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Demystifying Herd Immunity using Fish Models

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ABSTRACT

Herd Immunity refers to the indirect protection conferred to vaccine-naïve susceptible individuals by immune individuals during outbreak of communicable diseases as immune individuals act as hurdles in the spread of infection. This study was conducted to establish this concept using fish as the experimental models, to determine if the co-existence of immune koi carps (Cyprinus rubrofuscus) with naïve ones could confer protection against spread of pathogen Aphanomyces invadans, which caused Epizootic Ulcerative Syndrome (EUS) in fish. The experiments were so designed to have four replicate trials, where two sets of six-5 fish groups, acclimatized to laboratory settings, were subject to vaccination with crude pathogen extract and immuno-stimulation using ethanol extract of Solanum nigrum, proven to enhance immunity in EUS susceptible fish. The immune and naïve fishes were later challenged with the pathogen and the spread of disease, cumulative mortality, and antibody titer were measured. The cumulative mortality of naive fishes averaged across four trials was lower when they co-existed with immune fishes than when housed independently. The mortality in the aquaria with only vaccinated fish and only naïve fish were <4 % and ~85 % respectively, However, mortality among naïve fish in co-existence was only ~10 %, suggesting a protective herd effect.

Keywords: Herd immunity, Vaccination, Immunostimulation, Epizootic Ulcerative Syndrome, Cyprinus rubrofuscus, Solanum nigrum.

I. INTRODUCTION

Vaccination aims to provide both direct protection in the form of individual artificial immunity to the immunized individuals and indirect protection, as a population level consequence, to the naïve-susceptible individuals in the form of herd immunity. [1][2]

Herd immunity is an indirect effect that protects a population from the invasion of a new infectious disease by lowering the risk of infection among susceptible individuals in the population by the presence and proximity of immune individuals that occurs when a large percentage of a population has become immune to the infection. In this way, the benefits of the vaccine can be extended beyond the targeted groups. The benefit of indirect protection is caused by the interruption of transmission chain by immunized individuals. [1][4]

An outbreak can be aborted if the fraction of immunized individuals reaches a threshold level. When enough individuals are immunized, then most potential contacts are wasted and the infection fails to grow. Measles, mumps are some of the diseases that are believed to have been eradicated with the help of herd immunity. [3]

Infants, individuals immunocompromized as a result of repeated radiation and chemotherapy for cancer treatment, immunodeficient individuals, freeloaders who actively reject vaccines or parents who do not vaccinate infants as a result of vaccine illiteracy and vaccine hesitancy are among those who do not receive the direct protection offered by vaccines can be protected under the herd effect if a large proportion of the remaining population is immune. Such vaccine rejection can stand in the way of achieving the complete perceived benefits of vaccination.[10][11]

It is of interest to note that herd protection after mass immunization can eventually wane over time, especially where the immunogenicity of the vaccine does not allow sustained antibody protection. This calls for awareness about booster dose to vaccination. [5]

Nevertheless, gaps in our knowledge exist about how best to achieve herd immunity.

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II. MATERIALS AND METHODS

A.Collection, acclimatization and care of test fish:

The test fish, *Cyprinus rubrofuscus*, commonly known as koi carp, 60 in number (weighing between 8-18 g, SD ±5g) were acquired from Lemuria food company, Chennai-600068.

Table #1(Host responses to A.Invandas)

Species	**Source**	**Mean weight (g)**	**SD of Weight**	**EUS-Susceptibility**
Koi Carp ***Cyprinus rubrofuscus***	Lemuria food company	***18.34***	***±5***	**Yes**
SD = Standard Deviation				

All the test fish utilized for experimentation were clinically healthy and were maintained in translucent plastic tubs, 35 litres capacity, at a density of 5 fishes per tank, maintained at 21ºC. The aquaria was filled with de-chlorinated water, equipped with air stones and aquarium heaters maintained at 20°C.

The fishes were allowed to acclimatize over a fortnight and were fed twice daily to satisfaction with Optimum brand fish feed pellets containing around 28% proteins, supplemented with wild caught earthworms from the college nursery. Owing to accumulation of ammonia from fish waste, the pH and optical density of the tub water spiked every 3rd day, and Water quality was maintained by partial water changes twice weekly

B.Culture of oomycetes

a. ***Biopsy: The excision of affected tissue and Isolation of Aphanomyces invadens from EUS affected fish****:*

Fish with conspicuous red spots as a result of EUS, were obtained from M.G.R.Nagar Fish market. The affected fish so obtained were pinned on to the dissection board with the red spots facing the experimenter. The affected muscles in the region of interest of the spots were excised prudently up to 2 mm^3.

