PREVALENCE SURVEY OF NOROVIRUS IN PACIFIC WHITE SHRIMP *(Litopenaeus Vannamei)* IN THAILAND

SANIGAN THONGSAWAD

MASTER OF SCIENCE
IN VETERINARY PUBLIC HEALTH

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Thesis title Prevalence Survey of Norovirus in Pacific White Shrimp (Litopenaeus Vannamei) in Thailand

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ABSTRACT

Norovirus is an important gastroenteritis virus in humans; it is excreted by infected persons, and a virus containing faeces may contaminate water. Because of withstanding waste water treatment the virus has been found in fresh and sea water, mainly in sewage polluted areas. Norovirus is transmitted through water or by direct route from infected people to different kinds of food. Outbreak reports show that seafood is one important sources of norovirus.

Based on the accessible literature this thesis is the first study to investigate the contamination of shrimps with norovirus. The objectives of the survey were to determine the prevalence of norovirus in cultured Pacific white shrimps (Litopenaeus vannamei) which became the shrimp species of highest economic importance. Two hundred and forty (240) shrimp samples have been drawn at Talaythai auction market, the largest shrimp market in Samut Sakorn / Thailand, from December 2004 to January 2005.

The real time reverse transcriptase polymerase chain reaction (real time RT-PCR) was performed to detect norovirus genogroup II using QuantiTectTM Probe RT-PCR kit. With a detection line of 100 virus particles/extract no positive reactors were found. On the basis of the samples taken on this shrimp market the prevalence of norovirus in aquacultered Pacific white shrimps from Thailand, if present, can be assumed to be less than 1.2 percent ($\alpha = .05$).

Thus, during the study period farmed shrimps were not or, if at all, very low contaminated at the market level. Contamination from rearing, culturing, harvesting and transportation at the moment of sampling is considered very unlikely. Virological testing of shrimps seems to be a less effective tool to characterize the risk of human norovirus infection caused by shrimp consumption. Monitoring and preventive measures should concentrate on personal hygiene and supervision of the health status of the persons involved.

ชื่อเรื่องวิทยานิพนธ์ การสำรวจความชุกของเชื้อโนโรไวรัสในกุ้งขาวแปซิฟิก ในประเทศไทย

ผู้เขียน นางสาวศนิกานต์ ทองสวัสดิ์

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บทคัดย่อ

โนโรไวรัสเป็นเชื้อไวรัสก่อโรคทางเดินอาหารอักเสบที่สำคัญในคน ผู้ป่วยจากการติดเชื้อโนโรไวรัสสามารถแพร่เชื้อผ่านทางอุจจาระ และเนื่องจากเชื้อนี้ทนต่อการฆ่าเชื้อในกระบวนการบำบัดน้ำเสียได้ ดังนั้นจึงอาจพบการปนเปื้อนในแหล่งน้ำ ทั้งน้ำจืด และน้ำทะเล โดยเฉพาะในแหล่งน้ำเสีย โนโรไวรัสสามารถแพร่เชื้อผ่านน้ำ หรือ แพร่เชื้อโดยตรงจากผู้ป่วยไปยังอาหาร จากรายงานการระบาดของเชื้อโนโรไวรัสพบว่าอาหารทะเลเป็นแหล่งแพร่เชื้อที่สำคัญแหล่งหนึ่ง

จากการสืบค้นฐานข้อมูล การศึกษาครั้งนี้จะเป็นการศึกษาการปนเปื้อนของเชื้อโนโรไวรัสในกุ้งเป็นครั้งแรก จุดประสงค์เพื่อหาความชุกของเชื้อโนโรไวรัสในกุ้งขาวแวนนาไม (Litopenaeus vannamei) จากการเพาะเลี้ยงซึ่งเป็นกุ้งที่มีความสำคัญทางเศรษฐกิจมากที่สุดของประเทศ กุ้งจำนวน 240 ตัวอย่างได้ทำการสุ่มเก็บจากตลาดทะเลไทย จังหวัดสมุทรสาคร ซึ่งเป็นตลาดกลางกุ้งที่ใหญ่ที่สุดในประเทศ ในระหว่างเดือนธันวาคม 2547 ถึงเดือนมกราคม 2548

ตัวอย่างทั้งหมด ได้ทำการศึกษาโดยใช้ Real time reverse transcriptase polymerase chain reaction (Real time RT-PCR) เพื่อตรวจหาโนโรไวรัส จีโนกรุ๊ปสอง ไพรเมอร์ที่ใช้ได้แก่ NV107a (5'-AGCCAATGTTCAGATGGATG), NV119 (5'-TCGACGCCATCTTCATTCAC) และ TaqMan probe TM3 (GGII) (5'-TGGGAGGGCGATCGCAATCTGGC) จากจำนวนเชื้อต่ำสุด 100 อนุภาคไวรัสที่วิธีการนี้สามารถตรวจได้ ไม่พบเชื้อโนโรไวรัสในทุกตัวอย่าง จากการสุ่มเก็บตัวอย่างที่ตลาดกลางกุ้ง คาดว่าถ้ามีเชื้อโนโรไวรัสในประเทศไทย ความชุกของเชื้อโนโรไวรัสในกุ้งขาวแวนนาไมจากการเพาะเลี้ยงน้อยกว่า 1.2 เปอร์เซ็นต์ (α= .05) ดังนั้น ในการศึกษาครั้งต่อไปควรจะเพิ่มจำนวนตัวอย่างให้มากขึ้น

ดังนั้นอาจสรุปได้ว่าในระหว่างทำการศึกษา ไม่มีการปนเปื้อนของเชื้อโนโรไวรัสระหว่างเลี้ยงในฟาร์ม หรือถ้าจะมีการปนเปื้อนที่ตลาดก็อยู่ในระดับที่ต่ำมาก และไม่น่าจะมีการปนเปื้อนระว่างการเพาะเลี้ยง การจับรวมถึงการขนส่งในช่วงที่มีการเก็บตัวอย่าง การศึกษาทางไวรัสวิทยาจากกุ้งโดยตรง ยังไม่ใช้วิธีการที่เหมาะสมที่สุดสำหรับการหาความเสี่ยงของเชื้อนี้ที่ส่งผลต่อผู้บริโภค การตรวจติดตามและการป้องกันควรเน้นการสุขลักษณะที่ดีในการปฏิบัติ รวมถึงการตรวจสอบสุขภาพของบุคคลที่เกี่ยวข้องในกระบวนการผลิต

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

× g	gravity
µl	microlitre
CMS	Cramped muscle syndrome
dNTP	deoxynucleotidetriphosphate
DNA	Deoxyribonucleic acid.
ELISA	Enzyme-linked immunosorbent assay
EM	Electron Microscopy
HE	Hemacytic enteritis
IEM	Immune Electron Microscopy
IHHNV	Infectious Hypodermal and Haematopoietic Necrosis Virus
L. vannamei	*Litopenaeus vannamei*
M. rosenbergii	*Macrobrachium rosenbergii*
MBV	Monodon Baculovirus
MWCO	Molecular Weight Cut Off
NoV	Norovirus
P. monodon	*Penaeus monodon*
PCR	Polymerase Chain Reaction
ppt	parts per thousand
RDS	Runt-deformity syndrome
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SPF	Specific pathogen free
SPR	Specific pathogen resistant
TSV	Taura Syndrome
WSS	White Spot Syndrome

WSSV	White Spot Syndrome Virus
YHD	Yellow-head disease
YHV	Yellow-head virus

1. INTRODUCTION AND OBJECTIVES

Shrimps have been food for human for a long time. They are found throughout the entire world. They are not only a luxury-delicious food but also high in nutrient density. Because shrimps are an excellent source of selenium, vitamin D and B12 and are an unusually low-fat, low-calorie protein, shrimp as a nutrient helps to decrease the risk of cardiovascular diseases and is also useful in the prevention of cancer (De Oliveirae Silva *et al*., 1996; Bugel *et al*., 2001; Wang *et al*., 2005).

Thailand's frozen seafood exports fall into five main categories. Shrimps, prawns and lobsters are designed as one category, called crustacean. The other four categories are cuttlefish, squid, octopus and fish. Among them the category of shrimps, prawns and lobsters plays the most important role as a foreign exchange earner.

In 2003, about 80 percent of the total amount of shrimps in the world was produced by 5 countries. These were China (400,000 tons), Thailand (350,000 tons), Indonesia (168,000 tons), Vietnam (110,000 tons) and India (100,000 tons). Although China is the world's largest producer, Thailand is the major exporter in world shrimp export. It exports 10.11% of the world imports (Ministry of Commerce, 2004).

The major shrimp importing countries are the European Union, the United States and Japan with respective average shares of 37.06 percent (630,000 tons), 34.12 percent (580,000 tons) and 17.06 percent (290,000 tons) of the shrimp export from Thailand (Ministry of Commerce, 2004).

Before 1984, ninety percent of shrimps were captured from natural resources along the Gulf of Thailand. Since the early 1980's, shrimp farming in Thailand started and began to expand in the mid 1980s (Boromthanarat and Nissapa, 2000). By 1987 shrimp culture, focusing on Black tiger shrimp *(Penaeus monodon),* took off in Thailand, spreading quickly along the coast. As a result, the structure of shrimp production gradually changed, with half of the total production caught from the open

sea. This trend has steadily increased from that time and cultured shrimps now make up about 70 percent of the total production.

Pacific white shrimp *(Litopenaeus vannamei)* is a native to the pacific coast of Central and South America. It is the leading farmed species in the western hemisphere. It was introduced to Taiwan and China in 1998 and became a popular species for culture. In Thailand, it was introduced in 2002 and became a good candidate for aqua-culture since Black tiger shrimp *(P. monodon)* culture has the problems of not having a uniform size, antibiotic residues and market prices (Boromthanarat and Nissapa, 2000). Thus, farmers throughout Thailand have switched to this species because of its fast growth, high hatching survival, uniform growth rate and its large size. So, Pacific white shrimp *(L.vannamei)* is the dominant species in Thailand now.

Food-related illness is endemic world-wide, and bacterial pathogens have historically been associated with this mode of transmission. In recent years, however, the specific causes of most outbreaks of foodborne illness were still doubtful with a significant proportion presumed to be viral (Koopmans *et al.*, 2003).

Centers for Disease Control and Prevention (CDC) estimated 76 million cases of foodborne illness resulting in 325,000 hospitalizations and 5,000 deaths occurring each year (WHO, 2004). From 1993-1997, 32 percent of outbreaks reported to CDC had a laboratory confirmed etiology; of these 75 percent were bacterial, 17 percent were chemical, 6 percent were viral and 2 percent were parasitical in origin. Thus, 68 percent of foodborne outbreaks were categorized as having an unknown etiology. There are many pathogens that are not routinely tested by the laboratories. For the unidentified etiologic agents, the epidemiological profile using pathogen-specific patterns of symptoms and other epidemiological characteristics leads to the conclusion that of 712 outbreaks 47.8 percent matched the norovirus syndrome and 11.7 percent match the *Salmonella-like* syndrome. After combining information on known pathogens and epidemiological profiles, only 12.4 percent of the outbreaks

remained unclassified. Norovirus outbreaks appear as commonly as *Salmonella-like* outbreaks (Hall *et al*., 2002).

