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Study of Anti Bacteiral Activity in Mucus of Fresh Water Cat Fish *Clarias Batrachus*

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Abstract

Anti bacterial agents are potent, broad spectrum antibiotics which demonstrate potential novel therapeutic agents. These compounds are constitutively expressed and rapidly induced at different cellular levels to interact directly with infectious agent and pathogenic microorganism. Fish live in a microbe-rich environment and are vulnerable to invasion pathogenic or opportunistic micro-organisms. The mucus layer on the surface of fish consists of several agents that provide a first line of defense against invading pathogens. In the present study presence of antimicrobial property of microbial peptides in the mucous of the catfish, C*larias batrachus* were assayed. The fish mucus was collected ethically using the electrical pulse method. The potency of antimicrobial activity skin secretion of catfish was screened against four bacterial species *Escherichia coli, Staphylococcus aureus*, *Klebsiella,* and *Salmonella* using Hole plate diffusion method. The activity was measured in terms of zone of inhibition and expressed as millimeter (mm in diameter). The maximum inhibition zone of 13 mm was shown for *S. aereus* followed by *Salmonella, Klebsiella* and *E. coli*. This study first of its kind in *Clarias batrachus* affirms the presence to antimicrobial property in its mucus due to antimicrobial agents or peptides which may initiate future new drugs for the therapy of various infectious diseases and replace antibiotics and interferon.

Key Words: Anti bacterial activity, *Clarias batrachus*, mucus, inhibition zone, *E. coli, S.aereus, Klebsiella,* and *Salmonella.*

Introduction

Immergence of new diseases, antibiotic resistance, microbial mutations, tolerance to drug, are some of the challenges faced by present medical field. In quest for no side effect treatments we turn to our ancient system of herbal medicines (Anand 2008a, Anand and Amudha 2009). Innovation in biotechnology and medicine is viewed as any new active moieties if they can offer additional treatement options to patients (Woollett 2012). Even approval of new therapeutic agents via FDA's are accelerated (Nicholas, 2012). Regardless of the outcome new treatments are developed to improve both quality and span of human life (Anand 2008b, Woollett 2012). To achieve this global research is translated into explore medically significant agents to provide biologics and biosimilars to fix human disease. Antimicorbial agents in fish mucus opens a new window which can be a therapies of tomorrow due to its

unique properties like no side effect, animal origin and wide spectrum of action on bacteria, fungi, viruses and other pathogens.

Antimicrobial agents are host defense agents produced by animal kingdom (Hancock *et al* 2006). They are evolutionarily conserved components that are involved in immune response and are found among all classes of life (Beutler, 2004). These compounds are excellent candidates for development of novel therapeutic agents, as well as to complement conventional antibiotic therapy. Unlike antibiotics these do not induce resistance hence can be used lavishly as a therapeutic agent. Furthermore these have broad range of activity, as bactericidal as opposed to bacteriostatic and require a short contact time to induce killing (Ramasamy *et al*., 2011). Anti microbial peptides present in mucus has known to kill gram positive and gram negative bacteria, mycobacterium, enveloped viruses, fungi and even transformed or cancerous cells (Ramasamy *et al*., 2011).

Fishes are identified as the key exothermic vertebrates, with phylogenetic group including over 20,000 species, conquering frozen waters, hot springs, surface waters, dark oceanic depths withstanding tons of pressure, freshwater and extremely salty inhabitable waters. These species exhibit different *in vivo* as well as *in vitro* immune response to various antigenic stimuli. Many authors have recommend fish as the best animal model (Anand, 2002; Anand *et al*., 2009). Fish *Clarias batrachus,* which belongs to an air breathing catfish species, found primarily in Southeast Asia is robust species. It was suggested to be the best test organism due to its hardy nature and quick adaptability to lab environment (Anand *et al*., 2012).

Fish and products obtained from it contain potentially valuable and important proteins, enzymes and active compounds (Bauer, 1996). The mucus substances of fish act as a key component of innate immunity (Black and Pickering, 1982). Mucus secreted by fish play a major role in protection against infectious agents such as bacteria and fungi. However under such conditions the fish maintains a health state by defending itself against these potential invaders by a complex system of innate defense mechanisms (Cancre, 1999; Choncha, 2004). The mucus layer covering the integument of fish has mechanical protective barrier (Sangeetha, 2008). The mucus layer on the fish surface ensures disease resistance, respiration, ionic and osmotic regulation, locomotion, reproduction, communication, feeding and nest building (Negus 1963; Ingram 1980; Shephard, 1994). The mucus also plays a role in preventing colonization of bacteria, fungi and other parasites (Lemaitre 1996; Ebran, 2000).