The biopsied mass was inoculated in an isolating medium of GP agar in a clean, aseptic Petri dish. The media thus inoculated was subjected to incubation at controlled conditions; the temperature was set at 25ºC and was maintained for 12 hours. The incubated plates were examined under the microscope to visualize the budding tips of the hyphae. Repeated passaging on Glucose-Peptone Agar at 4 day intervals was performed to ensure that the fungal culture was devoid of any bacterial contamination.[13]

b. Media

The Glucose-yeast-peptone medium (ATCC medium: 1049)was prepared in distilled water, and sterilized by autoclaving at 121ºC at 2 bars for 15min to rid the medium of contaminants.[12]

c. Long Term Maintenance and Culture

The isolates were stored for future use in slope cultures and preserved for the duration of the project. The isolates were sub-cultures for experimental use by growing on GYP agar plates at room temperature.[13]

d. Sporulation and Germination

The sporulation of the mycelia was induced by washing the mycelia in autoclaved distilled water and placing overnight at room temperature.

The resulting germlings were retrieved after passaging onto GYP agar and culturing them overnight. The release of zoospores was visualized under the microscope.

C.Extraction of Immunostimulants:

The leaves of the *solanum nigrum* were collected, washed in distilled water and dried under shade. 20 g of dried leaves were weighed and were extracted in Soxhlet apparatus using ethanol as the solvent for 42 hours.[10]

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D. DOE: Design of Experimentation

This experiment involved 4 replicate trials housing naïve and vaccinated fish separately and in combination and exposing them to the pathogen, A.invadans.

The experimental fishes were maintained in 12 groups of five.

1. Set 1: 6 groups of 5 fish each : Immunostimulation

Round 1: 5 groups were administered with 0.5 ml of immunostimulant intra-peritoneally between the anal and the caudal fins using a 1 ml syringe. A 6th group of fishes were administered the same amount of saline under similar conditions and were regarded as the control group. Immunological assays were performed to determine the antibody titre.

Round 2: The 5 previously immunized groups were administered a booster dose once their antibody titre from the previous immunization had dropped.

2. Set 2: 6 groups of 5 fishes each : Vaccination

Round 1: 5 groups were immunized with heat-killed, crude extracts of *Aphanomyces invadans* intra-peritoneally between the anal and the caudal fins using a 1 ml insulin syringe. A 6th group of fishes were administered the same amount of saline under similar conditions and were regarded as the control group. Immunological assays were performed to determine the antibody titre.

Fish were anesthetized using eugenol at 150 ppm until movement ceased, prior to intraperitoneal injection with 200 µl of the immunostimulant suspension with an insulin needle.

Vaccinated individuals that would be housed in tanks with naïve individuals were marked with tags so that naïve and vaccinated could be identified.

Following the introduction of pathogen into the aquarium system, the experiment was run for 28 days during which each population was monitored for morbidity and mortality and for characteristic EUS clinical signs during the 28-days trial. Two fish from each tank were sampled at day 7, 14, 21, 28, post-injection (p.i.).

E.QUARANTINE: Prophylaxis by Chemotherapy

In order to contain fish for the next replicate trials, fish that survived the challenge were quarantined in pathogen-free water with an excess of lime and neem leaves to heal lesions.

F.Host Resistance Test:

The groups of 5 fishes that were administered immunostimulant and heat-killed A.inadans with 100% coverage were challenged on 21st day by intra peritoneal injection of live *Aphanomyces invadens*. Earlier, the challenge dose was standardized to give 100% mortality in control group. 96 hours mortality rate of each of the two groups was recorded. The relative percentage survival pertaining to the administered vaccine and immuno- stimulant was calculated using the formula

Relative Percentage of survival (RPS) =

{1 – (Cumulative experimental mortality/ Cumulative control mortality) X 100}

G. Raising and collection of antisera

The immunized fishes were bled using 1 mL syringe with 24 gauge needle from the common cardinal vein situated just below the gills at regular intervals of seven days for antibody response till 28th day and intervals of 2 days for lysozyme and neutrophil assay till 10th day.

H. Immunological assays:

a. Collection of macrophages

The macrophage isolation protocol was adapted from MEDOX, using sterile materials and techniques throughout. Viable macrophages were counted by mixing an aliquot of macrophage suspension with an equal volume of 0.1% v/v trypan blue in PBS and counting on haemocytometer. .

b. Determination of IgG concentration:

The protein A column was washed with 1X equilibration buffer. Equal volumes of serum and equilibration buffer were added to the column. 1mL of elution buffer was added to the column and elute containing IgG

was collected in tube containing neutralizing buffer (25µL/mL). This process was repeated a couple of times. The optical density of the elutes was measured at 280nm.

III. RESULTS AND DISCUSSION

This study was so designed to examine if aquatic herd immunity against A.invadans could be elicited in a laboratory setting. This report records the experimental methods undertaken to study the pattern and any differences, that might occur in the mode of spread of infection, when the immunity of the population at risk was varied by including, among the naïve and susceptible fish, a varying proportion of artificially immunized fish..

