 mm	Straight, Curved
Rats	50-75 gms	20	1", 1 1/2"	2 1/4 mm	Straight
	75-120 gms	18	1", 1 1/2"	2 1/4 mm	Straight, Curved
	100-200 gms	18	2", 3"	2 1/4 mm	Curved
		16	2"	3 mm	Straight, Curved
	150-300 gms	16	3", 4"	3mm	Curved
	200-350 gms	14	3"	4 mm	Curved
		13	3"	4 mm	Straight
Hamsters	60-200 gms	18	2"	2 1/4 mm	Curved
Guinea Pigs	250-300 gms	18	11/2", 2	2 1/4 mm	Curved
	350-450 gms	16	3", 4"	3mm	Curved
	400-600 gms	14	3"	4mm	Curved
		13	3"	4mm	Straight
Rabbits	1-3 kgms	16	3", 4"	3 mm	Curved

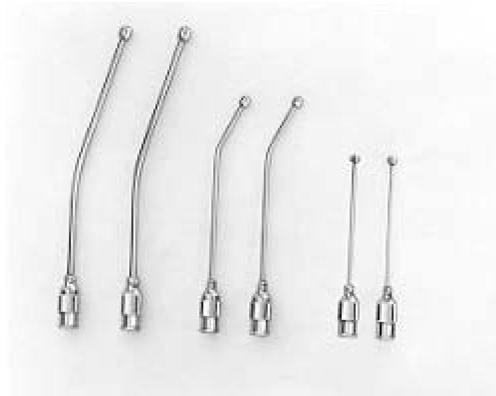


Figure 1: Feeding Needles. Examples of Different Sizes and Shapes of Feeding Needles.

Figure 2: Placement of Feeding Needle. Demonstration of Proper Placement of Feeding Needle for Gavage Technique.

2.3. Immunizations

Four sets of frogs underwent immunizations. The first set of 10 frogs was comprised of three orally immunized frogs (PO), three intraperitoneally (IP) immunized frogs, and four non-immunized frogs (control). The second group of 12 contained four PO, four IP, and four control frogs. The third group of 24 had eight PO, eight IP, and eight control animals. The fourth group contained 24 frogs with eight PO, eight IP, and eight control frogs. Groups 1, 2 and 4 were all boosted twice after the initial immunization and then harvested a week after the last boost. The third set of frogs was immunized only once and harvested 21 days later. Hapten dinitrophenol conjugated to the protein carrier keyhole limpet hemocyanin (DNP-KLH, CalBioChem) along with adjuvant cholera toxin, known to elicit a strong enteric mucosal immune response in mammals (Lycke and Holmgren 1986), comprised the immunogen. DNP-KLH orally immunized frogs received 2.5 mg DNP-KLH with 10 µg of cholera toxin as adjuvant. Intraperitoneally immunized animals received 200 µg of antigen and 200 µl of complete Freund's adjuvant for the first inoculation and 200 µg of antigen along with the same volume of incomplete Freund's adjuvant for the remaining inoculations (Tables 2-5). Muramyl dipeptide, the key factor in Freund's complete adjuvant has been described by McKenzie et.al (McKenzie and Halsey 1984). To prepare the emulsion for intraperitoneal injection, two 1.0 ml syringes and one 18 gauge double hub emulsifying needle were used. Each aliquot was delivered aseptically into 1 ml syringe and the emulsifying needle attached and the second syringe connected to the opposite end of the needle (Figure 3). The contents were mixed by forcing the material back and forth

through the needle for 10 minutes. As the formation of water-in-oil emulsion is initiated, the solution increases in viscosity and becomes more difficult to push through the emulsion needle. To test whether the emulsion is ready, a small drop of emulsion is placed into a beaker of water. The drop should hold together on the surface of water if prepared properly.

Figure 3: Emulsifying Needle. Demonstration of Emulsifying Needle with Two 1.0 ml Luer-lock Syringes for Intraperitoneal Antigen Delivery.

Table 2: First Group of Immunizations. Immunization Protocol, Routes and Dates of Immunization.

Frog ID	Route of Immunization	Protocol	Date of Immunization	Protocol	Date of Immunization	Date of Immunization	Date of Immunization	Euthanasia
40068609	PO	1.25 mg of antigen & 10 ug of toxin	10/28/09	2.5 mg of antigen & 10 ug of toxin	11/4/09	11/12/09	11/20/09	12/9/09
40106305	PO	1.25 mg of antigen & 10 ug of toxin	10/28/09	2.5 mg of antigen & 10 ug of toxin	11/4/09	11/12/10	11/20/09	12/9/09
40094344	PO	1.25 mg of antigen and 10 ug of toxin	10/28/09	2.5 mg of antigen & 10 ug of toxin	11/4/09	11/12/10	11/20/09	12/9/09
40061336	IP	200 ug of antigen & 200 ul of CFA	10/28/09	200 ug of antigen & 200 ul of IFA	11/4/09	11/12/10	11/20/09	12/9/09
40106346	IP	200 ug of antigen & 200 ul of CFA	10/28/09	200 ug of antigen & 200 ul of IFA	11/4/09	11/12/10	11/20/09	12/9/09
40062790	IP	200 ug of antigen & 200 ul of CFA	10/28/09	200 ug of antigen & 200 ul of IFA	11/4/09	11/12/10	11/20/09	12/9/09
40097857	control	No treatment	10/28/09	No treatment	11/4/09	11/12/10	11/20/09	12/9/09
40072280	control	No treatment	10/28/09	No treatment	11/4/09	11/12/10	11/20/09	12/9/09
40106625	control	No treatment	10/28/09	No treatment	11/4/09	11/12/10	11/20/09	12/9/09
40065537	control	No treatment	10/28/09	No treatment	11/4/09	11/12/10	11/20/09	12/9/09

Table 3: Second Group of Immunizations. Immunization Protocol, Routes and Dates of Immunization.

Frog ID	Route of Immunization	Protocol	Date of Immunization	Protocol	Date of Immunization	Date of Immunization	Date of Immunization	Euthanasia
48528626	PO	1.25 mg of antigen & 10 ug of toxin	4/2/10	1.25 mg of antigen & 10 ug of toxin	4/10/10	4/16/10	4/22/10	5/3/10
48529873	PO	1.25 mg of antigen & 10 ug of toxin	4/2/10	1.25 mg of antigen & 10 ug of toxin	4/10/10	4/16/10	4/22/10	5/3/10
48540782	PO	1.25 mg of antigen & 10 ug of toxin	4/2/10	1.25 mg of antigen & 10 ug of toxin	4/10/10	4/16/10	4/22/10	5/3/10
48548547	PO	1.25 mg of antigen & 10 ug of toxin	4/2/10	1.25 mg of antigen & 10 ug of toxin	4/10/10	4/16/10	4/22/10	5/3/10
48543284	IP	200 ug of antigen & 200 ul of CFA	4/2/10	200 ug of antigen & 200 ul of IFA	4/10/10	4/16/10	4/22/10	5/3/10
48530549	IP	200 ug of antigen & 200 ul of CFA	4/2/10	200 ug of antigen & 200 ul of IFA	4/10/10	4/16/10	4/22/10	5/3/10
48523067	IP	200 ug of antigen & 200 ul of CFA	4/2/10	200 ug of antigen & 200 ul of IFA	4/10/10	4/16/10	4/22/10	5/3/10
40075638	IP	200 ug of antigen & 200 ul of CFA	4/2/10	200 ug of antigen & 200 ul of IFA	4/10/10	4/16/10	4/22/10	5/3/10
40083849	control	No treatment	4/2/10	No treatment	4/10/10	4/16/10	4/22/10	5/3/10
40086381	control	No treatment	4/2/10	No treatment	4/10/10	4/16/10	4/22/10	5/3/10
40069038	control	No treatment	4/2/10	No treatment	4/10/10	4/16/10	4/22/10	5/3/10
40101269	control	No treatment	4/2/10	No treatment	4/10/10	4/16/10	4/22/10	5/3/10

Table 4: Third Group of Immunizations. Immunization Protocol, Routes and Dates of Immunization

Frog ID	Route of Immunization	Protocol	Date of Immunization	Euthanasia
48531097	PO	1.25 mg of antigen & 10 ug of toxin	7/9/10	7/30/10
48539881	PO	1.25 mg of antigen & 10 ug of toxin	7/9/10	7/30/10
45829561	IP	200 ug of antigen & 200 ul of CFA	7/9/10	7/30/10
48531588	IP	200 ug of antigen & 200 ul of CFA	7/9/10	7/30/10
48531614	control	No treatment	7/9/10	7/30/10
48513618	control	No treatment	7/9/10	7/30/10
48532124	PO	1.25 mg of antigen & 10 ug of toxin	7/24/10	8/13/10
48538598	PO	1.25 mg of antigen & 10 ug of toxin	7/24/10	8/13/10
48526365	IP	200 ug of antigen & 200 ul of CFA	7/24/10	8/13/10
48528096	IP	200 ug of antigen & 200 ul of CFA	7/24/10	8/13/10
48529602	control	No treatment	7/24/10	8/13/10
48539258	control	No treatment	7/24/10	8/13/10

Table 4, continued.

Frog ID	Route of Immunization	Protocol	Date of Immunization	Euthanasia
48513570	PO	1.25 mg of antigen & 10 ug of toxin	7/17/10	8/7/10
48550035	PO	1.25 mg of antigen & 10 ug of toxin	7/17/10	8/7/10
48520345	IP	200 ug of antigen & 200 ul of CFA	7/17/10	8/7/10
48380006	IP	200 ug of antigen & 200 ul of CFA	7/17/10	8/7/10
48541370	control	No treatment	7/17/10	8/7/10
48556023	control	No treatment	7/17/10	8/7/10
48371074	PO	1.25 mg of antigen & 10 ug of toxin	7/25/10	8/15/10
48518356	PO	1.25 mg of antigen & 10 ug of toxin	7/25/10	8/15/10
48534321	IP	200 ug of antigen & 200 ul of CFA	7/25/10	8/15/10
48531545	IP	200 ug of antigen & 200 ul of CFA	7/25/10	8/15/10
48532776	control	No treatment	7/25/10	8/15/10
48371541	control	No treatment	7/25/10	8/15/10

Table 5: Fourth Group of Immunizations. Immunization Protocol, Routes and Dates of Immunization.

Frog ID	Immunization Route	Protocol	Date of Immunization	Date of Immunization	Date of Immunization	Euthanasia
48519538	control	No treatment				10/25/10
48514844	control	No treatment				10/25/10
48532305	control	No treatment				10/28/10
48372541	control	No treatment				10/28/10
48375553	control	No treatment				11/12/10
48543086	control	No treatment				11/12/10
48372549	control	No treatment				11/18/10
48541798	control	No treatment				11/18/10
48530376	IP	200 ug of antigen & 200 ul of CFA	9/27/10	10/7/10	10/15/10	10/25/10
48554635	IP	200 ug of antigen & 200 ul of CFA	9/27/10	10/7/10	10/15/10	10/25/10
48531096	IP	200 ug of antigen & 200 ul of CFA	9/30/10	10/7/10	10/15/10	10/25/10
48372843	IP	200 ug of antigen & 200 ul of CFA	9/30/10	10/7/10	10/15/10	10/25/10

Table 5, continued.

Frog ID	Immunization Route	Protocol	Date of Immunization	Date of Immunization	Date of Immunization	Euthanasia
48532104	IP	200 ug of antigen & 200 ul of CFA	10/7/10	10/15/10	10/22/10	10/28/10
48547374	IP	200 ug of antigen & 200 ul of CFA	10/7/10	10/15/10	10/22/10	10/28/10
48549615	IP	200 ug of antigen & 200 ul of CFA	10/7/10	10/15/10	10/22/10	10/28/10
48522564	IP	200 ug of antigen & 200 ul of CFA	10/7/10	10/15/10	10/22/10	10/28/10
48537869	PO	2.5 mg of antigen & 10 ug of cholera	10/15/10	10/22/10	10/29/10	10/25/10
48528266	PO	2.5 mg of antigen & 10 ug of cholera	10/15/10	10/22/10	10/29/10	10/25/10
56271587	PO	2.5 mg of antigen & 10 ug of cholera	10/18/10	10/25/10	11/01/10	10/28/10
56265593	PO	2.5 mg of antigen & 10 ug of cholera	10/18/10	10/25/10	11/01/10	10/28/10
56114074	PO	2.5 mg of antigen & 10 ug of cholera	10/18/10	10/25/10	11/01/10	11/12/10
56276019	PO	2.5 mg of antigen & 10 ug of cholera	10/18/10	10/25/10	11/01/10	11/12/10
56278127	PO	2.5 mg of antigen & 10 ug of cholera	10/21/10	10/28/10	11/04/10	11/18/10
56272568	PO	2.5 mg of antigen & 10 ug of cholera	10/21/10	10/28/10	11/04/10	11/18/10

