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In vivo comparison of Edwardsiella ictaluri survival in kidneys of vaccinated and naïve rag1-/- zebrafish

Casey Janine Varner

IN VIVO COMPARISON OF *EDWARDSIELLA ICTALURI* SURVIVAL IN KIDNEYS OF VACCINATED AND NAÏVE *RAG1*$^{-/-}$ ZEBRAFISH

By

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NAÏVE *RAG1*$^{-/-}$ ZEBRAFISH

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This study used *rag1*$^{-/-}$ mutant zebrafish, which lack functional T and B lymphocytes, to investigate whether innate immune cells from vaccinated mutant zebrafish demonstrate enhanced survival compared to phagocytes from naïve mutant fish. *Edwardsiella ictaluri*, an economically significant aquatic pathogen and the causative agent of enteric septicemia of catfish (ESC), was used for the trials.

Quantification of live bacteria from sampled kidneys was accomplished via colony counts, luminescence readings, and differential DNA extractions using Ethidium Monoazide (EMA) and Propidium Monoazide (PMA) followed by qPCR. There was a general trend of less bacteria in vaccinated mutant fish. Additionally, the mortality in the vaccinated fish was less than the naïve group, suggesting that the vaccinated fish are better able to withstand the bacteria load. Giemsa-stained cytospins showed *E. ictaluri* exclusively within macrophages

from sampled kidneys, suggesting that the macrophages are the critical site of pathogenesis in *rag1*$^{-/-}$ zebrafish.

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CHAPTER I

INTRODUCTION

This study investigated whether innate immune cells from previously exposed (vaccinated) *rag1*$^{-/-}$ mutant zebrafish demonstrate enhanced bacterial clearance compared to fish that had never been exposed (naïve). *Rag1*$^{-/-}$ mutant zebrafish lack functional T and B lymphocytes and have been used in our lab to investigate enhanced protection following homologous bacterial re-exposure. The bacterial pathogen used in the current study was *Edwardsiella ictaluri*, an economically significant aquatic pathogen and the causative agent of enteric septicemia of catfish (ESC) (Hawke et al. 1981). This study also introduced a survival assay involving differential DNA extractions using Ethidium Monoazide (EMA) and/or Propidium Monoazide (PMA).

Overview of Teleost Phagocytes

To focus on phagocyte function in terms of innate immune system memory, *Recombination activation gene 1* (*rag1*$^{-/-}$) mutant zebrafish were used. The RAG1 protein is required for proper V(D)J recombination, which is essential for the development of T and B lymphocytes (Wienholds et al. 2002). Previous work has shown that *rag1*$^{-/-}$ mutant zebrafish, despite lacking an acquired

immune system, demonstrate protection against prior pathogen exposure (Hohn 2008).

The primary function of macrophages and neutrophils is to act as the first line of defense by phagocytizing antigens. Because of this ability, macrophages and neutrophils are integral in controlling bacterial diseases (Blazer 1991). The first cells to respond to local infection are tissue macrophages. Upon onset of a systemic inflammatory response, neutrophils are the first inflammatory cells recruited to the site of infection. Approximately one day later, monocytes arrive at the site of injury and are induced to differentiate into macrophages (Traver et al. 2003).

Both macrophages and neutrophils are able to recognize pathogens via cell-surface receptors that discriminate between pathogens and host cells. Once a pathogen is bound to the phagocyte surface receptor, it is surrounded by pseudopodia and internalized into membrane-bound phagosomes (Janeway et al. 2005; Secombes 1996). Macrophages phagocytize live *E. ictaluri* via receptor-mediated endocytosis and macropinocytosis (Booth et al. 2006; Hohn 2008). Unlike receptor-mediated endocytosis, macropinocytosis is not receptor-dependent and involves uptake of molecules into vacuoles. After the phagosome is formed, it becomes acidified and fuses with a lysosome, which is filled with enzymes, proteins, and peptides. In addition to the degradative agents found in the phagolysosomes, upon phagocytosis macrophages and neutrophils release reactive oxygen species (hydrogen peroxide, superoxide anion, and nitric oxide)

that help kill the engulfed bacteria (Janeway et al. 2005; Secombes 1996). With these functions, macrophages are able to engulf and kill pathogens.

Nutritional factors play an important role in the ability of macrophages to phagocytize and kill *Edwardsiella ictaluri.* When fish were fed three compositionally distinct feeds, significant differences in the phagocytic index of macrophages were observed (Blazer et al. 1989). Wise et al. (Wise et al. 1993) showed that increasing dietary vitamin E had an increasingly positive effect on the phagocytic capability of macrophages in both vaccinated and non-vaccinated channel catfish (*Ictalurus punctatus*). Additionally, ascorbic acid deficiency greatly reduced the phagocytic index of channel catfish macrophages challenged with *E. ictaluri*; however, the bactericidal index was not affected (Li and Lovell 1985). Finally, intracellular killing was found to be greatest when fish were fed diets high in n-3/n-6 fatty acids (i.e. menhaden oil) (Sheldon and Blazer 1991).

Macrophages and neutrophils are major contributors in protection against foreign antigens, especially bacterial pathogens, which is why they are considered to be the host's first line of defense. This protection, involving phagocytic as well as bactericidal activity, is influenced by environmental and nutritional factors; however, previous exposure to the pathogen seems to play a major role, at least in the phagocytic index.

Overview of the Fish Pathogen *Edwardsiella ictaluri*

Enteric septicemia of catfish (ESC) is a bacterial disease that primarily affects channel catfish (*Ictalurus punctatus*) and was first described in 1979 by J.

P. Hawke (Hawke 1979). The causative agent of ESC is *Edwardsiella ictaluri*: a gram-negative, oxidase negative, peritrichous, fermentative bacterium. The optimal growth temperature for *E. ictaluri* is 28ºC on blood agar plates, and colony formation typically takes about 48 hours (Hawke et al. 1981). ESC is characterized by both a rapid-onset septicemic form with high mortalities, and a chronic form that attacks the central nervous system (Shotts et al. 1986).

Edwardsiella ictaluri invades the host through oral (Baldwin and Newton 1993; Shotts et al. 1986), nasal (Morrison and Plumb 1994; Shotts et al. 1986), and gill (Nusbaum and Morrison 1996) routes and replicates within neutrophils and macrophages. Bacteria from fish that were orally challenged with *E. ictaluri* could be found in the head kidney and throughout the fish's body within one hour post infection. Additionally, phagocytes that contained bacteria early in the infection were associated with the vascular system, suggesting that the phagocytes carry the bacteria systemically via the blood (Baldwin and Newton 1993). Furthermore, when the olfactory organ of experimental fish is exposed to *E. ictaluri*, the organ begins to degenerate and leukocytes are recruited to the infection site (Morrison and Plumb 1994; Shotts et al. 1986). Additionally, *E. ictaluri* in fish challenged by immersion was continually present on the gills and was also found in high quantities in the liver (Nusbaum and Morrison 1996). Histological (Miyazaki and Plumb 1985), as well as microscopy-based (Booth et al. 2006), investigations have shown *E. ictaluri* replicating within phagocytic neutrophils and macrophages. Intracellular location and replication is also

evidenced by the inability of antibodies against *E. ictaluri* to provide protective immunity (Booth et al. 2006).

In addition to the information known about the intracellular invasion and replication of *E. ictaluri*, the virulence factors associated with its pathogenicity have also been evaluated. Thune et al. (Thune et al. 2007) used signature-tagged mutagenesis to investigate the genes responsible for *E. ictaluri* virulence. They identified a total of 50 genes that determine the virulence of *E. ictaluri*, including three genes involved in lipopolysaccharide biosynthesis, three genes involved in type III secretion systems (TTSS), and two genes involved in urease activity. The urease enzyme functions by increasing the surrounding environmental pH, thus enabling survival in acidic environments. This is important, especially for *E. ictaluri* because it replicates within macrophages. In addition, analysis of a TTSS apparatus mutant showed that it maintained its ability to invade host cells, but was unable to successfully replicate, indicating that the TTSS are responsible for both intracellular survival and replication. Knowing some of the virulence genes possessed by *E. ictaluri* enables investigators to understand the pathogenesis of the bacterium more completely.

Ethidium Monoazide and Propidium Monoazide

DNA-based quantitative techniques for detection of microbial pathogens tend to fall short because of the inability to distinguish live, viable cells from dead cells (Nogva et al. 2000). This could potentially lead to a significant overestimation of the presence of living microorganisms (Nocker and Camper

2006). An alternative molecular diagnostic technique is the use of RNA. Because of its rapid degradation, it is a good determinant for cell viability; however, RNA is difficult and technically demanding to work with because it is easily contaminated with RNA-degrading enzymes (Nocker and Camper 2006).

