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A Piscan Ulcerative Aeromonas Infection

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***Abstract*—**In the immunologic sense, clinical infection is a state of failure of the immune system to combat the pathogenic weapon of the bacteria invading the host. A motile gram negative vibroid organism associated with marked mono and poly nuclear cell responses was traced during the examination of a clinical material from an infected common carp *Cyprinus carpio*. On primary plate culture, growth was shown to be pure, dense population of an Aeromonas-like colony morphotype. The pure isolate was found to be; Aerobic, facultatively anaerobic, non-halophilic, grew at 0C, and 37C, oxidase positive utilizes glucose through fermentative pathway, resist 0/129 and novobiocin, produces alanine and lysine decarboxylases but non-producing ornithine dehydrolases. Tests for the *in vitro* determinants of pathogenicity has shown to be; Beta-haemolytic onto blood agar, gelatinase, casienase and amylase producer. Three *in vivo* determinants of pathogenicity were tested as, the lethal dose fifty, the pathogenesis and pathogenicity. It was evident that 0.1 milliliter of the causal bacterial cell suspension of a density 1 x 107 CFU/ml injected intramuscularly into an average of 100gms fish toke five days incubation period, then at the day six morbidity and mortality were initiated. LD50 was recorded at the day 12 post-infection. Use of an LD50 doses to study the pathogenicity, reveals mononuclear and polynuclear cell responses, on examining the stained direct films of the clinical materials from the experimentally infected fish. Re-isolation tests confirm that the re-isolant is same. The course of the infection in natural case was shown manifestation of; skin ulceration, haemorrhage and descaling. On evisceration, the internal organs were shown; congestion in the intestines, spleen and, air sacs. The induced infection showed a milder form of these manifestations. The grading of the virulence of this organism was virulent causing chronic course of infections as indicated from the pathogenesis and pathogenicity studies. Thus the infectious bacteria were consistent with *Aeromonas hydrophila*, and the infection was chronic.

***Keywords*—**Piscan, inflammatory respnonse, pure culture, pathogen, chronic, infection.

I. Introduction

MOST of the bacteria associated with disease in teleost fish are naturally occurring and gains wide distribution as saprophytes, which metabolize organics and minerals in the aquatic environment for the favor of building up their populations. Comparatively, *Aeromonas salmonicida*, *Haemophilus piscum* are acting principle fish pathogens and *Renibacterium salmonarium* as a potential fish pathogen. For saprophyte and opportunists, on transfer to pathogens, transition is in-variably initiated by one or more stress factors which augment the susceptibility of the fish to bacterial infections. Tracing back the stress factors reveals that they includes; poor water quality, temperature changes, over-crowding, trauma, transportation, nutritional deficiencies, parasitism and viral infections [1]-[9]. Aeromonads are spanning between principle and potential pathogens [3]. What so ever the consideration of the case, infection in teleost fish needs predisposing factors such as, port of entry, predilection site(s), building up population within the tissue-organ niche, communicate through quorum sensing signal peptide or signal homoserine that are helpful to evade local and systemic immune defense mechanisms. If virulence factors are well equipped to escape from these hosts immune mechanisms, then they will overweight them and produce clinical infections [10]. In the present work an attempt to report on, *Aeromonas hydrophila* fish natural and experimental clinical infection.

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II. Materials and Methods

A. Natural Infection

Around 140 common carp fish *Cyprinus carpio* were collected, during the period of June 2013 to Dec.2013 with an average of 100 gms body weight per each fish. These fish collect were made available from commercial fish aquacultures of the Middle East Company in Babylon Province. Some of the sampled fish appeared to be sluggish weak with signs of skin abnormalities indicating infections. These skin abnormalities were ulcer, haemorrhage and descaling. On evisceration, the internal organs were congested [4].

B. Processing

Direct swabs were made from the fish skin lesions, films were made, fixed and Giemsa stained. Infected fish were eviscerated under aseptic conditions and tissue samples were collected from; skin ulcer, spleen, liver, blood and intestines. Intestines opened longitudinally and contents evacuated then washed thoroughly with sterile saline till mucosa becomes clear. Composite sample was made from these tissue samples through morter grinding. The resultant tissue homogenate was centrifuged at 5000 rpm for10 min. The pellet was left and supernatant was kept for bacteriologic investigation. Duplicate sets of the supernatant cultures were made onto blood, macConkey and T.C.B.S. media. One set of the duplicate was incubated under aerobic incubation and the other was under the anaerobic condition with jar and anaerobic bags. The associated causal was identified by; manual methods [3], [12], API20 and Viteck [11].

