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THESIS

DETECTION AND IDENTIFICATION OF *PSEUDOMONAS* SPP. IN AQUATIC ANIMALS

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Pseudomonas spp. is one of the major bacterial diseases in aquaculture production. The aquatic animals subjected to some type of stress seem to be more susceptible to the disease. The infected animals are usually show haemorrhagic, septicaemia and whirling movement. These pathogen can spread rapidly in the farm. *P. fluorescens* is a dominant pathogen in this group and is economically significant. Monoclonal antibody (MAb) against a *P. fluorescens* was produced with the aim to develop rapid and practical method to detect this pathogen. Two MAbs were produced namely MAb 2E7 and MAb 9A1. To develop rapid method, peroxidase conjugation of the selected MAb (MAb 2E7 and MAb 9A1) was performed. The result showed MAb 9A1 conjugated with peroxidase still recognized *P. fluorescens*. Thus MAb 9A1 was considered to be used as a probe for strain detection and rapid diagnosis kit for *P. fluorescens* in the future.

Furthermore, DNA-based method was also developed for detection and identification purpose. The 16S-23S ribosomal DNA (rDNA) intergenic spacer was characterized and subsequently PCR was developed. The PCR was proven to be specific and sensitive to detect *Pseudomonas* spp. to the sub-clinical infection level. To identify upto species level, specific probes were studies and applied by reverse cross blot hybridization (RCBH). The method was highly specific for *Pseudomonas* spp.

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Student's signature Thesis Advisor's signature

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INTRODUCTION

Aquaculture has been a major sector contributing large income to Thailand. Farm management is a key of success to produce a good production to supply the world market. Improper farming practice can induce various disease problems, including parasitic, fungal, viral and bacterial diseases.

Pseudomonas infection is one of common bacterial diseases found in aquaculture. A number of aquatic animals including fish, frog and soft-shelled turtle are reported to be susceptible to the *Pseudomonas* spp. with moderate to high losses (Chinabut and Somsiri, 1998; Somsiri and Soontornvit, 2002). The infected animals often show opaque eyes and/or edema, especially in frogs infected with *P. fluorescens.* Whirling movement and head twisting are also common clinical signs of the *Pseudomonas* infection. The disease can be transmitted from parent to off-springs. When the aquatic animals are subjected to some type of stress, the symptom can easily be seen. This epidemic disease can spread rapidly in the farm. Farmer often use antibiotics for prophylaxis purpose and this inevitably causes some antibiotic resistant in the bacterium. Using overdose of antibiotics has become a consequence problem. The antibiotic residue may accumulate in aquatic animals and could threaten the public health. Thus the farm management could be a better way to prevent and control the disease. This is included early detection of the threatening pathogen.

Disease diagnosis is mainly based on a conventional biochemical test, which is time-consuming and requires a culturing procedure. This causes some delay of farm management, especially when the infection is in early stage and where the sensitivity of conventional methods is limited. Therefore, a rapid and more specific diagnosis method would be useful in term of disease control and farm monitoring. Monoclonal antibody (MAb) has become an extraordinarily important resource for medical

research, diagnosis, therapy, and basic science. MAb probe has made a significant impact on rapid diagnosis of numerous aquatic animal diseases (Adam *et al*., 1996). The present study aimed to develop MAb to *P. fluorescens* to be used as a probe for strain identification and a rapid diagnosis kit.

DNA-base technique is one of rapid technique to detect the pathogen in aquaculture. Thus we developed polymerase chain reaction to detect and identify *Pseudomonas* spp. This method was also aimed to improve sensitivity of the detection method. Moreover we described the use of a polymerase chain reaction (PCR) amplification of the intergenic spacer regions (ISRs) followed with a reverse hybridization technique to speciate *Pseudomonas* spp. up to species level.

We developed the MAb, PCR and PCR-RCBH to detect this pathogen. These techniques will help farmers detecting the pathogen in early stage of the infection. Therefore disease control can be highly effective. Consequently, the production cost will be lower.

OBJECTIVES

Overall objectives

The overall objective of this research project was to develop the rapid and simple method to detect *Pseudomonas* spp. in aquatic animals.

Specific objectives

1. To develop specific monoclonal antibody against *P. fluorescens*.
2. To develop detection method for *Pseudomonas* spp. by polymerase chain reaction.
3. To develop detection and identification method for *Pseudomonas* spp. by polymerase chain reaction-reverse cross blot hybridization (PCR-RCBH).
4. To develop the rapid and simple method to detect *P. fluorescens* in aquatic animals.