Other commonly used techniques for distinguishing live from dead cells are various dyes for use with microscopy (Blazer et al. 1989; Graham et al. 1988; Peck 1985; Sheldon and Blazer 1991; Shoemaker et al. 1997) as well as culture/plate count methods (Baldwin et al. 1988). Both of these methods are competent at distinguishing live from dead cells; however, they are not very efficient in terms of time and effort. The microscope-based method falls short in that it requires the researcher to extrapolate from a limited number of bacteria (Nocker and Camper 2006). Additionally, culture-based techniques can take days before obtaining results, and those results are highly dependent upon the culture media and incubation temperature (Nocker and Camper 2006).

Ethidium Monoazide (EMA) and Propidium Monozaide (PMA) overcome the limitations of the methods previously described. Both EMA and PMA are used in conjunction with DNA-based real-time PCR (qPCR) methods. The EMA and PMA can only enter bacterial cells with compromised cell walls or cell membranes; therefore, these chemicals function to differentiate live from dead bacteria by covalently linking, upon photoactivation, to dead bacterial DNA (Rudi et al. 2005). In addition, any unbound EMA or PMA is inactivated by reacting with water molecules during the photoactivation period. During real-time PCR amplification of EMA- or PMA-bound DNA is inhibited (Lee and Levin 2009;

Nocker and Camper 2006; Nocker et al. 2006; Nogva et al. 2003; Rudi et al. 2005).

Hypothesis

Several studies have suggested that non-lymphocyte cells demonstrate enhanced functions following homologous secondary pathogen exposure. For example, *Drosophila melanogaster*, when primed with a sublethal dose of *Streptococcus pneumoniae*, are protected upon secondary exposure to an otherwise lethal dose of the same bacteria (Pham et al. 2007). Furthermore, cockroaches (Faulhaber and Karp 1992) and bumblebees (Sadd and Schmid-Hempel 2006) were shown to produce long-term, specific responses upon secondary homologous bacterial exposures, while copepods exposed to a parasitic tapeworm displayed heightened secondary response against antigenically similar tapeworms (Kurtz and Franz 2003). Finally, earthworms *Lumbricus terrestris* (Laulan et al. 1985) and starfish *Asterias rubens (Brillouet et al. 1984)* were shown to produce inducible soluble defensins and an antibody-like molecule that provide specific protection. Invertebrates have largely been used for these types of studies, since they evolutionarily predate adaptive immunity.

Recombination activation gene 1 (*rag1*$^{-/-}$) mutant zebrafish lack functional T and B lymphocytes and, therefore, rely solely on innate immunity (Petrie-Hanson et al. 2009). *Rag1*$^{-/-}$ mutants demonstrated protection following secondary homologous bacterial exposure equivalent to wild-type zebrafish (Hohn 2008). Additionally, macrophages isolated from channel catfish previously

infected with *E. ictaluri* showed much higher bactericidal activity upon secondary exposure than macrophages isolated from naïve channel catfish (Shoemaker et al. 1997). Furthermore, larval channel catfish, that were vaccinated prior to development of acquired immunity, demonstrated specific protection upon secondary exposure (Mackey 2002). These findings, along with those above, suggest that following primary pathogen exposure, innate immune responses can provide enhanced protection upon secondary exposure to the same pathogen. This project was driven by the central hypothesis that, following homologous bacterial exposures with *Edwardsiella ictaluri*, innate immune cells from previously exposed *rag1*$^{-/-}$ mutant zebrafish demonstrate enhanced survival.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals and Conditions

For this study, *rag1*$^{-/-}$ mutant zebrafish were used. Prior to experimental use, they were housed in the Specific Pathogen Free (SPF) fish hatchery at the MSU-CVM. The Institutional Animal Care and Use Committee (IACUC) at Mississippi State University approved all experimental animal protocols.

Bacterial Strains and Growth Conditions

Frozen bacteria stock cultures in 20% glycerol were taken from -80°C and grown up overnight in 100 ml of Blood Heart Infusion (BHI) broth in a shaking incubator at 28°C. From the overnight culture, 1 ml was re-inoculated in 50 ml of fresh BHI broth and grown up to logarithmic phase (Optical Density of 0.4 at 540nm).

Two isolates of *Edwardsiella ictaluri* were used in this study: attenuated and wild type strains. RE-33, an attenuated *E. ictaluri* (Klesius and Shoemaker 1999), was used for primary (vaccination) exposures. For the plate assays, non-attenuated *E. ictaluri* isolate 93-146 that contained the pAK*gfplux*1 plasmid (Karsi and Lawrence 2007) was used.

Bacterial Exposure

A stock solution of Tricaine Methanesulfonate (MS-222) was made by combining 400 mg of Finquel (Argent Laboratories, Redmond, WA) and 97.9 ml double-distilled water and adjusting the pH, using 1M TRIS base, to pH 7 (Westerfield 2000). Anesthesia water was made by adding 4.2 ml of MS-222 stock solution to 100 ml of tank water. Adult zebrafish were placed in the anesthesia water until gill operculating considerably slowed. The zebrafish were then injected intramuscularly (IM) using a 0.3cc, 30 guage insulin syringe (BD Biosciences, San Jose, CA) (Hohn 2008). Fish were injected with 10 µl of 10^6 colony forming units (CFU) per ml of *E. ictaluri*, which delivered a total of 10^4 CFU/fish. After recovery from anesthesia, fish were moved to tanks with dechlorinated municipal water in a flow-through system and maintained at 27°C.

Kidney Extraction and Processing

One month following the primary injection with 10^4 CFU/ml RE-33, vaccinated and naïve *rag1*$^{-/-}$ zebrafish were injected with 10^4 CFU/ml non-attenuated *E. ictaluri*. During Trial 1, fish from both groups were sampled at 4 hours post injection, and 1, 2, 3, 4, 5, 6 and 7 days post injection. At each time point, kidneys from five fish were removed and placed into separate microfuge tubes with 300 µl of Hank's Balanced Salt Solution (HBSS; Sigma-Adrich, St. Louis, MO). Alternatively, fish from Trial 2 were sampled at 1, 2, 3, 4, 5, 7, and 9 days post injection. At each time point for Trial 2, kidneys from sampled fish were removed and placed into separate microfuge tubes with 500 ul of HBSS.

For both trials, each kidney was dissociated by pipetting up and down one hundred times with a P1000 pipettor. After dissociation, the kidney cell suspension was filtered through a 40 µm cell strainer (BD Biosciences, San Jose, CA) into a new microfuge tube.

Kidney Cell Counts

Cells from each sample were quantified using a hemocytometer (Fisher Scientific, Pittsburgh, PA). Twenty microliters of each suspension was mixed with an equal volume of 0.4% Trypan Blue (Sigma-Aldrich, St. Louis, MO), and 15 µl loaded into the counting chamber and cells counted.

In addition to quantifying the number of kidney cells from each sampled fish, a differential count was performed. Approximately 20% of the kidney cell solution was set aside for bacterial plate counts and cell quantification. The remaining 80% of the kidney cell suspension was diluted to a total volume of 1 ml, divided into two, 500 µl samples, and transferred to microscope slides via cytocentrifugation for 3 minutes at 800 RPM using a Cyto-Tek Centrifuge (Miles Scientific, Elkart, IN).

The slides used for differential kidney cell counts were stained with Wright's stain (Ricca Chemical Company, Arlington, TX), doubling the staining time listed by the manufacturer. The cells were viewed using an Olympus BX51 microscope (Center Valley, PA), connected to a Microfire Digitial Microscope Camera (Optronics, Goleta, CA), and imaged with PictureFrame Imaging Software (Optronics, Goleta, CA). Based on the staining patterns and

morphologies, cells were classified as monocytes/macrophages, red blood cells, neutrophils, eosinophils, lymphocyte-like cells, or precursors.

Luminescence and Bacterial Counts

Luminescence readings as well as plate counts were performed for bacterial quantification. After the kidneys were dissociated, 100 µl of the cell suspensions were added to a black, 96-well plate with a clear bottom; measurements were performed in duplicate. An IVIS Imaging System 100 Series with Living Image 2000 software (Xenogen Corp., Alameda, CA) was used to measure the bioluminescence from the Lux expressing *E. ictaluri* within the kidney cell suspensions. The temperature of the stage was adjusted to 27°C (temperature of the fish tanks), and xposure time and binning settings were set as sensitive as possible without resulting in an overexposed image.

To culture live bacteria located within the kidney phagocytes, each sample was centrifuged at 1000 x g for 5 minutes to pellet the cells. The supernatant was discarded and 500 µl of 0.2% Tween 20 (Sigma-Aldrich, St. Louis, MO) was added to lyse the kidney phagocytes in Trial 1, while 120 ul was added for Trial 2. Serial dilutions of the lysed kidney cells were performed and plated on Tryptic Soy Agar (TSA) + 5% sheep blood plates (BD Biosciences, San Jose, CA). The plates were incubated at 28°C and colonies counted after 48 hours.

