1.0. INTRODUCTION

1.1. Aquaculture

Fish, being a “protein rich food for poor people”, is well placed to make an important contribution to the millennium development goals. Aquaculture, the cultivation of fresh and saltwater fish, shellfish, and algae, is being increasingly recognized as an important method of food production and accounts for close to half of the fish consumed worldwide (FAO, 2010). Elsewhere in the world, notably in Asia, aquaculture has also grown and made important advances providing significant contributions to nutritional and food security demands, poverty alleviation and social wellbeing. Over the last 50 years the world aquaculture has increased significantly from less than 1 million tons in 1950’s up to 51.7 million tons in the year 2006 with 78.8 billion US$ in terms of value. In general it demonstrates that aquaculture has been continuously growing more rapidly than other animal food-producing sectors (The State of World Fisheries and Aquaculture, 2008). Recent developments in aquaculture are concerned mainly with the diversification of aquaculture activities, the production of high quality aquaculture species in large quantities and reducing the production cost of commercial farms (Citarasu, 2000).

The rearing of aquatic species is an ancient practice that can be traced back nearly 4000 years (Iwama, 1991). The intensification and rapid expansion of aquaculture on the other hand has a history of only a few decades and by the some serious environmental concerns have emerged. Shrimp farming is one of the most controversial crops of the rapidly expanding aquaculture industry because it has caused environmental degradation. The spectacular growth of shrimp aquaculture
industry in the 1980's as farmed shrimp has accounted for 25 percent of world shrimp supply. Most of the growth of world shrimp aquaculture production has reportedly been in Asia, where eighty percent of the world's cultured shrimp is produced. China is the largest producer (3.95,000 tonnes) followed by Thailand, Ecuador, Indonesia, India, Bangladesh and Vietnam (FAO, 2014). In terms of total area under shrimp culture, Indonesia, Vietnam and India were reported as having the largest number of hectares under production (Ahmed, 1997). In recent years, the growth and intensification of shrimp aquaculture in Asia has been explosive. While innovative aquaculture involves the applications of new and more effective ways or solutions for aquaculture management, there may be interventions related to breeding and farming techniques, aquatic environment management, disease control or animal health monitoring, nutrition and feed management of the cultured organisms, genetics and biotechnological tools which directly or indirectly play roles in the improvement of aquaculture production in an environment-friendly and sustainable manner (Ugoala, 2014). Recent developments in aquaculture are concerned mainly with the diversification of aquaculture activities, the production of high quality aquaculture species in large quantities and reducing the production cost of commercial farms (Citarasu, 2000).

1.2. Microbial diseases

Aquaculture production is expected to continue to grow through 2030, but at a slower rate than previously seen. In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens (Lundin, 1996). As is often the case in crustacean diseases, the causative agent in an outbreak is rarely known or unreported until the onset of the initial epizootic. By definition, an outbreak is the occurrence of a
pathogen at greater than baseline levels in a host population (Center for Disease Control and Prevention, 2007); thus it is important to know the baseline before one can ascertain the scale or effect of an outbreak.

Crustaceans (including crabs, shrimp, lobsters and crayfish) can play host to a wide range of pathogens and parasites. Viral infections have been encountered in both wild and cultured crustaceans; since their initial discovery in this host group in the 1960's (Vago, 1966), there have been over 50 viruses described from a diverse range of crustacean groups. Viruses with RNA genomes, some of which have devastating consequences for cultured populations, are also being increasingly described, particularly from intensively farmed penaeid shrimp hosts (Boonyaratpalin et al., 1993; Hasson et al., 1995; Poulos et al., 2006; Lightner, 2011). Whilst relatively little is known about viruses of wild crustaceans, it is critical that as much data as possible be collected on viral pathogens from wild decapod stocks (Johnson, 1984; Bonami and Lightner, 1991). Free-living bacterial infections of crustaceans are associated either with the exoskeleton (Getchell, 1989), or the haemolymph (Tubiash et al., 1975; Wang, 2011). Intracellular bacterial infections occur within specific types of host cell (Vincent et al., 2004; Eddy et al., 2007). Fungal pathogens (Phycomycetes, Ascomycetes and Fungi Imperfecti) have been implicated in catastrophic epidemics and mortality events in decapod crustaceans (Unestam, 1973).

The large volume of published literature on the association between infectious disease agents and farmed penaeid shrimp provides compelling evidence for the major constraint that these pathogens have imposed on efficient production and yield in intensive aquaculture systems (Lightner, 1993, 2011; Flegel, 2006a). Despite
difficulties in gathering accurate economic data on the effect of pathogens on crustacean production, approximately 40% of potential tropical shrimp production is estimated to be lost to infectious diseases each year (Lundin, 1996). Around 60% of current disease-associated losses in shrimp aquaculture may be due to viral pathogens with a further 20% to bacterial pathogens. By comparison, losses associated with fungal and parasitic agents are less (Flegel, 2006b, 2012).

