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Differences in virulence between two serotypes of *Ichthyophthirius multifiliis*

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ABSTRACT: Naïve channel catfish *Ictalurus punctatus* were infected by 2 isolates of the parasitic ciliate *Ichthyophthirius multifiliis* that differed in virulence. The isolates, NY1 and G5, Serotypes A and D, respectively, express different surface immobilization-antigens. The virulence of the 2 isolates was compared using tail-fin infections to quantitate parasite numbers and by analysis of the survival of infected fish. Although NY1 infected fish at a lower level than G5, all NY1-infected fish died, but 51% of G5-infected fish survived. The greater virulence of NY1 is apparently a consequence of its shorter life cycle, which results in overwhelming reinfection of fish before they can develop a protective immune response. This report represents the first experimental evidence for differences in virulence between serotypes of *I. multifiliis.*

KEY WORDS: Fish parasite virulence · *Ichthyophthirius multifiliis* · Immobilization antigen · *Ictalurus punctatus* · Channel catfish

INTRODUCTION

The ciliate *Ichthyophthirius multifiliis* is an obligate parasite of freshwater fishes that infects surface epithelia of the skin and gills. The life cycle of *I. multifiliis* consists of 3 stages: an infective theront, a parasitic trophont and a reproductive tomont (Nigrelli et al. 1976, Noe & Dickerson 1995). Free-swimming theronts invade the epithelium of fishes and rapidly differentiate into trophonts, which actively feed on the fishes for several days, growing to approximately 800 µm, and then leave the host and form encysted tomonts. Over the next 18 to 24 h, tomonts divide up to 10 times, producing as many as 10^3 daughter cells, which emerge as infective theronts, completing the life cycle.

Ichthyophthirius multifiliis expresses membrane proteins, referred to as immobilization antigens (i-antigens), which are a family of GPI-anchored surface glycoproteins ranging in size from 40 to 70 kDa (Dickerson et al. 1989, Clark et al. 2001). The i-antigens of *I. multifiliis* are analogous to those found in the free-living ciliate genera *Tetrahymena* and *Paramecium* but, unlike these ciliates, *I. multifiliis* does not appear to undergo i-antigen switching in response to environmental changes, such as changes in temperature or osmolarity (Clark & Forney 2003). The function of i-antigen proteins remains unclear. They are, however, involved in development of immunity against *I. multifiliis*, as vaccination of channel catfish with a purified i-antigen confers protection against infection by *I. multifiliis* expressing that i-antigen (Wang & Dickerson 2002, Wang et al. 2002).

Because *Ichthyophthirius multifiliis* isolates express different i-antigens, the i-antigens can be used to define serotypes of *I. multifiliis.* Serotypes are distinguished by monoclonal antibodies that bind a specific i-antigen and immobilize the free-swimming theronts (Dickerson et al. 1993). To date, 5 such serotypes have been characterized. The isolates used in this study, NY1 and G5, represent Serotypes A and D, respectively. NY1 expresses 3 i-antigens (56, 46 and 42 kDa), while the G5 isolate has a single 55 kDa i-antigen (Wang et al. 2002). As the serotype remains constant for an isolate, they are useful markers for comparative studies between serotypes.

Previous studies have suggested that G5 and NY1 isolates differ in their virulence (Wang et al. 2002). The experiments reported in the present study were carried out to determine the basis for this difference.

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Fish were quantitatively infected on their caudal fin with NY1 or G5, and the number of trophonts on the caudal fin was determined 5, 7 and 9 d after exposure. Days to death of fish infected with NY1 or G5 were compared by survival analysis. The results showed that *Ichthyophthirius multifiliis* G5 (Serotype D) infected channel catfish at an initially higher level than NYl (Serotype A), but all NY1-infected fish died during the course of the study, while only half of G5-infected fish died.

MATERIALS AND METHODS

Parasite culture. The isolation of clonal lines of the 2 *Ichthyophthirius multifiliis* serotypes used in this study has been previously described by Wang et al. (2002). NY1 (Serotype A) and G5 (Serotype D) were cultured separately on channel catfish *Ictalurus punctatus*. Tomonts harvested from infected fish were kept at room temperature (22°C) for 24 h; the theronts were collected and used to infect fish as previously described (Dickerson et al. 1981).