EMA and PMA Cross-linking

Propidium Monoazide (PMA; Biotium, Inc., Hayward, CA) and Ethidium Monoazide (EMA; Sigma-Aldrich, St. Louis, MO) were dissolved in *N,N*-

Dimethylformamide (DMF; Sigma-Aldrich, St. Louis, MO) to a stock concentration of 5 mM and stored at -20°C in the dark. The lysed kidney cell suspensions were divided into 3, 100 µl aliquots and PMA and/or EMA were added to each sample to a final concentration of 50 µM, leaving the third sample an untreated control sample. Samples were placed on ice, gently mixed and incubated in the dark, with intermittent mixing, for 5 minutes. Following incubation, the samples were subjected to a 2-minute light exposure using a 650-W halogen light (Utilitech 500W Portable Worklight with 650-W halogen light bulb) held 20 cm from the samples.

Isolation of Bacterial DNA

After cross-linking with EMA and PMA, bacterial DNA was isolated using a protocol adapted from Bilodeau *et al*. (2003) (Bilodeau et al. 2003). Briefly, following EMA/PMA treatment, all samples (including untreated controls) were centrifuged at 14,000 X *g* at 4°C for 2 minutes. Following centrifugation, the supernatant was discarded and the pellet resuspended in 200 µl of Cell Lysis Solution (Qiagen, Valencia, CA) and 1 µl of Proteinase K (20 mg/ml; Fisher Scientific, Pittsburgh, PA). The samples were incubated at 65°C for 1 hour. After incubation, 66.6 µl of Protein Precipitation Solution (NH_4OAC; Qiagen, Valencia, CA) were added to the samples; the samples were briefly vortexed and centrifuged at 14,000 X *g* at 4°C for 4 minutes. The supernatant was transferred to a new microfuge tube, to which 200 µl of 2-Propanol (Sigma-Aldrich, St. Louis, MO) was added. The samples were gently inverted 50 times and were

centrifuged at 14,000 X *g* at 4°C for 4 minutes to pellet the DNA. After centrifugation, the 2-Propanol was poured off and the pellet washed by adding 200 µl of 70% ethanol and centrifuging at 14,000 X *g* at 4°C for 4 minutes. The samples were vacuum centrifuged and resuspended in 27 µl of DNA Hydration Buffer (Qiagen, Valencia, CA).

Quantitative PCR

Quantitative PCR was performed using the protocol outlined in Bilodeau *et al*. (2003) (Bilodeau et al. 2003). Each amplification reaction (25 µl total) consisted of a DNA sample (5 or 10µl; zebrafish kidney and bacterial), 1X Platinum qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 0.2 µM of each primer, and 0.2 µM probe (Table 2.1). The amplification profile was: 2 minutes at 50°C; 2 minutes at 95°C; and 40 cycles of 15 s at 95°C and 1 minute at 60°C. All qPCR reactions were run on a Stratagene Mx3005P real-time PCR machine (Agilent Technologies, Santa Clara, CA), and results were analyzed using Stratagene MxPro QPCR software (Agilent Technologies, Santa Clara, CA).

Table 2.1 Primers and probe used in qPCR amplification reactions (from Bilodeau *et al*. (2003))

Primers and Probe	Sequences (5'-3')
Primers	
Forward	ACTTATCGCCCTCGCAACTC
Reverse	CCTCTGATAAGTGGTTCTCG
Probe	CCTCACATATTGCTTCAGCGTCGAC

Statistics

All statistics were run using IBM SPSS Statistics 18 (SPSS Inc., Chicago, IL). To determine the relationship between the qPCR and IVIS data and the colony counts, Pearson's Correlation was calculated. Additionally, one-way ANOVA analyzed data over the course of the sampling period within each group. Finally, Paired T-tests analyzed the possible differences between the vaccinated and naïve groups for each sampling timepoint.

CHAPTER III

RESULTS

Optimization of EMA and PMA

Effect of Water and 0.2%Tween 20 on EMA and PMA Efficiency. Both water and 0.2% Tween 20 are commonly used to lyse cells. Because the current project examined survival of intracellular bacteria, it was necessary to determine which lysing agent effectively lysed the zebrafish kidney cells while leaving the intracellular *Edwardsiella ictaluri* unharmed. *Edwardsiella ictaluri* was grown up overnight and an aliquot was heat-killed at 65°C for 30 minutes, while an equal volume of viable bacteria was placed on ice. Samples of both the viable and heat-killed *E. ictaluri* were treated with water or 0.2% Tween 20. Each of the water and 0.2% Tween 20 samples were then divided into three groups: EMA treatment, PMA treatment, and no treatment. To ensure the heat treatment actually killed all the bacteria, a sample was plated on Blood agar, incubated at 28°C, and checked for no growth after 48 hours.

After DNA extraction and quantitative PCR analysis, the difference in DNA yields from live and dead untreated samples (both water and 0.2% Tween 20) were not significant. Furthermore, the heat-killed bacterial samples that were treated with either EMA or PMA, with the exception of the 0.2% Tween samples

treated with PMA, showed a significant decrease in DNA yield. Although it appears that EMA is more efficient in both the water and 0.2% Tween 20 samples, there is no significant difference between the efficacy of EMA and PMA at linking dead bacterial DNA. Finally, there is no significant difference between samples resuspended in water versus 0.2% Tween 20 (Figure 3.1).

To ensure that the 0.2% Tween 20 was not harming the bacteria, dilutions of viable bacteria were performed in either 0.2% Tween 20 or water and were plated on blood agar and allowed to incubate at 28°C for 48 hours. Upon counting colonies, there was no significant difference between the water and 0.2% Tween 20 treatments.