The viruses of greatest interest are the group known as enteric viruses. Pathogenic enteric virus can be classified into different taxonomic groups. As they are also commonly found in water, they can potentially be transmitted by ingestion of water or other aquatic matrices contaminated with faecal waste from an infected individual. Today, the importance of viruses as agents of diseases is well known, and it is clear that the pollution of water with human wastes is a major potential source of serious diseases. Human pathogenic viruses that are commonly found in polluted water are Polio, Hepatitis A, Norovirus, Rotavirus, Reovirus, Parvovirus etc (Abel, 2000). Additional research established the importance of viruses, especially the human caliciviruses were most focused to the genus *Norovirus* (Koopmans and Duizer, 2004). As shrimp is an animal living in water, it may be contaminated by viruses. If they are not well cooked, the virus can infect humans as well. Additionally, a crossover contamination between raw shrimps and other food in the kitchen seems possible. The study of pathogenic enteric viruses in Thailand has not yet been established. This survey will be the first report to investigate epidemiological data which can further be useful.

The aim of this study is:

1. To determine the prevalence of norovirus in export Pacific white shrimp in Thailand
2. To compare the quantity of norovirus in shrimp from different areas

2. LITERATURE REVIEW

2.1 Introduction

Shrimps are animals that belong to the Phylum Arthropoda, Class Crustacea and Order Decapoda. They are small aquatic decapod crustaceans with 10 jointed legs on the thorax and well-developed swimmerets on the abdominal segments. Their bodies are compressed laterally. Globally, there are hundreds of species of shrimp that inhabit fresh, brackish and marine waters. A majority of these species is very small, rare and are not suitable for human consumption. All farmed shrimps and most of the wild ones belong to the Penaeidae and Palaemonidae families. Some of the important farmed shrimps are summarized in Table1.

Table1: Summary of farmed shrimp species

Family	Species	Common name
Penaeidae	*Penaeus monodon*	Black tiger shrimp, Giant tiger shrimp
	Litopenaeus vannamei	Pacific white shrimp, Western white shrimp, West Coast white shrimp, Whiteleg shrimp
	Litopenaeus stylirostris	Western blue shrimp
	Fenneropenaeus chinensis	Chinese white shrimp
	Fenneropenaeus indicus	Indian white shrimp
	Marsupenaeus japonicus	Japanese Kuruma shrimp
Palaemonidae	*Macrobrachium rosenbergii*	Freshwater prawn, Giant river prawn

Source: http://www.shrimpnews.com/Species.html, 2005

Most people have regarded shrimps as a luxury food for many centuries but now they have become more affordable and available since they can be produced with the help of industrial farming.

2.2 Shrimp farming in Thailand

2.2.1 History of shrimp farming in Thailand

In the past, Thailand harvested as much as 90 percent of its shrimps from natural resources, mainly the gulf of Thailand (Lavallee, 1997). Accidentally, it was discovered that salt fields could conveniently be converted into shrimp ponds because they are well linked to the sea through the network of canals. These conditions facilitate shrimp farming operations (Boromthanarat and Nissapa, 2000), and the farmers along the seacoast of the Inner Gulf of Thailand converted the salt fields into shrimp ponds and intensive shrimp farming was developed as from 1980s.

The shrimp culture industry first concentrated on coastal lands in the upper Gulf of Thailand provinces close to Bangkok which are named Samut Sakorn, Samut Songkram and Samut Prakarn (Boromthanarat and Nissapa, 2000). This region accounted for more than 40 percent of the country's total shrimp culture area (Lavallee, 1997). Later on shrimp farmers expanded into areas along the south-western coast adjacent to the Andaman Sea (Smith, 1999). During the market boom in the 1990s, shrimp culturing expanded very rapidly. At that time Black tiger shrimps were the only farmed crustacean and they spread quickly along the coast, Chao Praya Delta and eastern part of Thailand (Lindberg and Nylander, 2001; Szester, 2003).

In the last year, the production of cultured shrimp surpassed the number of shrimps caught from natural resources. In 2003, captured shrimps amounted to 67,000 tons and cultured shrimps to 330,000 tons (DOF, 2004). That means cultured shrimps accounted for 80 percent of the total production.

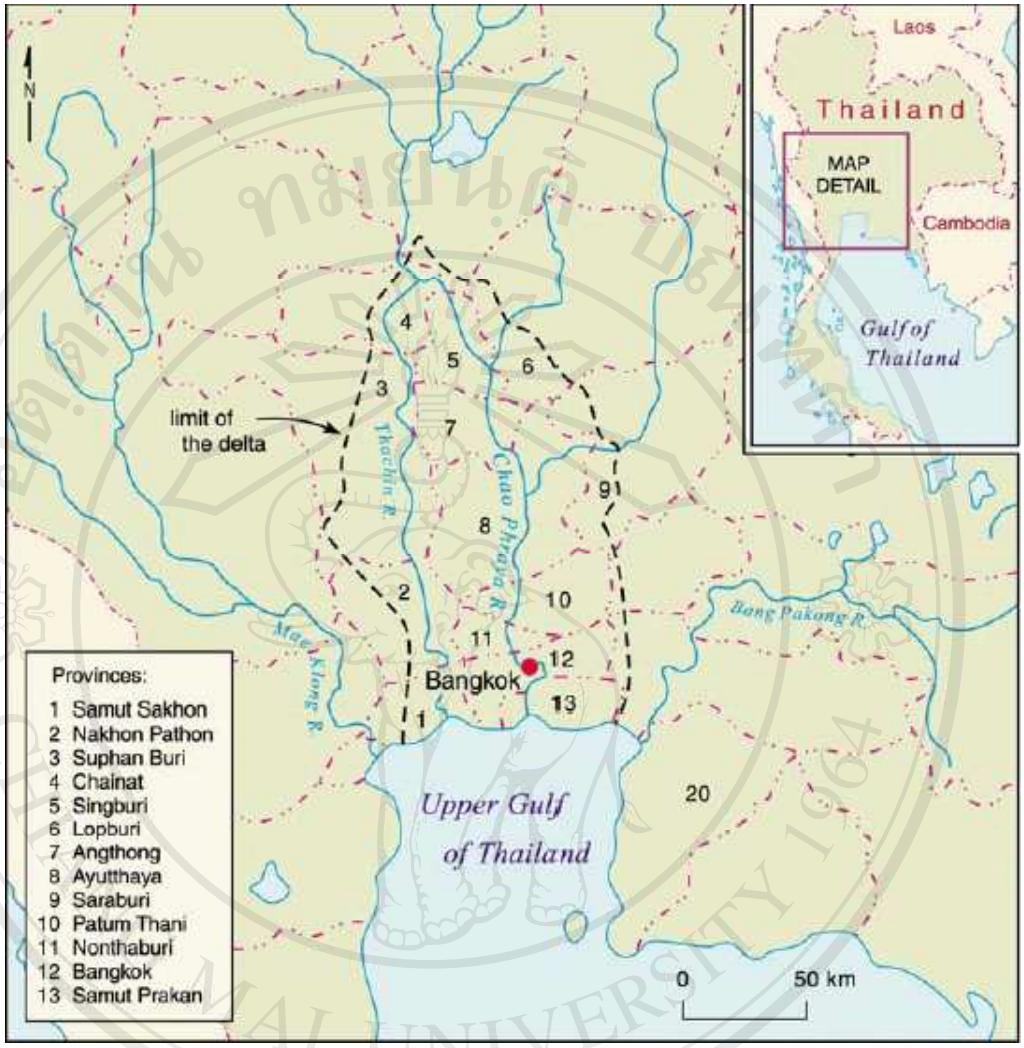

Figure 1: Chao Praya Delta, Thailand

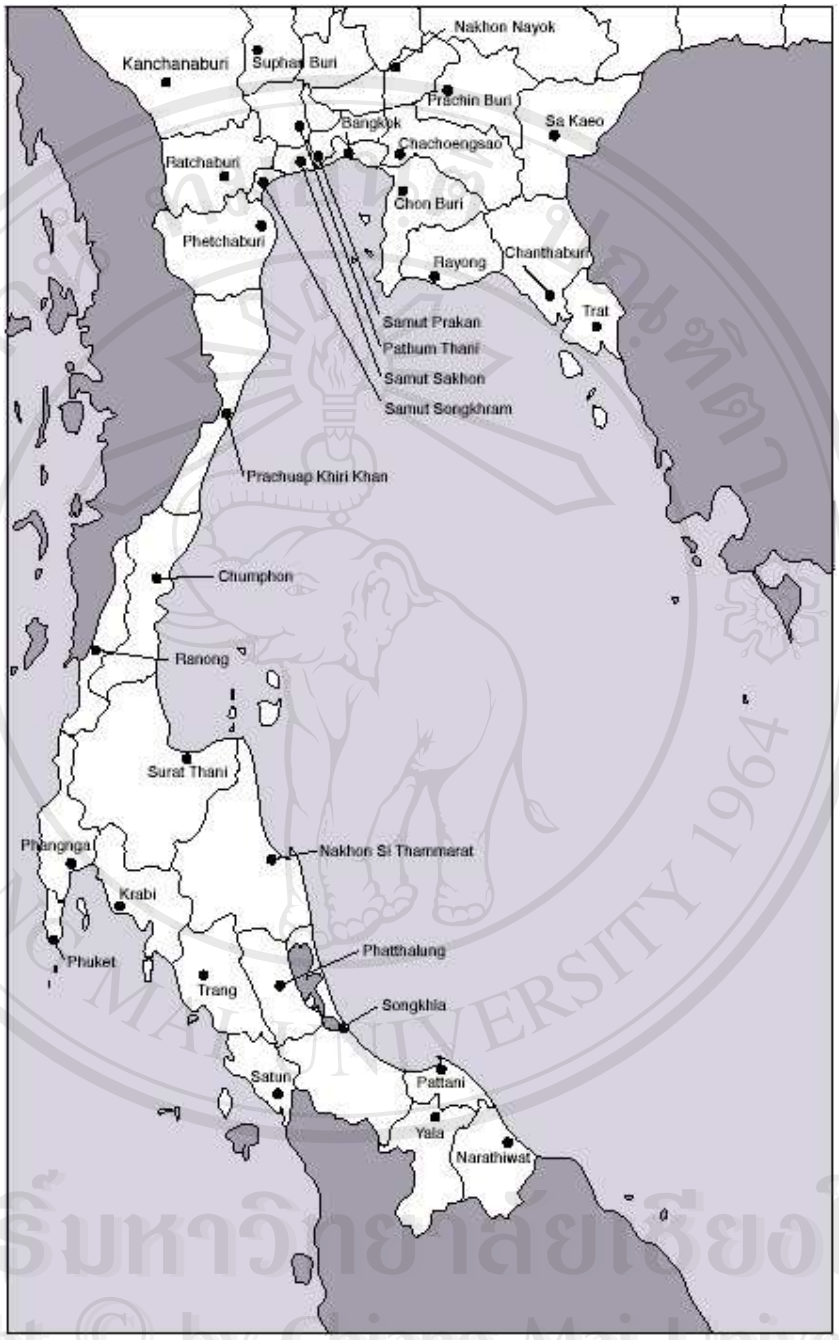

Figure 2: Shrimp farming region of Thailand

With increasing production profits, shrimp farming became the major economic product for Thailand, displacing the United States as the world's top seafood exporter with total sales of $3.4 billion in 1993 (Lavallee, 1997). Thai shrimp exports have increased to the extent that they have 40 percent of the international market (Lavallee, 1997).

Thailand remained the leader in both the production and export of shrimp for decade. However, in 2002 China overtook Thailand with an over all production of 310,700 tons compared to 240,000 tons of Thailand (Ministry of Commerce, 2004). The main reason that caused China to be the leader in shrimp production was the use of Pacific white shrimps. At same time Thailand faced problems with the Black tiger shrimp production. But since China has a high internal demand for shrimp consumption, Thailand is still leading in shrimp export. Thus, in 2002 Thailand exported 136,000 tons of shrimp while China exported only 72,000 tons (Ministry of Commerce, 2004).