2.4. ELISA

The purpose of the enzyme-linked immunoassay was to compare total Ig made by gut and spleen B cells of immunized frogs to non-immunized frogs regardless of antigen specificity (Figure 4). Serum was separated from whole blood collected from frogs. Using a 96 U well microtiter plate (BD Falcon), 100 µl of serial diluted (1:10^1 to 1:10^{11}, Figure 5) frog sera of one frog was placed in each row. Amphibian phosphate buffer saline (a-PBS, 65% 1X PBS and 35% de-ionized water), mammalian PBS adjusted for amphibian homeostatic salt, was added to the last column as control (Figure 6). The plate with sera was incubated at 37ºC for 1 hour. The contents of the plate were dumped, washed twice with a-PBS, blotted dry on clean paper towels and blocked with 2% casein overnight at 4ºC. Anti-*Xenopus* Ig hybridomas and the properties of their products have been described by Hsu 1984 at the Basel Institute for Immunology, Basel, Switzerland. Monoclonal antibodies 10A9, 14A11 and 14G1 are against heavy chain IgM. Antibodies 4110D5, 4110B3 and 408A10 are directed against IgX and 11D5 against IgY (Hsu and Du Pasquier 1984). The next day the plate was washed with PBS and 100 µl of mouse anti-frog IgM hybridoma supernatant (10A9) or IgX (408A10) at 1:100 dilution was added to all wells except the row with no primary antibody. The plate was incubated overnight at 4ºC. ELISA controls that contained no primary (IgM, 10A9) or no secondary antibodies (sheep anti-mouse IgG conjugated to horseradish perioxidase) were selected from the immunized group. 100 µl of sheep anti-mouse IgG conjugated to horseradish perioxidase (Sigma) was added after washing with PBS except for rows without secondary antibody. After incubation for an hour at room temperature,

the plate was washed twice with a-PBS and dried. 3, 3’, 5, 5’-Tettramethylbenzidine (TMB, Sigma) substrate solution was made by dissolving one tablet in 1 ml of DMSO and adding 9 ml of 0.05 M phosphate-citrate buffer, pH 5.0 (Sigma). Two µl of 30% hydrogen peroxide (EMD Chemicals USA) per 10 ml of substrate buffer solution was added immediately prior to use. The reaction was stopped by the addition of 100 µl of 2 M H_2SO_4 (BDH Aristar, 13.9 ml of 18 M stock H_2SO_4 solution was diluted in 86 ml of de-ionized water to prepare a 2 M solution) per well. Absorption was detected at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad).

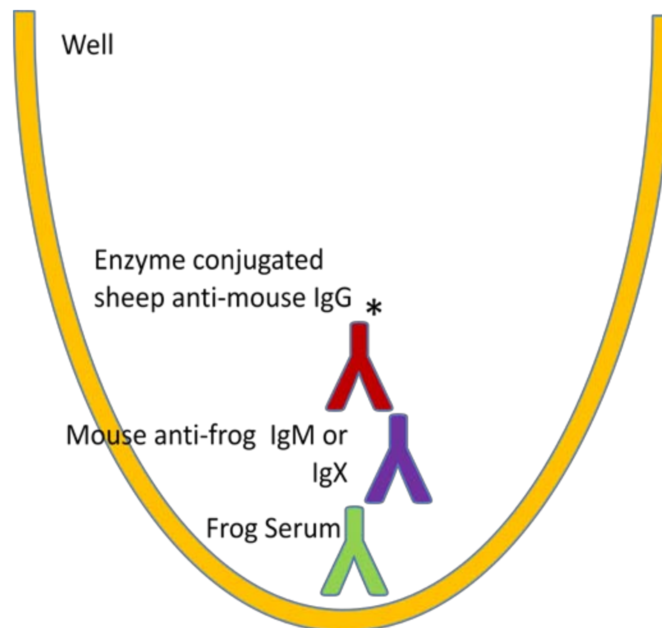


Figure 4: Total ELISA Protocol Diagram.

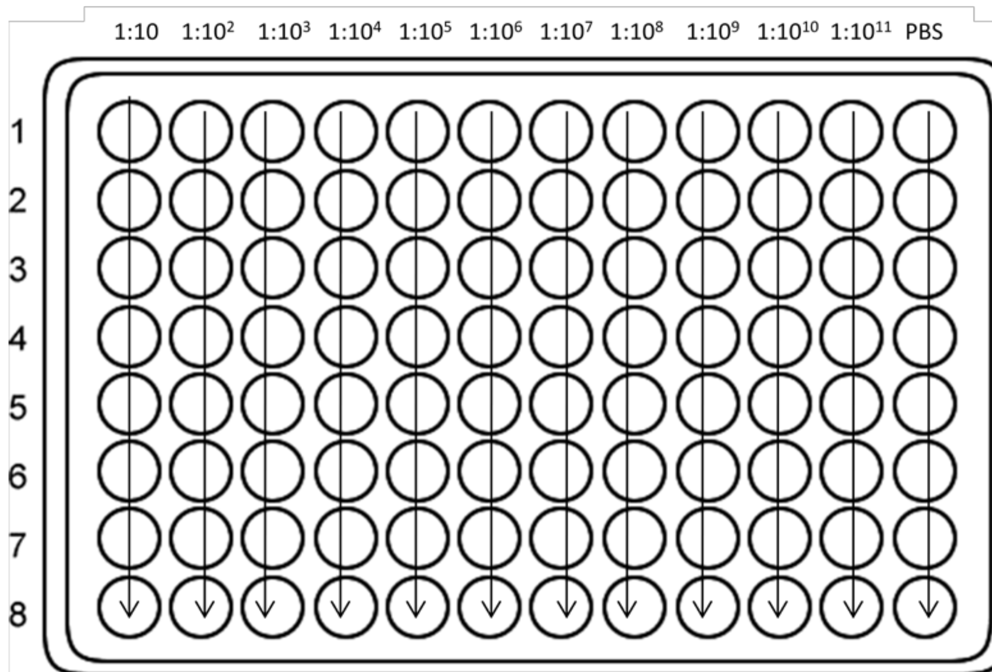


Figure 5: Total ELISA Protocol Serial Dilutions. Serum Titrations from $1:10^1$ to $1:10^{11}$ and PBS as Control.

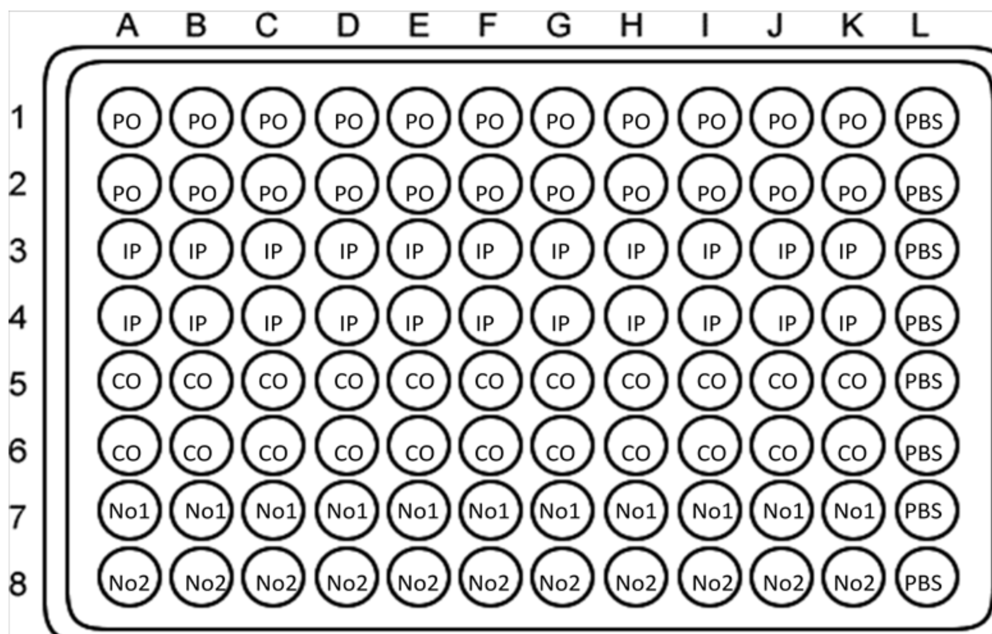


Figure 6: Total ELISA Protocol Plate Set-Up. Organization of Plate with Two PO, Two IP, Two Control Non-immunized, and Two ELISA Controls.

The second ELSA protocol was designed for measurement of antigen specific antibodies of a particular isotype. It started with 100 µl of 1 µg/ml DNP-KLH in each well (Figure 7). The plate was incubated at 37ºC for one hour, washed twice with PBS, and blocked with 2% casein overnight at 4ºC. The next day, after washing twice with PBS, *Xenopus* sera diluted from 1:3 to 1:243 were added to the wells and incubated for 2 hours at 37ºC (Figure 8). After washing with PBS, 100 µl of IgM (10A9) or IgX (4110B3) at a 1:100 dilution was added to all wells except the no primary antibody rows and the plate incubated overnight at 4ºC. Again as described in the first protocol, ELISA controls that contained no primary or no secondary antibodies were selected from the immunized group (Figure 9). Next, 100 µl of sheep anti-mouse IgG conjugated to horseradish perioxidase was added after washing with PBS except for rows without secondary antibody. This was then incubated for an hour at room temperature and after washing twice with PBS. 3, 3', 5, 5'-Tetramethylbenzidine substrate solution was made by dissolving one tablet in 1 ml of DMSO and adding 9 ml of 0.05 M phosphate-citrate buffer, pH 5.0. Next, 2 µl of 30% hydrogen peroxide per 10 ml of substrate buffer solution was added immediately prior to use. The reaction was stopped by addition of 100 µl of 2 M H_2SO_4 per well. Absorption was detected at 450 nm using an iMark Microplate Absorbance Reader.

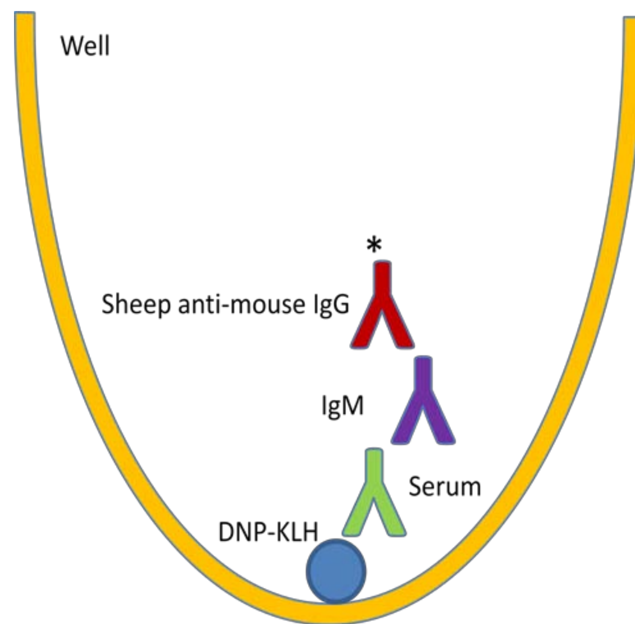


Figure 7: Antigen-Specific ELISA Protocol Diagram.

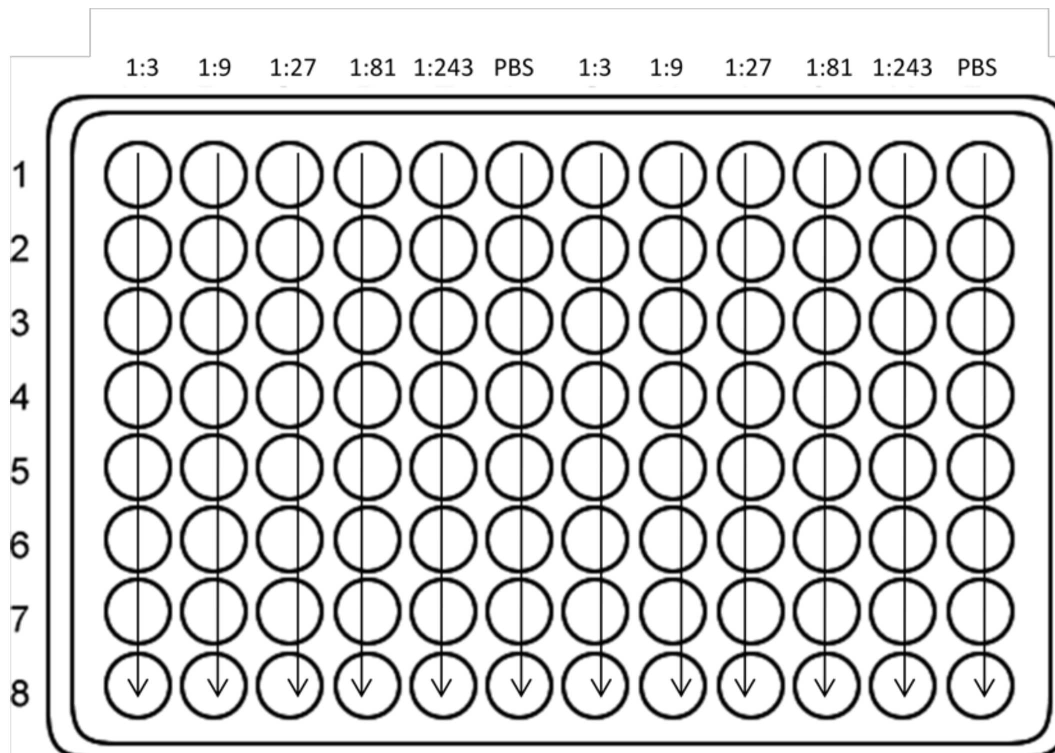


Figure 8: Antigen-Specific ELISA Protocol Serial Dilutions. Serum Titrations 1:3 to $1:3^5$ and PBS as Control. Multiple Samples Can Be Run Simultaneously.