Immobilization assay. Prior to experimental infection, immobilization assays using serotype-specific monoclonal antibodies (mAb) were conducted to ensure that each culture represented a single serotype (Dickerson et al. 1993). The mAb 10H3 identifies Serotype A, while mAb G361 was used for Serotype D (Lin et al. 1996). The mAbs were serially diluted in microtiter plates containing 50% phosphate-buffered saline in carbon-filtered tap water; 500 theronts were added to each well, and their motility was observed after 30 min by microscopy under low magnification. The theronts were incubated separately with both mAbs. Cultures were considered to be composed of only 1 serotype when all theronts were completely immobilized by a specific mAb.

Experimental fish. Outbred channel catfish juveniles with no history of exposure to *Ichthyophthirius multifiliis* were obtained from a local hatchery. The fish were held in aquaria with biological filtration and were treated with oxytetracycline and formalin prior to infection (Wang et al. 2002). Nitrite levels and pH were monitored daily and water temperature was maintained at 22 ± 1°C.

Quantitative caudal fin exposure. For each infection, 30 naive channel catfish with a mean weight of 25.3 ± 5.9 g were divided into 2 groups of 15 fish each. The groups were infected at room temperature with either *Ichthyophthirius multifiliis* NY1 or G5 as follows: sets of 5 fish were anesthetized with a 0.02% solution of Finquel MS-222 (Argent Chemical Laboratories). Once anesthetized, fish were covered in plastic wrap to maintain their body moisture, and their caudal fins were placed in plastic multi-channel pipette troughs (Labcor Products) containing 2.5×10^3 theronts in 50 ml of carbon-filtered tap water. Fish were exposed for 5 min before being returned to the aquaria. Infections were initiated with both serotypes on the same day to ensure standardization of conditions between groups. Parasites on caudal fins were counted 5, 7 and 9 d after exposure using a dissecting microscope (Olympus) at 10× magnification. Photographs were taken and used to measure parasite size at each time point. The time (d) to death of each fish was recorded; 3 replicate infections (designated I, II and III) were performed.

Statistical analysis. Parasite numbers on caudal fins 5, 7 and 9 d post-infection were compared between the 2 isolates. Upon residual analysis, the data was found to be non-normally distributed. A log-transformation normalized the data and allowed subsequent analysis using the Tukey-Kramer multiple comparison procedure to compare parasite numbers at each time point ($\alpha = 0.01$). Kaplan-Meier curves were generated to illustrate the mortality caused by the parasites after infection. Survival analysis using a log-rank test was conducted to verify the significance of the curves.

RESULTS

Initial infection

Ichthyophthirius multifiliis G5 initial infection was greater than that of NY1. The level of infection for each fish was determined by counting the number of trophonts on the caudal fin; 5 d after initial exposure, channel catfish infected with G5 theronts had on average a significantly greater parasite load than fish infected with NY1 theronts, bearing a mean of 82.6 ± 95.7 trophonts fish^{-1} for G5 compared to 11.0 ± 11.5 for NY1 (Table 1). The number of trophonts observed per fish was variable, however, both within and among replicates, even though the serotypes were derived from single-cell isolates (Table 1, Fig. 1). In Replicates I and III, fish infected with G5 had a mean parasite load of 126.9 ± 98.4 and 119.3 ± 94.7, respectively, but in Replicate II, only 4.0 ± 5.5 trophonts fish^{-1} were observed. Similar differences were noted for NY1. Only in Replicate II was an almost identical level of initial infection achieved for both isolates (NY1: 4.7 ± 6.1; G5: 4.0 ± 5.5). It is not clear why the extent of infection in Replicate II was lower than in the other replicates.