LITERATURE REVIEW

1. *Pseudomonas* spp.

The genus *Pseudomonas* was originally described by Migula in 1894. Pseudomonads are Gram-negative, straight or slightly curved rods motile by one or more polar flagella. They are oxidase positive, strictly aerobic chemo-organotrophs with a respiratory form of metabolism (Roberts, 1978). Palleroni *et al*. (1973) showed classification of *Pseudomonas* into five distantly related groups based on ribosomal RNA homology, called “RNA Similarity groups”. Over decades, classification of *Pseudomonas* had been poorly done. Later, re-classification of the organisms became a major interest of the bacteriologists. Several studies were made for assignment of members of the various RNA groups to different genera, leaving group I of Palleroni (1992) as *Pseudomonas* sensu strico (Table 1). Within this group the often reported pathogenic species including fluorescent group such as *P. aeruginosa*, *P. fluorescens* and *P. puitda*, nonfluorescent group such as *P. stutzeri* and *P. mendocina* and the plant pathogens, *P. syringae* and *P. cichorii* still remain in the Group I.

P. aeruginosa is a main species represent the Genus as it has been well characterized. It is also known as a genetical standard species since its genome has now been fully sequenced. In addition, *P. aeroginosa* is the most important as an opportunistic pathogen of humans. In contrast to *P. fluorescens*, *P. putida* is notable heterogenous species that can be subdivided by various taxonomic criteria into subspecies referred to as biotypes or biovars. Bossis *et al*. (2000) reported, *P. fluorescens* was subclassed into 5 biovars and *P. putida* was subclassed into 2 biovars.

The members of Group II mainly consist of animal and plant pathogens. One of the most important species of this group is *Pseudomonas (Burkholderia) cepacia*, know as plant pathogen and animal pathogen. The third RNA similarity group (Group III) is represented by five species. Two of the species, *P. acidovorans* and *P. testosterone* have been closely related to the new proposed genus *Comamonas* and currently they are classified as *Comamonas acidovorans* and *C. testosterone*.

Table 1 Some properties and present nomenclature of the principal species of *Pseudomonas* sensu stricto and of members of other RNA.

RNA group	Species	Comments	Present genus allocation
I	*P. aeruginosa*	Oxidase positive; fluorescent,	*Pseudomonas*
	P. putida	saprophytic, or opportunistic	*Pseudomonas*
	P. fluorescens	pathogens. Some of them can be	*Pseudomonas*
	P. chlororaphis	subdivied into a number of biovars.	*Pseudomonas*
	P. cichorii	Fluorescent; pathogenic for plants or	*Pseudomonas*
	P. syringae	mushrooms. *P. syringae* and	*Pseudomonas*
	P. viridiflava	*P. viridiflava* are oxidase negative.	*Pseudomonas*
	P. agarici	*P. syringae* comprises	*Pseudomonas*
	P. tolaasii	many pathovars.	*Pseudomonas*
	P. asplenii		*Pseudomonas*
	P. fragi	Nonfluorescent species	*Pseudomonas*
	P. stutzeri		*Pseudomonas*
	P. mendocina		*Pseudomonas*
	P. alcaligenes		*Pseudomonas*
	P. pseudoalcaligenes		*Pseudomonas*
II	*P. cepacia*	Most species are either plant or	*Pseudomonas*
	P. gladioli	animal pathogens.	*Pseudomonas*
	P. caryophylli		*Pseudomonas*
	P. mallei		*Pseudomonas*
	P. pseudomallei		*Pseudomonas*
	P. pickettii		*Pseudomonas*
	P. solanacearum		*Pseudomonas*
III	*P. acidovorans*		*Comamonas*
	P. testosteroni		*Comamonas*
	P. delafieldii		*Acidovorax*
	P. facilis	Facultatively autotrophic species ("hydrogen pseudomonas")	*Acidovorax*

Table 1 (Continued)

RNA group	Species	Comments	Present genus allocation
III	*P. saccharophila*	Facultatively autotrophic species	*Pseudomonas*
	P. flava	("hydrogen pseudomonas")	*Hydrogenophaga*
	P. pseudoflava		*Hydrogenophaga*
	P. carboxydoflava		*Hydrogenophaga*
	P. palleronii		*Hydrogenophaga*
	P. taeniospiralis		*Hydrogenophaga*
IV	*P. diminuta*	Very distantly related to group I	*Pseudomonas*
	P. vesicularis		*Pseudomonas*
V	*P. maltophila*		*Xanthomonas*

