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Trolling for the ideal model host: zebrafish take the bait

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Abstract

As little as 10 years ago, murine models of infectious disease were the host of choice for analyzing interactions between the pathogen and host during infection. However, not all pathogens can infect mice, nor do they always replicate the clinical syndromes observed in human infections. Furthermore, in the current economic environment, using mammalian models for large-scale screens may be less economically feasible. The emergence of the zebrafish (*Danio rerio*) as an infectious disease host model, as well as a model for vertebrate immune system development, has provided new information and insights into pathogenesis that, in many instances, would not have been possible using a murine model host. In this article we highlight some of the key findings and the latest techniques along with the many advantages of using the zebrafish host model to gain new insights into pathogenic mechanisms in a live vertebrate host.

Keywords

embryos; immune system; infectious disease; model host; morpholinos; pathogenesis; zebrafish

The ideal approach to accurately determine what stimuli are directing the course of an infection is to analyze the communication that occurs between the pathogen and the defense mechanisms of the host. This requires analysis of the infection process by examining the dynamic responses of both the pathogen and host to various stimuli. Obviously, in the case of human infectious disease, this type of thorough analysis is not practical. Therefore, a suitable host that mimics the human immune response to infection is needed. For many years, mammalian model hosts, ranging from nonhuman primates to murine models, have been used successfully for these purposes. However, there are many instances in which one of these previously utilized models is not ideal. This provided the motivation for the development of new host models. Recently developed models that allow analysis of the innate immune system during infection include flies (*Drosophila melanogaster*) [1–3], nematodes (*Caenorhabditis elegans*) [4,5], amoeba (*Dictyostelium discoideum*) [6,7] and even certain plants (*Arabidopsis thaliana*) [8]. All of

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these have been used quite successfully to examine infectious disease mechanisms where a specific question relating to innate immunity was being addressed.

To ask more complex questions relating to both innate and adaptive immune responses in a vertebrate model host, many infectious disease researchers have turned to the increasingly popular zebrafish (*Danio rerio*). The wealth of information available from decades of research on vertebrate development, toxicology and neurobiology has made the zebrafish an attractive host in which to examine infectious disease mechanisms. In the last 10 years, a large repertoire of both bacterial pathogens and viruses has been successfully examined for their ability to cause disease in the zebrafish infectious disease model (see reviews [9–11]).

What makes the zebrafish an ideal model host? Besides the obvious advantages of small size, low cost, easy maintenance and prolific breeding resulting in large numbers of progeny and *ex vivo* embryo development, the zebrafish has multiple additional benefits. For the first several days postfertilization, the embryos are transparent and for approximately 3 weeks the larvae are translucent. *Ex vivo* development of the embryos can be analyzed in real time under low-power microscopy, allowing researchers to exploit the optically transparent property to examine how mutations affect vertebrate organ and tissue development, including embryonic lethal mutations that could not be analyzed using traditional mammalian models. This property also lends itself well to infectious disease research using fluorescently labeled bacteria or viruses. Infection progression can be followed in real time with live embryos with the use of a fluorescent stereomicroscope to visualize the route of dissemination and the bacterial load over time. Recently, multiple transgenic zebrafish lines have been developed in which a fluorescent marker is placed under a tissue-specific promoter/enhancer elements allowing particular cell types to be tracked *in vivo* using fluorescent microscopy. Strains that have been particularly useful in examining host–pathogen interactions are a *fliI*:enhanced GFP (EGFP) strain [12] or a *lysC*:EGFP strain [13,14] for visualizing macrophages and a myeloperoxidase:GFP [15] or myeloid-specific peroxidase:EGFP [16] strain, facilitating the live visualization of neutrophils/heterophils *in vivo*. An additional strain with a GFP fusion to the *pu.1* promoter selectively labels all cells of myeloid lineage [17]. Research using fluorescently labeled bacteria in conjunction with zebrafish strains with fluorescently labeled immune cells to decipher key interactions between the host and pathogen has provided new insights into host–pathogen interactions [15,16,18–22]. However, one limitation of this kind of analysis is that it can only be performed in the first 3 weeks postfertilization before the development of melanization that disrupts the optical transparency and occludes visualization of internally expressed fluorophores. To circumvent this limitation, White and Zon recently developed a strain of transparent zebrafish, appropriately named ‘Casper’, that enables visualization of the interior of the fish into the adult stage [23]. The impetus behind the development of this strain was to monitor growth and metastasis of tumors *in vivo*. Previously, a very similar mutant was made by Ren *et al.* to perform a behavioral study on visual sensitivity using fully and partially pigmented zebrafish [24]. In the same manner, one can easily imagine the multiple advantages these fish could provide to the infectious disease researcher while using fluorescently labeled bacteria or viruses.