1.3. Shrimp bacterial diseases

Bacterial infections of shrimp have been observed for many years. Scientists have noticed that bacterial infection usually occurs when shrimps are weakened. Otherwise normal shrimp also may become infected if conditions favor the presence and the abundance of a particularly harmful bacterium (Getchell, 1989). If infected by bacteria capable of using shell for nutrition, the exoskeleton will demonstrate erosive and blackened areas. These bacteria typically attack edges or tips of exoskeleton parts, but if break occurs in the exoskeleton the bacteria are quick to enter and cause tenure damage. Filamentous bacteria are commonly found attached to the cuticle, particularly fringe areas beset with setae (Vanderzant et al., 1971).

The microbial flora of brown and white shrimp from the Gulf of Mexico and from pond reared brown shrimp has been studied (Vanderzant et al., 1970; Vanderzant et al., 1971). These studies indicated that the microbial flora of the Gulf shrimp and pond shrimp differ slightly. Bacterial counts of pond shrimp were reported to be much lower than those from Gulf shrimp (Vanderzant et al., 1970). The coryneforms (species of Corynebacterium, Arthrobacter, and Microbacterium) and to a lesser extent Vibrio were the predominant isolates from fresh pond-reared brown
shrimp. In contrast, the microbial flora of Gulf shrimp was dominated by coryneforms and species of Pseudomonas, Moraxella, and Micrococcus. Bacterial erosion of the shell is present in all penaeid shrimp, juveniles and adults alike. It manifests with the appearance of brown or black stains in areas that have been eroded through the action of chitinolytic bacteria, such as Vibrio sp., Aeromonas sp., Spirillum sp., and Flavobacterium sp. The disease is self-limiting and generally disappears when the shrimp is moulting and if left untreated; it becomes more serious and may become a systemic infection (Morales, 2004).

1.3.1. Vibriosis

Problems of diseases often accompanied intensification as environmental conditions deteriorated which brought the decline of the aquaculture industry. In recent years, Vibriosis has become one of the most important bacterial diseases in maricultured organisms, affecting a large number of species of fish and shellfish. The concept of “Marine Vibrios” requirement for Na\(^+\) and other ions (Baumann and Baumann, 1981). The first comprehensive study of bacteria digenous to the oceans was done by Fisher (1894) of the University of Kiel. His major conclusions that the majority of the heterophilic bacteria in the open oceans are Gram–negative, straight or curved rods or spirals that are usually motile by means of flagella (Baumann et al., 1971) have been confirmed by other researchers as well.

Both Vibrio and photobacterium are old genera that were described in the 1800s. The genus name Vibrio was coined by Pacini (1854) during his studies on cholera, and it is one of the oldest names for bacterial genus. Pacini coined the name the Cholera bacillus, which eventually have been named V. cholerae, the type of
species for the genus Vibrio. Much of the history and literature on Vibrio came from medical microbiology microbes that caused cholera and other similar vibrios that early bacteriologists had difficulty in differentiating from the true cholera bacillus. Mortality caused by vibrios in reared fish and shellfish is very common during early larval stages and can occur suddenly leading sometimes to death of the entire populations (Borrego et al., 1996; Diggles et al., 2000). Thiosulfate-citrate-bile salts-sucrose (TCBS) agar is a widely used medium for isolating and enumerating Vibrio species from marine and estuarine waters (Baticados et al., 1990). In several Vibrio sp. this technique has been used in diversity studies (Shangkuan et al., 1997; Somarny et al., 2002), and in Vibrio harveyi it has been used in differentiating pathogenic and non-pathogenic strains (Somarny et al., 2002; Hernandez and Olmos, 2004; Alavandi et al., 2006). The closely related species of V. harveyi and V. campbellii have caused disease in shrimp larvae while V. penaeicida and V. parahaemolyticus have infected in juveniles and adults.

Vibrio species are known to cause serious diseases in the tiger prawn (Penaeus monodon). V. harveyi is recognized as an important pathogen of cultured penaeid larvae throughout the Southeast Asian region (Lavilla-Pitogo et al., 1990; Jiravanichpaisal et al., 1994; Karunasagar et al., 1994). In the intensive culture of the black tiger prawn P. monodon, virulent strains of V. harveyi could cause devastating mortality in the hatchery stage (Sunaryanto and Mariam, 1986). The disease phenomenon caused by these strains is commonly referred to as luminous bacterial disease (Lavilla-Pitogo et al., 1990) or luminous vibriosis. This species has been recognized as pathogenic for several crustacean larvae, particularly, Penaeus sp. (Diggles et al., 2000; Liu et al., 1996; Robertson et al., 1998; Vandenberghe et al.,
1999). The genus *Vibrio* includes more than 30 species, at least 12 of which are pathogenic to humans and have been associated with food borne diseases (Chakraborty *et al.*, 1997).

Among them, *V. parahaemolyticus* has emerged as an important shrimp pathogen. Though there are many reports on the involvement of this bacterium in shrimp vibriosis (Ruangpan and Kitao, 1991; Xu *et al.*, 1994; Chanratchakool *et al.*, 1995; Alapide-Tendencia and Dureza, 1997). For example, the organism has been reported as a primary pathogen of cultured penaeid shrimp, especially in South America and Asia. The closely related species such as *V. harveyi* and *V. campellii* have caused disease in shrimp larvae while *V. penaeicida* and *V. parahaemolyticus* have infected juveniles and adults. However, some Vibrio species, or strains of certain species, have been identified as primary pathogens (Lavilla-Pitogo *et al.*, 1990). Pathogenic strains of *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus* have caused massive epidemics in the Philippines (Lavilla-Pitogo *et al.*, 1990). In order to control this bacterium, chemicals and antibiotics were widely applied in many years ago. However, some of their residues caused the serious impact in both environment and health of consumers. Additionally, the antibiotics resistant strains were also reported in many countries (Karunasagar *et al.*, 1994) and mass mortality caused by this pathogen is still occurring even today.