Source: Palleroni (1992)

The two genera in Group IV, *P. diminuta* and *P. vesicularis*, have been very distantly related to Group I in their properties, these bacteria more closely related to genus *Gluconobacter*. The last group (Group V) consists of *P. maltophilia* which currently has been classified as *Xanthomonas maltophilia*. *X. maltophilia* is a universal plant pathogen commonly found as a saprophyte in the natural habitats. It is also occasionally isolated from clinical specimens (Todar, 2004).

2. Pseudomonadaceae representatives in aquaculture

Bacteria belonging to the genus *Pseudomonas* are widely distributed in soil and water environments. A few members of *Pseudomonas* are pathogens of plants, fungi, animals and human. (Lyczac *et al*., 2000; Locatelli *et al*., 2002). Pathogenic species causing diseases in aquatic animals are including *P. anguilliseptica, P. chororaphis*, *P. fluorescens*, *P. plecoglossicida*, *P. pseudoalcaligenes* and *P. putida* (Austin and Austin, 2007). In addition, *P. aeruginosa* was reported in frog by Somsiri and Soontornvit (2002).

2.1 *Pseudomonas anguilliseptica*

P. anguilliseptica was originally described by Wakabayashi and Egusa (1972) as the bacterial causative agent of “Sekiton-byo”, or red spot disease of pond cultured Japanese eel, *Anguilla japonica*. It has been also reported in a wide rage of fish species, including Japanese eel *Anguilla japonica* (Wakabayashi and Egusa, 1972, Kuo and Kuo, 1978), European eel (Nakai and Muroga, 1982; Stewart *et al*., 1983), salmonid fish (Wiklund and Bylund, 1990), turbot *Scophthalmus maximus*, sea bass *Dicentrarchus labrax* (Berthe *et al*., 1995), sea bream *Sparus aurata* (Doménech *et al*., 1997; Doménech *et al*., 1999), black-spot sea bream *Pagellus bogaraveo* (López-Romalde *et al*., 2003) and cod *Gadus morhua* (Ferguson *et al*., 2004; Balboa *et al*., 2007).

Infected in rainbow trout showed haemorrhages at the fin bases. The petechial haemorrhages were observed in the peritoneum and occasionally in the liver and adipose tissue of the visceral organs (Wiklund and Bylund, 1990). Winter disease of sea bream, in which affected fish showed slow erratic swimming on one side at the water surface before sinking to the bottom of the cage and dying (Doménech *et al*., 1999). The moribund fish appeared lethargic or disoriented and showed ‘rolling’ or lack of balance. External lesions included bilateral flared opercula, opacity of the lens and cornea, and patchy reddening of the epidermis of the head. Internally there were haemorrhagic lesions on the liver although these were apparent in some moribund (Ferguson *et al*., 2004).

2.2 *Pseudomonas chororaphis*

P. chororaphis caused disease in Amago trout *Onchorhynchus rhodurus* (Hatai *et al.*, 1975). A high mortality occurred among advanced fry reared at a trout farm. In moribund fish the abdomen became distended with ascite fluid and haemorrhage were foundin various parts of the body (Hatai *et al*., 1975).

2.3 *Pseudomonas fluorescens*

P. fluorescens is likely to be spread through water, which will serve as the primary reservoir of infection. *P. fluorescens* is a dominant component of the freshwater ecosystem. At various times, *P. fluorescens* has been considered as a fish spoilage organism, a contaminant or secondary invader of damaged fish tissues, as well as a primary, but poor pathogen (Austin and Austin, 1987).

It has been reported to cause disease in a wide range of fish species, including gold fish *Carassius auratus* (Bullock, 1965), grass carp *Ctenopharyngodon idella* and black carp *Mylopharyngodon piceus* (Bauer *et al*., 1973), rainbow trout *Oncornynchus mykiss* (Li and Flemming, 1967; Li and Traxler, 1971, Sakai *et al*., 1989), carp *Cyprinus carpio* (Shiose *et al*., 1974), sea bream *Evynnis japonica* (Kusuda *et al*., 1974), tilapia *Sarotherodon niloticus* (Miyashita 1984), char *Savelinus pluvinus* (Yamamoto and Takahashi 1986), tench *Tinca tinca* (Ahne *et al*., 1982), silver carp *Hypophthalmichthys molitrix* and bighead *Aristichthys nobilis* (Csaba *et al*., 1981, Markovic *et al*., 1996), soft shell turtle *Trionyx sienensis* (Chinabut and Somsiri, 1998) and frog *Rana tigerina* (Somsiri and Soontornvit, 2002).