Since 1998, the production of Black tiger shrimps in Thailand significantly decreased due to the impact of viral conditions (Jonker *et al*., 2005). But, the import of Pacific white shrimp *(Litopenaeus vannamei)* to Thailand in 2002 (Limsuwan and Chanratchakool, 2004) reversed that trend. This upward trend has been promoted by the high preference of this species by the world's largest shrimp markets, the USA (Briggs *et al*., 2004). The low capital required for Pacific white shrimp farming also induces farmers to choose this new type of shrimp.

2.2.2 Economic impacts on Thai shrimp export

The shrimp industry faces a range of tariff and non-tariff barriers in export markets. For example, the new higher EU GSP on Thai shrimp and the preliminary tariff against anti-dumping announced by the USA (Josupeit, 2004) make Thailand a weak competitor compared to other exporting countries.

The main importing countries (US, Japan and EU) have put a ban on the use of certain antibiotics in shrimp production since 2002 (NFI, 2002), which has forced the industry to undergo costly inspections of antibiotic residues in all shrimp shipments. Since 1997 the outbreak of White Spot Syndrome and Yellow-Head Disease, as well as bacterial diseases repeatedly impacted the Thai shrimp production negatively. Even though these diseases cannot be treated with antibiotics, the farmers still used them frequently. Moreover the outbreaks caused a heavy decrease in shrimp production which led to the collapse of small-scale farms. To overcome this problem the Thai government prohibits imports of 17 antibiotics and chemicals for use in shrimp feeds, and has introduced other measures, to make sure that Thai shrimps are free of antibiotic residues.

2.2.3 Ecological impact

During the shrimp boom in the 1990s, Thai rice farmers converted their coastal fields, and the bordering mangrove forests into shrimp ponds. The areas just beyond the mangrove forest offer the right conditions for the production of pond shrimp and they have been extensively used for this purpose (Boromthanarat and Nissapa, 2000). Discharge water from shrimp ponds contains silt and organic substances from waste, feed and excretory products of the shrimp as well as some chemicals, for example, fertilizer, disinfectant, lime and other minerals. All these pollute the coastal environment (Potaros, 1995). As a result of this problem, the National Economic and Social Development Board banned production of shrimp cultures in mangrove forest areas (Lindberg and Nylander, 2001). Thus, shrimp cultures then moved to freshwater in inland areas, where farmers brought salt water from the open sea to fill their ponds. Unfortunately, this salt water ends up being released from the ponds and causes salinization of agricultural fields. An area in central Thailand which once produced rice and also shrimps has become an ecological desert (Briggs *et al*., 1993). Therefore, the low salinity shrimp farming technique (Szester and Flaherty, 2000) was developed since shrimp farmers had discovered that Black tiger shrimp post-larvae could be acclimatized to low-salinity environments. This method involves mixing the high salinity water with freshwater to give a final salinity of between 3-5 ppt.

There are several problems on low salinity shrimp farms: these include soil salinization, water pollution and competition for freshwater between agricultural and aqua-cultural farmers (Jenkins *et al.*, 1999; OEPP, 1996). The danger of soil salinization and water pollution caused by low salinity shrimp farming in agricultural areas started a public debate between shrimp farmers and anti-shrimp farming groups. Prawn culturing in freshwater areas was banned at the end of December 1998 as a result of its damaging effects on agricultural lands (Higano and Pitchitkul, 2000). In spite of the ban, shrimp production within the Chao Phraya Delta continued uninterrupted using less polluting systems (Szester, 2003). In the coastal provinces, brackish water areas where shrimp farming would be permitted and, freshwater zones where shrimp farming would be restricted, were identified and demarcated (The Nation, 2001).

2.2.4 The shrimp production chain

There are many stakeholders involved in shrimp production: broodstock and hatcheries, farms, auction markets, food and processing industries and infrastructure supporting industries (Figure 3).

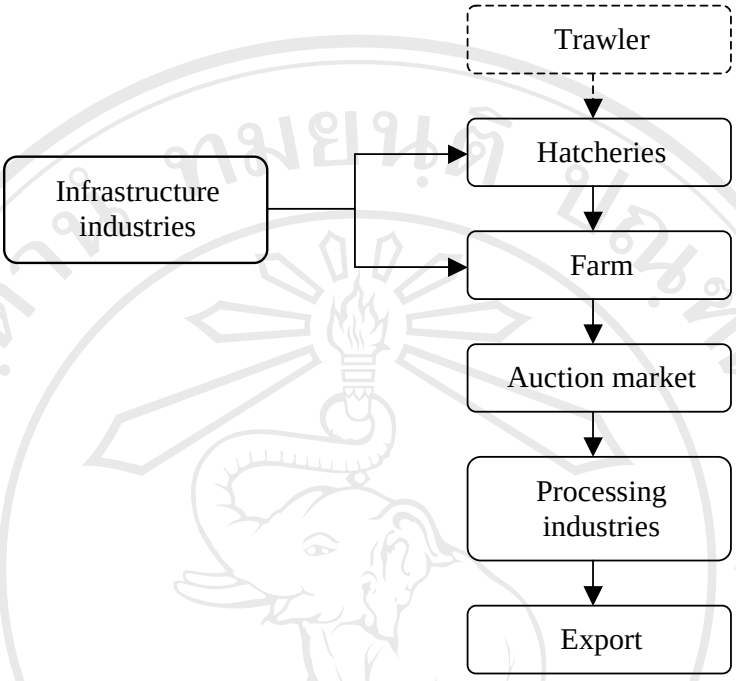

Figure 3: The stakeholders in shrimp production

2.2.5 Shrimp farming systems

There are two shrimp farming systems in Thailand. Based on stocking levels and pond sizes, the farming systems are classified as extensive, semi-intensive and intensive (Primavera, 1994). Further, the farming systems are divided on the basis of water exchange on open farms, re-circulation and minimal water exchange or semi-closed intensive ponds.

2.2.5.1 Farming systems according to stocking level and pond size

2.2.5.1.1 Extensive or traditional culture

The extensive system is the traditional shrimp aquaculture (Lindberg and Nylander, 2001). Ponds are normally filled with water using gravity during the high

tide period and with natural seeds. Stocking relies on the shrimp larvae that are naturally present in seawater. The density of shrimps is usually low and no control over species composition is possible (Boromthanarat and Nissapa, 2000). The tidal flows provide naturally occurring food organisms for the shrimps. Sometimes animal manures and chemical fertilizers are added to the pond water to accelerate the growth of plankton. Although a small number of farmers use water pumps, the system depends predominantly on the channeling of water in and out by the tides. The rate of flow is regulated by sluice gates. The farming technique requires no special skills or infrastructure. Input costs are minimal due to use of natural resources. These ponds are normally harvested partially. Yield is low and the cost of production is also low (Primavera, 1994; ASEAN, 1998; Lindberg and Nylander, 2001)

2.2.5.1.2 Semi-intensive culture

The natural seawater shrimp larvae are supplemented with hatchery-grown larvae to a moderate stocking density (Boromthanarat and Nissapa, 2000). Paddle aerators are used to maintain adequate oxygenation of the water, and pumps facilitate water exchange. Predator and pest elimination is carried out as necessary (Boromthanarat and Nissapa, 2000). Yield is increased by measures including raising the densities of larvae stocking, water management and other techniques, for example, feeding and health management. Cost of production is higher compared with the extensive culture. On the other hand yield increases, and more profit can be expected (Lindberg and Nylander, 2001).

2.2.5.1.3 Intensive culture

Intensive culture practices are to achieve the maximum yields and profit. Shrimp farms using intensive production methods produced around 90 percent of the total annual harvest in Thailand in 1991 (Lindberg and Nylander, 2001). Shrimp larvae from hatcheries are stocked with the possible highest density (Lindberg and Nylander, 2001). Addition of feed to the pond is essential and all factors affecting production such as water quality, water exchange and aeration require constant monitoring and

regulation. The pond sizes are usually smaller than those used in extensive and semi-intensive culture (Primavera, 1994, Boromthanarat and Nissapa, 2000). But, this system provides significantly high yields. In this system the culture of shrimp is carried in a two-step process. The latter is composed of a broodstock-hatchery phase for producing seed or post-larvae and a grow-out phase usually in earthen culture ponds for growing the fry to market size (Primavera, 1994).

2.2.5.2 Farm systems based on water circulation

2.2.5.2.1 Open farm system

In open farm systems, the water supply comes directly from good quality sources and large amounts of daily water must be exchanged to maintain suitable water quality (Lindberg and Nylander, 2001). This system has recently become less favorable to farmers, since the environmental conditions, especially the quality of water, tend to deteriorate over a time (ASEAN, 1998).

2.2.5.2.2 Re-circulation system

The re-circulation system is used to minimize contacts with poor quality water from outside the farm. This system needs more area and capital (ASEAN, 1998). Water reservoirs, sedimentation ponds, treatment ponds and drainage canals are needed. The clean seawater is pumped into the ponds and kept within the system. During the culture period, the effluent from the ponds is drained into the sedimentation pond, treated with chemicals and pumped into the reservoir for re-supply to the culture ponds (ASEAN, 1998). Normally in Thailand, since the shrimp farms are small-scale, this system is not favorable.

2.2.5.2.3 The minimal water exchange system or semi-closed intensive pond system

This system is the most commonly practiced particularly in Thailand (Lindberg and Nylander, 2001). It is suitable for small-scale farmers who cannot afford the construction of the water treatment ponds and reservoirs as in the case in re-circulation systems. Due to high farm densities, deterioration of the effluent water and the increase in viral diseases, there has been developing resistance amongst farmers to the use of the high water exchange. In order to avoid deterioration of the environmental conditions, several farmers have changed to the minimal water exchange system (Lindberg and Nylander, 2001). The water is treated in the grow-out ponds and there is no water exchange in the ponds during the first two months of shrimp culture. The system does not require water exchange. It is only the loss of water caused by seepage and evaporation that must be compensated (ASEAN, 1998).

2.3 Important farmed shrimps in Thailand

In Thailand, there are 3 important farmed shrimps for export: Freshwater prawn *(Macrobrachium rosenbergii de man)*, Black tiger shrimp *(Penaeus monodon)* and Pacific white shrimp *(Litopenaeus vannamei)*.

2.3.1 Freshwater prawn

The Freshwater prawn or Giant river prawn *(Macrobrachium rosenbergii de man)* is one of the major commercial aquacultural animals. They are mainly for domestic consumption. The natural distribution extends from Pakistan to southern Vietnam, across Southeast Asia and south to northern Australia (Mather and Bruyn, 2003). They are found in most inland freshwaters such as lakes, rivers, swamps as well as estuarine areas (New, 2002). The species is cultured in freshwater areas in every part of Thailand.

Freshwater is normally used for rearing Freshwater prawns from postlarvae to market size. However, the shrimp requires brackish water during its early life stages. Adaptation to freshwater happens later (Xianle and Yanping, 2003). Frehwater shrimp tolerate partially saline water up to 10 ppt, but they do not grow well at this level of salinity. Thus, the Giant river prawn develops much better in fresh water (New, 2004).