### 1.3.2. Clinical signs

Diseased prawn larvae are characterized by weakness, opaque colour, feeble and intermittent swimming movements and greenish luminescence (Lavilla-Pitogo *et al.*, 1990, Karunasagar *et al.*, 1994). Vibrio disease is described as Vibriosis or
bacterial disease, penaeid bacterial septicaemia, penaeid Vibriosis, luminescent Vibriosis or red-leg disease, and is widely distributed. Signs of Vibriosis include lethargy, tissue and appendage necrosis, slow growth, slow larval metamorphosis and body malformation, bolitas negricans, bioluminescence, muscle opacity, melanization, empty midgut and anorexia (Karunasagar et al., 1994; Lightner, 1996; Robertson et al., 1998). With bacterial septicaemia, large numbers of bacteria have been observed in microscopic wet mounts of the haemolymph. Necrosis and inflammation of organs (lymphoid organ, gills, heart, hepatopancreas, etc.), granulomatous encapsulation, are also present. Malacia can be evaluated easily by normal histological methods (Lightner, 1996).

1.3.3. Potential virulence factors of pathogenic Vibrio species

Adhesion of bacteria to host surface has been described as one of the initial steps in microbial pathogenesis (Home and Baxendale, 1983; Wang et al., 1998; Wang and Leung, 2000). The hydrophobicity of the microbial surface has been suggested to be a determining factor in the adherence of bacteria to host surface, which especially enables the bacteria to interact with animal cells and to survive within the host (Balebona et al., 1998).

Pathogenicity, or virulence, is the ability of a bacterium to cause infection, virulence factor (or mechanism of pathogenesis or virulence mechanism) indicates a bacterial product or strategy that contributes to virulence or pathogenicity (Salyers and Whitt, 2002). Virulence caused by factors such as the extracellular enzymes and stress proteins can be recognized by the loss of cell adherence, induction of cell lysis, or apoptosis. A clam primary cell culture, despite consisting of a heterogeneous cell
population, has been used to study the interaction between cells and a pathogenic Vibrio species (Choquet et al., 1999). Many extracellular bacterial proteases are suggested to play important roles in virulence. Several bacterial components or products have been suggested as virulence factors as they are an extracellular hemolysin/ cytolysin (Grey and Kreger, 1986), an elastolytic protease (Kothary and Kreger, 1987), a phospholipase (Testa et al., 1984).

1.3.3.1. Extra cellular products (ECP) toxins

The extracellular products (ECP) of the opportunistic pathogen Vibrionaceae strains are known to be virulent and toxic for fish and shrimp. Among these components, a toxin was purified showing a neurotoxic effect in fish, blocking the regulation of the central nervous system (CNS). This toxin displayed a 50% lethal dose of 50 ng /1 g fish, which is the lowest dose described for fish so far.

Vibrios are known pathogens with characteristic pathogenic and virulence properties (Thompson et al., 2004). Different ECP with toxic effects on shrimp have been identified and characterized from a variety of Vibrio species and strains isolated from marine organisms and the environment. These ECP have been proposed as virulence factors for shrimp and other marine organisms. Extracellular products such as chitinases, hemolysins, alkaline proteases, cysteine proteases alkaline metalchelator-sensitive proteases, serine proteases and metallo proteases have been isolated from cell- free culture supernatant (CFS) of *V. harveyi*, *V. anguillarum* (Bergeman), *V. alginolyticus* (Miyamoto) and other species. (Stensvag et al., 1993).

It has been well documented that extracellular products (ECPs) play important roles in the pathogenesis of vibrio in fish. The ECPs from various vibrios are highly
toxic to a great number of fish species which cause pathological changes that are similar to those elicited after inoculation of live bacteria (Inamura et al., 1984, 1985; Fouz et al., 1992a; Lamas et al., 1994; Esteve et al., 1995; Biosca and Amaro, 1996; Balebona et al., 1998; Wang et al., 1998; Zhang and Austin, 2000). Regarding the pathogenic mechanisms of ECPs, proteolytic activity and various hydrolytic activities of them have been considered as important virulence factors for the invasion, survival and proliferation of vibrios in host animals (Biosca and Amaro, 1996; Balebona et al., 1998). For example, the ulceration and degradation of tissue surrounding the site of injection could have been due to the hydrolytic action of ECPs (Balebona et al., 1998).

1.3.3.1.1. Proteases

In general, bacterial proteases have been suggested to be important virulence factors for a variety of organisms by causing massive tissue damage in the host, which may aid the bacteria in host cell entry (Finkelstein et al., 1983; Smith and Merkel, 1982). Proteases have also been implicated as virulence factors in various Vibrio strains pathogenic to fish (Norquist et al., 1990) or shrimp (Lee et al., 1997; Liu and Lee, 1999).