P. fluorescens is usually associated with haemorrhagic bacterial septicaemia. The clinical sign is indistinguishable from aeromonad septicaemias. The aeromonad infection is usually associated with concurrent environmental stress, especially high temperatures or intensive population. Pond fishes are most commonly associated with pseudomonad septicaemia. Generally aquarium tropical fish, marine fish and salmonids often showed, chronic symptom of the disease (Roberts, 1978). The symptoms of *P. fluorescens* infection in aquatic animals are included hemorrhagic opercula, eroded fin and ulcerated skin (Egusa, 1992) and whirling movement by adherence to nerve cells (Picot, 2001).

2.4 *Pseudomonas plecoglossicida*

P. plecoglossicida caused mass mortalities in pond-cultured ayu *Plecoglossus altivelis* (Temminck and Schlegel) during 1990's causing a huge economic loss in Japan (Wakabayashi *et al*., 1996). Diseased fish usually show pale gills and pale body as well as distended abdomen resulting from ascites. Lesion in spleen and kidney are commonly found. Necrotic lesion with haemorrhage, fibrin deposition and oedema is found in splenic pulp, sheathed tissue, and kidney. Liver also has necrotic lesions and abscess formations. However, no lesion is found in the brain (Kobayashi *et al*., 2004).

2.5 *Pseudomonas pseudoalcaligenes*

P. pseudoalcaligenes was reported by Austin and Stobie (1992) that caused disease in rainbow trout *Oncornynchus mykiss*. The fish display skin lesions extending over the entire flank, from the operculum to the tail. The skin and underlying muscle are eroded. In addition, moribund fish show haemorrhage in eyes and at the bases of fins. Rotten tail is also occurred (Austin and Stobie, 1992).

2.6 *Pseudomonas putida*

P. putida was isolated from rainbow trout *Oncorhynchus mykiss* (Walbaum) in Germany (Gierer *et al*., 1992), ayu *Plecoglossus altivelis* and yellowtail *Seriola quinqueradiata* (Kusuda and Toyoshima, 1976; Muroga, 1990; Wakabayshi *et al*., 1996), soft shell turtle *Trionyx sienensis* (Chinabut and Somsiri, 1998), frog *Rana tigerina* (Somsiri and Soontornvit, 2002), rainbow trout *Oncorhynchus mykiss* (Altinok *et al*., 2006). External clinical signs are included exophthalmia, dark pigmentation, frayed dorsal fin and ulceration on the dorsal surface of the fish (Altinok *et al*., 2006).

2.7 *Pseudomonas aeruginosa*

P. aeruginosa was isolated from diseased frogs (Somsiri and Soontornvit, 2002) in Thailand. Diseased frogs often reveal opaque eyes with/without edema, and emaciation.

2.8 *Pseudomonas diminuta*

P. diminuta was found in frog (unpublished data). Typical clinical signs are included opaque eyes, swollen feet, haemorrhagic lesions, abscesses, twisted head, and whirling movement.

Upto date, there are only four species causing problems in aquaculture industry of Thailand (Chinabut and Somsiri, 1998; Somsiri and Soontronvit, 2002). These pathogens are *P. aeruginosa*, *P. fluorescens*, and *P. putida* which belong to Group I, and *P. diminuta* which belongs to Group IV.

3. Diagnosis

Disease diagnosis is presently based on a conventional biochemical tests which are time-consuming, requiring a lengthy culturing procedure that need a selective Pseudomonas F agar following incubate at 22-28 ^{o}C for 24-48 h. Therefore, a rapid and more specific method of pseudomonad diagnosis would be useful for early detection in order to control the disease as well as for farm management. Identification of pseudomonads has been tedious since their phenotypic properties are highly uniform among the species. However, identification is valuable in terms of taxonomy and may lead to a better understanding of this genus.

The identification methods were mainly based on DNA analysis such as DNA sequencing and polymerase chain reaction (PCR) in conjunction with restriction fragment length polymorphism (RFLP) (Widmer *et al.*, 1998; De Vos *et al.*, 1998).