The water supply is very important to rear these prawns. Surface water of accessible rivers, lakes, reservoirs, irrigation, canals, etc. is most commonly used. The other choices are underground water and well water which are suitable due to their chemical and microbiological quality, less polluted and provide less likelihood of the introduction of unwanted insects into ponds (Drewes, 2002; Tidwell *et al*., 2002). Pond preparation follows the same technique as described for the Pacific white shrimp (section 2.3.3).

The cycle of Freshwater prawn culture can be summarized as follows: The prawn broodstocks are selected from pond-rearing or caught in the wild. Under natural conditions, mating occurs throughout the year, although there are sometimes peaks of activity related to environmental conditions. In tropical areas, these coincide with the onset of the rainy season, whereas in temperate areas they occur in summer. In hatchery conditions, female Freshwater prawns lay eggs 3-4 times per year (New, 2004). The number of eggs depends on the size of the female. Fully mature female Freshwater prawns lay between 80,000 to 100,000 eggs during one spawning. However, their first broods are often not more than 5,000 to 20,000 (New, 2000). Prawns breed and spawn in warm freshwater. Fertilizing takes place between a soft-shell female which has molted to shed her shell and a hard-shell male (New, 2000). Using its long claws, the male embraces the female and protects her for 1-2 days until her shell hardens. The male deposits sperm contained in a gelatinous mass between the walking legs of the female. A few hours after mating, the fertilized eggs are laid. The female incubates the eggs at the underside of her abdomen.

The eggs hatch into larvae and the process is normally completed for the whole brood within one or two nights. The larvae are then dispersed by rapid movements of the abdominal appendages of the mother. Freshwater prawn larvae require brackish water for survival (New, 2002). Those that hatch in freshwater will die unless they reach brackish water within a few days. Freshwater prawn larvae feed on plankton. They swim with the tail and ventral side up (upside down). The juvenile shrimp need 10-15 ppt salinity for 10 days, after that the salinity may decrease until the salt water becomes fresh water.

The larvae eat continuously. The feed varies widely at this stage but typically it includes brine shrimp or artemia (New, 2000). The prawns complete their larval life into post-larval (PL) stages, the latter swim normally dorsal side up and forwards. To reach the postlarval stage, the larvae must undergo 11 molts in approximately 10 days (Tidwell *et al*., 2002). Post-larvae can tolerate a wide range of salinities. This quality is a characteristic of Freshwater prawns (New, 2000).

When the fry is released in ponds it has been in the nursery for at least 30 days (Tidwell *et al*., 2002). To rear prawns, one-third of the water needs to be changed every 5-7 days to reduce wastewater. The shrimp molt when they reach new water. Freshwater prawns have to cast their exoskeleton or shell regularly in order to grow. This process is accompanied by a sudden increase in size and weight (New, 2000). Commercial feed is used, and the feed formula adjusted by the feeding company is done in each stage. The time for rearing Freshwater prawns is around 8-10 months. Freshwater prawns are harvested at different sizes dictated by market demands. If the grown up prawns have strong claws they are capable of defending their territory. At this age, prawns should be given enough area to prevent fighting. Overcrowding, bad water quality or poor diet seem to make prawns more aggressive, leading to injury or death, especially during molting (Spotts, 1981). The removal of large, dominate males increasing the chances of the growth of smaller prawns.

To date, Freshwater prawns are not popular with farmers because of their low yields compared to marine shrimp. Thailand produces only 50,000 tons of Freshwater

shrimps compared to 100,000 tons of Black tiger shrimps and 150,000 tons of Pacific white shrimps (Ministry of Commerce, 2004). There are many attempts to promote freshwater prawn cultures in freshwater areas in the central parts of Thailand, instead of the low-salinity cultures of marine shrimps which pollute the soils and the water sources (The Nation, 2001).

The main reason that Freshwater prawns can not be grown as intensively as marine shrimps is due to their fighting behavior. However, reduced risk of crop loss, low capital investment, less labor and minimal effluent discharge from ponds represent some of the advantages of more extensive aquacultural practices. In addition, few diseases associated with freshwater prawn cultures have been reported. The low incidence of prawn diseases may be due to reduced stocking densities and feed inputs, which generally improves water quality and minimizes stress. Freshwater prawn culture may well suit small-scale operators (New, 2000).

Figure 4: Freshwater prawns

2.3.2 Black tiger shrimp

Black tiger shrimps or Giant Tiger Shrimps *(P. monodon)* are named after their huge size and banded tails (Shrimpnews, 2005). Black tiger shrimp used to be the most wildly distributed and marketed shrimp species in the world, but, in recent times they have been superseded by Pacific white shrimps *(L. vannamei)*.

Black tiger shrimps are native to the Indian Ocean from Southeast Africa, through the Red sea and Arabian Gulf to the Indian subcontinent. They occur throughout the Malay Archipelago and the southwestern Pacific Ocean from Japan to Australia (AIMS research, 2000). They are commonly found in Southeast Asia. These shrimp are easy to culture, because they are quite adaptable and thrive in a wide range of salinity. About 80 percent of the Black tiger shrimps in the world market are farmed. They are mainly from various Asian countries.

In Thailand, farming of the Black tiger shrimp started in 1987 along the coastal areas. In the early 1980s, shrimp farms were concentrated in the upper Gulf provinces and mangrove areas. Following the discovery that shrimp farming destroyed mangrove forest areas, the Thai government imposed a ban on shrimp farming in those areas. After that, the low salinity shrimp farming techniques were developed (Szester and Flaherty, 2000) and the Black tiger shrimp cultures then moved to the inland freshwater areas. As far as 200 km from the Gulf of Thailand, Black tiger shrimp farms spread into the completely freshwater agricultural areas of the Chao Phraya Delta such as Nakhon Pathom, Suphanburi, Ayuthaya and Pathumthani by 1998 (Szester, 2003). Low-salinity was produced by mixing saline water trucked-in from the coast and freshwater drawn from irrigation canals or streams. Low salinity techniques provided the opportunity for producing two or even three shrimp crops per year.

Low salinity farming techniques are generally similar to those used in coastal operations and feature high stocking densities, aerated ponds, use of prepared feeds, fertilizers and chemotherapeutic agents. While coastal farms utilize naturally

occurring brackish seawater (15-30 ppt salinity) to fill and replenish pond enclosures, inland low salinity farm systems is prepare pound water by diluting saline solutions of 100-200 ppt, from coastal seawater evaporation ponds with fresh water. Most ponds have ionic concentrations similar to that expected for sea water diluted to the same salinity (Davis *et al.*, 2004). This approach achieves an initial pond salinity level between 4 and 10 ppt (Szester, 2003).

The cycle of Black tiger shrimp culture is summarized as follows: Broodstock shrimps are either captured in the wild or pond-reared (Verakulpiriya and Tattanan, 2002). All post-larvae in Thailand are hatchery-produced. Eyestalk ablation in female shrimps to stimulate ovarian maturation is common practice in Thailand (Limsuwan and Chanratchakool, 2004). One day after spawning, the eggs hatch into nauplii. These nauplii metamorphose into zoeae, myses and postlarvae. The entire process lasts approximately 18 -20 days. Postlarvae look like small adult shrimps. They are fed with zooplankton, detritus and commercial feeds (Szester, 2003).

Post-larvae are placed in nursery ponds where they are cultured for 30 - 45 days before being placed in grow-out ponds. In the latter, they reach harvest sizes. All shrimps are in a continuous cycle of molting as they grow. Commercial feeds, feed supplements, chemical and antibiotics are “appropriately” used.

In the recent past, Black tiger shrimps were the only important marine shrimp species in Thai shrimp culture industries. But, the Black tiger shrimps need high capital for growing broodstock caught in the wild. Hence, a new technique was developed to produce them in earthen ponds and concrete ponds but the quantities are still not sufficient to satisfy the high demands. Furthermore, shrimp farmers encounter diseases that adversely affect the production and slow the growth, leading to small sized shrimps. These problems forced farmers to search for an alternative shrimp for culture and the Pacific white shrimp seemed to be the better choice. This species took the place of the Black tiger shrimp.

Figure 5: Black tiger shrimp

2.3.3 Pacific White shrimp (*Litopenaeus vannamei*)

2.3.3.1 Introduction of Pacific white shrimp in Thailand

The Pacific white shrimp *(Litopenaeus vannamei)* is native to the Pacific coast of Central and South America. Its natural distribution extends from the Pacific coast of Mexico to northern Peru (shrimpnews, 2005) and is the leading farm-raised species in the Western Hemisphere. It accounts for more than 95% of the total production (Van Wyke *et al*., 1999). These Pacific white shrimps grow rapidly, tolerate high stocking densities, have a relatively low dietary protein requirement and tolerate a wide range of salinity (Van Wyke *et al*., 1999). Hatcheries in Latin America maintain captive broodstocks of *L.vannamei*, some of them pathogen-free, some of them and others pathogen-resistant (FAO, 2003).

Pacific white shrimps were introduced into Asia experimentally from 1978-1979 but, only for commercial purposes since 1996 in China and Taiwan. Later on their farming spread to most of the other coastal Asian countries namely, the Philippines, Indonesia, Vietnam, Thailand, Malaysia and India in 2000-2001 (Briggs *et al*., 2004).

Thailand began to import Pacific white shrimps in 1998 but, the commercial cultures remained mostly experimental and the results were unsatisfying (Limsuwan

and Chanratchakool, 2004). Until 2001, when problems with a decrease in the growth rate of Black tiger shrimp encouraged farmers to search for alternatives, the Thai government allowed official importations of the certified SPF (Specific Pathogen Free) broodstock, from the qualified and audited hatcheries. Before that, a risk assessment of the possibility of interbreeding with native species was done (Limsuwan and Chanratchakool, 2004). The first SPF broodstock was imported from Hawaii. Experimental culture in some farms together with Black tiger shrimp satisfied the producers. Since then, the spread of the Pacific white shrimp culture started (Briggs *et al*., 2004; Limsuwan and Chanratchakool, 2004). The Pacific white shrimps are currently the most produced in Thailand. For example, in 2002 the production was 100,000 tons followed by 200,000 tons in 2003 (Ministry of Commerce, 2003).

2.3.3.2 Pacific white shrimp culture

2.3.3.2.1 Pond preparation

The pond preparation is the most important step in Pacific shrimp culture. It comprises cleaning the pond and water preparation prior to releasing the shrimp fry. Following the last harvest of shrimps, the pond is drained and left to dry in the sun for 10-30 days (ASEAN, 1998). Then, the waste, which accumulates at the bottom of the pond during the previous crop, is removed. If the surface of the pond is clean, it is filled with water and left overnight before flushing out to remove debris and elevate the pH. This process is repeated until the pH of the water remains above 7 (ASEAN, 1998; Lindberg and Nylander, 2001). In the next step, the lime is applied. It is recommended that agricultural lime ($CaCO_3$) or dolomite [$CaMg(CO_3)_2$] should be used in a pond with water with a nearly neutral pH level and hydrate lime [$Ca(OH)_2$] should be used in a pond with water pH below 5. During the application, lime should be spread throughout the bottom of the pond and up to the top to the dike in addition to the surrounding areas, such as the feeding places (ASEAN, 1998; Lindberg and Nylander, 2001).

After liming, the pond should be filled to the maximum with water passed through a fine wire mesh to prevent the predators such as fish, crustaceae, invertebrates and other competitors from entering the pond. Some chemicals and disinfectants such as tea seed cake, CaO, hypochloride can be used to eradicate those animals in the ponds and to disinfect equipment and water before stocking (FAO, 1996; ASEAN, 1998).