Critically, these mucus compounds have no side effects; hence they can contribute to replace currently available antimicrobial agents. The antimicrobial molecule produced by the epithelial tissue serves as the first line of defense against microbial invasions in most of the vertebrates including humans (Gantz, 1999; Villarroel *et al*, 2007). Antibacterial activity in mucus has been demonstrated in several fish species (Austin 1998). Fish mucus also contain a variety of biological active substances such as lysozyme, lectins, flavoenzymes, immunoglobulins, c-reactive protein, apolipoprotein A-I and antimicrobial peptides which gives protection to fish from potential pathogens (Ellis, 2001; Kitani *et al*., 2007). One of the advantages of antimicrobial agent is that they can function without high specificity or memory (Rameshkumar, *et al*., 2009).

Dynamic antimicrobial agents like anti microbial peptides have been isolated from variety of organism in the last few years. AMP from several fish have been reported and studied namely: *Pleuronectes americanus* (Walbaum), *American plaice, Hippoglossoides platessoides* (Fabricius) and Atlantic halibut, *Hippogolossus hippogolossus* (Cole, 1997; Douglas, 2001). Misgurin from loach, *Misgurnus anguillicaudatus* (Cantor) (Park *et al*., 1997), Hagfish Intestinal Antimicrobial peptides (HFIAPs) from hagfish, *Eptatretus burgeri*

(Girard) (Hwang *et al*., 1994), Chrysophsins from red sea bream, *Chrysophrys major* (Iijima *et al*., 2003). Piscidin or Moronecidin from white bass, *Morone chyrysops* (Rafinesque) and striped bass, *Morone saxatilis* (Walbaum) (Douglas *et al*., 2003) and Hepcidins from several species of fish (Douglas *et al*., 2003; Shike *et al*., 2003).

The intensive literature survey has revealed that the most common Asian fish i.e. cat fish *Clarias batrachus* have not been ever screened for antimicrobial property in mucus. This work was conducted to know weather these fish produce antibacterial agents and also to know the efficacy of these compounds on some opportunistic pathogens.

Materials and methods

Animal

The Asian species of air-breathing catfish, *Clarias batrachus* lives in freshwater pools having low oxygen and carbondioxide content, which may dry up during summer). The genetically similar fingerlings obtained from one pair of Cat fish were procured from Ranipettai district in Tamil Nadu. These fifteen days old fingerlings were transported to the Environmental Biotechnology Laboratory in oxygenated bags.

Acclimatization

Acclimatization was carried out in a rectangular fish tank (2m X 4m X 3m). Before stocking fish in tank, the glass aquarium was washed by soap, previously disinfected with potassium permanganate and thoroughly rinsed thrice prior to filling with water. Fish were acclimatized to laboratory condition for four weeks before being used for experimental studies. The stock of fish was maintained at natural condition of photoperiod and ambient temperature and fed ad libitum with groundnut oil cake and rice bran (both are powdered form in the ratio of 1:2) once a day. Water was replaced for every 24 hrs and well aerated in order to reduce any accumulation of excretory. The

Experimental Setup

After the acclimatization period of ten days, twenty healthy fish were transported to glass aquaria of 50 liters capacity which served as control. Two sets of twenty healthy fish were kept in the two separate glass aquaria for the experimental work. The fish were allowed to adapt to the tank conditions for next ten days. Mucus secretion was induced by ethically proven method so that fish remains unaffected whereas the epidermis is stimulated to its secret the content. A special apparatus was designed as shown in Figure 1. The handling stress or suffocation was minimized during the experiment. Minute 5 Volt of current was passed through battery in order to give to induce mucus secretion (Ross *et al*.2000; Ramasamy *et al*., 2011).