Sequencing of the zebrafish genome is complete and although assembly of the genome is ongoing [101], numerous genetic markers have been mapped and databases of available expressed sequence tags/cDNAs, fish lines and antibodies are available, as well as a site for searching the zebrafish genome using BLAST [102]. Both genome searching and recent reports in the literature reveal high conservation between human and zebrafish immune systems, including both innate and adaptive immunity [11,25–30]. This has allowed zebrafish to emerge as a powerful tool for investigating immune system development, function and disease [9,11, 31–33]. Immunologists have utilized this new information to examine commonalities with mammalian immune functions, finding that nearly all cell types of the human immune system

are also conserved in the zebrafish [34,35]. Innovative techniques utilizing fluorescence imaging in real time have allowed for the study of inflammatory responses at damaged tissue sites [15,16,36,37]. Niethammer *et al.* utilized transgenic zebrafish expressing HyPer, a genetically encoded H₂O₂ ratiometric sensor, along with leukocyte-specific fluorescent tags to demonstrate that the epithelial dual oxidase (Duox) generates a tissue-scale gradient of H₂O₂ responsible for leukocyte recruitment in response to tissue injury [38]. Zebrafish have also been used to study the effects of environmental toxins on immune system development. Low-dose arsenic exposure resulted in an overall suppression of innate immune function and increased susceptibility to viral infection [39,40], possibly through disruption of the JAK/STAT pathway [40]. In addition, exposure to potent cyanobacterial toxins increased expression of genes essential for lymphocyte maturation and differentiation as well as oxidative stress response genes in zebrafish larva [41]; however, downstream developmental defects on immune function need to be investigated further.

An additional advantage of using the zebrafish embryo as an infectious disease model comes from the staged development of the immune system. Innate immunity is functional with macrophages and neutrophils active by 48 h postfertilization. However, the adaptive immune system does not gain full functionality until later time points [9,11,29,42], although the time at which the adaptive immune system becomes fully functional is currently under debate. However, no adaptive immune markers are observed during the first 4 days postfertilization [28]. This delayed development provides the unique opportunity to study the functional role of innate immune components on progression and/or clearance of a particular type of infection in the absence of adaptive immunity. Innate responses to foreign pathogens depend on complex networks of cellular receptors and adaptor proteins for recognition of pathogen-associated molecular patterns that initiate appropriate signaling response pathways. Signaling through these pathways results in activation of transcription factors, including NF- κ B, culminating in the synthesis of proinflammatory cytokines, such as TNF and interferons. Current studies demonstrate both the presence and functionality of these pathways in zebrafish, as well as the specific requirement and responses of these pathways to various pathogens [27,43–59]. Zebrafish infected with nervous necrosis virus in an age-dependent viral disease model revealed that a reduction in virus titer and mortality rate was dependent upon an active IFN- α response, and that virus replication could be inhibited in the susceptible zebrafish larval stage with IFN- α treatment offering insights into possible antiviral therapies [60].

Recent research has exploited the zebrafish model to ask key questions about the particular stimuli that give rise to specific interactions between the host and pathogen during infection. One particularly perplexing question faced by researchers studying *Streptococcus pyogenes* infection was the observed lack of polymorphonuclear leukocyte (PMN) infiltration in heavily infected tissues during necrotizing fasciitis infections (aka ‘flesh-eating bacteria’ infections). This question was recently addressed in a novel way using a combination of models, including zebrafish embryos, larvae and adult fish, as well as mice and *in vitro* assays to observe changes in neutrophil migration [61]. Infections in adult fish and embryos showed that a mutant strain of *S. pyogenes* missing a cytolytic toxin, streptolysin S, was less virulent than wild-type bacteria and, in addition, showed a greater amount of inflammation in the muscle tissue. Using Pu.1 zebrafish larvae (GFP-labeled myeloid cells) [17] and fluorescently labeled *S. pyogenes*, they were able to visualize greater numbers of GFP-labeled cells colocalized with the mutant bacteria compared with the wild-type strain, suggesting that the wild-type strain had the ability to inhibit PMN migration, which they subsequently confirmed using *in vitro* PMN migration assays [61]. Furthermore, using two-photon microscopy to visualize PMN migration in the mouse paw, they determined that there is a difference in timing of extravasation of the neutrophils from the vessel into the tissue that is dependent on the presence of streptolysin S of *S. pyogenes*. Importantly, they also observed that there were no differences in IL-8 concentration between the two strains, suggesting that this is not the main stimulus for

neutrophil recruitment in *S. pyogenes* infections and another unknown chemoattractant may be responsible [61].

