Immunological Diagnosis of Naturally EUS Infected Channa gachua

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Abstract: An immunological approach has been adopted to diagnose the causative agent/s of Epizootic Ulcerative Syndrome (EUS). It has been clearly established by the observation of syndrome under natural condition that it develops at three different consecutive stages i.e. the initial stage (red spots occur on whole body) followed by an advanced stage (a red circular ring, distinguished by the circular hemorrhagic ring), which ultimately terminated to an ulcer. To assess the involvement of various claimed pathogens in EUS, two serological tests, DIBA (Dot-Blot ELISA) and IEM (Immunoelectron Microscopy), have been conducted at the three different stages of syndrome. It is clearly revealed by both the tests that only the virus (SHRV-19E) has been found at initial stage. At the next stage involvement of bacterium (Aeromonas hydrophila) and fungus (Aphanomyces invadens) have been reported while at last ulcerative stage; only the involvement of bacterium (Aeromonas hydrophila) and fungus (Aphanomyces transformation test (LTT) and serum profiling have been conducted. The maximum transformation has been reported against the SHRV-19E followed by Aphanomyces invadens while least was against Aeromonas hydrophila. In serum profiling also, nearly similar results have been observed.

Keywords: SHRV-19E, IEM, Channa gachua, lymphocytes transformation test, Immunoelectron microscopy

1. Introduction

The conflicting views of various workers regarding the causative agent of EUS have created confusion rather than providing solution of the problem. It is observed that the scientists are working separately on different aspects of EUS and everybody is trying to prove the aspect of his study as the cause of the disease. The complicated nature of problem requires well-connected and co-operative efforts for this problem. Efforts have been made to develop symptoms of EUS through experimental infection trails since 1988 by various workers, based on which it has been considered of viral origin by Miller [1], Kanchanakhan [2]-[3], Lio-Po et al. [4] and John and George [5]; bacterial origin by Jhingran and Das [6], Pradhan and Pal [7], Pal and Pradhan [8], Ali and Tamuli [9] and Shariff and Subasinghe [10] and fungal origin by Roberts [11], Robert et.al. [12], and Saylor et.al., [13]. However, the exact symptoms of EUS, as happen in nature could be developed by Tripathi, [14]. Under present work it has been tried to isolate and identify the pathogens at various stages of syndrome.

During the previous study of the present authors, it has been revealed that in nature, the syndrome develops at three loosely demarcated consecutive stages. At the initial stage, the syndrome appeared as red spots spread over the whole body; followed by the appearance of red hemorrhagic ring which may be the future site of ulcer. This ring ultimately terminated into ulcer at chronic stage [14]. Thus, it is presumed that there may be more than one pathogens are involved rather than involvement of single causative agent. To work out the same, a different study plan has been designed. Under present study, the samples, for the recovery of pathogens have been collected at three different stages which then processed and tested by serological methods. To test the pathogenecity and virulency of individual pathogens against the experimental fish, *Channa gachua*, LTT and serum profiling have been conducted. Activation and proliferation is a frequent response of T lymphocytes to antigenic stimulation Pechhold and Kabelitz [15] and thus it can be taken as test for virulency of pathogen against any given host.

2. Materials and Methods

For the present study, the severely infected fish species of the region, *Channa gachua*, has been selected. To observe the development of syndrome under natural condition, six heavily polluted water reservoirs of the region, where the maximum incidences of EUS have been reported, are selected. Some 25-30 fish are caught from the same pond with help of local fisherman and kept in happa after proper tagging. A continuous observation has been made to see the development of syndrome and work out the proper symptomatology.

2.1 Immunization of Rabbit to Get PCA

Three female Rabbits, obtained from IVRI (Indian Veterinary Research Institute, Bhopal) were immunized to produce polyclonal antisera against identified antigens (Virus: Rhabdovirus, Bacteria: Aeromonas hydrophila and fungi: Aphanomyces invadans) respectively as per procedure given by Delves [16]. Each rabbit was injected subcutaneously in neck region in conjugation with Franck Complete and Incomplete Adjuvant. A total volume of 1 ml divided in different injection sets. This procedure was repeated three times in a month. Two weeks after last immunization, the rabbit were anesthetized and blood was collected by heart puncture and serum was stored at -20 °C until used. This has been cross checked by the serum obtained from AAHRI, Thailand.