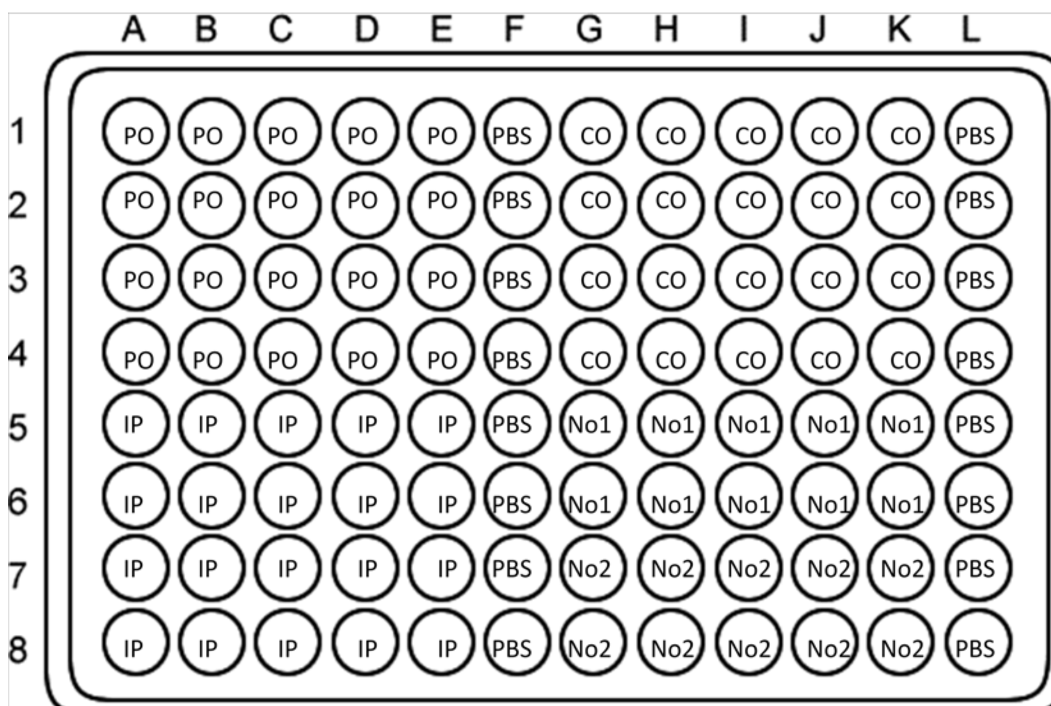


Figure 9: Antigen-Specific ELISA Protocol Plate Set-Up.

2.5 Phylogenetic Analysis

A set of amino acid sequences of immunoglobulin heavy proteins in non-mammalian tetrapods was compiled (Table 6). Alignments were initially made in Bioedit with ClustalW employing gap opening penalties of 10 and gap extension penalties of 0.1 for pairwise alignments, then 0.2 for multiple alignments and the protein weighting matrix of Blossum. Default alignment parameters were used. These alignments were then modified by hand, especially the entire C region alignment in making sure most homologous C regions were aligned to one another. MEGA was used to infer the phylogenetic relationships of immunoglobulin C regions and individual C domains. Neighbor-joining consensus trees were made from 1000 bootstrap replicates,

using pairwise deletion and collapsing internal nodes with less than 50% bootstrap support.

Table 6: List of Species Used to Generate Alignments.

Common Name	Species	Tree Abbreviation Example	Ig	C	Protein
Nurse shark	*Ginglymostoma cirratum*	M C1 shark	IgM	4	AAT76789.1
Zebrafish	*Danio rerio*	M C1 zebrafish	IgM	4	AAT67447.1
		T C1 zebrafish	IgT	3	AAT67446.1
Rainbow trout	*Oncorhynchus mykiss*	M C1 trout	IgM	4	AAW66972
		T C1 trout	IgT	4	AAW66981.1
Fugu	*Takifugu rubripes*	H fugu	IgH	2	BAD89297
Iberian ribbed newt	*Pleurodeles waltl*	M C1 newt	IgM	4	CAE02685
		X C1 newt	IgX/P	4	CAL25718
		Y C1 newt	IgY	4	CAE02686
Mexican axolotl	*Ambystoma mexicanum*	M C1 axolotl	IgM	4	A46532
		X C1 axolotl	IgX	4	CAO82107.1
		Y C1 axolotl	IgY	4	X69492
African clawed frog	*Xenopus laevis*	M C1 frog	IgM	4	AAH84123
		X C1 frog	IgX	4	S03186
		Y C1 frog	IgY	4	AAH97629
African clawed frog	*Xenopus tropicalis*	F C1 frog	IgF	2	MGC108125
Red-eared slider	*Trachemys scripta elegans*	M C1 slider	IgM	4	AAB03838
Chinese soft-shelled turtle	*Pelodiscus sinensis*	M C1 turtle	IgM	4	ACU45376.1
		Y C1 turtle	IgY	4	ACU45374.1
Green anole	*Anolis carolinensis*	M C1 anole	IgM	4	ABV66128
		Y C1 anole	IgY	4	ABV66132
Leopard gecko	*Eublepharis macularius*	M C1 gecko	IgM	4	ABY74510.1
		A C1 gecko	IgX/A	4	ABG72684.1
		Y C1 gecko	IgY	4	ACF60235.1
Duck	*Anas platyrhynchos*	M C1 duck	IgM	4	AAA68605.1
		A C1 duck	IgA	4	AAA68606.1
		Y C1 duck	IgY	4	CAA46322.1

Table 6, continued.

Common Name	Species	Tree Abbreviation Example	Ig	C	Protein
Common pheasant	*Phasianus colchicus*	M C1 pheasant	IgM	4	PMID 20398946
		A C1 pheasant	IgA	4	PMID 20398946
		Y C1 pheasant	IgY	4	PMID 20398946
Turkey	*Meleagris gallopavo*	M C1 turkey	IgM	4	PMID 20398946
		A C1 turkey	IgA	4	PMID 20398946
		Y C1 turkey	IgY	4	PMID 20398946
Quail	*Coturnix japonica*	M C1 quail	IgM	4	PMID 20398946
		A C1 quail	IgA	4	PMID 20398946
		Y C1 quail	IgY	4	PMID 20398946
Chicken	*Gallus gallus*	M C1 chicken	IgM	4	P01875
		A C1 chicken	IgA	4	AAB22614.2
		Y C1 chicken	IgY/G	4	S00390
Australian echidna	*Tachyglossus aculeatus*	M C1 echidna	IgM	4	AAN33013.1
		A C1 echidna	IgA	3	AAN33012.1
		G C1 echidna	IgG	3	AAM61760.1
Platypus	*Ornithorhynchus anatinus*	M C1 platypus	IgM	4	AAO37747.1
		A1 C1 platypus	IgA1	3	AAL17700.1
		A2 C1 platypus	IgA2	3	AAL17701.1
		Y C1 platypus	IgY/O	4	ACD31541
		G1 C1 platypus	IgG1	3	AAL17703.1
		G2 C1 platypus	IgG2	3	AAL17704.1
Gray short-tailed opossum	*Monodelphus domestica*	M C1 opossum	IgM	4	AAD24482.1
		A C1 opossum	IgA	3	AAC48835.1
		G C1 opossum	IgG	3	AAC79675.1
Mouse	*Mus musculus*	M C1 mouse	IgM	4	AAB59651.1
		A C1 mouse	IgA	3	AAB59662.1
		G C1 mouse	IgG	3	AAB59656
Human	*Homo sapiens*	M C1 human	IgM	4	CAA33070
		A1 C1 human	IgA	3	AAC82528.1
		G1 C1 human	IgG1	3	AAC82527.1

2.6 Ig Heavy/Ig Light Isotype Relationships

B cells were sorted based on IgL isotype using MACS magnetic cell sorting system (Miltenyi Biotec). Spleens were disassociated using a wire mesh with 1 ml of a-PBS, spun down and washed with MACS buffer [0.5% BSA (0.5g/100 ml) and 2mM EDTA (400 ug/100ml) into a-PBS]. After the supernatant was removed the B cells were resuspended in 1 ml of MACS a-PBS for counting. Since direct MicroBeads were not commercially available for frog immunoglobulins, the indirect magnetic cell labeling protocol for MACS was followed. The splenic B cells were spun down after counting and labeled with primary antibody in 1:2 dilutions for 1 hour at 4ºC. Cells were washed by adding 2 ml of buffer, spun down and the supernatant removed. This was repeated. In a second step, cells are magnetically labeling with MACS Microbeads. Anti-mouse IgG microbeads (20 µl) was added to 80 µl of resuspended cells and allowed to incubate for 15 minutes at 4ºC. After washing, cells were resuspended in 500 µl of MACS buffer. Positively labeled cells were separated following the positive selection strategy for MACS (Figure 10). A column is suspended onto a magnetic and the labeled and unlabeled cell suspension is added. The column is flushed with 3 ml of MACS buffer three times. The desired magnetically labeled cells remain in the column as cells negative for primary antibody flow through and are collected. These negative cells are used as control. The column is removed from the magnet and the positively labeled cells are flushed through the column and counted for comparison prior to labeling. Approximately 50,000 positively labeled cells were recovered using MACS sorting.

Figure 10: MACS Cell Sorting System. Photo Depicts Adding Cell Suspension into Column. Collected Cells Will Be Negatively Labeled and Used as Control.

Using RNeasy (Qiagen), RNA was purified from the positively collected cells. Buffer RLT (350 μl) was used to resuspend the pelleted cells. A 20 gauge needle was fitted to an RNase-free syringe and the cells were lysed by passing through the needle repeatedly. One volume of 70% ethanol was added to the lysate and pipetted to mix and 700 μl of sample was transferred to an RNeasy column and centrifuged for 15 s at 10,000 rpm. The flow-through was discarded and 350 μl of Buffer RW1 was then added to the column and centrifuged for 15 s at 10,000 rpm and flow through discarded. Then 10 μl of DNase I stock solution was added to 70 μl of Buffer RDD and mixed by inverting the bottle. The 80 μl was added to the RNeasy column and incubated at room temperature for 15 minutes. Buffer RW1 (350 μl) was then added to the column and

centrifuged for 15 seconds at 10,000 rpm and flow through discarded. 500 µl of Buffer RPE was added to the column and centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded. The spin column was placed in a new collection tube and 30-50 µl of RNase-free water was added and centrifuged for 1 min at 10,000 rpm to elute the RNA. Confirmation of RNA was performed using a nanodrop spectrophotometer. RNA was either stored in -80ºC or immediately converted to first-strand cDNA (Invitrogen).

For first strand cDNA synthesis, 5 µg of total RNA, 1 µl of random hexamer primer, 1 µl of 10mM dNTP mix, and DEPC-treated water were combined to make a total 10 µl solution in a 0.5 ml PCR tube. This was incubated at 65ºC for 5 minutes and immediately placed on ice for 1 minute. cDNA synthesis mix (2 µl of 10X RT buffer, 4 µl of 25mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNase OUT, 1 µl of SuperScript) was prepared by adding each component in the indicated order. cDNA synthesis mix (10 µl) was added to the RNA/pimer mixture, mixed gently and centrifuged briefly. The mixture was incubated for 10 minutes at 25ºC and immediately for 50 minutes at 50ºC. The reaction was terminated at 85ºC for 5 minutes and chilled on ice and1 µl of RNase H was added and incubated for 20 minutes at 37ºC. cDNA can be stored at -20ºC or used for PCR immediately.

cDNA results were tested using PCR with primers to the conserved WYRK motif and reverse primers to the N-terminal domains of the three IgH constant genes made by Invitrogen (Table 7). Master mix for PCR consisted of 10 µl of 5X buffer, 1 µl of dNTP (10mM), 1 µl of front and rear primers (10 mM), 5 µl of template (positive or negative), and 0.25 µl of Taq polymerase. Water was added to total a 50 µl mix. The gel prep

consisted of 0.4 g of UltraPure agarose, 60 ml of 1X TAE and 2.7 µl of gel green dye. The agarose was added to a 250 ml beaker, covered with plastic wrap and heated in the microwave for 1:30 minutes, making sure the solid dissolved. Gel green dye (2.7 µl) was added to the heated solution and stirred gently. The beaker was allowed to cool before pouring into a small gel cast with 1.5 mm ten tooth comb.

Table 7: Primers Used for PCR.