C. Experimental Infection

Seventy normal fish with an average of 100gm body weight were grouped into seven groups each of ten. Each ten in one

aquarium under standardized conditions of O2, feed, temperature, salinity and body weight surveillance [12]. These were left for adaptation for two weeks. Then infection were initiated by injection of 0.1 ml through intramuscular rout from the associated causal bacterial cell suspensions rated from 1x10 10 up to 1 x10 4 CFU/ml. The infected and control fish aquaria were watched daily for gross pathologic changes as well as death records [13]. The LD50 was calculated using mathematical formula;

$$\text{Log LD50} = 0.5 + \text{Log higher density} - \frac{\text{Total death percents}}{100}$$

A group of ten fish in one aquarium was infected through intramuscular rout in rate of 0.1 ml of 1 x 107 CFU/ml suspension to each fish and watched as above to check gross pathology changes caused by the causal from the onset till death.

D.Grading the Virulence

In this study the virulence was graded by fixing; aquarium conditions, dose, rout, time of watching and virulence parameter [14].

III. Results

The microscopic morphology of the associated causal organism was; vibroid, motile gram negative pleomorphic rods found in association with mononuclear and polynuclear inflammatory cell responses, Table I. A dense, pure, Aeromonas – like colony morphotypes was noted in the stained films of carp infected fish Table II. The pure isolate was found as; aerobic, facultative anaerobic, non-halophilic, grew at 0C and 37C, Table III. The presumptive identification tests performed to the isolate shown that it was oxidase positive, utilizes glucose through fermentative pathway, 0/129 and novobiocin resistant and produces alanine and lysine decarboxylases as well as non-hydrolase orinithine, Tables IV and V.

The *in- vitro* virulence factors have shown to be haemolytic on blood agar, gelatinase, caseinase and amylase positive as well as motile, Table VI. While, the *in vivo* determinants of virulence have shown that the LD50 was 1 x 107 CFU/ml. The doses 1 x10 4 up to 1 x10 6 CFU/ml were showing no death percentages up to 23 days post-infection. While the dose 1 x 10 7 has shown death of five fish, during the day 12 to the day 23 post-infection. In addition, the doses 1 x10 8 up to 10 10 CFU/ml were showing deaths of all test fish, Table VIII.

The manifestations of the natural clinical infection were with evident skin ulceration, haemorrhage and descaling. While, on evisceration of the affected fish; congestions of intestines, spleen, liver, air sacs and kidney were noted. In experimental infection, however, the clinical infection form appears as milder than in that noted in natural infection, Table IX, Fig. 1.

The death records were: zero, one, two, four, and five to the post-infection days; Five, six, eight, nine and twelve respectively. The causal bacteria is virulent and the course of the infection is chronic, Table IX-X.

The manual biochemical identification was confirmed by API 20 and by vitek as *Aeromonas hyrophila*. The LD50 dose was 1 x 10 7CFU/ml. The disease was successfully produced by the infectious dose but in a milder chronic form. The *causal* was re-isolated and re-identified as *A. hydrophila*, from the experimental chronic infected carp fish, Tables I-X.

TABLE I
DIRECT TRACING OF THE INFECTION

Infection nature	Infection associated causal	Inflammatory cell type
Natural	Gram Reaction: Negative Motility: Motile Morphology: Pleomorphic rods	Mixed mono and polynuclear cell responses.
Experimental	Gram reaction: Negative Motility: Motile Morphology: Pleomorphic rods	Mixed mono and polynuclear cell responses.

TABLE II
DIRECT RECOGNITION PROCEDURE: PRIMARY PLATE CULTURE RESULTS.

Criteria	Results
Colony morphotype	Aeromonas-like
Growth Density	Dense
Growth Purity	Pure

TABLE III
GROWTH CHARACTERISTICS OF THE ASSOCIATED CAUSAL

Criteria	Standard	Manual identification Natural infection	Manual identification Experimental infection
Aerobic growth	+	+	+
Microaerophilic growth	+	+	+
Anaerobic growth	-	-	-
Growth at 4C	+	+	+
Growth at 37C	+	+	+
Salt tolerance, NaCl 0%	+	+	+
Salt tolerance, NaCl5%	-	-	-
Salt tolerance, NaCl 7%	-	-	-
Growth onto TCBS	-	+	+

TABLE IV
THE PRESUMPTIVE IDENTIFICATION OF THE CAUSAL ISOLATE

Criteria	Results	Identity
Oxidase	+	
Glucose, oxidative/fermentative	fermentative	Aeromonas-like
Sensitivity to 0/129	Resistant	
Sensitivity to Novobiocin	Resistant	

TABLE V
THE AMINO ACID METABOLISM FOR THE ASSOCIATED CAUSAL

Amino acid	Standard	Manual, nat	Manual, exp	API20E,nat	API20E,exp.	Vitik,Nat.	Vitik,exp
ADC	+	+	+	+	+	+	+
LDC	+	+	+	+	+	+	+
ODL	-	-	-	-	-	-	-