Many methods have been documented for detection of pseudomonas using both DNA-based and antibody-based methods. For instance, De Vos *et al.* (1997) reported PCR method using primers of the two outer membrane lipoprotein genes (*opr*I and *opr*L). Kragelund *et al.* (1996) described antibody-based method by using dot blot to detect the group I Pseudomonas, whereas González *et al.* (1996) described the ELISA method using the polyclonal antibodies against protein F as a probe.

4. Treatment

Due to close association of pseudomonad infections with poor environment conditions, great improvements in the condition of infected fish may be achieved by reduction of stocking densities or improvement of water quality. Where therapy has been attempted, good results have been claimed for kanamycin injected intraperitoneally or for oxytetracycline orally however such infected fish will not usually feed (Roberts, 1978). Altinok *et al*. (2006) suggested that *P. putida* isolated from fish was resistant to most commonly used antibiotics; thus, it is hard to treat the *P. puitda* infection. Moreover, this bacterium is easy to develop resistant against the antibiotics resulting subsequent application of the drug are often not effective. Using overdose of the drug will accelerate the development of fully resistance for the bacteria to the drug.

5. Monoclonal antibody

Antibodies are immune system-related protein called immunoglobulins. Each antibody consists of two heavy chains and two light chains joined to form a “Y” shapes molecule. The two heavy chains are connected to each other by interchain disulfide bonds and each of the light chains are linked bond to heavy chains. The antigen binding region has great diversity. The constant region defines the five different class of immunoglobulin (IgG, IgA, IgE, IgM and IgD) distinguished by different heavy chains. Furthermore, there are four subclasses of IgG. Each class possesses different biological as well as structure properties (Nicholson, 1993). Antibodies made by a B-cell which can recognize and have a function to specifically

attach to foreign molecules calls antigen and alert the host's cellular immune response to destroy or neutralize them.

These cells are one of the final products of B-lymphocyte differentiation. The production of a strong antibody response is controlled by inducing and regulating the differentiation of B cells into plasma cells. During this differentiation, B cells go from virgin B cells, which have a modified antibody as a cell-surface antigen receptor and do not secrete antibodies, to activated B cells, which both secrete antibodies and have cell-surface antibodies, to plasma cells, which are highly specialized antibody factories with no surface antigen receptors. The steps in this differentiation process are controlled by the presence of the antigen and by cell-to-cell communication between B cells and helper T cells (Harlow and Lane, 1988).

It is important to realize that each antigen consists of a number of different epitopes. Therefore, an antigen may stimulate the proliferation of many different lymphocyte clones, each producing antibodies against a specific epitope. Thus, the immune response to an antigen is polyclonal with the resulting antisera containing a mixture of antibodies, each specific for different epitope (Nicholson, 1993). Mice, most commonly BALB/c, are immunized with an antigen, only one-tenth of the circulating antibodies are specific for the immunogen (Adams *et al*., 1996). Antibodies secreted by a B-lymphocyte clone are identical and therefore are a source of homologous antibodies. The splenic lymphocytes from the immunized mice are short lived and can not be grown in culture. In 1975, Köhler and Milstein developed a technique which allowed splenic lymphocytes to be fused with myeloma cells allowing the resulting hybridoma cells to grow indefinitely *in vitro*. Hybridoma cells are a hybrid of these cell types with the ability to grow in culture, but also to secrete an antibody with a defined specificity. These are known as monoclonal antibodies (MAbs). MAbs are powerful immunological tool because of the homogeneity of the antibodies from a particular hybridoma cell line (Harlow and Lane, 1988).

MAbs can be produced from impure antigen by selecting single cell clones after the fusion. The spleens of immunized mice are removed and suspension of single cells prepared. Individual splenocytes in the cell suspension are fused, usually by polyethylene glycol, with cells of a continuous mouse myeloma culture (Nicholson, 1993). Many of the commercially available myelomas have been isolated from BALB/c mice by injecting mineral oil into the peritoneum of the animal (Harlow and Lane, 1988). Myeloma cell lines used in fusions have been selected because they do not produce antibody molecules. For this reason P3x63.Ag8-653 and Sp2/0-Ag14 cell lines are the most frequently used lines because they do not secrete immunoglobulin heavy and light chains.