G5 infected fish continued to have a significantly greater parasite load than the fish infected with NY1 7 d after infection (Table 1). In Replicate II, in which the initial infection levels were similar between the 2 isolates, the number of NY1 trophonts had decreased to 1.2 ±

Table 1. *Ichthyophthirius multifiliis.* Mean number of trophonts observed on caudal fins of *Ictalurus punctatus* 5, 7 and 9 d post-infection. The 2 isolates NY1 and G5 were compared using the Tukey-Kramer multiple-comparison procedure. Asterisks indicate significantly different

Replicate	NY1	G5	p-value
5 d post-infection			
I	21.4 ± 11.3	126.9 ± 98.4	<0.0001*
II	4.7 ± 6.1	4.0 ± 5.5	0.9610
III	6.9 ± 8.7	119.3 ± 94.7	<0.0001*
Mean	11.0 ± 11.5	82.6 ± 95.7	<0.0001*
7 d post-infection			
I	1.9 ± 2.5	60.3 ± 70.5	<0.0001*
II	1.2 ± 1.5	5.3 ± 5.3	0.1052
III	4.1 ± 3.3	95.9 ± 82.8	<0.0001*
Mean	2.4 ± 2.7	54.0 ± 72.0	<0.0001*
9 d post-infection			
I	16.1 ± 37.1	15.7 ± 12.7	0.0922
II	38.1 ± 27.0	0.4 ± 0.8	<0.0001*
III	2.9 ± 2.5	36.8 ± 38.6	0.0007*
Mean	19.4 ± 30.1	16.6 ± 26.1	0.8378

1.5 fish^{-1}, by Day 7, while G5 numbers remained similar to those on Day 5. The number of G5 trophonts fish^{-1} observed on Days 5 or 7 was similar in Replicates II and III, but in Replicate I, numbers decreased 2-fold. In contrast, the number of NY1 trophonts observed on fish on Day 7 showed an approximately 2- to 11-fold decrease from that observed on Day 5 in all 3 replicates.

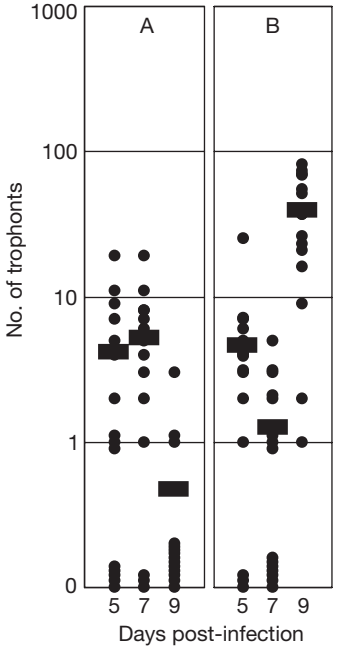

Fig. 1. *Ictalurus punctatus* infected with *Ichthyophthirius multifiliis.* Numbers of trophonts on caudal fins of fish infected with (A) Isolate G5, and (B) Isolate NY1, 5, 7 and 9 d post-infection. Data are for Replicate II. Each data point represents a single infected fish. Means are indicated by horizontal bars

Infective cycles

The infective cycle of *Ichthyophthirius multifiliis* NY1 was shorter than that of G5. The decrease in trophonts observed on the fish results from trophonts leaving the fish. Reinfection by theronts, the progeny of the initial infection, occurs approximately 18 to 24 h later, and the entire fish is infected, not just the caudal fin as in the initial exposure. For G5, the number of trophonts on Day 9 was less than that on Day 7 for all 3 replicates (Table 1), indicating that G5 trophonts had left the fish after Day 7 and reinfection had not occurred by Day 9. The pattern of infection with NY1 was strikingly different from that observed for G5. The average number of NY1 trophonts observed on Day 9 increased relative to Day 7 from 2.4 ± 2.7 to 19.4 ± 30.1 (Table 1), indicating that NY1 trophonts were leaving fish before Day 7 and that reinfection was generally occurring by Day 9. This demonstrates that the NY1 infection cycle took about 7 d to complete. In contrast, it was not until Day 9 that the number of trophonts decreased on G5 infected fish, indicating that the G5-infection cycle took approximately 10 d.