Over the years, many infectious disease researchers have had to rely heavily on *in vitro* assays to address virulence questions as live models were not amenable to manipulation or did not accurately demonstrate what happens during human infections. *Listeria monocytogenes* has been used for years as a model of an intracellular infection. However, the majority of data come from *in vitro* tissue culture assays as real-time *in vivo* imaging has not been possible. Levraud *et al.* recently exploited the zebrafish embryo model to visualize the rapid phagocytosis by macrophages *in vivo* of all GFP-expressing *L. monocytogenes* within 1 h of infection [62]. Moreover, using transmission electron microscopy of infected zebrafish larvae and a specialized staining technique, they were able to visualize the formation of actin comet tails used by the bacteria to invade adjoining cells, an event only observed in *in vitro* tissue culture models previously. *Listeria* phagocytosis *in vivo* has never been imaged in a rodent model of infection, showing one of the advantages of using this model for host–pathogen interactions [62].

Taking advantage of the developmental stages of the immune system in zebrafish embryos, Clatworthy *et al.* were able to show that both macrophages and neutrophils are required to successfully fight a *Pseudomonas aeruginosa* infection [63]. Infection at 50 h postfertilization, when both neutrophils and macrophages are present, resulted in an attenuated infection when using *P. aeruginosa* mutants defective in either type III secretion or quorum sensing, as has been demonstrated with murine models of infection. However, infection at 28 h postfertilization, when only macrophages are present, caused a lethal infection with these mutants. Investigating this phenomenon further, they were able to control the outcome of infection by using the technique of morpholino oligonucleotide technology. This technique uses micro-injection of nonionic oligos to prevent mRNA translation of the gene of interest, resulting in highly effective gene knockdown for up to 6 days postinjection [64]. Microinjection of a targeted morpholino into embryos can mimic the results from a transgenic knockout strain using a simple 30-s microinjection of an oligonucleotide. Clatworthy *et al.* used this knockdown technology to disrupt expression of host transcription factors that regulate expression of either myeloid or erythroid cell development. Knockdown of *pu.1* shifted progenitor cells to erythroid development, thereby eliminating the myeloid cells, which includes macrophages and neutrophils. Not surprisingly, these morphants were exceptionally susceptible to infection by both the wild-type strain and a mutant strain of *P. aeruginosa*. Knockdown of *gatal* expression resulted in increased numbers of myeloid cells (macrophages and neutrophils), and infection of these morphants resulted in increased survival of embryos compared with control embryos [63].

A similar technique was used by Clay *et al.* to determine if macrophages could limit mycobacterial growth early in infection [22]. Previously, mouse models of tuberculosis had suggested that mycobacterial growth is not inhibited early in infection but is only controlled after the onset of adaptive immunity. Using zebrafish embryos prior to the development of the adaptive immune response, they examined mycobacterial infection in the presence and absence of macrophages using morpholino knockdown technology to inhibit macrophage development. Results demonstrated that, indeed, macrophages limit growth of mycobacteria early in infection. However, phagocytosis by macrophages is critical for mycobacterial dissemination, and ultimate granuloma formation, all in the absence of adaptive immunity [22,65].

The delayed development of the adaptive immune system of the zebrafish embryo allowed Stockhammer *et al.* to perform a transcriptome analysis of the innate immune response to a systemic infection by *Salmonella enterica* serovar Typhimurium, using a strain that caused a lethal infection compared with a strain causing an attenuated infection [52]. Their analysis

covered four time points from 2 to 24 h postinfection, generating multiple sets of data, which then facilitated a gene ontology analysis. They observed conservation of expression of immune factors compared with infection profiles with other vertebrate models, as well as novel immune response genes. Using the data derived from their analysis, they further examined the TLR5 dependence and MyD88 dependence of expression of multiple immune genes by utilizing morpholino knockdown technology. Importantly, they found that flagellin acts as a ligand for TLR5, as in mammals, which is important for activation of specific host defense genes following stimulation. Using *myd88* morpholino knockdown demonstrated both MyD88-dependent and -independent pathways in response to *S. typhimurium* infection, mimicking what is seen in mammalian infections [52].