The fish in the two control groups, with 100% vaccination and immunostimulation, did not exhibit any behavioral or clinical symptoms pertaining to EUS throughout period of study.

A.Histopathology

Lesions characteristic of EUS were observed in all naïve fishes inoculated with *A. invadans*.

Mild localized swelling around the region of injection along with reddening was observed a day p.i. On day 4 p.i., deep reddening with loss in fish scales was observed. The profound reddening paved way for deep ulceration after day 6 p.i. Fungal hyphae were seen to be oozing out of the lesions into the water. Behavioral changes were observed wherein the affected fish showed labored and erratic swimming motion, some staying at the bottom of the tank. Some infected fish exhibited weakness and general loss of appetite, with progressive paleness at the site of infection after day 8 p.i.

B.Cumulative mortality:

The naïve fish housed alone (A & G) experienced the highest mortality in the experiment i.e. 100%. On the contrary, the lowest mortality in the experiment involved the immune fish stocked alone (F & L). In the 8 groups with the naïve fish housed with vaccinated fish (B-E, H-K), the cumulative mortality ranged from 0% to 100% with an average of 35.0% (RPS =80%) (Table 1).

C.Antibody response in fish:

In accordance with the finding of Hanifa et al , the antibody levels peaked on day 21 post vaccination/ immunostimulation and gradually dropped afterwards.

The vaccinated fish showed significantly higher antibody levels than the immuno-stimulated fishes.

The immune fish of groups B & H exhibited the highest antibody levels since they were housed with the maximum number of naïve-infected fish. The infected fish exuded live zoospores from the lesions and the vaccinated fish upon re-exposure to the lesions produced an overwhelming secondary response with high antibody levels.

Tank A with 0% vaccinated fish had no protective herd effect and all the naïve-susceptible fish contacted ulcers. This exhibits a scenario of a population of vaccine-naïve individuals experiencing an epidemic with no vaccinated individuals to act as barriers for the spread of the infection.

Similarly, tank G with 0% immunostimulated fish experienced 100% attack.

Small amounts of antibody were observed in the naïve-infected fish of tank A post infection as part of the natural defenses against invading pathogens. However, this was not sufficient to fight them off and prevent them from establishing infection.

These experiments are too few to allow of any but the most tentative conclusions being drawn. We believe, however, that the following inferences are allowable.

The small number of fish per tank likely also attributed to the variability in mortality, though utilizing numerous study replicates indicates a protective effect from comingled.

Active immunization of fish, in the manner indicated, confers a definite degree of protection against *A.invandans*. This shows that spread of infection occurs with difficulty among a population, each individual of which has been actively immunized.

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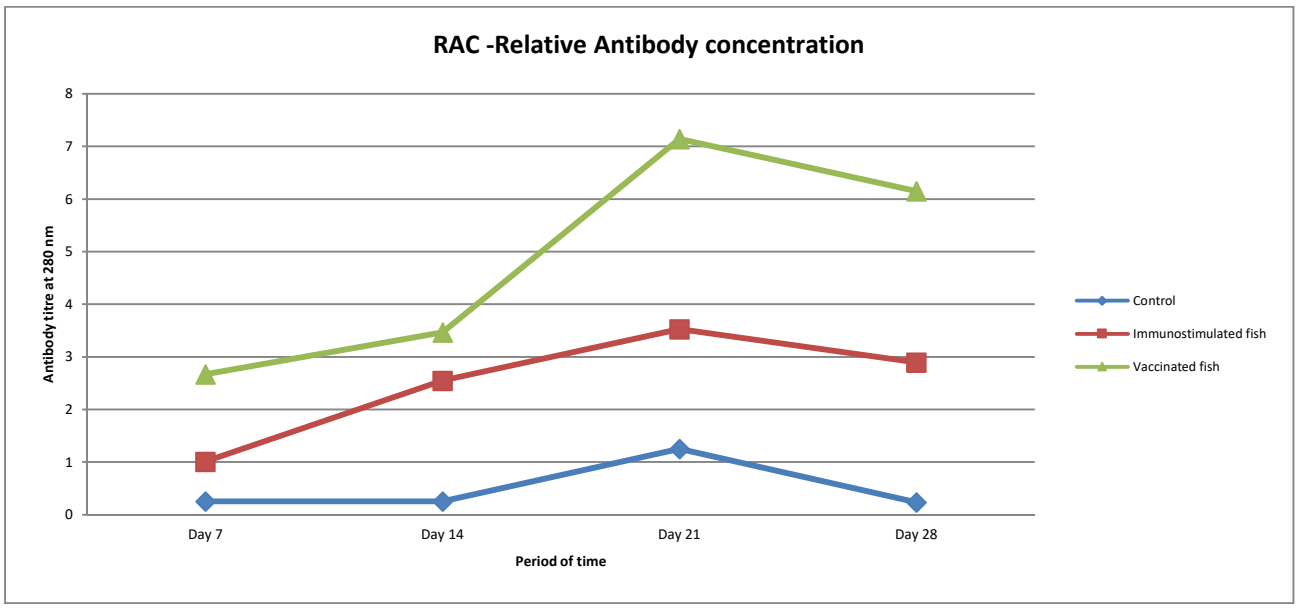