To increase the growth of natural food, organic and/or inorganic fertilizers are added to the culture pond. These stimulate the growth of plankton in order to provide shade in the bottom of the pond and utilize the nitrogenous or phosphate waste in the pond. Dry chicken manure is the most common organic fertilizer. Inorganic fertilizer such as urea and compound fertilizer may also be helpful (Limsuwan and Chanratchakool, 2004; ASEAN, 1998; FAO, 1996). The fertilizer must be dissolved in water before it is spread over the water surface to avoid precipitation at the bottom of the pond. After fertilization, the plankton should boom within a few days and the color of the water becomes slightly green. After that the fry can be stocked and cultured (Limsuwan and Chanratchakool, 2004; ASEAN, 1998; FAO, 1996).

2.3.3.2.2 Shrimp culturing

The steps followed during culturing of Pacific white shrimp are as follows. The broodstock is imported from Taiwan, China and the United State of America or other countries in South America (Limsuwan and Chanratcahkool, 2004). All the Pacific white shrimps raised in Thailand are from captive-bred, pathogenic-free postlarvae (Caseorbi, 2004). Eye ablation technique (cutting off one eye from the female) is used to initiate spawning in the female shrimps. The pond temperatures are maintained between 28-32^{o}C and salinity above 30 ppt, until postlarvae stages are at least reached (FAO, 2003). The seeds are fed with *Chaeotceros spp*., Artemia, Spirulina and commercial feeds. When juvenile shrimps reach postlarvae stages 3-4, the salinity is gradually reduced to 5 ppt so as to decrease the *Vibrio spp.* contamination until postlarvae stages of 7-8 are reached (Limsuwan and Chanratchakool, 2004). Postlarvae older than stage 12 are placed in grow-out ponds where they grow to

harvest size in 90 days (Limsuwan and Chanratchakool, 2004). So, the farmer normally harvests in cycles of 90-120 days (Limsuwan and Chanratchakool, 2004).

During culturing, fertilizers, bacteria and plankton are added to the pond. Antibiotics are used when the shrimps are stressed and show signs of disease. Prophylactically, they are also added in the last week of the first month. Chemicals like chloride, iodine, hydrogen peroxide, malachite green, formalin and potassium permanganate are used during the grow-out period. The shrimp feed pellets, and vitamins are added. Paddles are used to oxygenize the ponds. The techniques describe above are suitable for the production of small size shrimp because the culture period is limited (Lindberg and Nylander, 2004; ASEAN, 1998).

2.3.3.2.3 Low salinity shrimp culture

The marine shrimp culture in Thailand has 2 systems: low and normal salinity shrimp cultures. Low salinity inland shrimp culturing is done in freshwater areas in the central part of Thailand. The farmers truck in high salinity water (around 100-200 ppt) from salt pans and mix it with fresh water to reach a salinity about 3-4 ppt (Limsuwan and Chanratchakool, 2004). The salinity should not be less than 2 ppt all through the rearing period (Chanratchakool, 2004). The fries have been adapted to salinity before releasing. In low salinity cultures the production seems to be less than those in high salinity coastal culture ppt (Limsuwan and Chanratchakool, 2004).

The inland low salinity shrimp farms face the problem of the low quality of surface freshwater in some areas. For example water quality is poor in many parts of the Chao Phraya Delta as the main river system in this area passes through or close to many of the major population centers of the country, which are a major focus of industrial and agricultural activity within Thailand (ONWRC, 2003). Various pollutants arising from land-based sources, threaten the water quality of the Chao Praya Basin. Land-based sources of pollution include agriculture, sewage and industrial discharges, urban storm water and aquaculture. Major contaminants present in these sources are sediment, nutrients, toxic metals, pesticides, oxygen depleting

substances, pathogenic organisms, larvae of unwanted species, litter and others (Zafar *et al*., 2002).

The low temperature in the winter season results in slow growth of the shrimps. On the other hand in summer the temperature is very high and cause stress and low feed consumption (Limsuwan and Chanratchakool, 2004).

Another problem is there must be enough water for re-circulation. The salinity in all culture periods should not be lower than 2-5 ppt. Low salinity of the water is combined with low essential minerals. These minerals are essential for livability and growth rate. In addition, mineral content in the commercial feed is needed urgently when the shrimps change their exoskeleton and after heavy rain which results in lower salinity (Limsuwan and Chanratchakool, 2004).

2.3.3.2.4 Normal salinity shrimp culture

Normal salinity shrimp culture is found in coastal areas in the southern part of Thailand. The salinity is 10 ppt or more than. The seawater is treated as said in pond preparation. A screening for predators and water disinfection are done before releasing the fry. Normally the fly have been adjusted to high salinity from the hatcheries. The shrimps can be crowded and provide high production rates, because water circulation in coastal areas is better than in inland cultures (Limsuwan and Chanratchakool, 2004).

Pacific white shrimp is suitable for culturing in normal salinity. In spite of diseases, the normal salinity culture does not have any problem concerning salinity and minerals (Limsuwan and Chanratchakool, 2004).

Figure 6: Pacific white shrimp farm

Figure 7: Pacific white shrimp

2.4 Shrimp diseases

Several highly infectious viral and bacterial diseases affect either farmed or wild shrimps worldwide (Table 2). Sources of these infections to adult shrimp mainly originate from infected seedstock, broodstock or contaminated water supplies. The intensive farming that aims at increasing the production of shrimp has resulted in high stocking densities. The latter have led to increased stress levels in shrimp which plays an important role in the expression of diseases (Sritunyalucksana, 2001). In Thailand, the reported pathogens of serious concerns in shrimp farming are Vibriosis, White

Spot Syndrome, Infectious Hypodermal and Haematopoietic Necrosis, Taura Syndrome, Yellow-Head Disease, Monodon Baculovirus, Cramped Muscle Syndrome and Hemocytic enteritis (Table 2).

Table 2: Cosmopolitan shrimp diseases (including Thailand)

Name of disease	Agent	Affected shrimp spp.	Description
1. Vibriosis	*Vibrio spp.*	All cultured penaeids and cultured *Macrobrachium rosenbergii*	Vibriosis has been defined as a stress-related disease in shrimp. It is very common in hatcheries of both freshwater and marine shrimps. The unique clinical sign of this disease is the luminescence of infected larvae which can be observed at night. Infected larvae also show fouling, opacity, slow swimming, aggregation and death. Mortalities may reach 100%.
2. White Spot Syndrome (WSS)	White Spot Syndrome Virus, double stranded DNA baculovirus	All decapod crustaceans especially in penaeid shrimp. Experimentally, severe and lethal infections of WSSV from Thailand were produced in *Penaeus vannamei*, *Penaeus stylirostris*, *Penaeus aztecus*, *Penaeus duorarum* and *Penaeus setiferus*.	WSS has caused massive losses in the shrimp industry in Asia and Latin America since 1993 (Bondad-Reantaso, 2001). Horizontal transmission may be direct or vectorial. Water is the major abiotic vector. Rapid transmission occurs from infected shrimps through the water and by cannibalism of weak or moribund shrimps. Transovarial transmission occurs (OIE, 2003). Acutely infected shrimp show rapid reduction in food consumption, become lethargic and have a high mortality that reaches 100% within 3 to 10 days of the onset of clinical signs.

Name of disease	Agent	Affected shrimp spp.	Description
3. Infectious Hypodermal and Haematopoietic Necrosis (IHHN)	Infectious Hypodermal and Haematopoietic Necrosis virus (IHHNV), Single-stranded DNA virus	Considered highly contagious and infectious to many penaeids*: Penaeus stylirostris, Penaeus vannamei, Penaeus monodon, Penaeus chinensis, Penaeus occidentalis, Penaeus californiensis, Penaeus semisulcatus* and hybrids of *Penaeus monodon and Penaeus esculentus, Penaeus setiferus, Penaeus japonicus, Penaeus aztecus, and Penaeus duorarum, Penaeus indicus and Penaeus merguiensis*	IHHNV is enzootic in Taiwan, Singapore, Malaysia, Thailand, Indonesia, Australia and Philippines and possibly also enzootic in Ecuador, Peru, and Central America. This disease is now widely distributed in cultured penaeids in the southeast United States, Caribbean, Brazil, Hawaii, Guam, Tahiti, New Caledonia and Israel. Affected shrimps have reduced food consumption, cannibalism, and high mortality. In some cases, shrimp repeatedly rise slowly to surface, roll over and sink to bottom. Disease is severe among juveniles in high density tanks cultures. Some members of the population which survive carry the virus for life and pass it onto their progeny and other populations by vertical and horizontal transmission. In Pacific white shrimps IHHNV is a chronic disease called 'runt-deformity syndrome' (RDS) which is characterized by low crop production and variable sizes, reduced growth rates and cuticular deformities. Individuals may develop bent or deformed rostrums, wrinkled antennal flagella, cuticular roughness and other cuticular deformities. Coefficient of variation of size distribution of stocks with severe RDS is greater than 30% and may approach 90% (OIE, 2003).

Name of disease	Agent	Affected shrimp spp.	Description
4. Taura Syndrome (TSV)	Taura syndrome virus, a small picorna-like RNA virus	Susceptible are blue shrimp (*Penaeus stylirostrus*) and Pacific white shrimp (*Penaeus vannamei*)	Most important shrimp disease of Pacific white shrimp in Thailand. The imports of Pacific white shrimps from unreliable hatcheries are the main sources of TSV (Briggs *et al.*, 2004). It is a disease of the nursery phase when the postlarvae are transferred into grow-out ponds or tanks. The mortality is 40-90% (OIE, 2003). Transmission is horizontal by cannibalism of infected shrimps, cohabitation of infected and noninfected individuals and via infected transport water and equipment (OIE, 2003). The selection of Pacific white shrimp that are resistant to TSV may be one way of getting this disease under control.
5. Yellow-Head Disease (YHD)	Yellow-head virus (YHV)	Cultivated penaeid shrimp, natural infected is Black tiger shrimp (*Penaeus monodon*)	YHD is one of the most common diseases affecting farmed shrimps in Thailand. It can affect cultured shrimp from the late postlarval stage onwards, but high mortality is usually encountered from early to late juvenile stages. The disease is characterized by high and rapid mortality, typically accompanied by the pathognomonic signs of yellowing of the cephalothorax and general bleaching of body colour (OIE, 2003). Although the economic impact of YHD outbreaks on farms can be significant, losses are somewhat reduced because prepared feeds are not heavily applied

Name of disease	Agent	Affected shrimp spp.	Description
			during the first month of the grow-out cycle. No effective treatment for YHD exists. Farmers often attempt emergency harvesting followed by cleaning and disinfection of the grow-out pond (Szester, 2003).
6. Monodon Baculovirus (MBV)	*Penaeus monodon* type baculovirus (MBV), double-stranded DNA virus	*Penaeus monodon, Penaeus merguiensis, Penaeus semisulcatus, Penaeus kerathurus, Penaeus vannamei, Penaeus esculentus, Penaeus penicillatus, Penaeus plebejus, Metapenaeus ensis*	MBV-type baculoviruses are widely distributed in cultured and wild penaeid shrimp and prawns in the Eastern Hemisphere, Australia, East Africa, the Middle East, many of the Indo-Pacific countries, and in South and East Asia. Lethargy, anorexia, dark coloured, and with heavy surface fouling. Acute MBV causes loss of the hepatopancreatic tubule and midgut epithelia resulting in, dysfunction of these organs, often followed by secondary bacterial infections. MBV has been associated with high mortalities (over 90%) in late postlarvae and juvenile shrimps. It causes heavy mortalities (70% of all stages of *P. monodon* in the Philippines and 90% of postlarval *P. monodon* in Madras, India). It is partly, responsible for the collapse of the shrimp culture industry in Taiwan in the late 1980s. In *P. monodon*, juvenile and adult are more resistant to MBV than larval shrimp.