Code	Name	5'-3' Sequence	T_M C
MFC 201	X1 IgM C3 F1	AACACACAGCTGGCTTCA	58.4
MFC 202	X1 IgM C4 R1	AGCATCTCAAGGTGGCAGTT	58.4
MFC 203	X1 IgX C3 F1	GTGTTTGTGCTGAGGTGGCAGTT	60.5
MFC 204	X1 IgX C4 R1	TAGTTCTTGAGCGGATGGTG	58.4
MFC 205	X1 IgY C3 F1	CACCCTGATCTTCCATCACC	60.5
MFC 206	X1 IgY C4 R1	TAAAGATTAAGTAGTAGA	42.4
MFC 207	X1 IgY C4 R2	GTCGTACGTATTCTG	43.5
MFC 208	X1 IgY C4 R3	CTATAGAACCCCACACTTC	55

3. RESULTS

3.1 Development of Frog Experimental Techniques

The optimal sedation concentration for the young post-metamorphosis frogs was 2 g/L of MS-222 buffered solution. At 2 g/L, frogs ranging from 20-40 g were adequately sedated to handle without extensive animal movement which minimized trauma and risk of dropping. Sedation was adequate at 10 minutes, but not at 5 minutes duration which was assessed by cessation of movement, especially swimming motion of the rear feet and delayed righting reflex if turned over. At 500 mg/L and 1g/L, the frogs continued to move 20-30 minutes after immersion. At 5 g/L, some frogs stopped moving after 2 minutes and the righting reflex returned after 60 minutes in the recovery tanks. Based on this finding, it was determined to be an adequate euthanasia dose (Table 8).

Table 8: Results of MS-222 Sedation.

Frog ID	Weight (g)	Concentration (g/L)	Time to sedation (min)
1	20	0.5	25
2	28	1	> 30
3	22	2	10
4	26	5	2

Blood collection by cardiac exsanguination was a quick, reliable method of collecting 1.0-1.5 ml of blood from each frog. Cardiac puncture, venous cut-down and toe-clipping did not result in consistent blood volume collection. Problems in the first

and second groups of immunizations included hemolysis and clotting, but were not issues in the 3rd and 4th groups of immunizations.

3.2 Gavage Technique

The optimal method of antigen delivery was empirically determined to be with a 20 gauge 1.5 inch stainless steel feeding needle (Figure 11). Although longer stainless steel and disposable plastic gavage needles were adequate for the demonstration during necropsy, they were too flexible for ease of guidance in the mouth at the commissure of the jaw (Table 9). Also, a more heavily sedated frog was ideal for antigen delivery. The jaw and swallow reflex were still intact in sedated frogs immersed in 2 g/L MS-222 at 10 minutes and antigen delivery was not consistent (Figure 12). Frogs sedated for approximately 15 minutes, relaxed their jaw and allowed insertion of the feeding needle and proper delivery of antigen.

Table 9: Comparison of Feeding Needle Material, Length and Frog Sedation.

Needle length (inches)		Material		Sedation (minutes)	
3	1.5	Stainless steel	Plastic	15	10
-	+	+	-	+	-

Figure 11: Placement of Feeding Needle. The Ball of the Needle Can Be Seen Through the Stomach. (The Stomach Has Been Opened to Demonstrate Placement).

Figure 12: Antigen Leaking Out of Frog. A Picture Showing Antigen Leaking Out of the Mouth of a Lightly Sedated Frog.

3.3 Immunizations

The duration of sedation was not sufficient for the first group of frogs. Oral innoculant leaked out of some frogs' mouths. Approximately, 50% of frogs did not receive the complete oral dose. It was also difficult to open the mouth to guide the feeding needling down into the stomach. The increase in sedation aided in animal handling and feeding needle delivery, but spontaneous regurgitation and leakage of antigen/adjuvant continued in the second group with approximately 50% of oral immunization failure. The second set of frogs developed granulomas in the peritoneum presumably as a consequence of intraperitoneal injections. This was not seen in any other group of animals or recorded in literature. It was then proposed that the DNP-KLH the second group of frogs was injected with was contaminated. It had a rancid

sulfur-like smell. Subsequent bottles of lyophilized powder were reconstituted with de-ionized water, aliquoted and stored in -20ºC. Due to this granuloma finding the third set of frogs was only given the initial immunization and harvested three weeks later. No lesions were found prior to blood collection. In the third group, slightly larger frogs were used (30-40 g). Oral immunizations in the third group only had ~20% of leakage of antigen/adjuvant. The rational was that they had a larger stomach and could hold more volume. Increasing the concentration of the DNP-KLH and decreasing the dose further assisted in orally antigen delivery in the fourth and last group. The fourth group of frogs was immunized with less volume, more concentrated antigen, more heavily sedated and boosted two more times than the third group before harvest. No antigen leaked out of the mouth. No lesions were observed during harvest. The concentration of antigen intraperitoneally immunized remained the same as originally stated in materials and methods throughout the immunization schedule for all groups. A summary is provided in Table 10.

Table 10: Summary of Group, Sedation, Concentration and Immunizations.

Group	Length of sedation	Larger frog	Concentrated antigen	Antigen leaked out
1	-	-	-	+
2	+	-	-	+
3	+	+	-	+
4	+	+	+	-

3.4 ELISA

Dissolving the TMB tablets in DMSO and phosphate-citric buffer solution was a challenge in the initial ELISA trials. Even after several hours, the tablet remained intact in the solution. Crushing the tablet would leave flakes of TMB in solution. This causes uneven color change and high well absorbance in certain wells. Eventually, the protocol was established to leave the tablet in 1 ml of DMSO in a 15 ml centrifuge tube and to incubate it at 37ºC for 10 minutes and pulse vortex a few times to remove any flakes of TMB. This method took less than 15 minutes to completely dissolve the tablet. Nine ml of citric phosphate buffer was then added to the dissolved tablet-DMSO solution. Two µl of H_2O_2 was added immediately prior to plating to complete the TMB substrate solution.

The first ELISA protocol (measuring total relative levels of a particular isotype) used frog serum in a 1:10 dilution as the primary antigen. The purpose of this protocol was to test for changes in total response of IgM or IgX due to immunization through a particular route. Twenty eight samples were tested: nine orally immunized frogs, nine systemically immunized frogs and 10 control non-immunized animals. Six frogs in the third group were not tested. This decision was based on the results of the first eighteen animals and was made to conserve serum for future testing. Only the first three groups were tested using the first ELISA protocol. Results showed no significant difference between non-immunized controls and orally immunized and intraperitoneally immunized frog, although there was a higher systemically immunized response than oral or control IgM response in much of the titrating portion of the curve (Table 11, Figure 13). ELISA controls were as expected.

Table 11: Average IgM Group 1 Total ELISA Protocol.

	1:10	$1{:}10^2$	$1{:}10^3$	$1{:}10^4$	$1{:}10^5$	$1{:}10^6$	$1{:}10^7$	$1{:}10^8$	$1{:}10^9$	$1{:}10^{10}$	$1{:}10^{11}$	PBS
IgM Avg PO	0.331	0.284	0.337	0.343	0.321	0.201	0.144	0.12	0.089	0.082	0.075	0.077
IgM Avg IP	0.272	0.289	0.289	0.29	0.294	0.177	0.119	0.113	0.094	0.087	0.071	0.073
IgM Avg cntrl	0.32	0.303	0.334	0.382	0.307	0.201	0.175	0.13	0.132	0.158	0.078	0.091

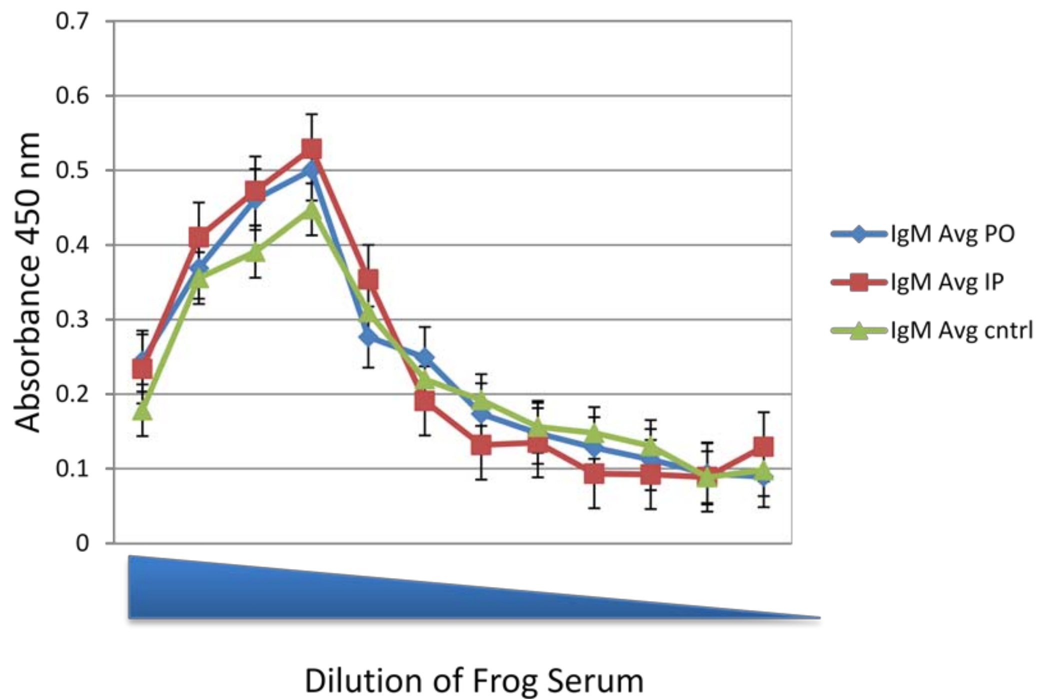


Figure 13: IgM Group 1 Total Ig ELISA Protocol.

Results for the IgX total ELISA for the first immunized group showed induction of IgX after oral immunization, not seen in the IP group (Table 12, Figure 14). This is an exciting finding because I hypothesized that IgX, a proposed analog of IgA, would be specifically produced with oral immunization compared to systemic immunization. This is the first evidence that oral immunization of *Xenopus* elicits an IgX response, consistent with the idea that it is (like our IgA) a dedicated mucosal isotype. Only the first group of immunized frogs had an increased oral response. The response was not seen in the second group, where oral responses were lower than intraperitoneal or control non-immunized responses. Group 4 was not tested for IgX using the first ELISA protocol.

Table 12: Average IgX Group 1 Total ELISA Protocol.

	1:10	$1{:}10^2$	$1{:}10^3$	$1{:}10^4$	$1{:}10^5$	$1{:}10^6$	$1{:}10^7$	$1{:}10^8$	$1{:}10^9$	$1{:}10^{10}$	$1{:}10^{11}$	PBS
IgX Avg PO	0.294	0.364	0.323	0.291	0.218	0.201	0.205	0.212	0.155	0.119	0.096	0.1
IgX Avg IP	0.211	0.188	0.198	0.179	0.195	0.131	0.142	0.167	0.156	0.121	0.091	0.094
IgX Avg cntrl	0.199	0.231	0.212	0.189	0.194	0.201	0.165	0.171	0.158	0.126	0.109	0.097

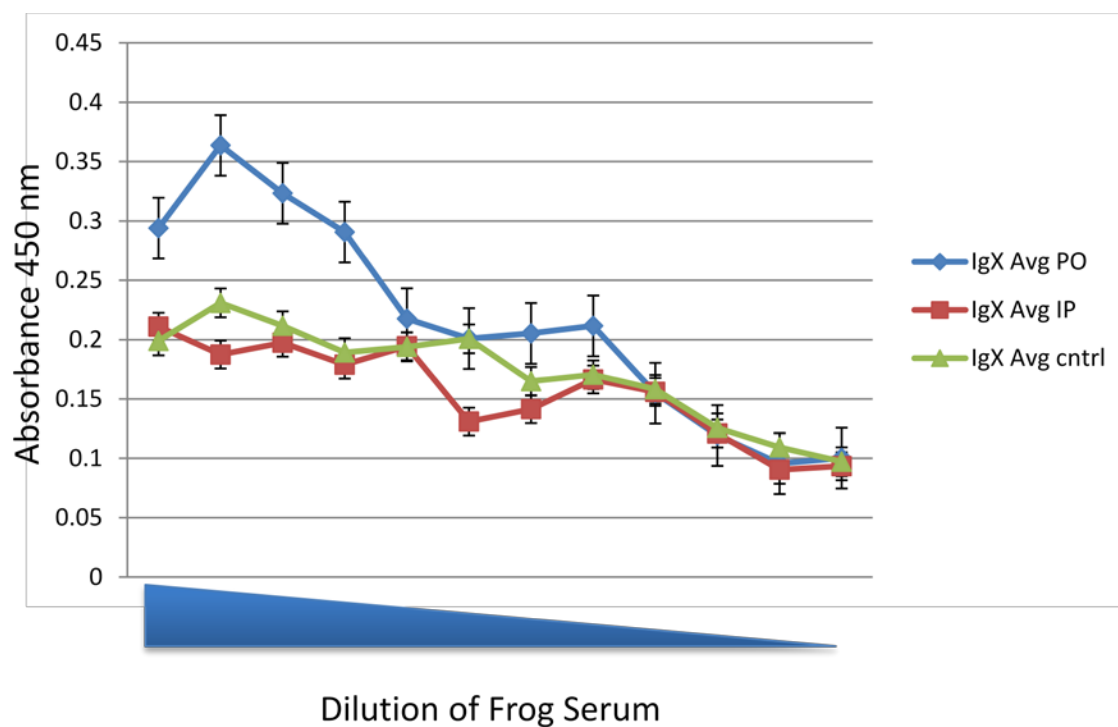


Figure 14: IgX Group 1 Total Ig ELISA Protocol.