Viron particles were cultured on two fish cell lines CGK and CGL; and isolated as per procedure followed by Plumb [17]. The bacterial isolates were inoculated into 50 ml of Brain Heart Infusion Broth (Difco) for 24 hrs at 20°C. The culture was centrifuged for 10 mins. at 2325 x g and pallet resuspended in PBS. The suspensions were diluted until the transmission was 50% read spectrophotometrically at 520 nm, corresponding to the cell density of 10^3 colony forming units (cfu/ml). The fungal spores were cultured on sun hemp seed and counted according to method given by Mac Farrland Standard [18].

The PCA of SHRV, Aeromonas hydrophila and Aphanomyces invadans are cross checked with antiserum obtained from AAHRI, Thailand; IMT Chandigrah, India. The antisera and spleen at different stages of naturally infected fish were taken and processed as per procedure given by the Bendayan [19]. The dissected spleen were fixed with Mc Dowells fixative (4% formaldehyde, 1% glutraldehyde, 80µM sodiumhydrogenphosphate and 68µM NaOH) and post-fixed in 2% osmium tetrachloride. The antibodies so produced by the immunization were conjugated with gold. Float the grids, section side down on 1% gelatin in PBS for 10 mins. Resin sections were stained with 1% methonolic uranyl acetate and lead citrate according to routine electron microscopy procedures. Ultrathin sections were stained with 2% aqueous uranyl acetate, neutral uranyl acetate, and embedded in methyl cellulose. Sections were observed at different magnifications at AIIMS, New Delhi.

2.2 Dot-Blot ELISA (DIBA)

To assess the presence of the three different pathogens (Virus: Rhabdovirus, Bacteria: Aeromonas hydrophila and fungi: Aphanomyces invadans) at three different stages of syndrome i.e. red spot stage, red circular ring stage and finally at ulceration stage. Infected fish were collected from the happa which was kept in natural water bodies. To assess the presence of various pathogens spleen, skin and liver are selected. Fishes with above mentioned symptoms were brought into laboratory and dissected aseptically to take out spleen, skin and liver. The dissected organs were thoroughly washed in PBS and sonicated separately with help of ultrasonicator. This was followed by centrifugation of tissue at 300xg to remove tissue debris. Supernatants were taken and again filtered by various pore size filter papers to segregate the pathogens. For the purpose of Dot-Blot ELISA, the procedure given by Yamaura [20] was followed.

2.3 Immuno Electron Microscopy (IEM)

Samples at three different stages of syndrome were selected. The infected fish were brought to the laboratory and sacrificed to dissect out spleen under aseptic condition. Spleen from various samples were thoroughly washed by the PBS and preserved.

For the purpose of Immuno Electron Microscopy (IEM), PCA of SHRV, Aeromonas hydrophila and Aphanomyces invadans and samples at different stage of naturally infected fish and artificially infected fish were taken and processed as per procedure given by the Beasley [21]. For the purpose fish were dissected in the laboratory and their spleen was selected for the study. The ultra structural observations were made on different magnifications at AIIMS, New Delhi. The gold markers are conjugated with three different suspected antigens (Virus: Rhabdovirus, Bacteria: *Aeromonas hydrophila* and fungi: *Aphanomyces invadans*) to observe the presence of pathogens at different stages of the syndrome.

2.4 Paper Electrophoresis

For the purpose of electrophoresis the serum was separated from blood of healthy and naturally infected fish as per procedure given by Gupta and Arunen [22]. The electrophoresis was done with the help of standard markers (IgM and Transferine from Sigma) at 200 V for 90 mines. This was followed by staining of strip with bromophenol blue as per procedure given by the Plummer [23]. The quantification of protein was done with the help of densitometry.