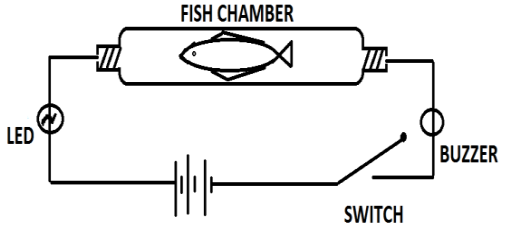

Figure1 Schematic diagram of an apparatus specially designed for the stimulation of mucus secretion using electric shock

Skin mucus collection and prevention

Mucus was collected based on Ross *et al*. (2000) with slight modification. Epidermal mucus was collected carefully scrapping the dorsal body surface using a plastic spatula. Mucus was not collected in the ventral side to avoid intestinal and sperm contamination (Kuppulakshmi *et al*., 2008). After collecting, the mucus it was pooled and immediately frozen to prevent any external bacterial contamination then lyophilized and stored at 20°C.

Purification of the mucus

Lyophilized mucus was resuspended in 1ml of 100mM (w/v) ammonium bicarbonate (pH 8.2) at 1mg/ml and centrifuged at 9500 xg for 10min at 4°C (Ramasamy *et al*., 2011). The mucus retenate was freeze dried and resuspended in water for testing antimicrobial activity.

Antibacterial assay by Hole plate diffusion method

The antimicrobial activity of the mucus was assayed by agar diffusion method. One percent agar was autoclave, and was kept in 44ºC water bath for 30 minutes for stabilizing the temperature. Individual agar were prepared for *Escherichia coli, Staphylococcus aureus*, *Klebsiella,* and *Salmonella* by adding 100- 150 µl of pure culture in 50 ml broth and left overnight. 5ml of this agar with bacterial culture was poured in Petri plates to solidify. After solidification 6 wells of approximately 5 mm in diameters and 2.5 mm deep were made on the surface of the solid medium using ethanol dipped tip of plastic pipette. Sterile dimethylsulfoxide (DMSO) was used as negative control. For positive control 10 µl of Kanamycin (prepared from stock solution, 50 mg/ml stored at -20ºC) at concentration of 50µl/100 ml in deionized water was used. Each well was then filled with 10 µl of the test mucus. Plates were incubated at 37°C for one day. The radial zone of inhibition was measured by using a ruler and the diameters of inhibition zone were determined in millimeters.

Statistical analysis

The statistical analysis was made using student t test, individually on each pooled samples and the mean value of five individual observations was taken for each parameter.

Results

Fish are one of the organisms that have managed to survive in a milieu of pathogenic organisms. The primary interference of fish with their environment happens through a mucus layer that covers their entire body. Research has demonstrated that the mucus layer is composed of biochemically diverse secretions from epidermal goblet cells and epithelial cells. It was reported that epithelial tissues produce antimicrobial molecules which serve as the first line of a host defense against microbial invasion in a variety of vertebrates.

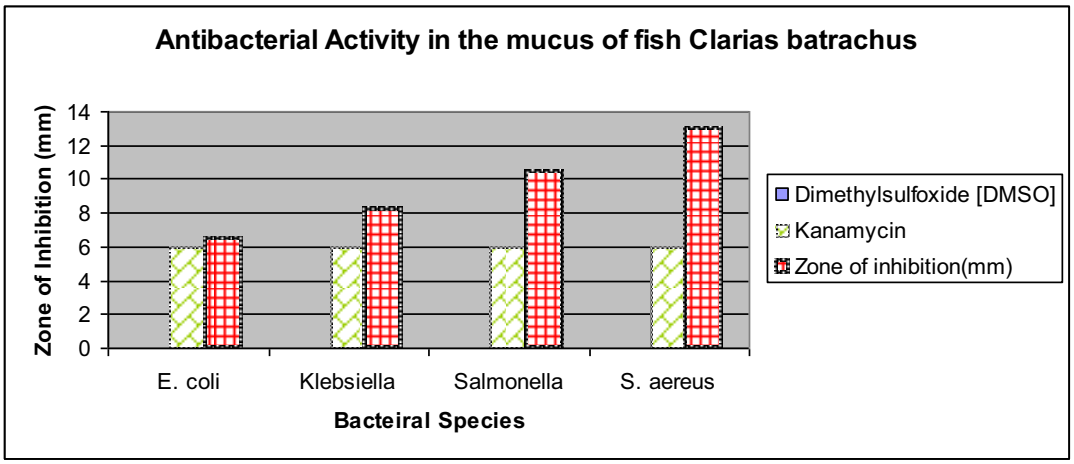

Figure 2 The bar graph of zone of inhibition produced by mucus of fish *Clarias batrachus* in contrast to Kanamycin as positive control and DMSO as negative control against the bacteria *E.coli, S.aereus, Klebsiella,* and *Salmonella*.