The second ELISA protocol, using DNP-KLH as the primary antigen, tested for a specific response to DNP-KLH instead of total IgM or IgX levels in serum. A total of 18 orally immunized, 18 intraperitoneally immunized and 18 non-immunized frog serum samples were compared by this ELISA. This set of ELISA experiments had nine no primary antibody controls and nine no-secondary controls. Some samples were omitted due to insufficient serum for dilution (Table 13).

Table 13: Omitted Frogs from Antigen-specific ELISA Protocol.

48371074	PO
48518356	PO
48534321	IP
48531545	IP
48532776	Control
48371541	Control

In the first two immunized groups, all frogs did not receive the full antigen dose. This may have resulted in a poor oral response compared to control and systemic immunization in both groups. However, in the third group, there were fewer frogs that refused the oral immunization and a stronger oral response is seen compared to systemic and control (Figure 15). Also there is no significance between systemic and control. The fourth immunization is stronger on the Y axis due to a full immunization with the concentration increased and the dose decreased. Again oral immunization is significant over control and systemic immunizations. The average of all four immunizations is show in Table 14 and Figure 16 and shows a significant increase in systemic IgM serum levels in the intraperitoneally injected group of animals compared to IgM serum levels of orally immunized animals and non-immunized animals. However, there was no significance for IgX Ag-specific ELISA. In fact, immunized groups 1-3 showed no correlation between orally immunized, intraperitoneally immunized or control animals (Figures 17-18). The no primary and no secondary controls were as expected.

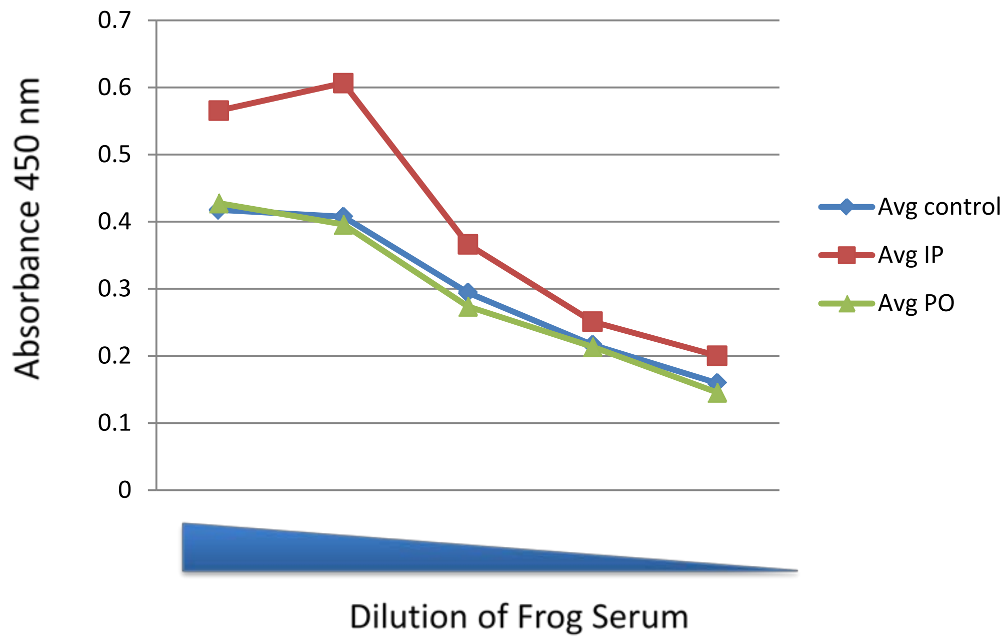


Figure 15: IgM Group 3 Antigen-specific ELISA Protocol. Average IP Has Significantly Higher Response Than Oral or Control Animals.

Table 14: IgM Average Data for Antigen-specific ELISA Protocol.

Serum dilution	1/9	1/10	1/27	1/81	1/243	PBS
Avg of control	0.518333	0.51425	0.440611	0.341833	0.286667	0.195333
Avg of IP	0.6485	0.640833	0.547333	0.455389	0.394	0.191944
Avg of PO	0.533167	0.53275	0.458833	0.361	0.303111	0.176889
Avg of no primary	0.164667	0.181333	0.191222	0.189222	0.175444	0.165
Avg of no secondary	0.087333	0.1055	0.094778	0.091111	0.090667	0.092778

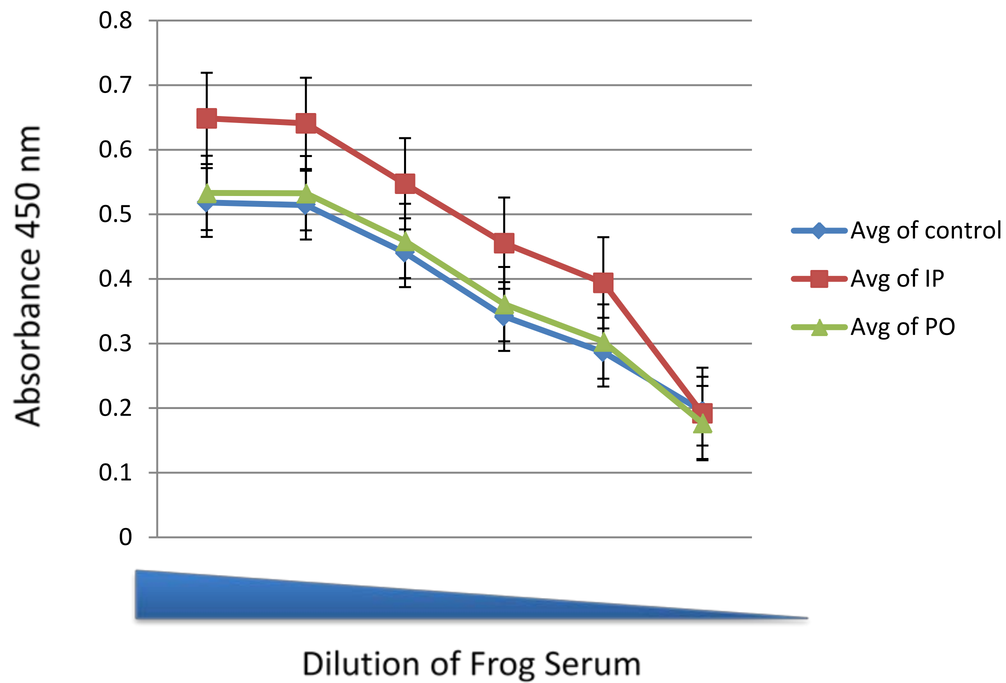


Figure 16: IgM Average Antigen-specific ELISA Protocol.

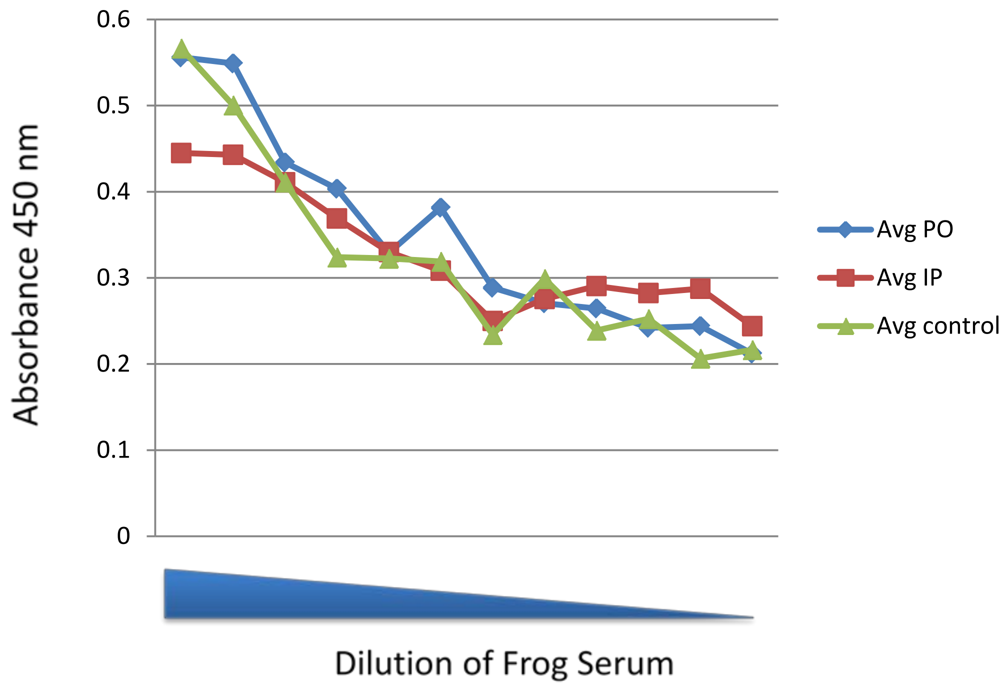


Figure 17: IgX Group 3 Antigen-specific ELISA Protocol.

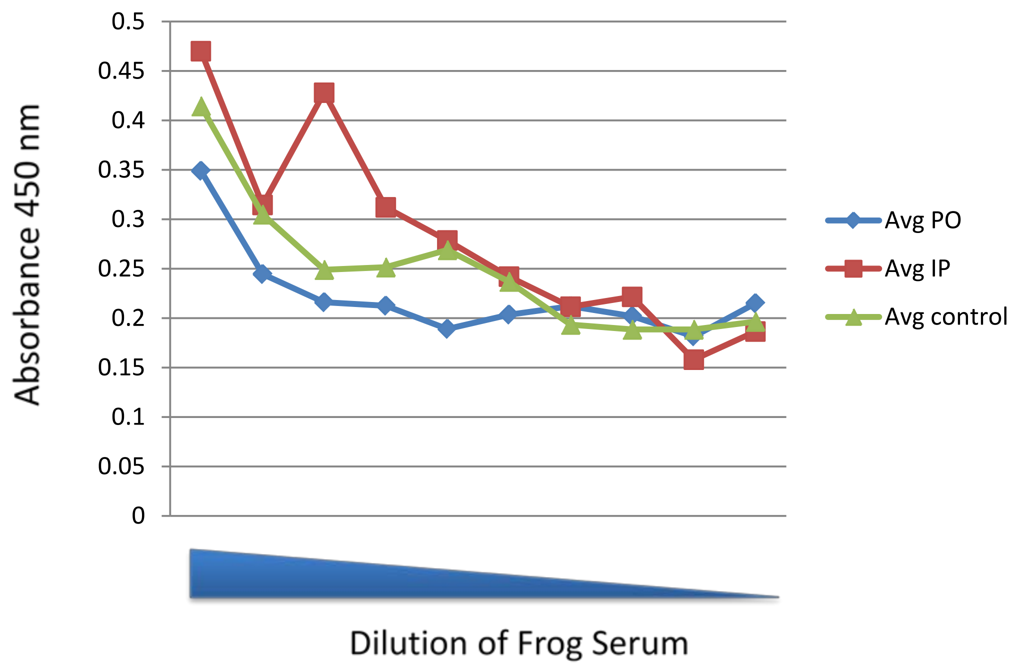


Figure 18: IgX Group 4 Antigen-specific ELISA Protocol.

3.5 Phylogenetic Analysis

Results of ELISA showed that oral immunization elicits an IgX response. To support my hypothesis further, we wanted to combine this physiological data with comparative immmunogenetics analyses. Immunoglobulin heavy chain C region sequences from other animals may help inform us of the relationship between frog IgX and human IgA. Recent research in several higher vertebrates suggested that we should revisit the natural history of these immunoglobulin heavy chain C region loci, as a more representative phylogeny could now be created. . For example, in Choi et al 2010a, the authors looked at pheasant, turkey and quail heavy chain genes and found that avian IgA is more closely associated with mammalian IgA than previously believed (Choi et al. 2010a). This analysis used the complete C region from C1 to C4 depending on species. Transmembrane or secreted domains were not included in this analysis. Our C region tree analysis shows a tree of the C regions of many isotypes from sharks to man. Looking at the data from IgX and IgA of bird and mammal (Figures 19-20), IgX did not cluster with IgM. Unlike what was found in any previous phylogeny, IgX and IgA were closely related.