Proteases from pathogenic Vibrio species in fish have been associated with invasive mechanisms, damage and lethal effect in the host (Inamura et al., 1985; Kanemori et al., 1987; Kothary and Kregar, 1987; Nottage and Birkbeck, 1987; Nishina et al., 1992; Naka et al., 1995; Morita et al., 1996; Miyoshi et al., 1998).

1.3.3.1.2. Hemolysins

Hemolytic and cytotoxic activities of ECPs have also been considered as important virulence factors contributing to the pathogenicity of the infection process.
Marked hemolytic activity has been clearly demonstrated in the ECPs in vitro and in vivo, a phenomenon which is responsible for the severe hemorrhaging symptoms found in vibrio-infected fish (Thune et al., 1993; Lamas et al., 1994; Biosca and Amaro, 1996; Balebona et al., 1998; Zhang and Austin, 2000). Regarding the cytotoxic activity present in the ECPs, its precise contribution to the pathogenicity of vibrios is still obscure, because cytotoxicity of the ECPs is highly dependent on the cell lines chosen for the test (Toranzo et al., 1983). Moreover, cytotoxicity to fish cell lines has also been reported in the ECPs isolated from some non-pathogenic reference strains (Toranzo et al., 1983). For example, V. salmonicida and V. ordalii are poor producers of proteases and hemolysins, and the mechanisms of their hemorrhagic activity are still unknown (Toranzo and Barja, 1993).

### 1.4. Current treatment protocols

Health management of cultivable organisms in aquaculture is of paramount importance in order to prevent mortality leading to heavy economic loss. However, due to the declining catch from capture fisheries, shrimp farming was initiated to maximize profits. Problems of diseases often accompanied this intensification and deterioration of environmental conditions have caused the decline in aquaculture industry. Pressure to ensure production led to reliance on antibiotics, which are administered to farmed shrimp primarily to prevent or treat bacterial diseases. Chemotherapy, vaccination and other prophylactic measures are generally adopted in this regard. Utilization of chemotherapeutic agents in aquaculture has been extensively studied (Aoki, 1992; Plumb 1995).
In the field of aquaculture, both therapeutic and environmental problems have been addressed, as antimicrobial agents are released into the surrounding water during treatment of bacterial fish diseases. Vaccines can be divided into live attenuated vaccine, killed vaccine, DNA vaccine and live vaccine. In recent years, live vaccines have received increasing attention as they have many advantages over any other vaccines (Schreuder et al., 1996; Ueda and Tanaka, 2000; Sommerset et al., 2003). Although some live attenuated vaccine and killed vaccine have been developed for the prevention or cure of the bacterial disease in marine animals, live vaccine against bacterial infection in marine animals are still not available (Zhu et al., 2004).

1.5. Shrimp immune system

Being an aquatic animal, shrimp are constantly exposed to a variety of bacteria and viruses. While most of them are harmless bacteria, some are pathogenic. Shrimp have several known defense mechanisms to protect themselves against pathogen invasion. The external cuticle provides an effective physical and chemical barrier against the attachment and penetration of pathogens (Martin et al., 2004). Unlike foregut and hindgut the mid gut (equivalent to intestine) is not lined by cuticle (Lovett and Felder, 1990) and therefore provides a favorable site for invasion of pathogens (Ruby et al., 1980; Jayabalans et al., 1982). In most cases, the defense mechanisms of the cuticle and that of the digestive tract are sufficient to protect against even highly virulent pathogens. However, when pathogens are able to break these defense barriers and enter into the body, they will encounter the innate immune systems. This system consists of cellular and humoral responses and the well-known mechanisms of these responses are phagocytosis, blood coagulation, nodule formation, and encapsulation (Jiravanichpaisal et al., 2006). Some hydrolytic enzymes are also involved in these
processes such as lysozyme (Somboonwiwat et al., 2006; Burge et al., 2007). Several innate immune response systems have been characterized in crustaceans. In *P. monodon*, the prophenoloxidase activating system (proPO) is induced which in turn will lead to melanization and generation of factors for immune reactions such as the cell adhesion factor, peroxinectin (Cerenius et al., 2008). As of today most knowledge on the invertebrate immune response has been elucidated from the analysis of host reactions after direct injection of bacteria into the body cavity or tissues of these animals. Although this approach has been shown to be effective for identifying virulence factors and host defense mechanisms, it bypasses the natural entry of microbes through oral routes of infection and subsequent persistence within the organism (Vodovar et al., 2004).

### 1.6. Difficulties of current treatment methods

However, extensive use of antibiotics and other chemotherapeutic agents has resulted increased drug-resistant bacteria in aquatic environments. More and more antimicrobial compounds (amikacin, ampicillin, kanamycin, penicillin G, streptomycin and tetracycline etc.), which have been among the most commonly used drugs in clinical and agricultural treatments, are now partially or completely ineffective in controlling vibriosis, as well as other bacterial diseases of fish (Austin and Austin, 1993; Li et al., 1999).