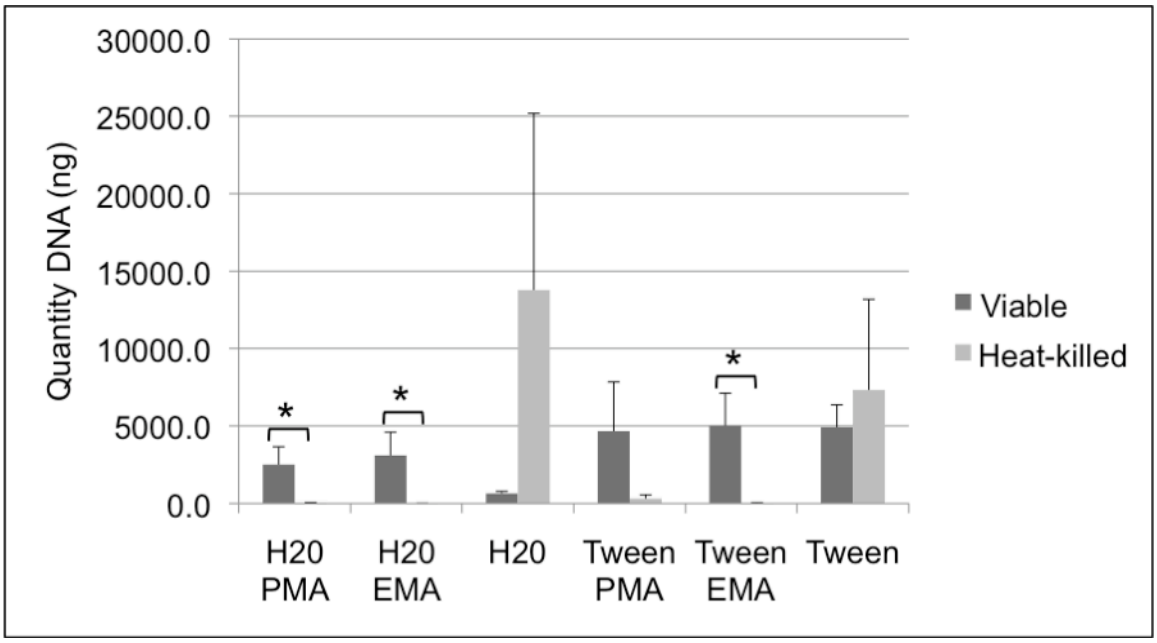


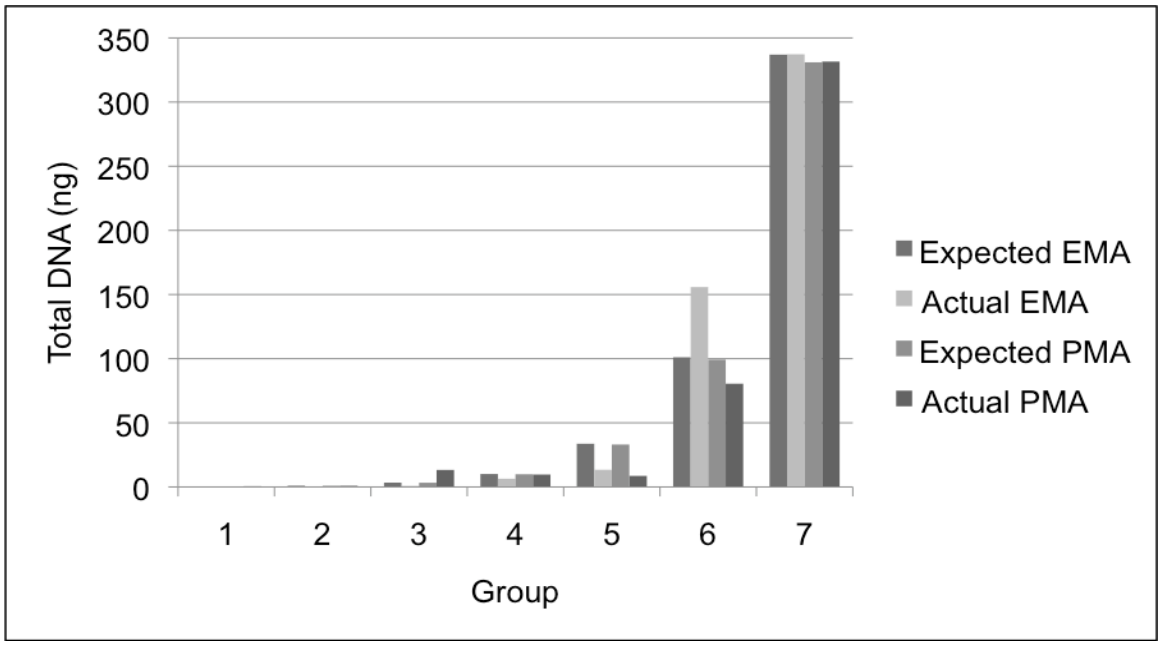
Figure 3.1 EMA and PMA versus water or 0.2% Tween 20 using viable and heat-killed *E. ictaluri* (*n*=3). An asterisk denotes significant difference ($p<0.05$). Error bars indicate standard deviation.

Differential DNA Extraction Using EMA and PMA. To evaluate the efficiency of EMA and PMA, predetermined ratios of live and dead *E. ictaluri* were treated with each of the agents and resulting qPCR signals analyzed. Again, an aliquot of *E. ictaluri* was heat-killed at 65°C for 30 minutes, while viable cells were stored on ice. After the heat-treatment, live and dead cells were mixed in defined volumetric ratios consisting of 0%, 0.3%, 1%, 3%, 10%, 30%, and 100% viable cells (Table 3.1).

When the amount of DNA obtained from each of the treated groups was compared to the expected DNA yield (based on Group 7 DNA yield), the correlation for each of the treatments was very high (EMA: Pearson's Correlation Coefficient=0.970; PMA: Pearson's Correlation Coefficient=0.954) (Figure 3.2).

Table 3.1 Defined ratios of viable and heat-killed *E. ictaluri* (in µl) for the evaluation of differential DNA extractions using EMA and PMA.

	1	2	3	4	5	6	7
Live	0	3	10	30	100	300	1000
Dead	1000	997	990	970	900	700	0



Group	Expected EMA (ng)	Actual EMA (ng)	Expected PMA (ng)	Actual PMA (ng)
1	0	0.4	0	0.59
2	1.011	0.29	0.993	0.95
3	3.37	0.79	3.31	13.3
4	10.11	6.38	9.93	9.7
5	33.7	13.42	33.1	8.64
6	101.1	155.85	99.3	80.5
7	337	337.26	331	331.6

Figure 3.2 Comparison of expected and actual DNA yields from EMA- and PMA-treated defined ratios of viable and heat-killed *E. ictaluri*.

Bacteria Survival

Assessment of the ability of *Edwardsiella ictaluri* to survive in kidneys of naïve and vaccinated *rag1*$^{-/-}$ zebrafish was accomplished using three different methods. These methods included counting colonies on agar plates, performing differential DNA extractions using EMA and PMA, and measuring luminescence of the bacteria using IVIS imaging system.

Throughout the seven days of sampling during Trial 1, both the naïve and vaccinated groups experienced mortalities. Of the 60 fish injected in each group, 5 fish from the vaccinated group (8.3%) and 13 from the naïve group (21.6%) died. These mortalities started at 3 dpi and persisted throughout the rest of the trial with the vaccinated group experiencing the bulk of their mortalities 4 dpi and the naïve group on 3 dpi (Figure 3.3).

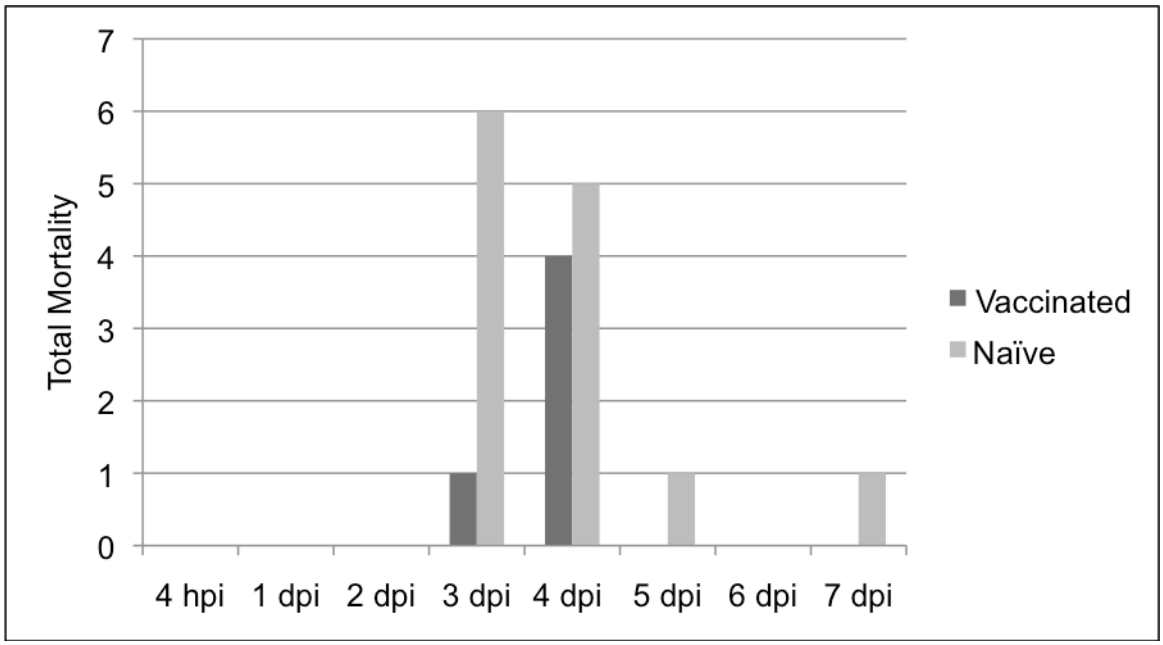


Figure 3.3 Daily mortality in the vaccinated and naïve mutants in Trial 1.

Similar to Trial 1 mortality data, infected fish from Trial 2 started dying on day 3 post infection. Additionally, the vaccinated group only had mortalities on 3 and 4 dpi, while the naïve group's mortalities persisted throughout the trial (Figure 3.4). Of the 100 fish injected from the naïve group, a total of 15 died (15%), whereas of the 80 fish injected from the vaccinated group, only 5 died (6.25%).

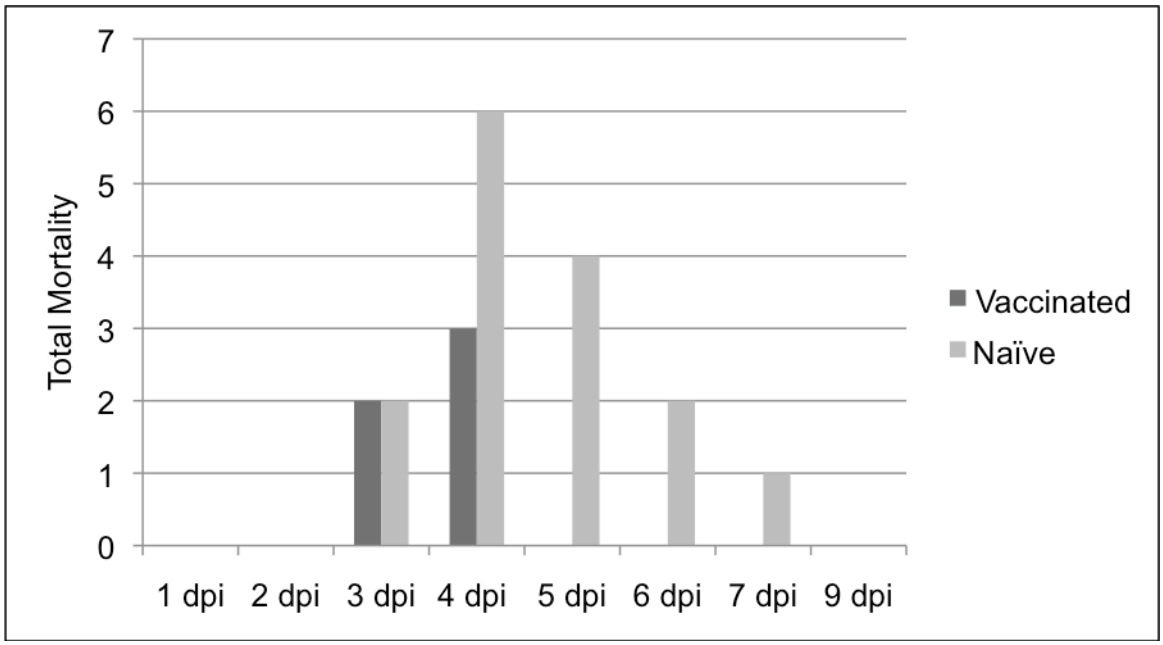


Figure 3.4 Daily mortality in the vaccinated and naïve mutants in Trial 2.