Antimicrobial property of mucus secretion of the fish *Clarias batrachus* was different for *E. coli, S. aereus, Klebsiella* and *Salmonella*. Figure 2 show the size of zone of inhibition in the bacterial lawn. The clear zone indicated that the mucus secreted by fish *Clarias batrachus* had the antibacterial activity. The maximum inhibition zone of 13 mm was shown for *S. aereus* followed by *Salmonella, Klebsiella* and *E. coli*.

Discussion

The genetic information in organisms is designed to finding solutions to all challenges which nature put before it (Anand and Amudha 2009). Aquatic organisms are under tremendous selection pressure including space competition, predation, surface fouling and reproduction. Aquatic animals are continuously exposed to various microenvironments. All species with low tolerance are eliminated, while those species best suited for survival enriched habitats become extensively dominant (Anand *et al*., 2012). All living organism have genes which express proteins to help them survive in most inhabitable environments (Anand, 2010). These proteins have industrial applications and holds solutions to many human problems.

Fish are in constant interaction with their aquatic environment, which contains a wide range of pathogenic and non-pathogenic microorganisms (Vennila *et al* 2011). The epidermis and the epidermal mucus secretions act as biological barriers between fish and the potential pathogens of their environment (Shephard, 1993). Expression of one or more of the above mentioned antimicrobial components in fish epidermal mucus has been observed following microbial stress (Aranishi and Mano, 2000; Patrzykat, 2001), thus supporting the role of epidermal mucus in protecting fish from infectious pathogens. This preliminary information suggests that the mucus from these fish species may be a source of novel antimicrobial agents for fish and human health related applications. The antimicrobial property of epidermal mucus against infectious pathogens has been demonstrated previously in rainbow trout (*O.mykiss*) (Kanno *et al*., 1989), ayu (*Plecoglossu altivelis*), turbot (*Scopthalamus maximus*) and carp (*Cyprinus carpio*) (Lauth *et al*., 2002). In the present study the mucus isolated from *Clarias batrachus* shows a strong inhibiting effect on the tested microorganisms. This may be due to the pore forming properties against several bacterial strains. Fish secrete antibacterial proteins which is able to permeablize the membrane of the

target cell and thus act as a defense barrier (Kuppulakshmi *et al*., 2008). The antibacterial activity may be due to the antibacterial glycoprotein present in the mucus which kills bacteria by forming large pores in the bacteria's membrane (Ebran *et al*., 1999). Fish mucus is believed to play an important role in the prevention of colonization by parasites, bacteria and fungi and thus acts as a chemical defense barrier (Pickering, 1974).

The results of the present study support the folkloric usage of fishes and suggest that the mucus of fishes possess certain constituents with antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens.

Future prospects

The presence of antibacterial agents in nature attests to their overall importance in building the defense strategies of most organisms. They are considered part of the humoral natural defense of invertebrates against infections and have thus also been termed "natural antibiotics". The mucus collected from fish *Clarias batrachus* or any organism which shows broad spectrum of antibacterial activity can be subjected to further evaluation, by separating cocktail of proteins and compounds aiding the anti bacterial property. Each component can be further evaluated for its mode of action on bacteria or their effects on various combinations. In vivo studies can be conducted with permission from Institutional Biosafety Committee as these peptides are very potent against bacteria, but have no toxic or hemolytic effect on host cells. The taxonomic distribution of antimicrobial peptides or anti bacterial agents can be pooled which can commence of a new era in studies of animal antimicrobial agents to replace antibiotics and interferon.

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***Authors' contributions: Nathiya.T. and Alphonse Maria. A** (M. Sc Biotechnology Students), helped in standardizing the protocols and contributed in preparation of the manuscript and **Dr. Anand Prem Rajan** (Associate Professor) designed and executed the work, contributed in writing, final editing and corresponding author of manuscript.*

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