Name of disease	Agent	Affected shrimp spp.	Description
7. Cramped Muscle Syndrome (CMS)	Unknown	Often found in Pacific white shrimp	The agent of CMS has not been identified up to now. But low feed and water quality or sudden changes of environment may be responsible for this disease.
8. Hemocytic Enteritis (HE)	Blue-green algae toxin	All shrimp	HE occurs when Blue-green algae boom in grown-out ponds. Shrimps that consume toxic blue-green algae will develop enteritis (Briggs *et al.*, 2004).

2.5 Norovirus

2.5.1 Introduction

There are many groups of foodborne viruses which infect persons and cause diseases. Of these, the genus norovirus (NoV) is currently recognized as one of the most important causes of human foodborne illness (Koopman and Duizer, 2004). The genus was recently approved as the name for the group of viruses that were provisionally described as Norwalk-like virus. It belongs to the family of Caliciviridae. Currently, the human norovirus is comprised of three groups, namely (GI, GII and GIV). Each of them is divided into more than 25 genetic clusters (CDC, 2005).

2.5.2 Morphology

Norovirus is a non-enveloped single-stranded RNA with a diameter of 35-39 nm (Büchen-Osmond, 2003). It has a simple round structure with 32 cup-shaped depression capsid. It is round and exhibits icosahedral symmetry (Büchen-Osmond, 2003). The capsomer arrangement is clearly visible. The complete genome is 7300-7700 nucleotides long and is characterized by a guanine-cytosine content of 48-55.8% (Büchen-Osmond, 2003).

2.5.3 Epidemiology

Norovirus is a highly infectious pathogen. It is the most common cause of gastroenteritis in people of all age groups. The infections occur both as sporadic cases and as outbreaks. The food handlers infected by norovirus are a particular risk group to healthy humans. They can contaminate food and drinks that many other people consume. Outbreaks of norovirus illness mainly happen in institutions such as restaurants, nursing homes, schools, day care centers, cruise ships, etc (CDC, 2001).

Most foodborne viruses are more resistant to heat, disinfection and pH changes than vegetative bacteria. Since norovirus is uncultivable in the laboratory, little is known about the length of time it remains infectious in the environment. Thus, it may be useful to consider survival data of norovirus from other enteric viruses, which have similar survival characteristics and can be cultivated. The length of survival of enteroviruses in the environment is affected by a number of issues including temperature. It can survive in high levels of chlorine and varying temperatures and other environmental factors facilitate its spread through recreational and drinking water, as well as food (CDC, 2001). Norovirus can survive in 60^{o}C for 30 minutes and in frozen conditions (EC, 2002). Available evidence suggests that salinity is of little significance to survival of norovirus (EC, 2002).

2.5.4 Symptomatic features

Norovirus causes foodborne illness in humans of any age (EC, 2002). The average incubation period of norovirus is 12-48 hours with duration of the disease for 12-60 hours (EC, 2002). A common clinical symptom is the gastroenteritis which is characterized by an acute onset of nausea, vomiting, abdominal cramps and non-bloody diarrhea (CDC, 2001). Vomiting is relatively more prevalent among children while, diarrhea is more in adults (CDC, 2001). Accompanying symptoms such as low-grade fever, headache, chills, myalgia, malaise and joint pain are frequently reported (CDC, 2001; EC, 2002). But, asymptomatic infections are also common. Immunosuppressed persons or transplant recipients may suffer persistent diarrhea (Kaufman *et al*., 2003). Unusual symptoms were described in an outbreak of norovirus infection in a military field hospital in Afghanistan. They included neck stiffness, photosensitivity, confusion and disseminated intravenous coagulation (CDC, 2002). Dehydration and stress due to work and environmental conditions, such as high temperature and low humidity might have contributed to the severe disease among the soldiers (Hutson *et al*., 2004).

Hospitalization and fluid therapy may be required due to severe dehydration caused by vomiting and diarrhea. Fatal cases are known from susceptible persons

only. Normally, norovirus infection is a short-term and self-limiting disease that should be treated with rest, oral rehydration and intravenous replacement of electrolytes, if necessary (EC, 2002).

2.5.5 Transmission mode

CDC estimates that 23 million cases of acute gastroenteritis are due to norovirus infection, and at least 50% of all foodborne outbreaks of gastroenteritis are attributed to this virus (Widdowson *et al*., 2005). It is transmitted by both direct and indirect contact. Analysis of epidemiological data between January 1996 to November 2000 found that 39% of the outbreaks of norovirus gastroenteritis were foodborne, 12% by person-to-person transmission, 3% waterborne and 18% could not be linked to any specific transmission modes (CDC, 2001). Principally, the fecal-oral route is considered the major transmission mode while, person-to-person is the secondary mode (CDC, 2001). Frequently, primary outbreak starts with an infection due to a faecally contaminated vehicle (e.g. food or water), whereas the secondary and tertiary outbreaks result from person-to-person transmissions (CDC, 2001). Since the infection dose sometimes is lower than 100 particles, secondary infection by norovirus may be spread by droplets e.g. form food handlers. This route plays an important role in outbreaks of norovirus in daycare, school, food catering, cruises, hospitals, nursing homes, etc (CDC, 2001). Alternative ways of contaminating food include gross environmental contamination following vomiting and diarrhea.

Norovirus contaminates food, water or the environment and it is both persistent in the environment and resistant to disinfection (Meschke, 2004). Norovirus is one of the important viruses that commonly contaminate water. In the latter, it is able to withstand the process of sewage treatment and pollutes public water. The polluted water may distribute viruses over long distances and introduce contamination to food materials that are grown in or prepared using that water (Laverick *et al*., 2004; Fleet *et al*., 2000).

2.5.6 Norovirus as food infection

Norovirus is suspected to be one of the most common causes of gastroenteritis originating from the consumption of food. The norovirus outbreaks are linked to many different food items, either seafood from faecally contaminated areas or other food categories which may be contaminated during production, handling and preparation (EC, 2002). CDC reported that between 1997 to 2000 more than 57% of the outbreaks were foodborne (CDC, 2001).

2.5.6.1 Seafood

Norovirus does not propagate in living marine animals. It only contaminates seafood either at the source, principally through sewage pollution of the sea, or during seafood processing through inadequate hygienic practices of operatives or systems (EC, 2002). Thus, mainly seafood from sewage-polluted areas is potentially contaminated with human enteric viruses (CDC, 2001). But, sometimes it can be proved that the matrix was contaminated by a food handler. Many outbreak reports show that seafood is one of the important sources of norovirus. The outbreaks are associated with seafood items such as oysters and shrimps (Stafford *et al.*, 1997; Alcamo, 2001; Ng *et al.*, 2004). One major epidemiological factor which should be taken into consideration is the question of whether the viral contamination remains on the surface of the consumed food or becomes internalized and, if so, whether such contaminated organs are consumed or are removed during food preparation. It is also important on how thoroughly the seafood is cooked before consumption (CDC, 2001).

2.5.6.2 Other food categories

Apart from seafood, many types of foods are associated with outbreaks of norovirus infections. Contamination happens at any step in the food chain, but the infected food-handler is found to be the most frequent source. Outbreaks have been traced back to many food items including sandwiches, bread rolls and other bakery products, cake icing, cold meats and hamburgers, mixed salads vegetables, fruits,

juices, and ice (EC, 2002). Most of these foods are ready-to-eat, defined as “food that is edible without washing, cooking, or additional preparation by the consumer or by the food establishment and that is reasonable expected to be consumed in that manner” (Sair *et al*., 2002). Handling cooked products with bare hands has been identified as a major factor for pathogen transfer to ready-to-eat foods (Sair *et al*., 2002). In addition, drinking water contaminated with norovirus is an important source of norovirus infections, both as sporadic disease and as outbreaks (CDC, 2001).

2.5.7 Diagnostic methods

In the past, bacteria have been incriminated as causes of most disease outbreaks. Nevertheless, viruses have also been important, but routine methods to detect them have been lacking until in recent times. Advances in analytical techniques for detecting viruses have changed our understanding of the epidemiology of these viruses. These techniques include electron microscopy, ELISA and PCR.

2.5.7.1 Electron Microscopy (EM) and Immuno-Electron Microscopy (IEM)

Old methods for diagnosing viral infections include direct and immune electron microscopy of fecal samples. Electron Microscopy is the first method used in norovirus studies (CDC, 2001). This technique is useful for specimens collected during the early stages of illness because approximately 106-107 viral particles per milliliter in stool are required for visualization by EM (CDC, 2001). Using this technique, norovirus can be identified by its characteristic morphology.

Immuno-electron microscopy (IEM) improves the sensitivity of the EM by a factor of 10-100 (CDC, 2001). In the convalescent-phase serum from a patient is coated on the examination grid of the microscope before stool specimens are applied. The antibodies on the grid trap homologous virus and the diagnostic yield of virus is increased (EC, 2002). However, IEM has specific problems. The greatest disadvantage is that its success mainly depends on the skill and expertise of the

microscopist. Furthermore, the virus may be totally masked if a large amount of antibodies are present, resulting in a false-negative results (CDC, 2001).

2.5.7.2 Enzyme Linked Immunosorbant assays

The capsid proteins of norovirus are capable of automatically grouping into stable virus-like particles in baculoviruses. This phenomenon has allowed the detection of these viruses with the help of ELISAs (CDC, 2001). Initially, these ELISAs were developed to measure total anti-norovirus immunoglobulin in human sera, but in recent times they have been adapted to detect anti-norovirus immunoglobulins IgA, IgG and IgM (Sair *et al*., 2002).

The earliest assays targeted antisera between acute and convalescent phases (CDC, 2001). Certain adults possess preexisting immunoglobulin G (IgG) antibodies to norovirus. A single serum sample is insufficient to indicate a recent infection. This is because seroconversion is defined as greater than a 4-fold raise in the IgG antibody titer in a blood sample between these 2 phases. For IgG assays, the acute-phase serum should be drawn within the first 5 days and the convalescent-phase serum during the third to sixth week (CDC, 2001). While serum IgM enzyme immunoassays have been used to detect recent infections, in certain cases where diagnosis is critical (e.g., when a food-handler is implicated as the source of an outbreak), a single assay of serum immunoglobulin A (IgA) antibody can be successful if the specimens are collected 7-14 days after exposure (EC, 2002). Anyhow, these ELISA-assays have been reported to detect the presence of 104-106 viral particles in clinical specimens (CDC, 2001; Mäde *et al*., 2005). To date, these assays are type-specific but some experiments have shown progress in the development of more broadly reactive serum enzyme immunoassays for the detection of norovirus (Sair *et al*., 2002).

2.5.7.3 PCR and RT-PCR

Polymerase chain reaction (PCR), and especially Reverse transcriptase polymerase chain reaction (RT-PCR), are suitable methods to detect the norovirus

genome. It has revolutionized the detection of DNA and RNA, allowing the detection of as little as a single copy of a given sequence. The method is commonly used for the detection of norovirus in both clinical and environmental specimens. It is a sensitive and specific tool for norovius outbreak investigations (EC, 2002).