In practice, a considerable amount of antibiotics, administered in the feed, are unabsorbed by the fish and enter into the environment in antimicrobially active forms. In addition, spatial and temporal differences always result in significant discrepancies in antimicrobial susceptibilities of vibrios (French et al., 1986; Li et al., 1999).
Therefore, in order to reduce the indiscriminate use of these antibiotics, drug susceptibility tests should be carried out prior to the use of any antibiotics in fish farms (Li et al., 1999). Although chemotherapy is currently the most commonly used method in controlling and treating bacterial diseases in fish farms, limitations such as posing extra economical problem to fish farmers and influencing surrounding ecosystem (Li et al., 1999). Therefore, development of methods other than chemotherapy is much desired. Prophylactic treatment of vibriosis by vaccination is highly recommended, and recent reports have shown that this area is highly promising (Qin and Pan, 1996; Woo et al., 2001). More evidence indicates that application of probiotics in aquaculture is effective against vibriosis by improving culture conditions, inhibiting potential pathogens, enhancing nutrition, and stimulating host immunity. Details on this subject have been adequately reviewed elsewhere (Iranto and Austin, 2002).

1.7. Alternative approaches

In total, environmental concerns, regulatory constraints, cost, and pathogen resistance have greatly diminished the appeal of antibiotic use and other chemotherapeutical methods in aquaculture (Palm et al., 1998). Considering the potential threat of diseases on one hand and the environmental issues on the other, diseases management aspects should concentrate on environment friendly biotechnological methods like development of rapid procedures for detection of diseases, prophylaxis (vaccines, immunostimulants, edible antibody, passive immunization, bioremediation of environment including the methods of administration) and disease treatment protocols.
1.7.1. Vaccination

Methods of vaccination include injection, oral administration and immersion. Intraperitoneal (IP) injection and the most effective method of immunizing shrimp is intraperitoneal (IP) injection. This technique allows the use of adjuvants, which enhance the magnitude of the immune response. Injection ensures that each shrimp receives the exact dose of vaccine. However, it is very labor intensive and stressful to the fish since it requires anesthetization and handling. This method is not considered practical for fish with weights less than 15g (Ellis, 1988; Smith, 1988).

Immersion vaccination consists of two methods, namely dip and bath vaccinations. The main portal of vaccine in this method is the gill tissue. In the bath method, vaccine is added directly into the holding tanks, cohere by handling stress to the fish is minimized. This technique is not stressful; but consumes more vaccine and since the vaccine solution is more dilute than the former method, it requires a long exposure time period, approximately 1-2 hours, and requires oxygenation of the water. Immersion methods permit mass vaccination of the fish below 5g (Ellis, 1988; Home and Ellis, 1988).

Oral vaccination, which involves the incorporation of antigen into the feed over a suitable time course, is the only method economically suited to extensive aquaculture. This method offers a significant advantage for it reduces labor cost, time saving, no handling and therefore limits stress to the fish, decreases the possibility for cross contamination with needles, does not require disposal of treatment water and allows mass vaccination of fish of any size. However, oral vaccination requires larger doses of vaccine than the injection or immersion methods to achieve protection.
Further disadvantages are poor stability of the antigen in the digestive system and lack of control on the dosage for each fish, which is dependent on the feeding rate (McLean et al., 1999; Home and Ellis, 1988; Hart et al., 1988). Research is still continuing to optimize oral vaccination conditions and also how to increase the potency of this method.

1.7.2. Natural herbal medicines

The natural plant products have been reported to have various effects like antistress, growth promoting, appetizing, tonic immunostimulation, aphrodisiac and antimicrobials in finfish and shrimp larviculture due to the active principle natures such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils (Citarasu et al., 1998; Citarasu et al., 2002; Sivaram et al., 2004). Plants have been a rich source of medicines because they produce a variety of bioactive molecules most of which are probably evolved as chemical defense against predation or infection. Medicinal plants contain active constituent like terpenes, alkaloids, steroids, saponins, tannins, phenols, quinines and flavonoids (Leven et al., 1979; Harborne, 1982; Bever, 1986).

1.7.3. Immunostimulants

Various chemical compounds, called "immunostimulants", are known to increase resistance of livestock and humans, as well as fish and shellfish infections. These include bacteria, microbial products, complex carbohydrates, animal extracts, plant extracts, cytokines and lectins, synthetic compounds such as dipeptide bestatin, and a number of muramyl and lipopeptides (Raa, 1996; Galeotti, 1998). Similar (Yano et al., 1991). Immunostimulants have been shown to induce non-specific and humoral defense mechanisms such as oxidative activity of neutrophiles, engulfment
potential of phagocytic cells and activities of cytotoxic cells (McLean et al., 1999; Anderson, 1992). De Baulny et al., (1996) found no reduction in mortality in a challenge study after oral administration of β-glucan; however, an increase in white blood cell count was observed. Increase in total antibody level and non-specific protection capabilities in bacterial challenge of rainbow trout following administration of glucan has also been reported (Anderson and Jenny, 1993).

1.7.4. Antibodies

Antibodies, on the other hand, inhibit pathogens by forming an antibody-antigen complex that inactivates the binding sites of bacteria to the host's cells. They produce no toxic metabolites and have no side effects. There is no possibility of overdosing with the continuous use of antibodies (Coleman, 1999). The use of antibodies is not restricted to bacterial and fungal diseases but also has application to viral infection and neutralization of venom (Hatta et al., 1997). All these factors suggest antibodies may have the potential to partially replace antibiotics in the future.