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused. This leaves a large number of unfused cells, both spleen and myeloma cells, still present in the culture. The spleen cells from the mouse die with 3 days of culture and are therefore not a problem. However, the myeloma cells quickly adapt to the culture conditions and will outgrow the hybridoma cells resulting from the fusion. Removal of the myeloma cells is therefore essential and is achieved by drug selection. Typically, myeloma cells are constantly diving B-tumor cells which are defective in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis (hypoxanthine-guanine phosphoribosyl trasferase gene, HGPRT mutants). These hybridomas Addition of aminopterin to the culture medium blocks the *de novo* nucleotide synthesis pathway and forces the cell to use the salvage pathway in which HGPRT uses exogenous hypoxanthine and thymidine. Myeloma cells defective in HGPRT are unable to use this pathway and therefore die in culture. The only cells able to grow in HAT culture medium (hypoxanthine, aminopterin, thymidine) are the hybrids between myelomas with nonfunctional HGPRT and cells with functional HGPRT, which are unable to synthesis DNA via *de novo* nucleotide synthetase pathway and rely on the salvage pathway for DNA synthesis, will be able to grow (Fig. 1 and Table 2) (Harlow and Lane, 1988).

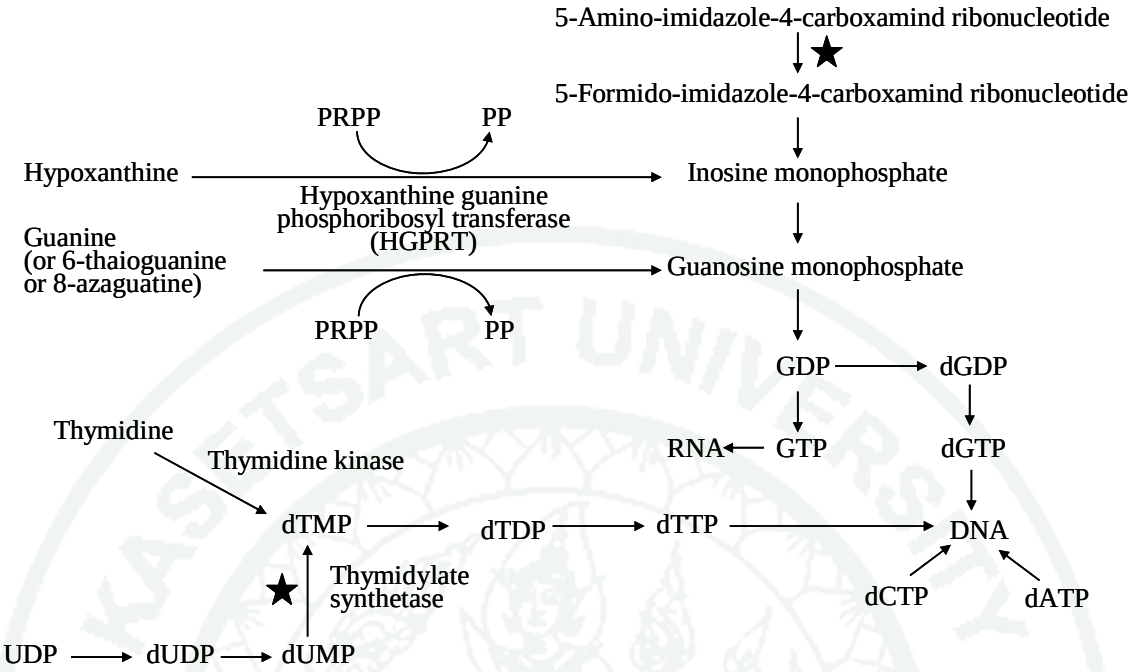

Figure 1 De novo and Salvage nucleotide synthesis pathways. The main synthetic pathway are blocked with the folic acid analogue aminopterin (★).

Source: Goding (1996)

Table 2 Growth of cells cultured in HAT medium.