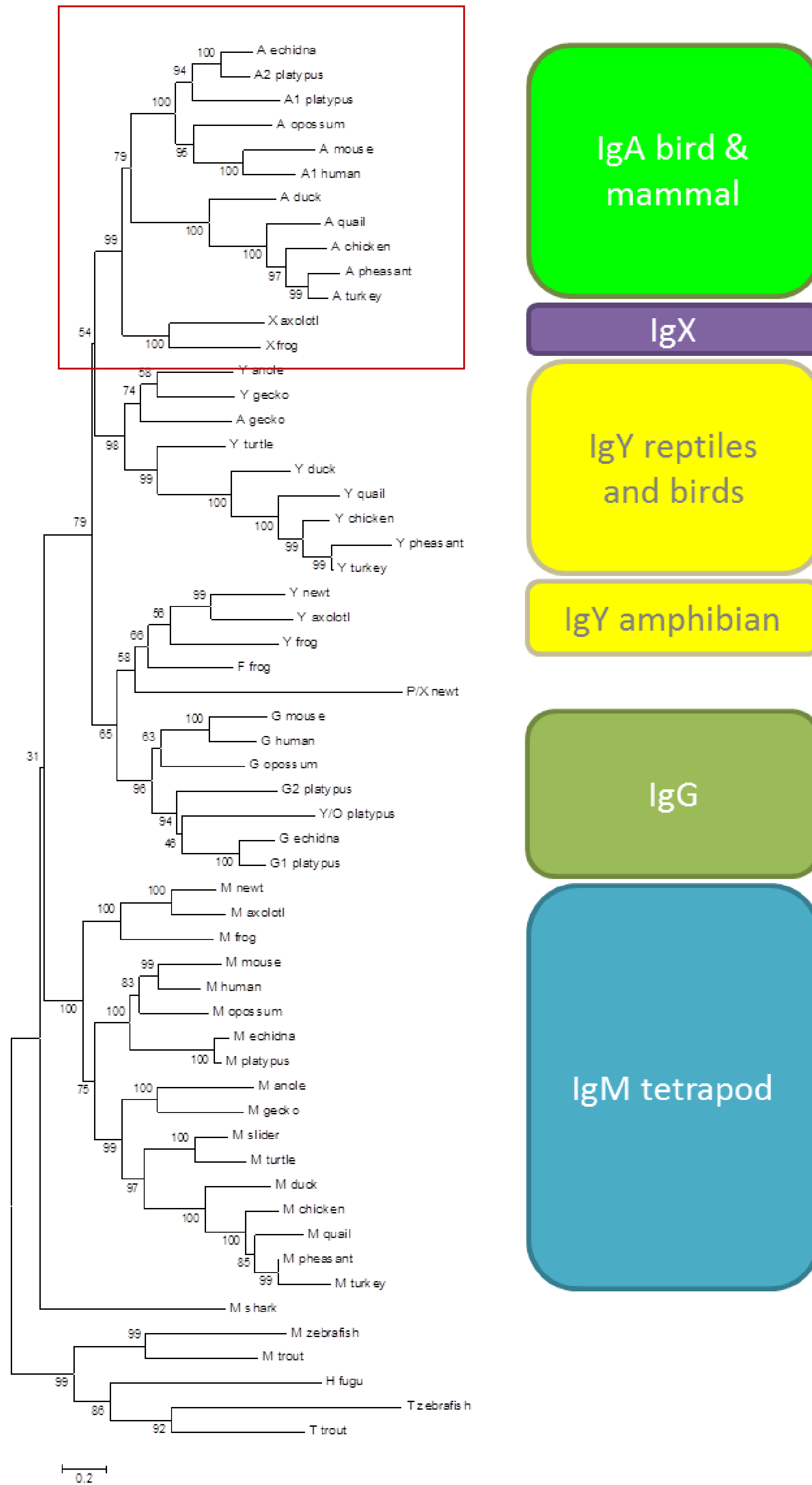


Figure 19: Constant Region Tree.

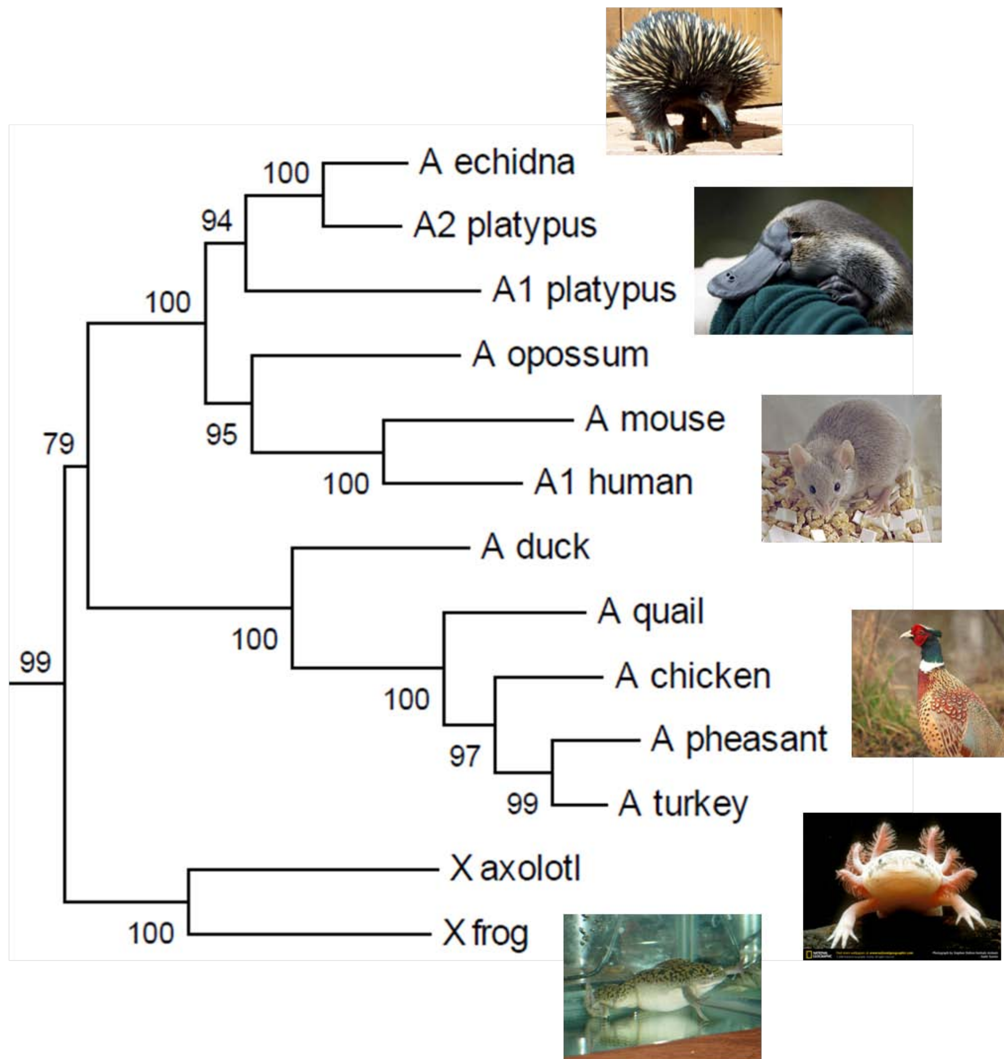


Figure 20: Closer Look at Constant Region Tree.

The recent divergence of IgX and IgA is an unheralded finding and the tree suggests that IgM gave rise to IgX that gave rise to IgA. Physiologically, the data in this project show that oral immunization gave rise to an IgX immune response. Secondly this tree suggests that IgX is more closely related to IgA than to any other isotype, with high statistical support depicted in the 99 bootstrap value at the node linking them. Sequence identity and reactivity to oral immunization are shared suggesting that IgA and IgX may actually be of same clan, but more work is needed to support this idea. This story may prove to be similar to how shark IgW has been reclassified as IgD. In that case it also took a more complete phylogeny to "connect the dots". Similar results were attained by maximum parsimony, although neighbor-joining produced greater statistical support.

3.6 Ig Heavy/Ig Light Isotype Relationships

To see if Ig light chain in *Xenopus* has a preference for Ig heavy chain, *Xenopus* B cells from spleen were sorted using MACS system. For the first PCR, only κ and σ positive and negative cells were tested against IgM, IgX and Igκ primers. For κ negatively sorted cells, there were positive bands with IgM, IgX and Igκ primers as expected. For κ positively sorted cells, we expected a positive band with IgM primer and a cleaner band at the IgX primer. Results for both σ positive and negative sorted cells as well as all negative controls for positive and negative cells were negative as expected (Figure 21).

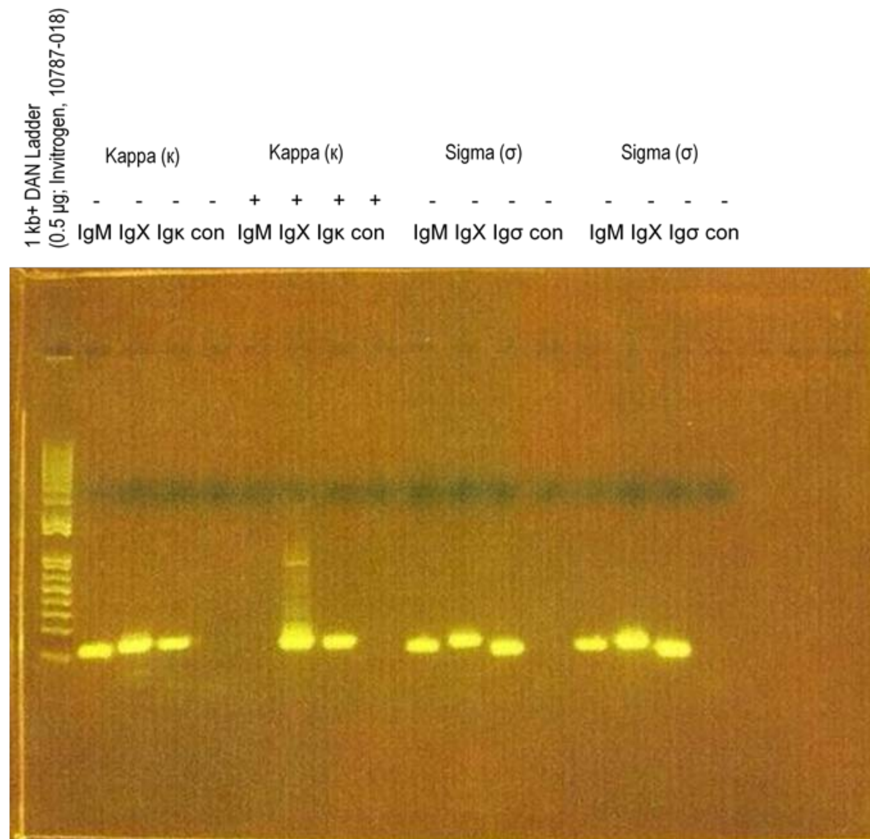
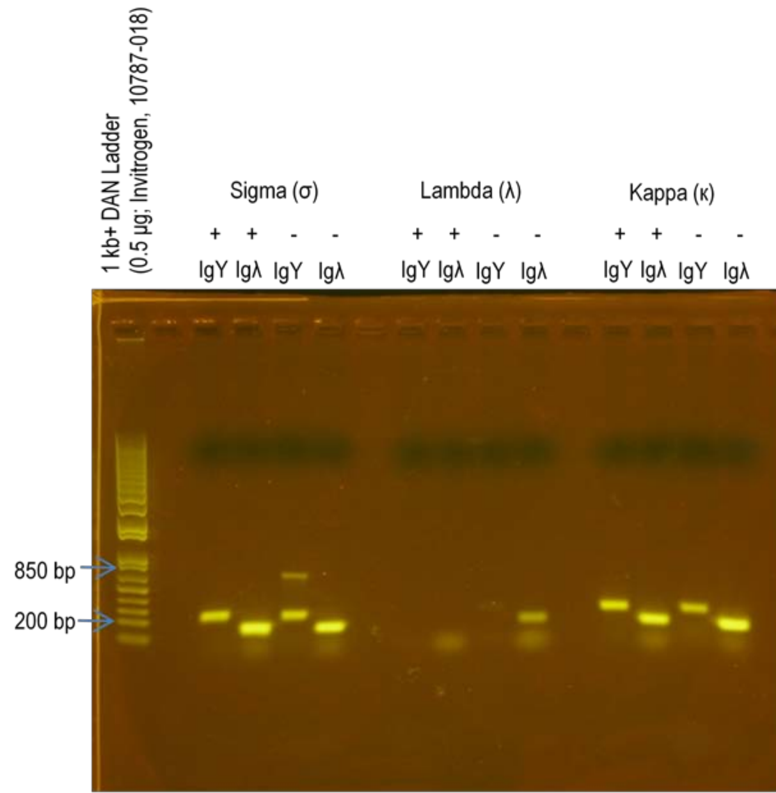


Figure 21: cDNA (σ & κ) with IgM, IgX, Igκ and Igσ Primers. The (-) Are Negatively Sorted Cells and (+) Are Positively Sorted Cells.

In the second PCR, the Igσ positive and negative sorted cells resulted in positive bands. Although, primers were made to be 200 bps, the bands for IgY varied slightly from Igλ. Also, Igσ negatively sorted cells had another positive band at approximately 850 bp. Igλ positive and negatively sorted cells with IgY and Igλ primer were not as robust as expected for positively sorted IgY cell. However, Igκ sorted cells had anticipated bands for IgY and Igλ (Figure 22).



1° PCR (30 µl): Xenopus laevis Spleen (σ, λ & κ) cDNA with IgY (MFC230-MFC231) & Igλ (MFC232-MFC233) Primers
Annealing Temp: 48°C for IgY & 52°C for Igλ
0.8 % agarose (20,000X GelGreen), 108 V/50 Min
F 6.3, 8", ISO 100
12-20-2010

Figure 22: cDNA (σ, λ & κ) with IgY & Igλ Primers. The (-) Are Negatively Sorted Cells and (+) Are Positively Sorted Cells.

4. DISCUSSION

4.1 Development of Frog Experimental Techniques

The ideal concentration for sedation of frogs weighing 20-40 g for immunizations was determined at 2 g/L of MS-222 for 15 minutes. This concentration provided the quickest time to sedate the frogs with the widest safety margin against overdose. At this concentration and duration of immersion, there were no complications in handling the animal, inserting the microchip identification, guiding the gavage needle and delivering the oral innoculant. Euthanasia was determined to be less than 15 minutes based on 5 g/L concentration. Death was always confirmed by a secondary method of euthanasia such as pithing, exsanguination or decapitation. Incision of the base of the heart, provided the most consistent and reliable method of terminal blood collection in 20-40 g *Xenopus* frogs.