Leukocyte differential counts. Differential cell counts were performed as a way to examine the composition, in terms of different cell types, of the sampled kidneys (Table 3.2). Within the values obtained for the monocyte/macrophage cell type, the control values were significantly different than the vaccinated values on day 3 post infection, and there were no significant differences between the control and naïve values. Additionally, the naïve and vaccinated monocyte/macrophage counts were significantly different at 2 dpi (Figure 3.5A). For the red blood cell counts, the controls were not significantly different than the vaccinated group; however the controls were significantly different from the naïve group at 3 dpi, and the naïve and vaccinated groups were significantly different on day 3 post infection as well (Figure 3.5B). On day 2 post infection, the controls were significantly different from the vaccinated group for neutrophil

counts, and the vaccinated and naïve groups were significantly different on these days as well. Additionally, the controls were significantly different from the naïve at 3 dpi (Figure 3.5C). There were no significant differences in the eosinophil counts for any of the groups (Figure 3.5D). The lymphocyte-like cell counts were significantly different between the control and vaccinated groups on day 4 post infection, and the control and naïve groups at 3 dpi (Figure 3.5E). Finally, the precursor counts were significantly different between the control and vaccinated groups on all sampling days except days 1 and 3 post infection, and the control and naïve groups on all sampling days. The naïve and vaccinated groups had significantly different precursor counts on day 3 post infection only (Figure 3.5F).

Table 3.2 Differential kidney cell counts (*n*=5). Counts are based on 100 cells per kidney. Values are means plus or minus standard deviation.

	Percentage of Cells					
	monocyte macrophage	Red blood cell	Neutrophil	Eosinophil	Lymphocyte-like cell	Precursor
Control	39 ± 9	27.2 ± 13.7	4.4 ± 2.1	6.6 ± 3.8	5.4 ± 3.0	17.4 ± 2.8
Vaccinated						
1 dpi	38.3 ± 5.0	26.3 ± 10.9	1.5 ± 1.9	4.5 ± 3.1	3.8 ± 3.4	25.8 ± 7.8
2 dpi	27 ± 9.5	24.8 ± 15.8	0.8 ± 1.3	5.6 ± 1.3	5 ± 1.2	36.6 ± 9.7
3 dpi	36 ± 3.7	29.8 ± 9.6	1.3 ± 0.5	4.5 ± 2.4	1.5 ± 1	27 ± 4.5
4 dpi	39 ± 7.1	12.8 ± 6.9	2 ± 1.2	5.6 ± 1.7	2.4 ± 1.5	38.2 ± 5.5
5 dpi	38.2 ± 3.7	14 ± 7.1	3 ± 0.7	7 ± 2.8	2.2 ± 1.1	35.6 ± 7.4
7 dpi	39.4 ± 1.1	16 ± 5.4	2.8 ± 1.9	6.4 ± 5.2	2.2 ± 1.5	33.2 ± 8.2
9 dpi	38 ± 13.8	12.4 ± 6.1	2 ± 1.6	3 ± 1.9	3.4 ± 2.3	40.8 ± 12.2
Naïve						
1 dpi	35.2 ± 8.1	23.6 ± 6.3	2 ± 1.2	4.4 ± 4.6	4 ± 1.6	29 ± 6.7
2 dpi	40.8 ± 7.4	13.2 ± 21.3	2.4 ± 1.8	6.4 ± 6.7	3.2 ± 2.9	34 ± 10.9
3 dpi	40.4 ± 8.1	9.2 ± 3.6	2.2 ± 2.3	3 ± 0.7	1.8 ± 1.5	43.4 ± 7.0
4 dpi	44.8 ± 5.6	13 ± 10.1	2.2 ± 1.1	4.2 ± 3.6	2.2 ± 1.3	33.6 ± 2.6
5 dpi	38.6 ± 11.4	9 ± 6.0	2.6 ± 1.5	6.2 ± 1.3	2.4 ± 0.5	41.2 ± 7.9
7 dpi	40.6 ± 4.4	9.6 ± 6.3	2.6 ± 1.3	3.8 ± 3.9	2.8 ± 1.8	40.6 ± 7.3
9 dpi	36.2 ± 9.7	18.4 ± 13.2	2.2 ± 1.6	1.8 ± 0.8	2.6 ± 1.5	38.8 ± 6.7

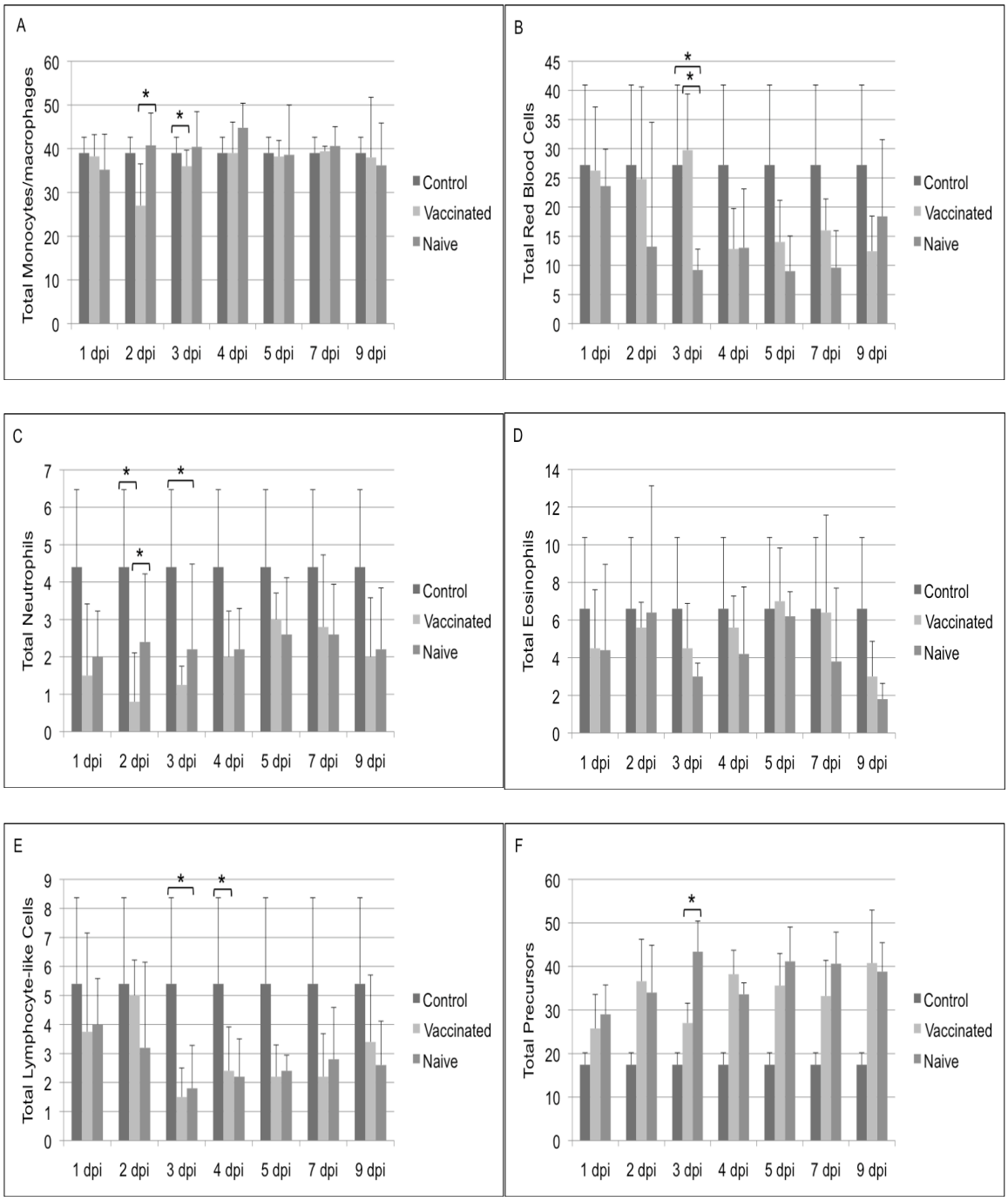


Figure 3.5 Differential kidney cell counts from control, vaccinated and naïve fish (*n*=5) for each of the different cell populations in the kidney. An asterisk denotes significant difference ($p<0.05$). Error bars denote standard deviation.