Cell type	DNA synthesis		Survival in HAT medium
	Salvage pathway	De novo pathway	
Myeloma	HGPRT$^-$	Aminopterin sensitive	Die (No DNA synthesis)
Spleen	HGPRT$^+$	Aminopterin sensitive	Die (Finite survival in vitro)
Myeloma-spleen hybrid	HGPRT$^+$	Aminopterin sensitive	Live
Myeloma-myeloma hybrid	HGPRT$^-$	Aminopterin sensitive	Die (No DNA synthesis)
Spleen-spleen hybrid	HGPRT$^+$	Aminopterin sensitive	Die (Finite survival in vitro)

Source: Liddell and Weeks (1995)

The ability to generate hybridoma cell lines *in vitro* produced of unlimited quantities of MAbs, homogenous populations of immunoglobulin molecules specific for a single antigenic epitope. Moreover, hybridoma cell lines have the advantage of providing an unlimited supply of the antibody in the cell supernatant. All these have become an extraordinarily important resource for medical research, diagnosis, serology, therapy, and basic science. MAb probes have made a significant impact on the rapid diagnosis of numerous fish diseases (Nicholson, 1993; Adam *et al.*, 1995).

Many MAbs have been developed against microbial pathogens such as *Staphylococcus* species (Yazdankhah *et al.*, 1998), *Ureaplasma urealyticum* (Naessens *et al.*, 1998), *Burkholderia pseudomallei* (Pongsunk *et al.*, 1999), *Yersinia pestis* (Prior *et al.*, 2002), iridovirus (Shi *et al.*, 2003), *Edwardsiella tarda* (Kumar *et al*., 2008), *Vibrio* spp. (Longyant *et al*., 2008) and spring viraemia of carp virus (Chen *et al*., 2008) and others. These MAbs were mainly developed for the pathogen diagnostic purpose, which several as rapid and specific method. Some of those were developed the MAbs for identification purpose.

Thus, developing specific monoclonal antibody against *P. fluorescens* coupled with this technology make it possible to develop an efficient diagnostic kit for detection of the bacteria in aquatic farms and could possibly be used as an probe for identification purpose.

6. Ribosomal RNA

Ribosomal RNA (rRNA) is the central component of the ribosome, the protein manufacturing machinery of living cells. In prokaryotes a small 30S ribosomal subunit contains the 16S rRNA. The large 50S ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Normally ribosomal RNA genes located in the order 16S, 23S, and 5S rRNA genes, which are separated by intergenic transcribed spacer (ITS) regions in bacteria. The rRNA genes are organized in a ribosomal operon (*rrn*) and the *rrn* operon is a multigene family that found in most bacterial genomes. Thus,

analysis this multigene family, *rrn* operons and tRNA genes have been demonstrated to be highly useful among genotyping methods.

The rRNA-coding regions (16S rDNA) have been extensively used to underpin phylogenetic relationships at the species level or above (Woese, 1987). The 23S rDNA sequences are available for a few bacterial species and this variation has been used for typing clinical isolates (Ludwig *et al.*, 1994; Anthony *et al.*, 2000). However, intergenic spacer regions (ISRs), especially those located between the 16S and 23S rDNAs have been proved to be under less evolutionary pressure (Rijpens *et al.*, 1996; Smart *et al.*, 1996; Sawada *et al.*, 1997; Berridge *et al.*, 1998; Chun *et al.*, 1999). The ISRs were widely used in bacterial differentiation and identification of closely related microorganisms or stains (Xian-Yu *et al*., 2006) for instance the study of *Streptococcus thermophilus* (Mora *et al*., 2003), *Aeromonas* sp. (Laganowska and Kaznowski, 2004), *Vibrio vulnificus* (Xian-Yu *et al*., 2006), *Streptococcus phocae* (Hassan *et al*., 2008) and *Proteus* sp. (Cao *et al*., 2009).

7. Reverse cross blot hybridization

This method is another branch of hybridization assay, which the probes are bound to a membrane and the target DNA is applied in solution to the membrance (Dattagupta *et al*., 1989; Saiki *et al*., 1989). Hybridization with labeled target DNA in lines perpendicular to the probe lines enables simultaneous testing of several samples to several probes. In recent year, a PCR-reverse cross blot hybridization method have been reported to detect several bacteria such as in *Brucella* spp. (Rijpens *et al.*, 1996), *Mycobacterium* spp. (Kox *et al*., 1995; Posteraro *et al*., 1998; Sanguinetti *et al*., 1998; Puttinaowarat *et al*., 2002a) and in freshwater bacterial groups (Zwart *et al*., 2003) that have been more specific and more sensitive.