1.7.5. Passive immunization

Passive immunization involves use of an antibody raised in other animals to provide extended disease resistance to the target animals or humans. This approach has been investigated as an alternative to vaccination for prophylactic purposes, and to antibiotic therapy for therapeutic effects (Carlander et al., 1999; Bartz et al., 1980; Ebina et al., 1990; Marquardt, 1999; Hatta et al., 1993b; Yokoyama et al., 1992; Gutierrez et al., 1994). In fish, especially with respect to certain species, passive immunization may be considered as a potential alternative to vaccination (Hatta et al., 1997). Oral or systemic administration of specific immunoglobulins to certain antigens (bacteria, virus, venom, toxin, etc.) may be applied to neutralize biological
activities of the antigens. However, administration of large amounts of antibody may be required in passive immunization (Hatta et al., 1997). Akhlaghi (1999) passively immunized rainbow trout using anti-\textit{V}.anguillarum antibodies raised in sheep, rabbits or rainbow trout via intraperitoneal or oral routes.

1.8. Edible antibody

New generation vaccines such as recombinant, antigen purified and DNA vaccines are poorly immunogenic due to lack of an innate immune stimulus. Therefore, search of new adjuvants for these vaccines has become a topic of interesting. In new adjuvant development, saponins are outstanding candidates. Many of the saponins have been found to have adjuvant effects on purified protein antigens. The chemical structures of the saponins are related to their adjuvant activities, and influence the nature of the immune responses. (Songa and Hua, 2009). As these plant-originated adjuvants may promote different branches of the immune system, the adjuvants have the potential to be used in design of new vaccines so as to induce a desired immune response.

The discovery and use of antibiotics and vaccination in animal agriculture have evolved from the management of small poultry flocks in the era prior to 1890s (Wehman, 1892) to the large consolidated units of today (Cook, 2000). The antibodies present in egg yolk have been termed IgY (Hatta \textit{et al.}, 1990). Thus, it is possible to obtain pathogen-specific IgY antibody from eggs laid by hens immunized against antigen (Bartz \textit{et al.}, 1980; Shimizu \textit{et al.}, 1988). Since poultry farming is carried out on a large scale globally, eggs may be a suitable source of antibody for passive immunization, which requires large amounts of antibodies.
In a period of 6 weeks, one immunized hen produces 298 g of IgY, which is much higher than the serum antibody (16.6 mg) obtained from one rabbit (Nakai et al., 1994). Moreover, due to the phylogenetic distance between birds and mammals (Jensenius et al., 1981), chickens produce more specific antibodies against mammalian antigens than do mammals. For example, BSA (Ermeling et al., 1992) and human serum (Loösch et al., 1986) are more antigenic to avian species than to mammals. The IgY is superior to serum antibody due to higher levels of specific antibodies (Orlans, 1967; Rose et al., 1974) and relative ease of purification (Akita and Nakai, 1992) with low cost (Polson et al., 1980). The egg yolk antibody has also an advantage over the serum antibody because of its compatibility with modern animal protection regulations (Gottstein and Hemmeler, 1985).

1.8.1. Chicken egg yolk antibody (IgY)

Immune responses by producing antibodies (immunoglobulins) against foreign materials (antigen) are important for chickens to protect themselves from infection. In laying hens, the immunoglobulin, IgG in the blood is efficiently transferred across the follicular epithelium of ovary and accumulated in the yolk during oogenesis (Rose and Orlans, 1981). Chickens store high contents of IgY in the yolk and are considered to be efficient antibody producers (Gottstein and Hemmeler, 1985). Laying hens can transfer large amounts of immunoglobulin from serum to egg yolk of their eggs, where it serves as a means of passively protecting the developing chicks (Kariyawasam et al., 2004; Rosenberger et al., 1985). The availability of large amounts of relatively inexpensive IgY from egg yolks makes it feasible to use these antibodies for passive immunization by oral administration or injection (Carlander et al., 2000).
When producing antibodies from chicken eggs, it is common to immunize the chickens when they start laying eggs. Bollen and Hau (1999a) compared the productivity of young chickens immunized at the beginning of the egg-laying period with older chickens immunized during the latter half of the egg-laying period. The older chickens had consistently higher titers than the younger chickens, although the difference was not always significant (Bollen and Hau, 1999a). Arasteh et al. (2000) has clearly shown that apparently therapeutic levels of IgY can be attained in salmonids when orally treated with this immunoglobulin.

Specific IgY development and production can be achieved by immunizing laying hens with the target antigen. However, the resulting immune response of the immunized hens cannot be very predictable. Mainly five factors influence this response: the antigen (dose and molecular weight), the type of adjuvant used, the route of application, the immunization frequency, and the interval between immunizations (Schade and Hlinak 1996).