To determine the host response to *Mycobacterium* infection, Volkman *et al.* examined host gene expression of zebrafish larvae infected with wild-type *Mycobacterium marinum* compared with an RD1-deleted *M. marinum* strain [66]. The RD1 locus has been shown to be involved in the ability of mycobacteria to induce granuloma-forming events [67]. An RD1-dependent induction of *mmp9* was observed at 5 days postinfection. Knockdown of *mmp9* expression by injection of antisense morpholinos against *mmp9* during infection with the wild-type strain showed an attenuated infection similar to that observed with the RD1 mutant, demonstrating a deficiency in granuloma formation. Microscopy of infected tissues illustrated that the granuloma-associated *mmp9* expression was not from macrophages but, surprisingly, from adjacent epithelial cells. Subsequently, they determined that the signal for *mmp9* induction was a bacterial-specific factor, ESAT-6, which acts to promote granuloma formation by interacting directly with epithelial cells. This study is an excellent example of how the zebrafish model can be used to examine host–pathogen interactions during infection [66].

Lastly, multiple aspects of the zebrafish make them amenable to large-scale mutagenesis screens. The low cost and small size of the zebrafish embryo make them ideal for doing screens in a 96-well plate format. Looking for clearance or persistence of a mutagenized pathogen that is fluorescently labeled could be very efficiently accomplished using a fluorescent stereomicroscope and infected zebrafish embryos arrayed in a 96-well plate. Adult zebrafish have also been used successfully for large-scale mutagenesis screens. Two species of streptococcal pathogens were analyzed using signature-tagged mutagenesis and then screened through adult zebrafish [68,69]. In both reports, previously identified virulence genes were pulled out, confirming the utility of the screens in zebrafish to identify factors involved in pathogenesis, as well as novel factors that had not previously been shown to be virulence related.

Although the zebrafish now has a proven track record as a model for infectious diseases, there are some limitations to the model. In comparison to mammalian models, currently there is a lack of zebrafish-specific antibodies available for the zebrafish model. Although some human antibodies have been found to cross-react with the same factor in zebrafish [70], this is not often the case. However, as more researchers take advantage of this system, the availability of new tools and resources is bound to expand. The website ZFIN: The Zebrafish Model Organism Database has an antibody database listing over 400 antibodies currently available for zebrafish research that will most likely continue to expand [102]. In addition, the NIH has developed a Trans-NIH Zebrafish Initiative “to promote the use of zebrafish as a model organism for the study of vertebrate development and disease” lending their support with funding opportunities as well as resources for using zebrafish as a research model [103]. Second, the optimal temperature for maintaining zebrafish is 28°C, whereas for most human pathogens the optimum temperature is 37°C. Some virulence genes have been shown to be regulated by temperature; therefore, the researcher needs to be cognisant that temperature may affect the response of the pathogen to this host. However, several mutagenesis studies have shown multiple virulence

genes that were identified in mammalian models of pathogenesis behave similarly in the zebrafish model [63,68,69,71].

Future perspective

What does the future hold for the zebrafish infectious disease model? As new reagents are developed, researchers will be able to go beyond the current limitations of labeling host-specific factors. The availability of transparent adult zebrafish will allow the visualization of pathogen dissemination and accumulation in real time in a live animal, providing new information on the mechanism of dissemination and the tissue tropism for a particular pathogen; information that could be directly applicable to treatment of human infections. One area that will provide new knowledge to the infectious disease research community and that is beginning to be explored is doing large-scale screens with mutagenized zebrafish embryos to find strains that are either more or less susceptible to infection. Such a screen could identify previously unknown factors of the host immune response. In addition, the 96-well plate format is ideal for determining the efficacy of new drugs to fight disease, by treating infected embryos with newly synthesized or modified antimicrobials to determine those most effective against specific pathogens. Researchers have just begun to tap the many possibilities available with this model.

Executive summary

- Advantages of using zebrafish as a model host include: low cost, small size, easy maintenance, sequenced genome and high rate of reproduction.
- Transparency of embryos allows real-time imaging of infections *in vivo*.
- Zebrafish have a well-developed immune system with both innate and adaptive immunity that have many similarities to that of humans.
- Morpholino technology in embryos allows knockdown of any host gene, allowing the researcher to manipulate the innate immune response to elucidate key factors in response to infection.

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