Theoretically, PCR is capable of amplifying a single specific nucleic acid sequence up to a million-fold. The method is particularly attractive for detection of non-culturable infectious agents (Sair *et al.*, 2002). There is a quantitative relationship between the amount of starting material and the PCR product at any cycle (Provenzano *et al*., 2001). However, it is a common experience for replicate reactions to yield different amounts of PCR product. Furthermore, at the end of a PCR reaction in which the numbers of cycles are empirically pre-determined, different amounts of starting material can yield similar amounts of amplification products due to the consumption of the reagents (Provenzano *et al*., 2001).

After amplification, many techniques can be used to detect and confirm the identity of the amplified product. These include electrophoresis and real-time amplicon detection.

2.5.7.3.1 Electrophoresis

Electrophoresis is normally the simplest and most commonly used method. The electrophoresis of an aliquot of the PCR on an agarose or polyacrylamid gel followed by visualization after staining with ethidium bromide, a fluorescent dye, that intercalates into the DNA of amplicon detection. After staining, ultraviolet trans-illumination allows virtualization of the DNA in the gel (Newton and Graham, 1997).

2.5.7.3.2 Real-time amplicon detection

Real-time detection is an alternative of quantitative PCR that detects the amount of the final amplified product at the end-point. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. The increasing of this signal

increases in direct proportion to the amount of PCR product in a reaction (Newton and Graham, 1997). By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template, this technique is so-called “real-time” (Newton and Graham, 1997). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product (Newton and Graham, 1997).

For the RNA detection, it is required to reverse RNA into cDNA before amplification. In a two-step RT-PCR, the two steps of reverse transcription and PCR are performed separately in different tubes. The development of One-step real-time PCR uses RNA (as opposed to cDNA) as a template and performs these two steps in a single buffer system and in one tube. This is the preferred method if the RNA solution has a low concentration but only if singleplex reactions are run.

2.5.7.3.3 Limitations of PCR

While a very powerful technique, PCR also has some limitations. Primer design is extremely important for effective amplification. The primers for the reaction must be very specific for the template to be amplified. Cross reactivity with non-target DNA sequences results in non-specific amplification of DNA. Also, the primers must not be capable of annealing to themselves or each other, as this will result in the very efficient amplification of short nonsense DNAs.

The reaction is limited by the sizes of the DNAs to be amplified. Also, Taq polymerase has been reported to make frequent mismatch mistakes when incorporating new bases into a strand (Slish, 2003).

The most important consideration in PCR is contamination. If the sample that is being tested has even the smallest contamination with DNA from the target, the

reaction could amplify this DNA and result in a false positive identification (Slish, 2003).

In the last 10 years, diagnosis of norovirus in outbreaks has improved with the increasing use of reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR can be used to test both stool and emesis samples (EC, 2002). Identification of the virus is best made from stool specimens taken within 48 to 72 hours after onset of the symptoms (EC, 2002), although good results are obtained by analyzing samples taken as long as 5 days after the onset of symptoms. Virus can sometimes be found in stool samples taken as late as 2 weeks after recovery (EC, 2002). However, in many countries these methods are not available for routine diagnosis because of the high price of machine and reagents.

These molecular methods have been adapted for the detection of norovirus in food and water too. Since the foods are one route of norovirus transmission, the routine diagnoses of this virus in food and environmental matrices have been developed in some laboratories (Mäde *et al*., 2005). When norovirus is detected in food, the comparison with those viral strains that are detected in human stool samples enables one to make an epidemiological linkage (Sair *et al*., 2002).

3. MATERIALS AND METHOD

This study aimed at determining the prevalence of Norovirus in export Pacific white shrimp (*Litopenaeus vannamei*) in Thailand. The quantitative result was to be used in establishing the prevalence of Norovirus in Pacific white shrimps (*L. vannamei*) in Thailand and for risk assessment.

3.1 Study design

3.1.1 Study area

The study started at the "Talaythai auction market", the biggest shrimp wholesale market for export in Thailand. The shrimps were pooled from shrimp farms and distributed to shrimp freezing factories. Their origins were from central, eastern and the southern part of Thailand. Pacific white shrimp (*L. vannamei*), is the most dominant shrimp for export. The samples were collected from December 2004 to January 2005.

3.1.2 Sampling Technique

A Modified cross-sectional survey was used in this study. The collection of dates allowed a systematic random sampling pattern with a randomly determined starting date. A convenient 14-day interval was used.

Around 250 containers having 3-10 tons each were brought to Talaythai market everyday. Ten containers, which arrived at Talaythai market on sampling date, were conveniently chosen and 5 Pacific white shrimp (*L.vannamei*) were drawn from each container. Shrimps were loaded from 200-l bucket into 30-kg baskets at the market. Out of these baskets, one shrimp was systematically chosen as a sample. There were 5 sampling rounds and 50 shrimps were sampled per round giving a final sample size of 250.

The sample size of 250 was determined based on a 50% prevalence and a 95% confidence interval.

The shrimp samples were separately put into plastic bags and maintained at-20^{o}C until laboratory processing.

3.1.3 Questionnaire

A questionnaire was administered at the market level. For each container, information was recorded from a certificate which officially was issued by The Department of Fisheries. The document provided information on the location, volume of shrimps, date of harvesting and collecting. Shrimp culture information was recorded by interviewing shrimp owners, if possible. The questionnaire gathered information as categorized in Table 3.

Table 3: The sets of questionnaire

Topic	Description
1. Farm of origin	Location of farm; district or province, name of owner or farm, farm size; number of ponds
2. Management	Sources of pond water, draining interval, sources of shrimp larvae
3. Harvesting	Date of harvesting and date of collection, certificate needed, volume of shrimp and average weight
4. Transportation	Transportation conditions, distance and time from farm to market

3.2 Method

3.2.1 Sample Preparation

The frozen shrimps were thawed and the shell was removed from them. Five grams of each sample were put into a stomacher bag. Ten milliliters of phosphate buffered solution of pH 7.4 was added and homogenized in the stomacher for 1 minute. The mixture was stored for 24-48 hours at +4^{o}C. After centrifugation of the liquid phase at 3000g for 10 minutes, the projection was separated from the firm particles. The supernatant was filtered through 0.45 and 0.20 micrometer filters, respectively. The clean filtrate was transferred into a 4 ml Amicon® concentrator 50000 MWCO (Millipore GmbH, Schwalbach, Germany) and centrifuged at 3000g at 15^{o}C for 20 minutes. The centrifugation step was repeated until an end volume of 140 ml was reached. The concentrated samples were used for RNA extraction.

3.2.2 Viral RNA extraction and purification

Viral nucleic acids were purified from concentrated samples using QIamp® RNA viral mini kit (QIAGEN® GmbH, Hilden, Germany), following the company protocol. Briefly, the sample was lysed under high denaturing conditions to inactivate RNase and to ensure isolation of intact viral RNA. Lysis buffer (AVL) with RNA carrier (560 µl) was added and mixed by pulse vortexing for 15 seconds and incubated at room temperature for 10 minutes. Briefly centrifugation was done to remove drops from the inside of the lid. Buffering conditions were then adjusted by adding 560 µl of absolute ethanol and mixed by pulse vortexing for 15 seconds to provide optimum binding of the RNA to the QIamp membrane.

The sample (650 µl) was loaded onto the QIamp spin column in a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. The procedure was repeated 2 times. The RNA attached to the membrane and contaminants were washed away in two steps using two different washing buffers. Viral RNA was eluted in a special RNase-free

buffer, suitable for the PCR step. The RNA samples were kept at -70^{o}C prior to viral RNA quantitation.

3.2.5 Quantitative PCR

Real time RT-PCR was used as a quantitative method to detect norovirus. It was performed by using the QuantiTect^{TM} Probe RT-PCR kit (QIAGEN, Hilden, Germany). The kit consists of 2x QuantiTect Probe RT-PCR master mix (with HotstarTaq DNA Polymerase, QuantiTect Probe Rt-PCR buffer, dNTP mix including dUTP, ROX as a passive reference dye, and 8 mM $MgCl_2$); QuantiTect RT mix (containing Omniscript reverse transcriptase and Sensiscript reverese transcriptase), and RNase-free water.

PCR master mix was carried out in 30 µl (for 1 sample) reaction volumes combining 25 µl 2x QuantiTect Probe RT-PCR Master Mix, 0.5 µl QuantiTect RT-Mix, 2.5 µl primers and 2 µl H_2O.

Primers for Norovirus Genogroup II used in this study were described by Höhne and Schreier (2004). There are NV107a (5'-AGCCAATGTTCAGATGGATG) (20 pmol/µl) and NV119 (5'-TCGACGCCATCTTCATTCAC) (20 pmol/µl). Each primer was used at a final concentration of 400 nM, and a specific fluorescence labeled TaqMan probe TM3 (GGII) (5'-TGGGAGGGCGATCGCAATCTGGC) was used at a final concentration of 100 nM. The master mixes were kept on ice at all times.

The single-tube RT-PCR was carried out in a 0.2 ml 96 tube plate. The master mix was mixed thoroughly and 25 µl was dispensed in each tube. The template RNA was added to the individual PCR tube. The tube was covered with a sealing film and kept on ice before the start of the cycling procedure.

The amplification was carried out in a BioRad® My I cycler RT-system (BioRad®, California, USA). Reverse transcription time was programmed at 50^{o}C for 30 minutes to eliminate secondary structure in the template RNA. The thermal profile

for dissociation consisting of the initial activation step to activate HotStarTaq DNA polymerase for 15 minutes at 95^{o}C, and was followed with 45 cycles of denaturation (15 seconds, 94^{o}C) and annealing/extension (60 seconds, 60^{o}C).

Optic graphs showing the fluorescence intensity of each reaction of the PCR cycles were generated. RT-PCR results were produced as sigmoidal-shaped amplification plots, in which the fluorescence was plotted against the number of cycles. Data were collected and viewed using the software and graphics programs provided with the BioRad® My I cycler.

3.2.3 Positive control

Positive control was prepared from shellfish tissue artificially contaminated with norovirus. The viral RNA was extracted and purified as described in sections 3.2.1 to 3.2.2.

3.2.4 Negative control

RNase free water and Master Mix were used as negative controls to ensure no contamination occurred during the preparation steps.

3.3 Data management and Statistical analysis

All the data were managed and analysed using the Microsoft®Excel program. Prevalence of Norovirus was to be calculated and Chi-square and Fischer exact tests were to be used for descriptive analysis. ANOVA was to be performed to compare viral quantities from different shrimp sources.

4. RESULTS

The study was meant to detect norovirus in shrimps in Thailand. Two hundred and forty samples of shrimps were collected at the Talaythai auction market in Samut Sakorn. They were processed for RNA and tested using real-time PCR. All were negative for norovirus (Table 4).

The amplification curve of fluorescence against cycle number was plotted (Figure 8). The results of shrimp samples gave flat lines, while positive control showed an increase in fluorescence. The line of the negative control was similar to the test samples.