1.8.2. IgY technology

IgY technology is a highly innovative and expanding branch of biotechnology which offers many advantages: it is produced by a non-invasive method which does not cause pain to animals or lead to their death, since it is based on the simple act of collecting eggs. Hens are inexpensive to keep than rabbits and, furthermore, the IgY production of a hen corresponds to that of a large mammal (Schade et al., 2007). Recent research has concentrated on methods of isolation, purification, and identification of specific antibodies from egg yolk. These applications get into the medical and veterinarian fields. For example, hens were exposed to a specific strain of enteric microorganisms to produce antibodies to the organism in the egg yolk (Mime and Yoshimasu, 1998).
The first step of IгY separation from egg yolk usually involves in the
extraction of IгY from yolk. One of the major obstacles in isolating IгY from egg yolk
is a high concentration of lipids and lipoproteins (Hansen et al., 1998; Verdoliva
et al., 2000). The water dilution method yielded IгY in the highest level (91%), purity
(31%) and with same level activity to that obtained by using other methods as well.
Deignan et al. (2000) evaluated the dextran sulphate, the phosphotungstic acid, the
polyethylene glycol and the isopropanol-acetone methods. The dextran sulphate, the
phosphotungstic acid methods were both the better methods with regard to IгY
recovery, followed by Polson method (Polson et al., 1985). The use of isopropanol-
acetone method gave the lowest recovery.

The choice of suitable IгY extraction method is mainly influenced by the
quality of extraction (preservation of antibodies activity, purity and recovery of
antibodies), scale of extraction (laboratory or industrial), cost effectiveness and
technology. For example for a wide use of IгY in food application, large-scale
production of IгY with high recovery and purity are necessary. Such a separation
process should be simple, economical and requiring few chemicals. In view of these
requirements, the water dilution method appears to be the most appropriate technique.

Lipids and proteins are the major constituents of egg yolk. The lipid fraction,
including triglycerides, phospholipids, and cholesterol, constitutes approximately one
third of the yolk. Proteins consist 15 to 17% of the yolk, which can be separated by
centrifugation into two main fractions, the granule (precipitate on centrifugation) and
the plasma (clear fluid supernatant on centrifugation) (Stadeklman and Cotterill,
1977).
1.8.3. IgY in aquaculture

In aquatic species, IgY against *Edwardsiella tarda* was administered orally to passively immunize Japanese eels (Mine and Kovacs-Nolan, 2002). These studies demonstrated that IgY could serve as an effective means against bacterial and viral infections (Van Nguyen *et al.*, 2010). Country fowl, *Gallus domesticus* was immunized with inactivated WSSV and produced anti-WSSV IgY by with and without hot water extracts of the immunoadjuvant, *Asparagus racemosus*. IgY and anti-WSSV IgY coated with artificial diets and fed to the shrimp *P. monodon* juvenile for 30 days after growth characteristics are improved. And the immunological parameters such as phenol oxidase, NBT assay and lysozyme activity increased (Kumaran *et al.*, 2010).

The egg yolk has been shown to be a convenient source of polyclonal antibodies. Egg-yolk IgY can be used as an inexpensive and effective source of antibodies for the passive immunization of animals suffering from intestinal diseases (Marquardt *et al.*, 1999). In aquatic animals, specific IgY protection against *Edwardsiella tarda* in Japanese eel (Gutierrez *et al.*, 1993) and *Yersinia ruckeri* in rainbow trout (Lee *et al.*, 2000) by oral passive immunization has been demonstrated. In shrimp, only one species of which effect of passive immunization of anti-WSSV IgY derived from truncated fusion protein of WSSV was initially reported to provide a good protection in *Penaues chinenis* from the viral infection (Kim *et al.*, 2004).

The success of oral administration appears to be dependent on the gastric conditions of the animal, since a significant amount of IgY given orally is considered to be degraded and inactivated at this stage (Shimizu *et al.*, 1988). Parenteral administration of IgY to shrimp has been reported to prevent mortality successfully after bacterial challenge for a 17 days period (Lee *et al.*, 1997). It is to be expected
that studies on the therapeutic or prophylactic use of IgY Abs will be intensified in future. In particular, because of the increasing resistance to microorganisms to antibiotics, research on all aspects related to the development of specific IgY against pathogenic microorganisms will have to be intensified. IgYs can be used both in veterinary medicine and in human medicine. Recently, chicken antibodies have been widely used as primary antibodies for ELISA, western blotting and immunohistochemistry techniques (Hatta et al., 1997).

Akhlaghi (1999) obtained significant protection against vibriosis up to a month post IP injection of rainbow trout with anti-\textit{V. anguillarum} antibodies, while non-immune sera did not confer any protection. Passive immunization using pathogen-specific antibodies raised in chickens is a potential method for the protection of teleost against diseases. The oral delivery route offers technical and economic advantages. In this study, the water-soluble fraction (WSF) of the egg yolks of vaccinated hens was used as a source of anti-Vibrio anguillarum IgY in intraperitoneal (IP) injection, oral intubation, or feeding of rainbow trout. IP-injected anti-Vibrio IgY was transferred into the trout system in high enough levels to confer protection against vibriosis in an experimental challenge. This protective effect was retained at least 14 days post IgY injection and proved the efficacy of pathogen-specific IgY in enhancement of disease resistance. Oral intubation and feeding of anti-Vibrio IgY resulted in different levels of protection against vibriosis (Nikoo Arasteh \textit{et al.}, 2004).