The second slide from the cytospins performed on all sampled kidneys were stained with giemsa and evaluated for *E. ictaluri*. On all the stained slides, *E. ictaluri* was observed exclusively within macrophages; no extracellular *E. ictaluri* was observed (Figure 3.6).

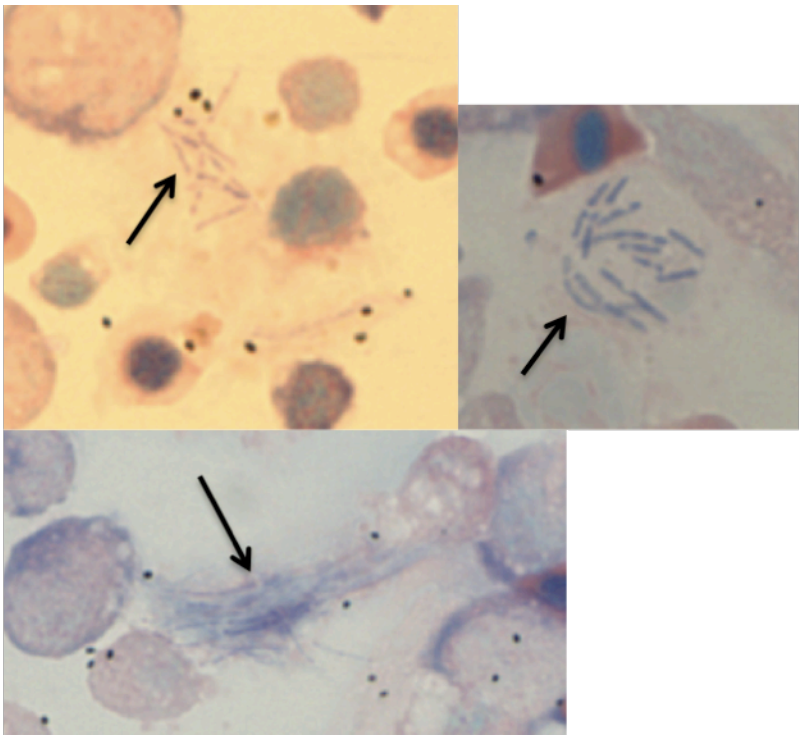


Figure 3.6 Cytospins stained with Giemsa showing intracellular *E. ictaluri* (black arrows).

Cell and colony counts. Total kidney cell counts increased throughout the sampling period. Although the fish were not symptomatic, around 3 and 4 dpi some of the kidneys from naïve fish were enlarged, pale, and had undefined borders compared to kidneys from vaccinated fish. With respect to cell counts, there were statistical differences within the naïve group between 1 and 7 dpi and within the vaccinated group between 3 and 7 dpi over the seven-day sampling

period. Furthermore, comparison between naïve and vaccinated cell counts from 1 and 3 dpi were significantly different (Figure 3.7A).

Colonies of *E. ictaluri* obtained from the lysed kidney cells were enumerated after a 48-hour incubation, colony forming units of *E. ictaluri* per milliliter (CFU/ml) was calculated, and the data was log transformed. The values obtained from the naïve fish throughout the sampling period indicated day 3 post infection was significantly different than the CFU/ ml from 4 hpi, 1 dpi, 2 dpi, and 7 dpi. There was no significant difference in the CFU/ml obtained from the vaccinated fish over the duration of the sampling period; however, when the naïve and vaccinated fish were compared to each other, there was a significant difference on day 1 and 4 post infection. Overall, the most bacteria were isolated from naïve fish on days three and four post infection than from vaccinated fish (Figure 3.7B).

Finally, colony forming units of *E. ictaluri* were calculated as a proportion of total kidney cells counted, and the resulting data was log transformed. There were significant differences in CFU of *E. ictaluri*/kidney cell between naïve and vaccinated groups on 1 and 4 days post infection. However, there were no significant differences within each group over the duration of the sampling period (Figure 3.7C).

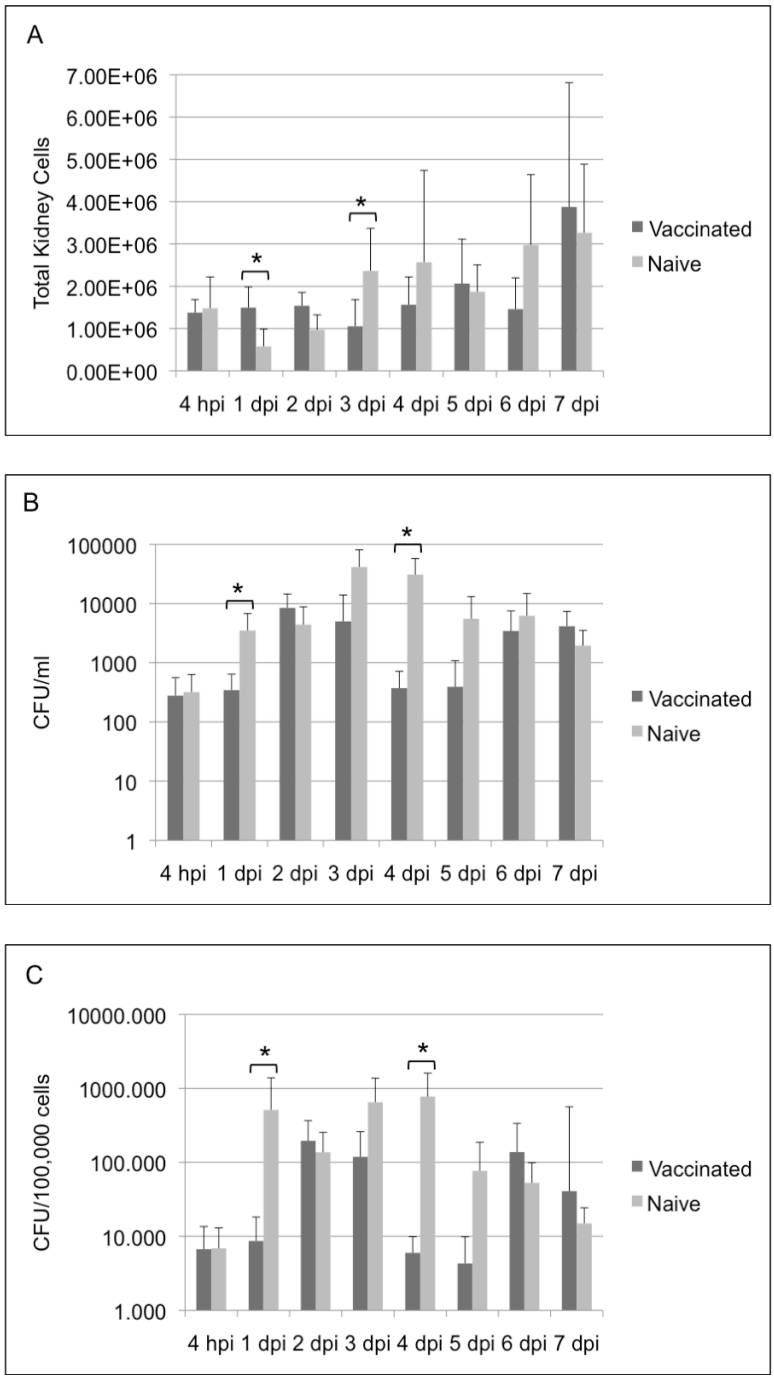


Figure 3.7 Trial 1: A) Comparison of kidney cell counts from vaccinated and naïve *rag1*$^{-/-}$ mutant zebrafish (*n*=5). B) Comparison of bacterial counts from dissociated kidney leukocytes from vaccinated and naïve mutants (*n*=5). C) Comparison of number of bacteria per kidney from vaccinated and naïve mutant fish (*n*=5). An asterisk denotes significant difference ($p<0.05$). Error bars denote standard deviation.

For Trial 2, there was a significant difference between the kidney cell counts from vaccinated and naïve fish at 5 dpi. However, within both the naïve and vaccinated groups, there was no significant difference in cell counts over the course of the sampling period (Figure 3.8A). The colony forming units of *E. ictaluri* per milliliter data was log transformed and there was no significant difference when the naïve and vaccinated groups were compared to each other. However, within the vaccinated group, day 4 post infection was significantly different than 1, 2, 3, 5, 7, and 9 dpi. Additionally, day 5 post infection was significantly different from 7 dpi. Likewise, within the naïve group day 4 post infection was significantly different than the other sampling time points, and 5 dpi was significantly different from 3 and 7 dpi (Figure 3.8B).

Because *E. ictaluri* was only observed within macrophages from the Giemsa-stained kidney samples, the differential count data from Trial 2 was used to determine the number of macrophages in each kidney sample, thus allowing for the calculation of number of colony forming units *E. ictaluri* per kidney macrophage. After log transforming the data, there were no significant differences within the naïve or vaccinated groups in terms of CFU *E. ictaluri*/kidney macrophage. Likewise, there was no significant difference between the two groups for any of the sampling days (3.8C).