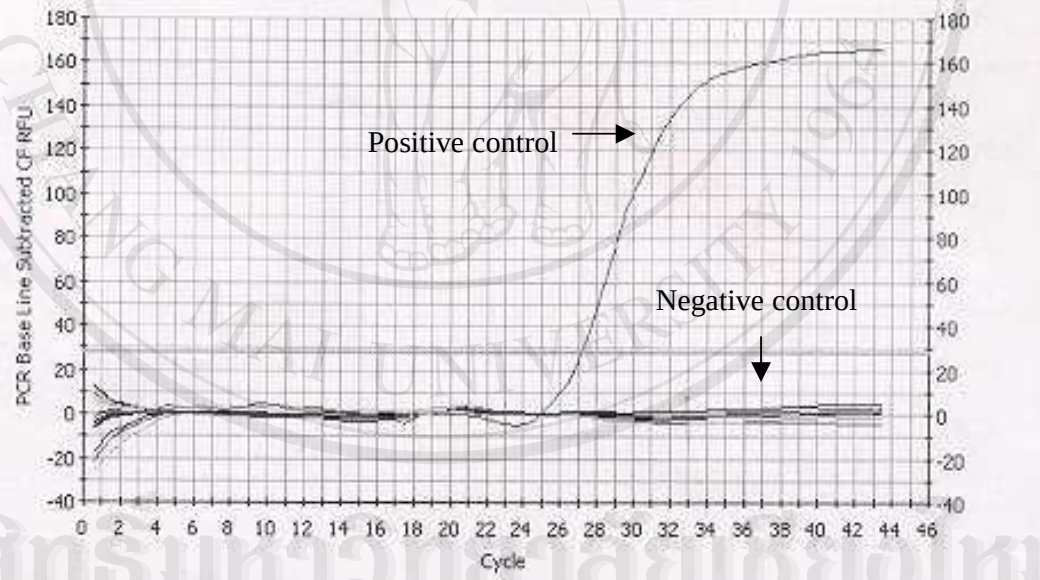

Figure 8: Real time PCR detection results of norovirus

The shrimp came from different regions in Thailand (Table 4). The transportation distances and times to the Talaythai auction market, located in Samut Sakorn, varied by regions and provinces. The shrimp farms located in the central region had the shortest distances and times followed by those in the eastern and southern regions, respectively.

Similarly, the sizes of shrimps varied by provinces but had similar sizes by regions. Regionally, these were 10.57 cm in central, 11.97 cm in the eastern and 12.48 cm in the southern. Equivalent patterns were also observed in weights, where shrimps from the central region had an average of 9.44 gm followed by the eastern with 12.90 gm and the southern with 19.93 gm. The shrimps from the southern region had the largest sizes.

Table 3: Summary results of Norovirus detection in shrimp samples from Talaythai auction market, Samut Sakorn, Thailand

Region	Province	Mean Distance (km)	Mean Time (hr)	Weight (gm)			Length (cm)			Norovirus neg/pos
				Mean	SD	Range	Mean	SD	Range	
Central										
	Nakhon Pathom	48	1.00	11.66	3.33	7.7-17.2	11.53	1.23	9.0-13.3	15/0
	Samut Sakhon	15	0.20	9.45	2.41	5.4-12.8	10.84	0.95	9.0-12.1	15/0
	Petchaburi	88	1.30	12.13	2.17	10.0-13.4	11.05	0.98	9.5-12.2	10/0
	Ratchaburi	78	1.00	5.10	3.85	2.3-12.6	9.25	2.32	6.0-12.6	10/0
	Samut Prakan	65	1.00	8.85	1.47	6.1-10.6	10.18	0.76	9.0-11.4	10/0
	Overall	**58.8**	**1.26**	**9.44**	**2.65**	**6.30-13.32**	**10.57**	**1.25**	**8.5-12.32**	**(60)***
Eastern										
	Chachoengsao	118	1.40	11.12	2.76	6.9-18.1	11.71	1.82	8.8-20.5	45/0
	Prachin Buri	171	1.40	13.97	3.57	8.1-20.3	12.15	1.11	10.0-14.1	20/0
	Chanthaburi	289	4.00	12.12	1.89	9.1-15.2	11.58	0.76	10.5-13.0	20/0
	Rayong	215	3.00	14.36	1.67	11.9-18.5	12.43	0.66	11.5-14.0	15/0
	Overall	**198.25**	**2.45**	**12.90**	**2.47**	**9.0-18.03**	**11.97**	**1.89**	**10.2-15.4**	**(100)***
Southern										
	Surat Thani	609	7.00	12.07	3.16	8.6-16.3	11.17	1.07	9.5-12.7	20/0
	Nakhon Si Thammarat	746	11.00	11.98	0.49	11.5-12.8	11.08	0.19	10.8-11.3	5/0
	Trang	793	11.30	20.06	2.02	16.6-21.9	13.62	0.31	13.1-13.9	5/0
	Phuket	827	12.00	16.42	1.19	14.8-17.9	12.66	0.77	12.1-14.0	5/0
	Rayong	532	7.40	18.63	3.71	13.9-25.6	12.83	1.10	11.5-14.8	10/0
	Prachuab Khiri Khan	245	3.30	15.10	3.11	10.2-22.3	11.76	1.90	6.0-14.3	30/0
	Phangnga	753	11.00	20.66	2.21	17.8-23.4	13.40	1.06	12.0-14.8	5/0
	Chumphon	428	6.00	21.48	1.58	19.8-23.3	14.28	0.89	13.0-15.0	5/0
	Krabi	778	11.30	16.00	3.29	10.7-19.3	11.52	1.64	9.9--14.0	5/0
	Overall	**634.56**	**8.92**	**16.93**	**2.31**	**13.77-20.31**	**12.48**	**0.18**	**10.87-13.87**	**(90)***

SD = Standard Deviation neg = negative

( )* Sample sizes per region pos = positive

5. DISCUSSION

It is well known that pollution of water with human waste is a major potential source of both pathogenic bacteria and viruses. Of the viruses, norovirus is now recognized as an important cause of human gastroenteritis, associated, with an increasing number of outbreaks (CDC, 2001). This virus can be excreted with human feces and has been found not only in wastewaters but also in rivers, recreational waters and seawater. It is able to withstand wastewater treatment and hence may contaminate surface water (Abel, 2000; Pusch *et al*., 2005). To date, there has not been any official report of noroviral gastroenteritis outbreak in Thailand. But, the few studies of sporadic cases which had been caused by noroviruses, show that the norovirus does exist in Thailand (Guntapong *et al*., 2004; Hansman *et al*., 2004). From the study by Hansman *et al*. (2004) norovirus and sapovirus were detected in 12% of stool specimens from infants admitted to hospital in Chiang Mai, Thailand between July 2000 and July 2001. The hazard of norovirus in water may cause a contamination of shrimps, which are an important economic aquatic-animal in Thailand (Ministry of Commerce, 2004). The chances for norovirus contaminating shrimps exist at any step from farm to market. It may come from the contaminated water, feed, ice, vehicle and equipments as well as infected people who handle the shrimps.

The current study took place at the Talaythai auction market in Samut Sakhon, the biggest shrimp auction market in Thailand. The working hours were around 12pm to 6am, but could be adjusted according to the shrimp quantity that was received. The shrimps were transported to this market in closed containers, or sometimes in open trucks. They were kept cool by using water and ice. Even though the shrimps came from the central, eastern and southern parts of Thailand, the times of harvesting to unloading at the market had the same average range of 8-12 hours.

The questionnaire forms were not filled up completely since it is very difficult to interview the busy managers in a place as busy as this market. Also, most of the data

from the questionnaires did not imply anything especially because it was impossible to get deeper details of farm management at the market level. For future research a direct questionnaire at the farm level is emphasized. However, the researchers should be aware of hidden or wrong information since the farmers normally think that the researchers are interested in detecting antibiotic residues.

The results of this study showed that there was an "actual" zero prevalence of norovirus in Pacific white shrimps, which are reared in Thailand at this time. The failure to detect norovirus is unlikely to be caused by low sensitivities of the test used. In this study, the protocol used for recovery of the RNA virus was developed by the Institut für Lebensmittel, Arzeimittel und Tierseuchen (ILAT), Berlin for routine diagnosis of norovirus in food samples and used since 2003. It has been shown that the test procedure is effective in detecting norovirus. Polymerase chain reaction (PCR), the most sensitive and specific method to detect norovirus in food and environment (Höhne and Schreier, 2004), was used. A distinct positive curve of the PCR technique was given by the positive control which was prepared by artificial contamination of shellfish with norovirus. The same protocol was done with the positive controls and with the shrimp samples. Only the latter gave negative results. PCR was high in both sensitivity and specificity. Using ELISA plus Transmission electron microscopy (TEM) as the reference test, PCR was shown to have a sensitivity of 94.1% and a specificity of 92.4% (Rabenue *et al*., 2003). The two attributes of PCR are high enough to routinely detect the presence of norovirus in samples.

Furthermore, it is unlikely to give negative results because of using the wrong primers. The primers used in this study were described by Höhne (2004) and they detect a broad range of norovirus genogroup II. The norovirus genogroup II shows a global distribution (Gallimore *et al*., 2004; Kageyama *et al*., 2004) and is detected most frequently in clinical and environment samples. The studies of norovirus in sporadic cases in Thailand also reveal that the majority of isolates in Thailand belong to norovirus genogroup II (Guntapong *et al*., 2004; Hansman *et al*., 2004).

Moreover, the failure to detect norovirus in shrimp in this study does not mean that there is no norovirus contamination in shrimps and shrimp farms in Thailand. The reason for the negative results may be from the low prevalence of norovirus. According to the results the prevalence of norovirus in Thailand is supposed to be lower than 1.2 percent (α=0.05). Thus, the sample size should be increased accordingly in the subsequent studies if more information is required for validating the status of norovirus in the Thai shrimps. However, it can be surmises to suggest that future attention on investigating norovirus be focused on people working with shrimps and shrimp farms. Further, continual active surveillance of norovirus in marketed shrimps is highly recommended.

Low viral contamination of shrimps may be another explanation since shrimps are not filter feeding animals like shellfish, the most common matrix of norovirus outbreaks (Guyader *et al*., 1996; Hale, 1999; Dubois *et al*., 2003). Thus, norovirus accumulation in shrimps may be too low to be detected by conventional laboratory methods. The positive control showed the cycle threshold (Ct) at cycle 26 of the real time PCR, while, all of the test samples did not show the Ct in the fluorescence curve. They gave the same patterns as the negative control. These findings indicated that samples did not contain norovirus or the real time PCR detector could not detect the low copy number of norovirus. PCR technique allows determination of norovirus not fewer than 10^2 particles from food extract. On the other hand the infection dose is said to be 10-100 particles, which represents the detection line.

The negative results lead to the conclusion that during the period of this study shrimps were not or had very low contamination at the market level. Therefore, there existed no load of virus during rearing, culturing, harvesting and transportation at the moment of sampling.

Shrimp farming in Thailand is of high benefit to the owners but also needs high capital input. The farmers, who run the shrimp farms have to work very carefully, because a small mistake may lead to high loss. Ponds and equipment have to be cleaned and disinfected before use. The shrimp fries and feed have to come from

reliable sources. Nevertheless, has been found from a review of the literature that the farm cleansing system does not eliminate the norovirus since a virus can survive a long time in the environment, is a wide range of pH and many kinds of disinfection (Barker *et al*., 2004). Also many reports show that norovirus was detected from various water sources especially polluted water (Abel, 2000; Van den Berg, *et al*., 2005; Pusch *et al*., 2005), and that the main shrimp farming areas in Thailand are also polluted (Szester, 2003). On the other hand there is no signal from this study that norovirus must be taken into account as a "serious" severe and "frequent" hazard in shrimp farming.

Mainly infected people are an important source of transmission. Norovirus is found in the stool and vomit of ill people. Persons involved in shrimps from farm to market, such as the farmers and their employees, workers and brokers in the auction market, as well as the visitors who are sick with norovirus gastroenteritis are particular risk groups for the spread of the virus. It is estimated that as many as half of all norovirus outbreaks are connected directly with contamination by an ill person (Widdowson, 2005). However, that kind of contamination did not happen during this study.

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DECLARATION

I, the undersigned, declare that the thesis is my original work and has not been presented for a degree in any University.

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