1.9. Immunoadjuvants

Adjuvants were initially thought to be agents capable of promoting and sustaining antibody response. However, new evidence has shown that adjuvants
influence the titer, duration, isotype and avidity of antibody, and affect the properties of cell-mediated immunity (Hunter et al., 1995). Adjuvants can be used to improve the immune response to vaccine antigens for several different purposes, including: (1) increasing the immunogenicity of weak antigens; (2) enhancing the speed and duration of the immune response; (3) modulating antibody avidity, specificity, isotype or subclass distribution; (4) stimulating cell mediated immunity; (5) promoting the induction of mucosal immunity; (6) enhancing immune responses in immunologically immature or senescent individuals; (7) decreasing the dose of antigen in the vaccine to reduce costs or (8) helping to overcome antigen competition in combination vaccines (Singh and O'Hagan, 2003).

In most vaccines for mammals and poultry, adjuvants are a crucial ingredient for efficacy. The development of furunculosis for fish clearly demonstrated the necessity of using adjuvant injectable vaccines to obtain an acceptable level of protection. In salmonids, a single injection of vaccines with aluminium based adjuvant or glucans induced an acceptable protection, however, the duration is relatively short (Midtlyng et al., 1996).

1.9.1. Demerits of chemical adjuvants

However, the immunization of hens without use of any adjuvant also results in acceptable antibody (Ab) titres (Calzado et al., 2001). There are many adjuvants, which differ in their chemical characteristics, their efficacy in stimulating the immune system, and their secondary side-effects in the inoculated hens. The most common route for antigen injection in hens is the intramuscular (i.m.) route. Extensive studies by Schwarzkopf et al. (2000) compared immunisation by the i.m. or the subcutaneous
(s.c.) routes. It could be shown that the s.c. antigen injection provoked a higher Ab titre than injection via the i.m. route. The intravenous route (i.v.) should only be used without adjuvants (Calzado et al., 2001).

1.9.2. Herbal immunoadjuvants

Recently, herbal immunoadjuvant is highly influenced to enhance the antibody production in animal models (Patwardhan, 2000). Moreover, the alternative herbal substances possessing the interesting properties like non-toxic, biodegradable and biocompatible (Citarasu et al., 2003). Medicinal plants including *Withania somnifera, Tinospora cordifolia* and *Asparagus racemosus*, demonstrated significant immunostimulatory activity particularly at humeral level in experimental systems with (or) without included immunosuppression (Ziauddin et al., 1996). The production of anti WSSV yolk antibody, IgY using inactivated WSSV and herbal immunoadjuvant extract, *Asparagus racemosus* through the hen *Gallus domesticus* (Kumaran et al., 2010).

1.9.2. Immunoadjuvant saponin

Experiments demonstrating the physiological, immunological and pharmacological properties of saponins have aroused considerable clinical interest in these substances. In animal system, various studies have shown the effect of saponins on cell membrane, animal growth and feed intake, protein digestion, cholesterol metabolism, animal reproduction, the immune system, nervous system functioning or cytostatic effects on malignant cells or molluscicidal effect or virucidal activity or effect on protozoa (Francis et al., 2002).
Saponins have been widely used as adjuvants for many years and have been included in several veterinary vaccines. The adjuvant action of saponins was, however, not so pronounced in some of the non-mammalian species tested (Cossarini-Dunier, 1985; Grayson *et al.*, 1987). Triterpenoid saponins have been detected in many legumes such as soybeans, beans, peas, lucerne, etc., and also in alliums, tea, spinach, sugar beet, quinoa, liquorices, sunflower, horse chestnut and ginseng. Saponins from soybean have been separated into six (Khalil and El-Adawy, 1994) or eight (Oda *et al.*, 2003) fractions of soyasapogenol and soyasaponins groups. Oda *et al.*, (2000; 2003) found that the soyasaponins exhibited high adjuvant activity while the soyasapogenol group exhibited low activity.

New vaccine technology has led to vaccines containing highly purified antigens with improved tolerability and safety profiles, but the immune response they induce is suboptimal without the help of adjuvants. However, there are no reports available on an immunoadjuvant effect of egg yolk antibodies against pathogenic *Vibrio* sp. In addition to their role in infectious disease prevention, current and future adjuvants are likely to play a valuable role in therapeutic vaccines for vibriosis.
Objectives of research

The research presented here tested the hypothesis that immunoadjuvant saponins isolated from *Asparagus racemosus* and *Glycine max* (soybean) would enhance growth and improve immune responses in shrimps.

The hypothesis of this study is as follows

- To isolate the virulent Vibrio sp such as *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* from the infected shrimp farms and marine tidal sediments.

- To identify and purify the virulence factors from the above pathogens.

- To immunize the proteins in chick, *Gallus gallus domesticus* by with and without suitable herbal adjuvant and get the edible antibody IgY.

- To purify the edible antibody IgY using suitable methods, coated with artificial shrimp feeds and to be fed at different stages of shrimps.

- To study the efficiency of the IgY as a laboratory trials against the pathogenic virulent Vibrio sp. to the challenged and analyze the biochemical, haematological and immunological parameters.

- Field trails for the edible antibody IgY fed shrimp post larvae for different intervals.