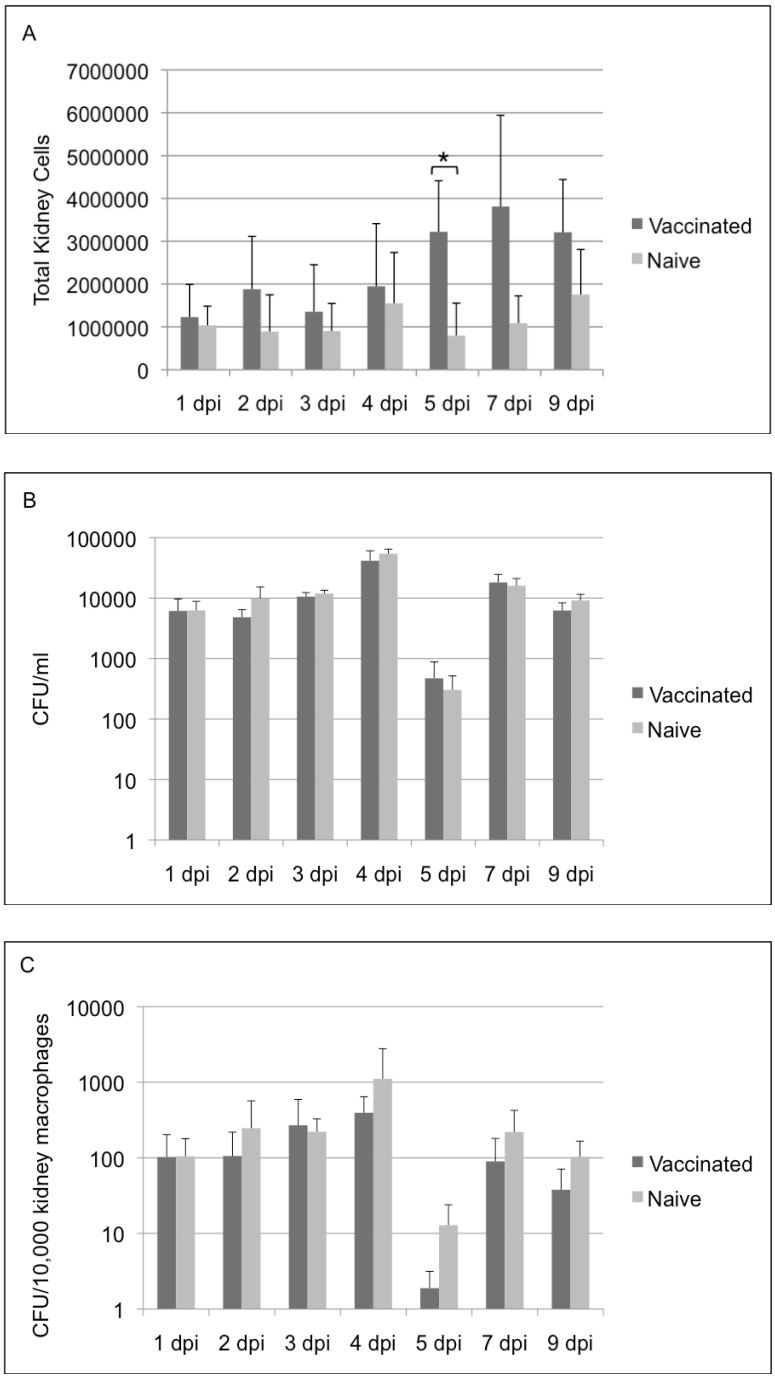


Figure 3.8 Trial 2: A) Comparison of kidney cell counts from vaccinated and naïve *rag1*$^{-/-}$ mutant zebrafish (*n*=5). B) Comparison of bacterial counts from dissociated kidney leukocytes from vaccinated and naïve mutants (*n*=5). C) Comparison of number of bacteria per kidney macrophage from vaccinated and naïve mutant fish (*n*=5). An asterisk denotes significant difference ($p<0.05$). Error bars denote standard deviation.

Quantitative PCR. The qPCR results showed amplification of several samples; however they were inconsistent with the data obtained from the plate counts (Figure 3.9). Within the samples with amplification, there was a decrease in the amount of amplification between the untreated sample and the sample treated with EMA or PMA. Taking into consideration that there were approximately one million cells in each sample, the expected DNA yield should be around 7 ug. However, DNA quantification revealed an average of 0.27 ug of DNA per sample, which is more than a 10-fold decrease in DNA yield.

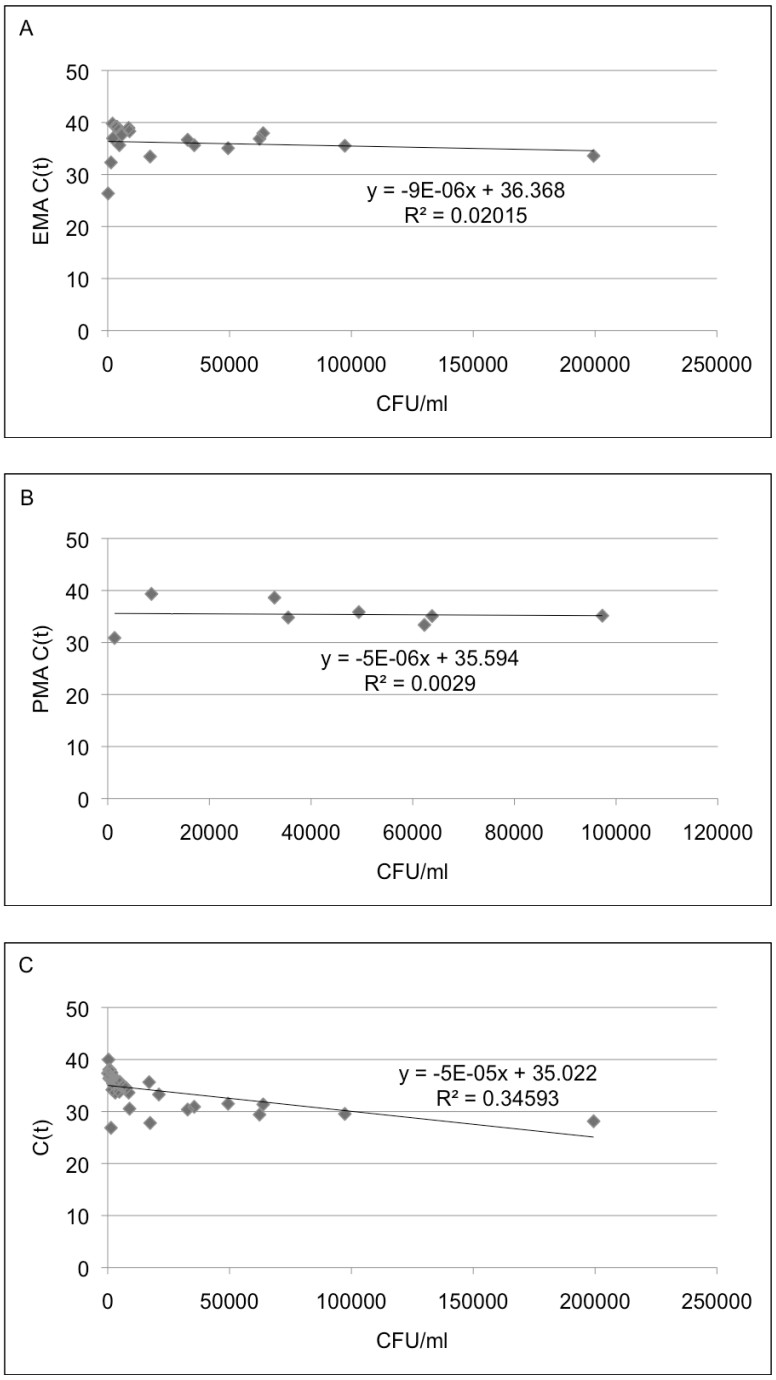


Figure 3.9 Scatter plots and regression analysis demonstrating the relationship between CFU *E. ictaluri*/ml and threshold cycle (C(t)) from quantitative PCR from A) EMA-treated, B) PMA-treated, and C) untreated samples from Trial 1.

Comparison of luminescence with colony count data. The final step in the survival assay was to use the IVIS system as a way to quantify the live bacteria in the kidney cells from sampled fish. Similar to the qPCR data, the data obtained from the IVIS showed luminescence in several samples, but it was inconsistent with the plate count data (Figure 3.10).