2.5 Lymphocytes Transformation Tests

The lymphocytes transformation tests were done as per procedure given by Cummingham-Rundles et al., [24]. For this purpose three antigens were selected from three different suspected pathogens i.e. SHRV (RV-19E), Aeromonas hydrophila (RS-III) and Aphanomyces invadens (AH-III). The separation and purification of these antigens were done with the help of PAGE and gradient ultra centrifugation as separated for the generation of polyclonal antisera. The β counting was completed at CDRI, Lucknow. In order to evaluate the response to a given antigen the degree of transformation in unstimulated culture was compared to degree of transformation in stimulated culture (SR ratio).

3. Observations

3.1 Rising of Polyclonal Antisera

The polyclonal antisera generated into the rabbit from SHRV, Aeromonas hydrophila, and zoospores of Aphanomyces invadens were obtained from rabbit by bleeding from juguler vein. These antisera were cross tested by captured ELISA, with the standard antisera RE-19 and RE-20 obtained from AAHRI, Thailand. The test was positive with the isolate RE-19 only. Four bacterial isolate were recognized from infected fish R-3, R-8, R-9 and R-11, out of which only R-9 were found to be virulent. These have been cross checked with the polyclonal antisera obtained from Chandigrah. Similarly the antisera of spores of Aphenomyces invadens was also cross checked with antiserum obtained from AAHRI, Thailand. The PCA generated against zoospores of Aphenomyces invadens was due to the cell wall of zoospores. On cross checking with standard antisera it was found to be related with IN-01.

3.2 Dot-Blot ELISA

Dot-blot ELISA is one of most frequently used method, employing now a days for qualitative detection of viral, bacterial, fungal and protozoan in diagnostics. Dot Blot ELISA were conducted with antigens obtained from infected fish and polyclonal antisera raised against suspected and

identified antigens *i.e.* SHRV, *Aeromonas hydrophila* and wall of secondary zoospores of *Aphanomyces invadens*.

A sum of total 27 tests of Dot Blot ELISA was conducted for this purpose. The first nine tests were conducted with the three antiserum raised against three claimed pathogens (Rhabdovirus: SHRV-19E; bacterium: Aeromonas hydrophila and fungus: Aphanomyces invadans) and the antigen/s obtained from three different samples of skin, spleen and liver, recovered at initial stage of syndrome. This test was positive only with virus (Rahabdovirus: SHRV-19E) in all the samples of skin, spleen and liver. The next nine tests were conducted at circular ring stage of syndrome with the same antigen/s and antisera. In this case, fungus was reported from all three samples, virus was reported from spleen and liver only while bacterium couldn't be recovered from any sample. Similarly, the last nine tests were conducted at ulcerative stage of syndrome and with the same combination of antigen/s and antisera. In this case fungus was reported from all samples, bacterium reported from skin and liver while virus reported from spleen only (Table.1).

 Table 1: Results of DBELISA conducted in naturally infected fish

S.	Antigens obtained	Antibodies			
No.	Stages	Organs	RV-19 E	RS-III	AH-III
A1	Initial stage (with red spot)	Skin	+	+	+
2.		Liver	+	-	-
3.		Kidney	+	-	-
4.		Spleen	-	-	-
B1	Advance stage (With red circular ring)	Skin	+	-	+
2.		Liver	+	-	+
3.		Kidney	+	-	+
4.		Spleen	+	-	+
C1	Chronic stage (with ulcer)	Skin	-	+	+
2.		Liver	-	+	+
3.		Kidney	-	+	+
4.		Spleen	-	+	+

3.3 Immuno Electron Microscopy (IEM)

Under immunoelectron microscopic test the cellular structures couldn't recognize properly partially due to high magnification and rest is due to damaged cellular components due to natural or artificial infections and only conjugated gold spots could be seen in many cases. The immunoelectron microscopy test conducted on the spleen of fish in the initial stage of infection was found positive only with the viral antiserum (Fig. 1). The test was positive with virus (Fig.2) and fungus (Fig.3) in the sample obtained at the circular ring stage. In chronically infected fish, the IEM was positive with the antisera of bacterium (Fig.5) and fungus (Fig.6) and exhibited gold spots. In case of virus, the maximum spots were observed in cytoplasm while in case of bacterium and fungus these were scattered in disintegrated intracellular spaces (Table.2).