Inflammation

G5 infection resulted in greater inflammation. The heavier infection by G5 trophonts on Day 5 appeared to elicit a more pronounced inflammatory response than NY1, which was qualitatively assessed as epithelial erythema. On Day 5, the NY1 and G5 trophonts were similar in size (mean: 400 µm, range: 200 to 700 µm, n = 100), but the gross morphology of the caudal fin in fish infected with G5 differed from that of fish infected with NY1 in which little epithelial erythema was observed. On Day 7, inflammation was observed in both NY1- and G5-infected fish, although fewer NY1 trophonts were observed. Trophonts were larger than on Day 5, averaging approximately 700 µm for both isolates (range: 300 to 1100 µm, n = 100).

On Day 9, inflammation resulting from infection again differed between the 2 isolates, and was more pronounced in fish infected with G5. The inflammatory response appeared to correlate with the difference in the length of the life cycle of the 2 isolates, as G5 trophonts continued to increase in size from 700 µm on Day 7 to about 800 µm (range: 500 to 1100 µm, n = 35). In contrast, NY1 trophonts averaged only 300 µm (range: 200 to 500 µm, n = 25), which was less than on Days 5 or 7 of infection. On Day 9, no large trophonts were observed on NY1-infected fish and, conversely, no small trophonts were found on fish infected with G5. The difference in size of the infective trophonts between the 2 isolates on Day 9 supports the idea that

larger G5 trophonts represent the initial infection, while the smaller NY1 trophonts represent the second round of infection.

Mortality

Infection with the *Ichthyophthirius multifiliis* NY1 isolate resulted in complete mortality. The severity of infection was determined by measuring fish mortality for a period of 42 d following initial exposure. The data are significantly different, as all 45 fish infected with NY1 died by Day 19, but 23 of 45 fish infected with G5 were still alive at 42 d post-infection (Fig. 2A). Prior to Day 10, 1 NY1- and 5 G5-infected fish died, most likely as a result of stress from handling during initial infection, and not from infection per se. The curves for the 2 serotypes cross on Day 14 post-infection. A log-rank test was performed, which assumed that the study ended on Day 14, allowing analysis of all deaths occurring before Day 14 independent of the deaths occurring after this point. This demonstrated that for the first 13 d of infection, the difference in survival between NY1 and G5 was not significant ($p = 0.0941$). In contrast, as clearly indicated by the survival curves, a similar analysis beginning on Day 14 showed that fish infected with the G5 isolate had a significantly higher survival rate than fish infected with the NY1 isolate ($p < 0.0001$), as all 44 remaining NY1 infected fish died by Day 19, but only 17 of the remaining 40 G5 infected fish died by Day 42.

The Kaplan-Meier survival curve for the second replicate infection illustrates most clearly the differences in the course of infection between these 2 isolates (Fig. 2B). In this replicate, the number of trophonts on infected fish on Day 5 was almost identical, yet the effects of the infection were strikingly different. The initial infection with G5 led to the death of 1 fish on Day 5 and a second fish on Day 7, most probably as a result of handling during the initial infection. The remaining G5-infected fish survived until Day 25 post-infection, after which 8 fish died between Days 26 and 34, towards the end of the third cycle of infection. The remaining 5 fish survived up to Day 42. All 15 fish infected with NY1 died on Day 14, which corresponded with the start of the third cycle of infection for NY1.