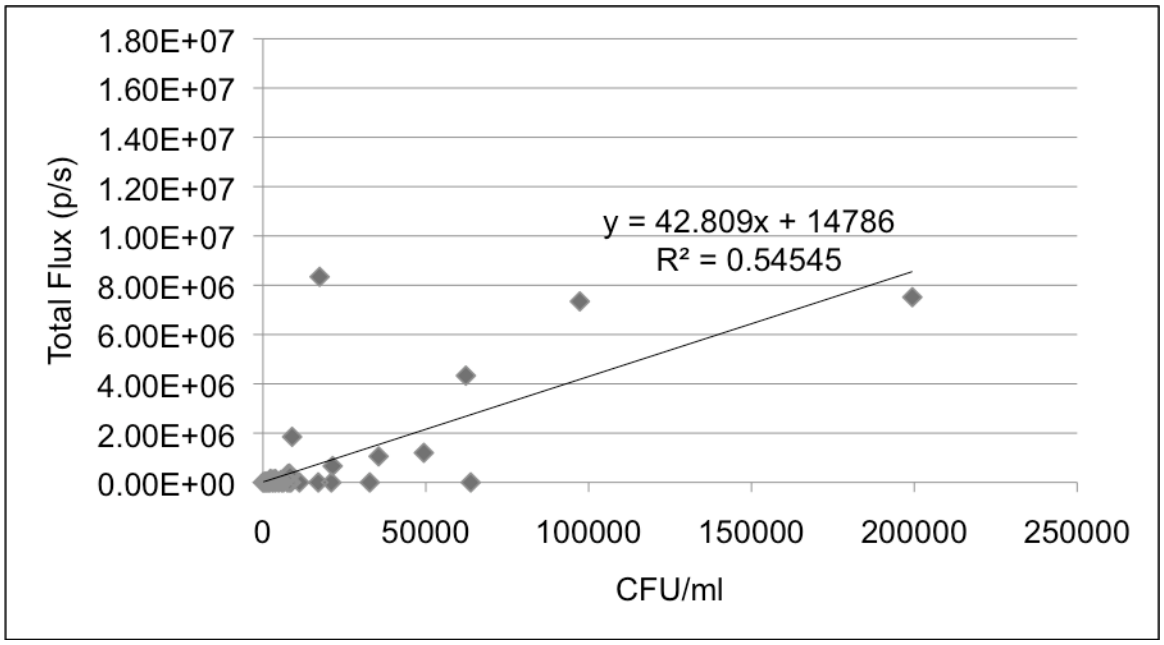


Figure 3.10 Scatter plots and regression analysis demonstrating the relationship between CFU *E. ictaluri*/ml and total flux (photons/second) from IVIS data from Trial 1.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The current project was multipartite, but dealt foremost with the investigation of the survival of *Edwardsiella ictaluri* in kidneys from vaccinated and naïve *rag1*$^{-/-}$ zebrafish. Additionally, the current study examined three different methods for enumerating the differences described above. The first method, which is the method historically used, was to count colonies on TSA + 5% sheep blood agar. The other two methods were to measure the luminescence of the bacteria in kidney samples and to perform differential DNA extractions using EMA and PMA. To evaluate whether these new methods are as efficient as the standard colony count, the luminescence and qPCR data were compared to the colony count data. Additionally, microscopic examination of tissues and cells were used to correlate the findings to mortality.

Ethidium Monoazide and Propidium Monoazide were evaluated in terms of their efficacy to remove DNA from dead bacteria. We also evaluated if water or 0.2% Tween 20, both commonly used to lyse cells, had an impact on either chemical's efficiency. Previous macrophage killing assay studies used 0.2% Tween 20 to lyse kidney-derived phagocytes (Graham et al. 1988; Shoemaker et al. 1997); since there was no significant difference between the ability of EMA or

PMA to bind dead bacterial DNA in the presence of water or 0.2% Tween 20, the latter was used in the current study.

It appears that days 3 and 4 post infection are critical in terms of survival and bacterial load for the naïve and vaccinated mutants. For both of the trials, both groups of fish experienced their highest mortalities on these days. Additionally, the naïve mutants experienced steady mortalities following the mortality spikes at 3 and 4 dpi, which indicates that this group is either less efficient at clearing the bacteria, or is more sensitive to *E. ictualuri* infections than the vaccinated mutants. Additionally, the data from Trial 1 shows that during these two days as well as 1 dpi, the number of CFU/kidney cell in the naïve mutants was higher than in the vaccinated mutants. On day 1, this is a result of the number of kidney cells in the naïve mutants being significantly less than in the vaccinated mutants compounded with the fact that there was also significantly more bacteria in the naïve fish.

Identification of *E. ictaluri* within kidney macrophages, as well as the differential leukocyte counts performed in Trial 2 enabled a more accurate calculation of the ratio of bacteria to kidney cell (CFU/kidney macrophage). The data show the number of CFU/kidney macrophage is higher in the naïve mutants than the vaccinated mutants on days 1, 2, 4, 5, 7, and 9 post infection. This could be a result of the vaccinated mutants' total cell counts being consistently higher throughout the trial even though the bacterial counts obtained from the two groups were similar. Overall, these results, as well as those from Trial 1, suggest that the vaccinated mutants are able to mount an immune response

faster, thus keeping the proportion of bacteria to kidney cells lower, resulting in lower mortality.

Although there appears to be a difference in bacterial clearing between the naïve and vaccinated zebrafish, the results are only statistically significant on days 1 and 4 post infection for Trial 1. This is due in part to the high amount of variability between the fish in each group. Presumably, a larger sample size for each day would overcome this problem; if more fish are sampled each day, outliers will not make as big of an impact. Additionally, the results on 6 and 7 dpi from Trial 1 are unexpected. On these days, the vaccinated mutants have more bacteria per cell than the naïve mutants. The vaccinated fish effectively cleared the bacteria on days 4 and 5, but on days 6 and 7 of Trial 1 the bacteria counts were higher than the naïve mutants. This could be a result of the surviving fish shedding the bacteria and re-infecting the others. However, we would expect a similar trend to appear in the naïve group as well. Another possibility is that the most susceptible naïve mutants had already been sampled or died, leaving only naïve fish that were naturally more resistant to the bacteria.

Unfortunately, the differential DNA extraction using EMA and PMA did not yield the results that were expected, and were not consistent with the data obtained from the plate counts. The confounding aspect is that the differential DNA extractions were initially successful. The only difference between the samples in the initial trials and the samples from Trial 1 was the presence of kidney cell DNA as opposed to pure bacterial cultures. Upon further investigation, it was found that the DNA yield from the Trial 1 samples was

significantly less than what would be expected. Therefore, it can be assumed that the inconsistency with the qPCR results was not so much in the qPCR reaction, but more in the DNA extraction procedure. It is possible that the cells were not sufficiently lysed, therefore causing a low DNA yield. This is unlikely because the proper volumes of reagents were used based on the total number of cells in the sample. Another possible explanation is that the DNA was not adequately precipitated during the isopropanol step. However, the exact same procedures were used, with success, for the DNA extraction during the initial trial.

The third method used to quantify live bacteria contained within the kidney samples was to measure the luminescence of *E. ictaluri* via the IVIS imaging system. An obstacle encountered with the IVIS was that it wasn't sensitive enough to pick up the luminescence from samples with only a small amount of *E. ictaluri*. Another problem encountered was that the machine could only be set as sensitive as the sample with the highest luminescence; if the settings are too sensitive the image becomes supersaturated, which requires the user to reduce the sensitvity. So, theoretically, samples with a small amount of bacteria may not have given off a signal because of another sample with a high amount of bacteria was dictating less sensitive settings. One way to overcome this problem is to image each sample individually so the settings can be personalized to the individual sample. Additionally, whole fish could be imaged rather than only kidney cells. This would also provide an image of where in the body the bacteria are most concentrated and the migration of the bacteria throughout the zebrafish body during the infection cycle.

Based on the results of the study, it appears that there is a significant difference between the naïve and vaccinated mutants at clearing *Edwardsiella ictaluri* (Trial 1, days 1 and 4 post infection). However, there was a high amount of variation between the sampled fish within each group. Additionally, at this point, it seems the most efficient and accurate way to quantify the live bacteria from the infected zebrafish is via colony counts on agar plates. Both the qPCR and luminometer methods, although promising, did not prove to be as reliable as the colony count method. The qPCR method, in theory, should be more sensitive, and more accurate, than the plate count method. At the very least, the qPCR should be comparable to the plate count method. Because of the huge potential the qPCR has, it is important that further effort be made to optimize the DNA extraction procedure. Finally, further trials should be run to increase the robustness of the colony and cell count data. More trials and/or larger sample sizes should decrease the standard deviations of the samples and thus, enable statistical significance between the groups.

The observation that the vaccinated mutants survived and cleared *E. ictaluri* more efficiently than the naïve mutants is very exciting. The data reported in this study imply that, as a result of previous exposure to *E. ictaluri*, the vaccinated zebrafish are better able to react to and clear the bacteria upon secondary exposure. Classically, this reaction has been attributed to lymphocyte-driven immunity, however, the *rag1*$^{-/-}$ zebrafish lack functional lymphocytes; therefore these results must be attributed to cells of the innate immune system.

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