 Table 2: Results of Immunoeletron Microscopy conducted

 in naturally infected field

In naturally infected fish							
S.	Antigens	Antisera					
NO.	Stage of disease	RV-19E	RS-III	AH-III			
1.	Initial stage (with red spot)	+	-	-			
2.	Advanced stage of disease (With red	+	-	+			
	circular ring)						
3.	Chronic stage of disease	-	+	+			
	(With ulcer on body)						



Figure 1-6: immunoelectron microscope of naturally infected *Channa gachua* (for detail ref. text)

3.4 Lymphocytes Transformation

Lymphocytes Transformation: The degree of transformation was measure as "count per min."(cpm) of H1count incorporated into lymphocytes and results are expressed in terms of SR ratio which may be defined as "the ratio of cpm in stimulated culture to the cpm in unstimulated culture. The increase in number of transformed cells is recorded in all the three experimental antigens. The net cpm is observed against the G-Protein of SHRV and it showed a dose dependent raise. The maximum cpm is observed at 100 μ g concentration while lowest in case of 25 μ g concentration but the maximum count in this case is recorded after 144 hrs of exposure. In case of the antigen obtained from *Aeromonas hydrophila* the dose dependent cpm is observed and it was the maximum at 100 μ g concentration while lowest at 25 μ g concentration. Similarly in case of *Aphanomyces invadans* a

dose dependent change is noticed. On comparing the above results the most severe response was have due to G-Protein from the SHRV while least in case of LPS obtained from *Aeromonas hydrophila* (Fig.7-9).





3.5 Serum Protein Profile

The serum protein profile of healthy naturally infected and artificially infected fish was conducted on acetate cellulose paper electrophoresis (Figs.10-11). The total protein value was the highest in healthy fish, *i.e.* 5.0. Among the naturally infected fish, it was the lowest in the fish caught at hemorrhagic stage which was 4.12. The albumin and globulin ratio was found to be the lowest in naturally infected fish caught at ulcer stage and it was 0.45. The albumin fraction was the lowest in naturally infected fish at ulcerative stage while globulin was the maximum in the fish with ulcerative stage and it was 1.43.



Figure 10: graph showing the total serum protein (TP) content and serum albumin/globulin ratio



Figure 11: graph showing the ratios of different serum proteins percentage at different stage of infection

4. Discussion

From the observation of development of syndrome in ambient condition, it has been clearly revealed that syndrome develops at three different stages [14]. Accordingly, under present study the EUS has diagnosed at the same stages. Immunodiagnostics is one of the most authentic tools for the diagnosis of pathogens. The Dot-blot ELISA test and immunoelectron microscopic analysis clearly indicate that in the initiation of EUS, the virus is the first to enter the body of fish which gives rise to red circular rings which may be appeared due to inflammation. The fungus is the secondary invader at the site and then the bacteria and other pathogens make their entry into the hemorrhages and the same have been reported under the present study.

Though, the lymphocyte transformation is very old test for the assessment of immune efficiency but it gives the basic idea about the host pathogen interaction. Viral antigen induced transformation has been established with the mixed results. However Thopson *et al* [25] for adenovirus type 2, influenza and herpes simplex type 1. Bacterial induced transformation is well established by many workers *e.g.* Nilsson [26], Pearmain *et al.* [27] Miller and Jones [28] etc. In case of fungal induced transformation, the experiments were done with the antigens from *Candida albican*, reported by Cunningham-Rundles *et al* [24]. In the present experiment the results was favorable with viral and fungal antigens, which again support the findings of the work.