4.2 Gavage Technique

Using a straight 20 gauge 1.5 inch stainless feeding needle provided the best delivery method for gavage of 20-40 g *Xenopus*. Frogs sedated at 2 g/L for 15 minutes were easier to orally immunize than frogs sedated for only 10 minutes at the same concentration. As a method of avoiding noxious stimuli, frogs can quickly expel the contents of their stomach. It is also a common sign of illness in amphibians, especially frogs and toads. Animals sedated in both concentrations recovered without complications or regurgitation of stomach contents. Problems reported in small animals

include perforation of the esophagus, gavage into the trachea or lungs or even death. There were no adverse effects observed from the gavage method seen in *Xenopus*, although problems reported in small animals include perforation of the esophagus, gavage into the trachea or lungs. Sequelae of these events include sepsis or even death (Atcha et al. 2010) .

4.3 Immunizations

For the immunization protocol, the increase in sedation time, decrease concentration of innoculant and larger frog all helped in oral immunization delivery by preventing innoculants from leaking out of the mouth. Although, this was a novel procedure, sham immunizations before the initial experimental group would have been ideal to determine the best variables for antigen delivery. Control immunizations should have been immunized with inert substance such as sterile saline instead of receiving no treatment. Using the baseline serum as control would have helped make observations as to whether immunizations were eliciting significant immune response. Also, withholding food may have helped empty the stomach and prepare for antigen acceptance.

4.4 ELISA

Using the first total ELISA protocol, a higher but not significant serum IgM level was detected between the orally immunized frogs and the intraperitoneally immunized frogs. However, total IgX levels were higher in orally immunized frogs compared to

systemically immunized animals and non-immunized controls. The result of the first trial of oral immunization delivery supports my hypothesis that IgX, a proposed analog of IgA, would be specifically produced with oral immunization compared to systemic immunization. Again this is the first evidence that oral immunization elicits an IgX response.

In the second, antigen-specific ELISA protocol, results showed significant serum IgM response between intraperitoneally immunized frogs compared to orally and non-immunized animals. This contrasts with serum IgX antigen-specific ELISA protocol in which no significant difference was measured between any of the three immunizations.

Incubating the tablet in DMSO at 37ºC provided rapid and accurate results and proper substrate solution to use for ELISA. Other variables that would have decreased variance or increased sensitivity of ELISA results are pre-bleeding of frogs to use as controls instead of using non-immunized control frogs. The baseline serum of each frog would have served as an internal control for each PO or IP immunization. Unfortunately, the amount of serum collected via survival method in a 20-40 g frog (100-150 µl) would have been a limiting factor for this method, although this method has been subsequently developed in the lab (publication pending). Also, repeating each frog immunization ELISA in triplicate would have given an average response and eliminated any outliers caused by inconsistencies in ELISA procedures.

4.5 Phylogenetic Analysis

Recent data now allow more rigorous studies of the natural history of tetrapod genes, and more immunogenetic studies and genome projects will only help further define immunoglobulin evolution. Sequence similarities along with structural resemblance to IgM, suggest that IgX might be thefunctional analog, not the ortholog of IgA. The IgA of Aves appears to be a mucosal functional analog of mammalian IgA (Mansikka 1992) and there is high sequence identity suggesting orthology (Choi et al. 2010b), although there are four C domains in avian IgA suggesting a deletion occurred to yield the mammalian IgA of three (Aveskogh and Hellman 1998).

Immunoglobulin heavy constant regions were analyzed. Alignments were initially made in Bioedit with ClustalW and MEGA was used to infer the phylogenetic relationships of immunoglobulin C regions. Trees were drawn employing pairwise deletion of gaps and the Dayhoff rate matrix. Neighbor-joining consensus trees were made from 1000 bootstrap replicates and for the first time, results showed IgX and IgA were closely related.

In addition to phylogenies using the entire C region, the relationships of these isotypes are being studied with phylogenies of individual C domains. The tree of the individual domains used in the previous C region tree show in (Figure 23), uses different colors to show isotype and shapes to show domains.

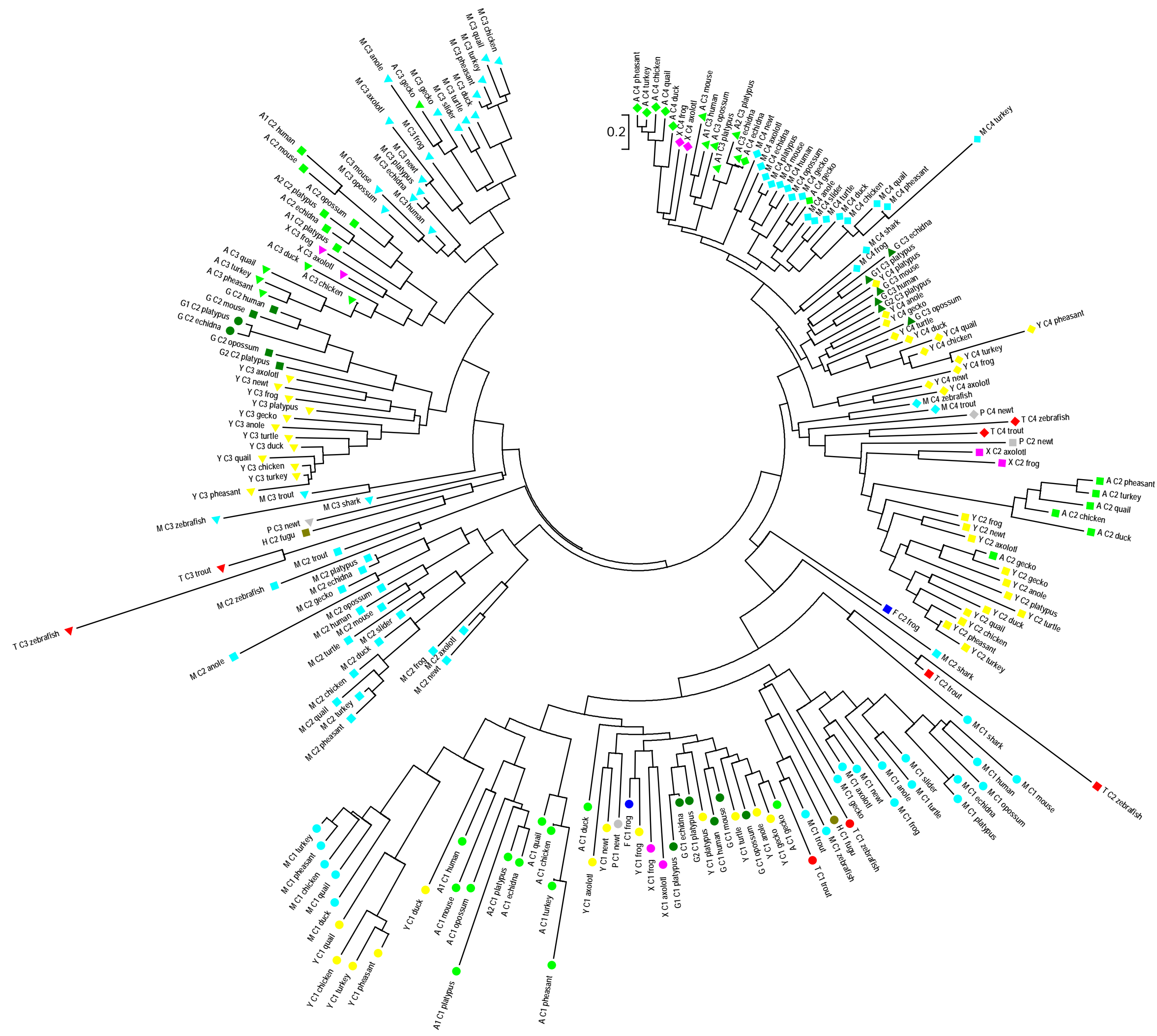


Figure 23: Individual Constant Region Tree.

A rectangular representation of the area in the red box is depicted in Figure 24. The third constant domain of IgX groups with not only IgA C3 but also IgA C2 of mammals, suggesting exon duplication or homogenization. Therefore, the C2 domain of mammalian IgA may not come from the same ancestor of C2 of amphibian IgX or C2 reptilian IgA, but rather a more recent duplication within the mammalian IgA loci. Thus the actual history of the genes that encode the isotypes may be too complex to explain as descent of the entire C region *en block*.

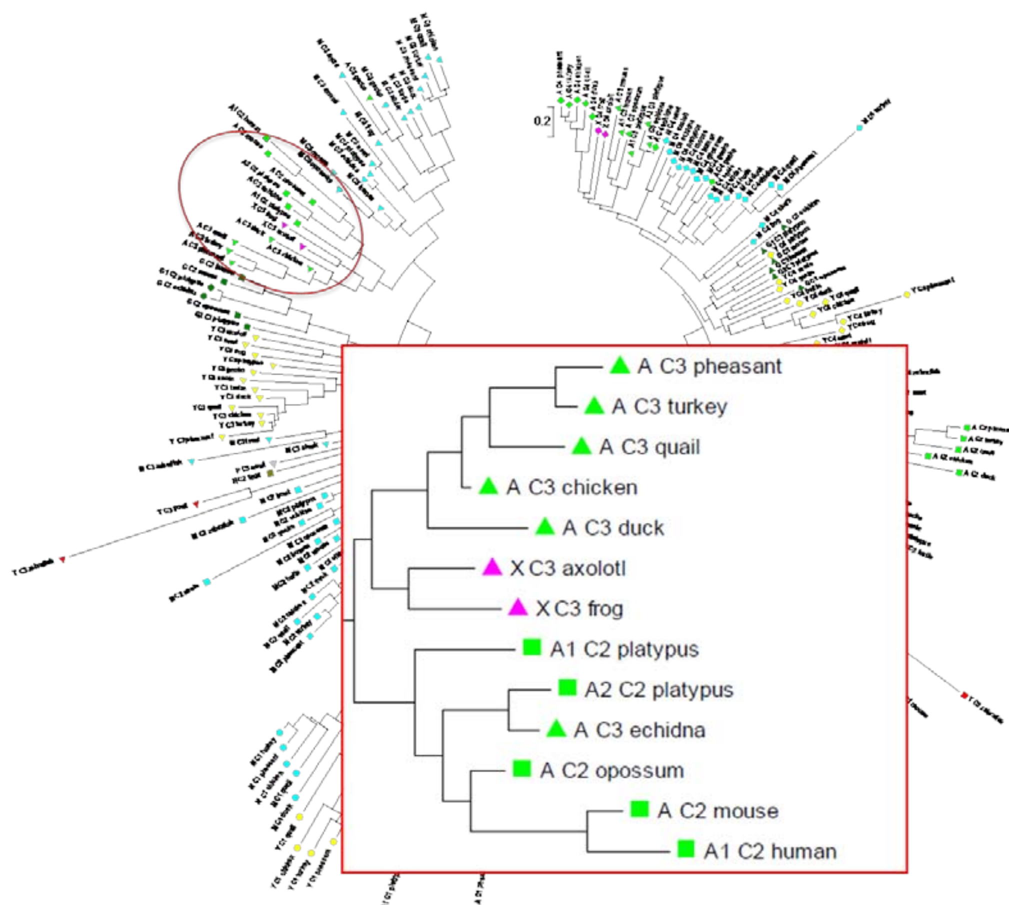


Figure 24: Closer Look at Individual Constant Region Tree.

4.6 Ig Heavy/Ig Light Isotype Relationships

In mammals, Igκ and Igλ light chain can be found on any of the five major classes of immunoglobulin heavy chain. In lower vertebrates some heavy/light chain isotype preference has been shown at the protein level (Miracle et al. 2001). In *Xenopus* previous literature has shown that an isotype found in cold- blooded vertebrates, Igσ has a preference for the two T cell independent IgH isotypes found in gut: IgM and IgX, but not IgY (Hsu et al. 1991) (Criscitiello and Flajnik 2007). Although, these isotypes have been identified, there is still little evidence to indicate their distinct functional roles.

To follow up on this protein observation, we wanted to sort B lymphocytes based on light chain and quantify heavy chain expression by PCR. The first objective was to sort labeled B cells from the spleen and the second to determine immunoglobulin heavy chain to immunoglobulin light chain association. Cell sorting by the MACS system averaged 50,000 positively labeled cells per sort and 2.0×10^6 unlabeled cells as control. In the first PCR attempt, only Igκ and Igσ positive and negative cells were tested against IgM, IgX and Igκ primers. Unfortunately, the Igλ positive and negatively sorted cells with IgY primer were not as robust as expected for positively sorted IgY cells in the second PCR. This was perhaps due to poor cell sorting technique. Our results show that for positively and negatively sorted Igσ and Igκ cells in the first PCR and Igσ, Igλ and Igκ in the second PCR positive bands as expected. This is because only one cell can cause a band. For this reason, real time PCR is a more sensitive and specific test to quantify a positive response.