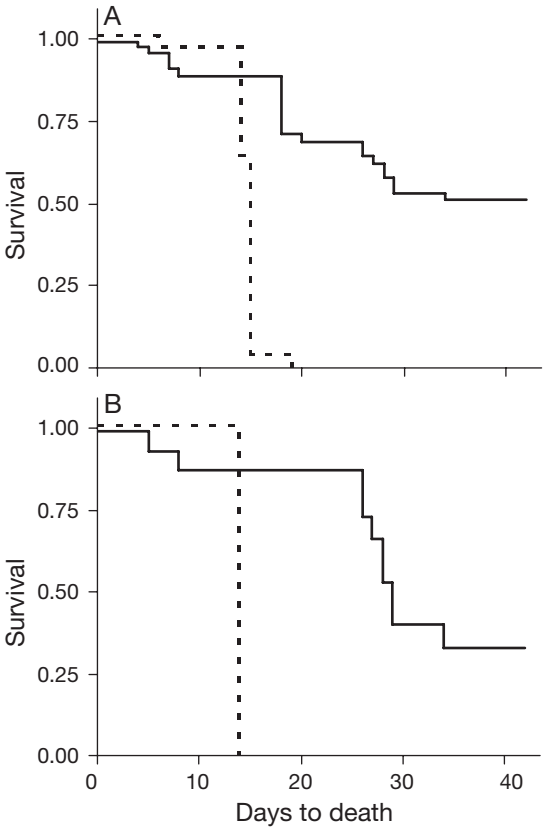

Fig. 2. *Ictalurus punctatus* infected with *Ichthyophthirius multifiliis*. Kaplan-Meier survival curves of fish infected with Isolates G5 (continuous line) and NY1 (dashed line). (A) Pooled data for Replicates I, II and III; (B) Replicate II data only

DISCUSSION

Quantitative tail infection of channel catfish with *Ichthyophthirius multifiliis* G5 (Serotype D) resulted in a heavier initial infection than with NY1 (Serotype A) in 2 of 3 replicates and a similar level of infection in a third replicate. Although fish exposed to NY1 initially became infected with fewer *I. multifiliis*, all NY1-infected fish died by Day 19 of infection, while 23 of 45 fish infected with G5 survived for 42 d. The higher mortality following infection and reinfection demonstrates that NY1 is more virulent than G5. Our results suggest that this differential virulence was not a function of the initial level of infection, but rather, resulted from the interplay between the differences in time required for the 2 *I. multifiliis* isolates to complete their infective life cycle and the time required for the fish to develop protective immune responses.

This is best illustrated by Replicate II, in which the initial infection, measured on Day 5, was essentially identical for NY1 (4.7 ± 6.1 trophonts fish^{-1}) and G5 (4.0 ± 5.5 trophonts fish^{-1}), and thus the course of the infection represents the response of fish to similar initial parasite loads. On Day 7, the number of trophonts observed on G5-infected fish was similar to that on Day 5, while the number of NY1 trophonts had decreased, indicating that NY1 trophonts had begun to exit the fish before Day 7. Infective theronts emerge about 18 h later. On Day 9, the results of this second cycle of infection were observed on NY1-infected fish, as the num-

ber of trophonts increased to 38.1 ± 27.0 trophonts fish^{-1}, about 8 times greater than observed on Day 5 of infection. This demonstrates the increased level of reinfection expected, but is a substantial under-representation of the total extent of secondary reinfection, as only trophonts on the caudal fin were counted. From the second infection onward, the entire fish was exposed to reinfection, unlike the initial exposure in which only the caudal fin was infected. In contrast, the number of trophonts observed on G5-infected fish on Day 9 had decreased to 0.4 ± 0.8 trophonts fish^{-1}, indicating that most trophonts had left the fish, but reinfection had not yet occurred.

During the natural course of infection, as modeled in these experiments, *Ichthyophthirius multifiliis* leaves and reinfects fish over a period of several days, and the estimated times required to complete the life cycle are averages. A life cycle of 7 d for NY1 predicts that the second round of infection would occur over Days 7 to 8 and the third round of infection over Days 14 to 15. Each tomont has the potential to produce as many as 10^3 infective theronts (MacLennon 1937). Assuming that each tomont produced 500 theronts and 10% succeed in reinfecting fish, then an initial infection of 5 NY1 theronts fish^{-1} could result in reinfection with 250 theronts on Days 7 to 8 and with 12 500 theronts fish^{-1} from Days 14 to 15 in these closed systems. On Day 14, 43 of the remaining 44 NY1 fish infected died, with the last fish succumbing on Day 19. This suggests that the exponential increase in the magnitude of reinfection following 3 rounds of infection, occurring over 14 d (even starting with a modest infection, as in Replicate II), can overwhelm fish and lead to total mortality of the infected population. Exposure to a dose of 15 000 NY1 theronts fish^{-1} causes complete mortality in naive fish, which is in agreement with the predicted levels of infection (Wang et al. 2002).