IV. CONCLUSION

In this study, we examine the concept of "herd immunity" using fish in an aquatic system. The cumulative results indicate that coexistence of immunized fish with naïve individuals indeed provides a protective effect to the latter. The mortality in the aquaria with only vaccinated fish and only naïve fish were <4 % and ~85 % respectively, However, mortality among naïve fish in co-existence was only ~10 %, suggesting a protective herd effect**.** From the findings of the current study, it was concluded that, though vaccination cannot, in general, be considered a preventive measure that completely eliminates the risk of infection of a population with a specific pathogen, the protection achieved may significantly reduce the number of susceptible individuals. The resulting herd immunity may effectively prevent

Immunization strategy -Vaccination	Percentage immunization	0% A	20% B	40% C	60 % D	80% E	100% F
	Total number of fish in the group	5	5	5	5	5	5
	Number vaccinated	0	1	2	3	4	5
	Antibody titer at 280 nm/ Concentration of IgG (mg/ml)						
	Day 7	0.252	2.710	2.679	2.682	2.668	2.685
		3.80	40.21	39.60	38.97	39.71	39.68
	Day 14	0.254	3.498	3.462	3.469	3.471	3.469
		3.83	41.12	40.25	40.52	40.65	40.83
	Day 21	1.249	7.189	7.138	7.145	7.120	7.134
		18.56	59.92	59.34	59.32	58.96	59.15
	Day 28	0.232	6.194	6.145	6.150	6.143	6.152
		3.49	46.79	46.29	46.23	46.27	46.19
	Day 35	1.320	7.096	7.124	7.156	7.204	4.112
		19.35	59.14	59.89	60.05	61.92	47.13
	Probability of challenged fish encountering a vaccinated fish and wasting a potential contact w.r.t the spread of infection	NA	1/5	2/5	3/5	4/5	NA

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	Number of fish expected to develop red spots i.e. Number of naïve-susceptible fish	5	4	3	2	1	0
	Number of fish that actually developed red spots i.e. Number infected	5	3	2	1	1	0
	Attack Rate	100%	60%	40%	20%	20%	0%
	Number of naïve fish protected indirectly by the vaccinated fish	0/5	1/4	2/3	1/2	0	NA
	Relative Percentage of escape from infection	NA	40%	60%	80%	100%	100%

Table 1:Statistical analysis of the experiment for Groups A-F

Table 1:Statistical analysis of the experiment for Groups G-L

Immunization strategy: Immuno-stimulation	Percentage immunization	0% G	20% H	40% I	60 % J	80% K	100% L
	Total number of individuals in the group	5	5	5	5	5	5
	Number subject to immunostimulation	0	1	2	3	4	5
	Antibody titer at 280 nm/ Concentration of IgG (mg/ml)						
	Day 7	0.249	1.005	1.001	1.006	1.010	1.003
		3.75	14.81	14.85	14.83	14.88	14.80
	Macrophage activity, OD at 450 nm	0.453	3.121	3.130	3.126	3.133	3.126
	Day 14	0.250	2.547	2.550	2.549	2.552	2.543
		3.77	37.69	37.72	37.70	37.67	37.71
	Day 21	1.244	3.528	3.530	3.526	3.532	3.529
		18.46	37.42	37.44	37.40	37.41	37.38
	Day 28	0.237	2.895	2.888	2.890	2.893	2.899
		3.41	42.86	42.88	42.82	42.81	42.83
	Day 35- Booster Dose	2.113	3.459	3.412	3.420	3.423	3.427
		36.23	37.17	36.98	37.01	37.09	37.10
	Probability of challenged fish encountering an immunostimulated fish and wasting a potential contact w.r.t. the spread of infection	NA	1/5	2/5	3/5	4/5	NA
	Number of fish expected to develop red spots i.e. Number of naïve-susceptible fish	5	4	3	2	1	0
	Number of fish that actually developed red spots i.e. Number infected	5	3	2	1	1	0
	Attack rate	100%	60%	40%	20%	20%	0%
	Number of naïve fish protected indirectly by the vaccinated fish	0/5	1/4	2/3	1/2	0	0
	Relative Percentage of escape from infection	NA	40%	60%	80%	100%	100%

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