5. CONCLUSION

I proposed that IgX, a functional analog of our IgA, will increase with oral inoculation compared to intraperitoneal injection and that IgM will be increased in systemically immunized animals compared to orally challenged animals. My results support an increase in serum IgM from systemic immunization compared with serum IgM levels from oral immunization. Although this hypothesis was stated in by Hsu (Hsu 1998), this is the first experiment that compared orally immunized animals with systemically immunized animals.

Improvements for future studies include sham immunizations before the initial experimental group. This would have been ideal to determine the best variables for antigen delivery. Control animals should have been immunized with inert substance such as sterile saline instead of receiving no treatment. Other improvements include taking baseline serum (or prebleed) for each frog to use as control. Also, withholding food may have helped empty the stomach and prepare for antigen acceptance. As the studies continue, the use of larger immunization sets will provide statistical significance. Future research also, includes refining the immunization and both total and antigen-specific ELISA protocols. Ideally, the IgX antibodies in the gut mucosal epithelium should be compared to serum IgM in systemically immunized frogs. This protocol is currently being refined. Ongoing studies will focus on determining if oral immunization of the frog gut epithelium will elicit a specific B cell immune response without T helper cell activation. This is currently being performed by Sara Mashoof in Dr. Criscitiello's

lab. Around day 10 of larval development, we can ablate the thymus with cauterization under the dissecting microscope (Figure 25). At this stage common lymphoid progenitors have not seeded the thymus and the post metamorphosis frog immune system can be studied, never having had T cells.

T cell deficiency results from thymectomy of larval *Xenopus* before stem cell migration to the developing thymus occurs (Horton et al. 1998). These animals have decreased anti-tumor response (Robert et al. 1997) and decrease allograft immunity (Horton et al. 1998). Thymectomized frogs have no peripheral T cells but B cells and NK cell numbers are increased (Horton et al. 2000). However, thymectomized adult frogs express IgM and IgX, but not IgY in sera (Hsu 1998).

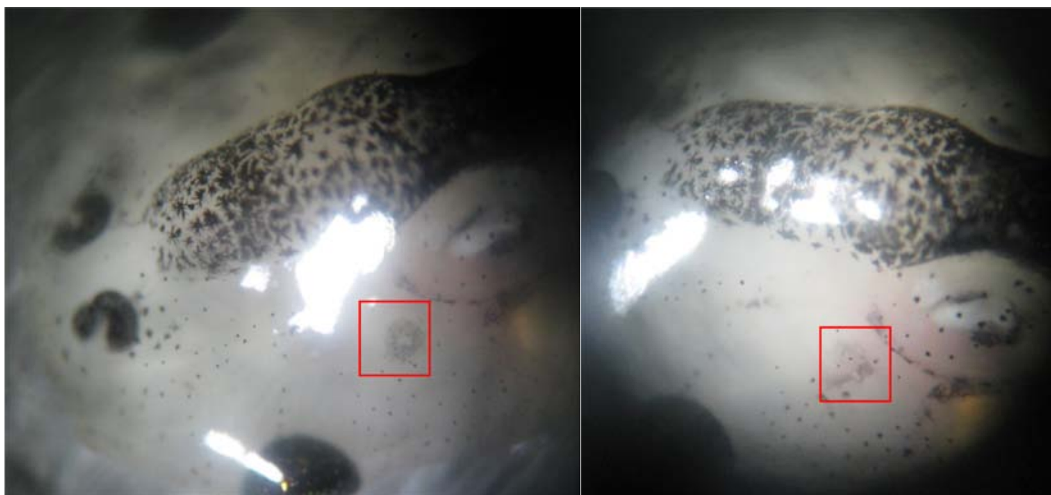
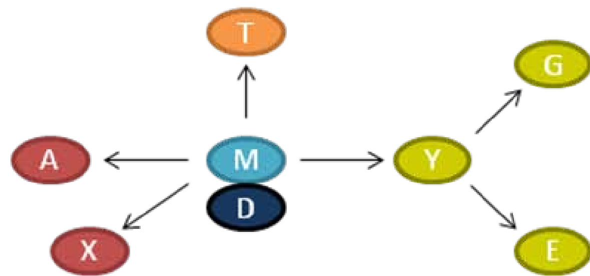
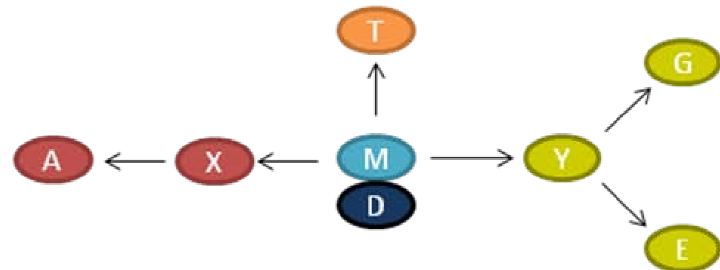


Figure 25: Larval Thymectomy. The Red Box in the Photo on the Left Shows the Thymus Before Cauterization. The Photo on the Right Shows After Cauterization.

In summary, oral and systemic immunizations were refined in the amphibian model, the most primitive animal to share our Ig heavy chain switch mechanism. Methods of sedation, oral gavage, immunization and ELISA were techniques developed for this study. Results of ELISA show a promising significant IgX response to oral immunization, but not a significant IgM response to systemic immunization. The entire C domain phylogeny reveal an intimate relationship between amphibian IgX, avian IgA and mammalian IgA, supporting the theory that IgX gave rise to IgA. Real time PCR will study light chain association in the cells that were sorted by MACS. Ongoing studies will continue to resolve functional and evolutionary roles of the heavy and light chain isotype of the first vertebrate to use class switch. Larval thymectomized *Xenopus* are currently being immunized orally and systemically.

Whereas we used to think IgX was a mucosal innovation of only amphibian lineage descendent of IgM, we now have evidence that it in fact gave rise to our IgA and perhaps one day the nomenclature will be condensed (Figure 26). It is satisfying that the organization of the known tetrapod Ig heavy loci do not complicate this hypothesis as loci encoding IgA and IgX share similar syntenic relationship from frog to man (Figure 27). To support this idea further, we only need to look at the five isotypes we commonly study. They may be derived from two sources; the very genesis of our adaptive system in the cartilaginous fish (IgM and IgD), and then a radiation after the innovation of class switch in amphibians, with IgX going on to our IgA and IgY spawning IgG and IgE (Figure 28).





Previous Consensus

New Model

Figure 26: Natural History of Our Antibody Isotypes.

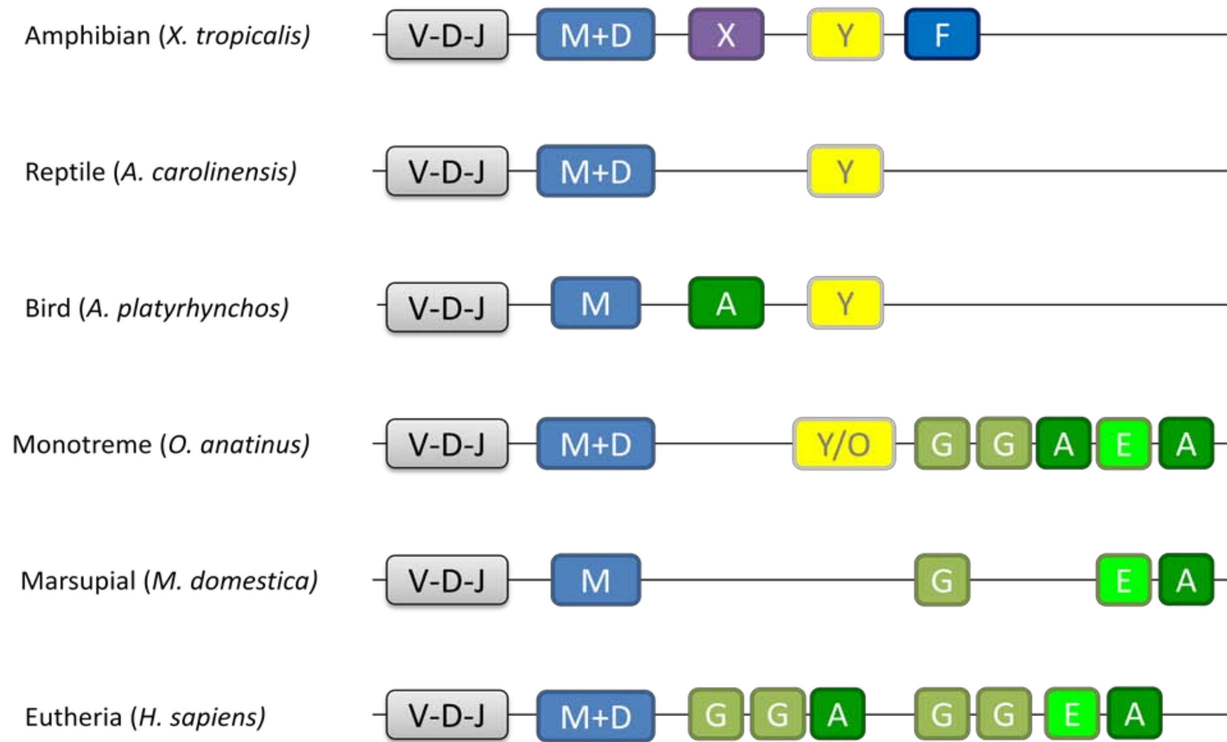


Figure 27: Syntenic Relationships Between IgH. These Heavy Chain Loci Depict the Arrangement of Constant Region Encoding Exon Clusters of Common Tetrapods.

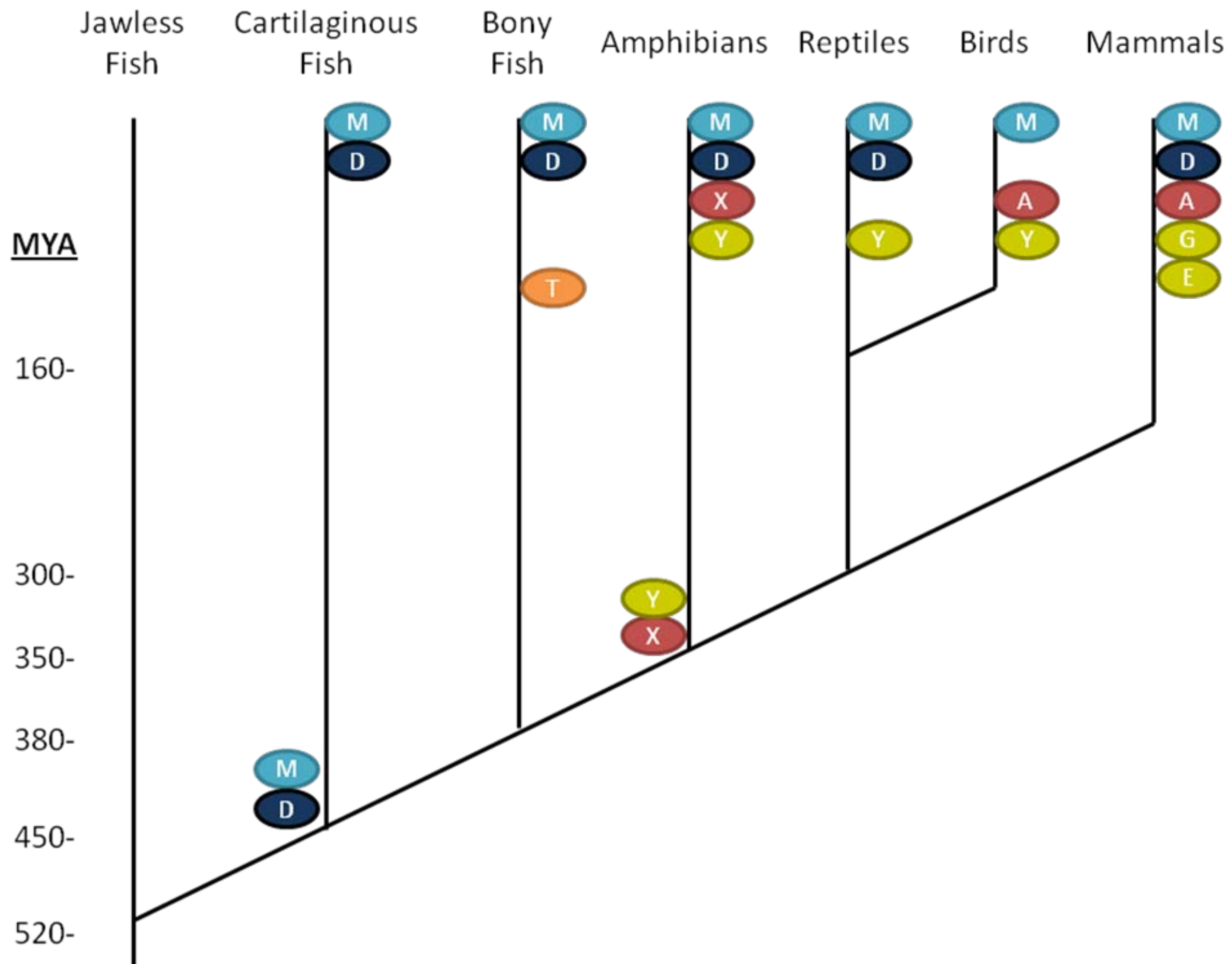


Figure 28: Proposed Origin of IgG and IgE and IgA.

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