In contrast to NY1, the number of trophonts found on fish infected with G5 was similar on Days 5 and 7 in 2 replicates and decreased on Day 7 in 1 replicate. On Day 9 post-infection, fewer trophonts were observed than on Days 5 or 7 for all 3 replicates. As G5 trophonts had left the fish by Day 9, their progeny could be expected to reinfect fish in the second cycle from Day 10, and the third cycle of infection could begin around Day 20. In Replicate II, the majority of deaths in fish infected with G5 (7 fish) occurred on Days 26 to 29, near the end of the third round of infection, and in Replicate III, the majority of deaths occurred on Day 18 (8 fish), near the end of the second round of infection. No G5-infected fish died in Replicate I.

Fish infected with a sublethal dose of *Ichthyophthirius multifiliis* are protected against subsequent infection (Hines & Spira 1974). Numerous studies have demonstrated the prominent role of humoral immunity in this protection, as titers of anti-*I. multifiliis* serum antibody rise following infection, and naive fish can be completely protected against lethal challenge by injection of immobilizing mAbs, which trigger rapid parasite exit through i-antigen cross-linking (Clark et al. 1988, 1996, Lin et al. 1996). In addition, antibodies secreted from the skin of immune fish immobilize theronts *in vitro* and render them unable to infect naive fish (Xu & Klesius 2002, Xu et al. 2002). Consequently, the time required for fish to develop a robust humoral immune response to *I. multifiliis* in relation to the time that they are exposed to exponentially increasing numbers of theronts is critical to the ultimate outcome of the infection. Induction of adaptive immune responses to *I. multifiliis* has been observed within days following initial exposure. The earliest response correlated with infection is an increase in the level of total IgM mRNA, detected by reverse transcription-PCR, in skin or head kidney samples 4 d after infection of rainbow trout *Onchorhynchus mykiss* with *I. multifiliis* (Sigh et al. 2004). In channel catfish, antibodies specific for *I. multifiliis* can be detected in serum within 7 to 12 d following infection and within 12 d in the skin (Wang et al. 2002, Xu et al. 2004). However, antibody titers do not peak until at least 4 wk after infection (Wang et al. 2002, Maki & Dickerson 2003). Although antibodies recognizing *I. multifiliis* can be detected within 1 to 2 wk, the results of these experiments clearly demonstrate that initial phases of the humoral response are not sufficient to prevent complete mortality caused by the massive third round of infection by NY1 around Day 14. The response, however, was able to provide protection in some fish against G5, with its longer cycle of infection and higher level of inflammation, as deaths did not occur until Day 18, at the end of the second round of infection, or until Days 26 to 29, near the end of the third round of infection.

The difference in virulence observed between these 2 isolates of *Ichthyophthirius multifiliis* is best explained as resulting from the interplay between the time needed by fish to develop protective immune responses following infection and the time required for *I. multifiliis* serotypes to complete their life cycle. The death of fish infected with *I. multifiliis* is believed to be a result of loss of gill function caused by the high numbers of parasites found in gills. For NY1, the infective cycle took 7 d and, compared to G5, induced less severe epithelial erythema, an indicator of host inflammatory responses, during the first round of infection. The third round of NY1 infection occurred about Day 14. Although anti-*I. multifiliis* antibodies were present at this time, the magnitude of this third cycle of infection was sufficient to overwhelm the fish. In contrast, G5 had both a longer 10 d infective life cycle and

induced a more pronounced epithelial erythema, presumably indicative of a more pronounced inflammatory response. As shown in Replicate II, in which NY1 and G5 initial infections were essentially identical, these differences allowed some fish in the G5-infected population to survive, presumably because they had time to generate antibody titers sufficient to protect against the third and subsequent rounds of infection.

An important observation stemming from these results relates to the design of vaccine trials in catfish. These results suggest that at 2 wk following infection or immunization, the antibody levels are enough to provide only modest protection against infection. This work suggests that a longer 3 wk cycle of immunization and challenge may prove a more effective method for assessing vaccine efficacy in catfish.

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