Nat=Natural infection isolate;exp=experimental infection isolate

TABLE VI
IN VITRO TESTS FOR VIRULENCE FACTORS OF THE ASSOCIATED CAUSAL

Factor	Standard	Manual, nat	Manual, exp	API20E,nat	API20,exp	Vitik, nat	Vitik, exp
Beta haemolysis	+	+	+	.	.	.	.
Gelatinase	+	+	+	+	+	+	+
Casienase	+	+	+	.	.	.	.
Motility	Motile	Motile	Motile	.	.	.	.
Amylase	+	+	+	+	+	+	+

TABLE VII
THE STUDY OF LD50 OF THE ASSOCIATED CAUSAL

Dose /colony forming units/ml.	Fish death percents
1 x 10^{10}	10:10(100%)
1x10^{9}	10:10(100%)
1 x 10^{8}	10:10(100%)
1 x 10^{7}	5:10(50%)
1x 10^{6}	0:10(0 %)
1 x 10 ^{5}	0:10(0%)
1x 10 ^{4}	0:10(0%)
LD50	1x 10 7

TABLE VIII
DEATH/LIVE PERCENTAGES IN FISH INFECTION EXPERIMENT USING 0.1 ML OF 1X10 7 CFU/ML. PER AVERAGE 100 GM FISH BODY WEIGHT

Days Post infection	Live fish %	Dead fish %
1 to 5	10:10(100%)	0:10(0%)
6 to 7	9:10(90%)	1:10(10%)
8	8:10(80%)	2:10(20%)
9 to 11	6:10(60%)	4:10(40%)
12 to 22	5 :10(50%)	5:10(50%)

TABLE IX
PATHOGENICITY OF THE ASSOCIATED CAUSAL IN CARP FISH

Manifestation	Natural	Experimental
Skin: erythema	+++	++
Ulceration	+++	++
Descaling	+++	++
Haemorrhage	Near the head, dorsal part near fin	Near the site of infection, extend to head, gills
Swelling	-	At the site of injection and extend to head
Congestion in visceral organs	++++	++

TABLE X
GRADING OF THE ASSOCIATED CAUSAL VIRULENCE

Grade	Incubation peroid	Course	Parameter	Dose	Pathogen	Reference
Highly virulent	12 hour	Acute	Tissue invasion	0.1 ml.,1 x 10^{6}	*F. colanar*	21
Low virulent	12 hour	Acute	Tissue invasion	0.1ml., 1 x10^{6}	*F. colanar*	21
Virulent	48 hour	Subacute	Tissue invasion, death	0.1 ml,1 x10^{8}	*A. hydrophila*	19
Virulent	5 days	Chronic	Tissue invasion, death	0.1 ml.,1x10^{7}	*A. hydrophila*	This study

Fig. 1 (a) Normal healthy common carp *Cyprinus carpio*

Fig. 1 (b) Natural *Aromona sulcerus* infection

Fig. 1 (c) Experimentally produced infection in carp *Cyprinus carpio*

IV. DISCUSSION

The presence of an associated causal that coexisted with inflammatory cell responses both in the natural and experimental infections stay as an initial mark for the causal relationship Table I. The colony morpho-type, density and purity may be a further mark for this causation. The possession of virulence factors via *in vitro* parameters like motility, haemolysin (s) and proteases, LD50 and pathogenicity in same host fish became strengthening evidences towards the consideration of the pathogenic relationship. The causal is now proved to be a virulent pathogen based on the following parameters; Dose 0.1 ml, density 1x10^{7} to 100 gm body weight, incubation period within five days, morbidity and mortality starts at the sixth up to the twelve day post-infection. In the first five days, the

immune defense mechanisms combat the pathogenic weapon of the bacteria invading the host. During the days 6 up to 23 a collective number of the deaths were five. Within these days the pathogen grow in population, produce virulence factors, quorum sensing peptides or homoserine [22], these materials may augment the pathogen abilities to overweight the ability of the immune system to combat the pathogen effect(s) leading to tissue damage that might be terminated by the host death. The course of infection is chronic. There was an individual variation in susceptibility, morbidity and mortality. Hence, a definite causal relation do exist [4], [8], [14]-[21] and, *A. hydrophila* natural and experimental infection are being reported Tables I-X. Andaman [19] classifies *Aeromonas* fish diseases into; typical and atypical, this infection form is being in a match with the typical type C which is a chronic ulcerus form [18]. The findings in this report are important for fish commercial producers, since stress factors were mostly encountered where ever fish aquaculture is practiced [17].

V. Conclusion

Natural *A. hydrophila* disease in common carp is reported in Babylon Province/Iraq. The LD50 was 1 x 10 7CFU/ml. An experimental piscan model is being developed and the recovered isolate has the grade of virulent with an inherent chronic infectivity nature.

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