Under present work, interestingly dose dependent results in form of straight line curves are obtained indicating thereby a proportional relation with the pathogen and the overall impact exerted on the immune system of hosts. Viral antigens have been somewhat less extensively investigated than bacterial antigens, although transformations with vaccinia [29]-[30] and poliomyelitis vaccine [31] were among the earliest reports of antigen-induced lymphocyte transformation. In some instances, the addition of live virus (rubella, Newcastle disease, polio, and influenza) to human lymphocyte cultures depresses lymphocyte transformation. Olsen et al. [32] have demonstrated impaired DNA, RNA, and protein synthesis in measles-infected lymphocytes following stimulation with PHA or PWM. This experimental situation has been viewed as an in vitro mimic of congenital rubella, where a lack of response to PHA has often been correlated with clinically identifiable infection. In addition, Smitbwick and Berkovich [33] observed depressed response to PPD in vitro with lymphocytes taken from children with measles and active tuberculosis. Virus-induced suppression may result from cell surface changes induced by replicating virus within the cell. Thompson et al. [25] have demonstrated lymphocyte activation of fresh cells from donors by their own lymphocytes infected in short-term cultures by adenovirus type 2, influenza, and herpes simplex type-1. Viral replication does not always produce suppression and may occur simultaneously with transformation (e.g. parainfluenza). Inactivation of viruses alone does not always permit lymphocyte transformation. Antigen preparation (and hence antigen presentation) must be determined for each system [34]-[36]. The specificity of lymphocyte transformation has virus-induced been established in a number of instances. Herpes simplex (type 1) will stimulate the lymphocytes of individuals having histories of repeated herpetic infections [37], [38]. The specificity of lymphocyte responses to herpes simplex antigen can be demonstrated in experimental animals. Rabbits, which have been preimmunized with herpes simplex, have been shown to respond to herpes, but not to vaccinia. The reciprocal experiment yielded equivalent results [39]. The use of specific antigens in clinical assessment of immune deficiencies has particular application in cases where disease-specific or disease-related antigens may be developed. Such antigens (e.g., herpes virus in herpetic infections) [37], paramyxoviruses in multiple sclerosis [24] may reveal specific immune deficiencies in otherwise immunocompetent individuals. Activation followed by cell-cycle progression and DNA synthesis can sensitize T cells to undergo AICD. This in turn can result in an overall increase in [³H]TdR uptake, while at the same time viable cell numbers decrease due to AICD Pechhold and Kabelitz [15].

The serum protein profile of naturally EUS affected fish was found to be in accordance with the interpretation given by Laurell [40] for the stress conditions. The total protein of naturally infected fishes was found quite low as against the healthy fish. Albumin, a transport protein was found to be quite reduced in naturally infected fish as compared to the healthy fishes which might be due to the conversation of albumin in to protective protein (γ globulin). The acute phase protein, CRP having $\alpha 2$ mobility, was found too high in naturally infected fish indicating thereby the extreme stress condition. The increase in γ globulin was a clear cut sings of infectious nature of disease. The protein profile of naturally infected fishes was found comparable to the protein profile of fishes experimentally inoculated with virus and fungus of set S1e except γ globulin [18]. However, the globulin produced by viral and fungal pathogens was nearly equal to that produced in naturally infected fish. The results of serum protein profiles when compared with those of Laurell [40], it has supported the views of authors regarding the stages of infection and involvement of pathogens. However, Malcahy [41] also stated the similar findings from his experiments on a fish, *Atlantic salmon*.

By this different approach under present study, it has been revealed that the EUS symptoms have been produced in fish under laboratory condition without touching the fish as has been happening in nature. It has been observed that the syndrome develops in sequential order. Thus, the present study concluded that the virus is the triggering factor for EUS in presence of thermal and chemical stress, resulting in the damaging of physiological and immunological barriers of host which reflect in form of "red circular ring" (inflammation) and predisposed the fish for the fungal attack. After fungal invasion and their proper vegetative growth, zoospores are liberated and making the host as a receptor for all the opportunistic pathogens reflected as a "severe granulomatic ulcer" (EUS).

On the basis of experiments conducted and observations made consistently for a period of three years the EUS defined as "seasonal epizootics of inland fishes with chronic mycotic granulomatous ulcers on the body, initiated with red spots followed by red circular ring due to the SHRV under thermal and chemical stress".

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Volume 3 Issue 6, June 2014 www.ijsr.net

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