MATERIALS AND METHODS

1. Study on monoclonal antibody production

1.1 Culture and preparation of bacterial isolates

P. fluorescens and non-Pseudomonas were grown on tryptic soy broth (TSB) at 30^{o}C for 18 h. The bacteria were harvested by collecting the suspension. It was centrifuged at 3,500 x g for 30 min and pellet was washed once with 50 mL sterile saline (0.85 % NaCl). It was then resuspended and adjusted to 1.0 x 10^{7} cells mL^{-1} with sterile saline and stored at 4^{o}C. The *P. fluorescens* (AAHRI 01419) suspension was subjected to UV at program sterile (GS Gene Linker, BioRad) for 9 min. Then the suspension was divided into two: one was kept for the next step and another was sonicated for 5 min (Sonopuls HD 70, Bandelin). Both set were checked their viability by growing on tryptic soy agar (TSA) and cultured at 30^{o}C for 18 h.

1.2 Immunization of mice

Four 8 week-old BALB/c female mice were set into two groups: one was for the UV-treated antigen and another was for the sonicated antigen. Each two mice were intraperitoneally (ip) injected with 100 μL of *P. fluorescens* (AAHRI 01419) suspension (1.0 x 10^{7} cells mL^{-1}) mixed 1:1 with TiterMax Gold adjuvant (CytRx Corporation). Twenty-eight days after the first injection, the second injection was given. Ten days later, blood was collected from tail for antibody titer. The serum was serial diluted to test against *P. fluorescens* at 1.0 x 10^{7} cells mL^{-1} by indirect enzyme-linked immunosorbent assay (ELISA). Four weeks after the second injection, the third injection was given. The antibody titer test was done 10 days after the injection. Four weeks after the third injection, a mouse was intravenously (iv) injected with 100 μL of bacterial suspension (0.5 x 10^{6} cells mL^{-1}) without adjuvant. The spleen was collected at day 3 after the injection.

1.3 Hybridoma production

Hybridomas were produced by the method of Cambpell (1984) with modifications (Adams *et al*., 1992). The process are including maintenance of myeloma cells, preparation of mouse red blood cell feeder layer, preparation of mouse spleen cells, fusion, screening hybridoma ELISA procedure, second screening prior to the expansion, expansion positive clones, cloning of positive clones, expansion into flasks, antibody harvesting, cryopreservation of cell lines and thawing cell lines.

1.3.1 Maintenance of myeloma cell line

A mouse myeloma cell line (SP2/0-Ag-14) held storage in liquid N_2, was thawed out eight days prior to the fusion. The cells were placed in 37 ^{o}C water bath for 5 min approximately. Immediately, place into 9 mL DMEM with supplement of 2 mM L-glutamine, 100 i.u. mL^{-1} penicillin, 100 μg mL^{-1}streptomycin, 0.5 mM sodium pyruvate, 10% foetal bovine serum (FCS, heat inactivated for 30 min at 56 ^{o}C). The cell resuspension was centrifuged for 7 min at 150 x g and the cell pellet was resuspended gently in 5 mL of DMEM with additives and 20% FCS. This was transferred in a 25 cm^3 tissue culture flask. The flask was placed in an incubator at 37 ^{o}C and 5% CO_2. Five days prior to the fusion, the cells were counted (using a haemocytometer) and the cell concentration was adjusted to 1.5 x 10^5 cells mL^{-1} with the addition of fresh medium. The cells were maintained in the mid-log phase.

On the day of fusion, the cells were counted again, and approximately 1.0 x 10^7 cells mL^{-1} were used.

1.3.2 Preparation of mouse red blood cell feeder layer

Non-immunized mouse was killed by exposure to chloroform. This mouse was bled immediately by cardiac puncture using a 1 mL syringe containing 10 i.u. of sterile heparin connected to a 23G needle. Approximately, 0.7 mL of blood was taken from the mouse and then added directly in 200 mL of DMEM

with the additives (hypoxanthine, aminopterin, thymidine) and placed in the 37 ^{o}C incubator.

1.3.3 Preparation of mouse spleen cells

Three days after the final booster injection, the immunized mouse was killed by exposure to chloroform. The mouse was bled immediately by cardiac puncture. The blood was placed into 1.5 mL eppendrof tube and left for 2 h at room temperature (RT) to allow it clot. Then centrifuged at 300 x g for 15 min and collected serum. This serum was used to establish the antibody response of the mouse at the time of fusion. The mouse was swabbed with 70% alcohol and carefully cut the gut opened. The spleen was removed and quickly placed into petridish containing 20 mL of DMEM (serum free warmed to 37^{o}C). Fatty tissue was trimmed and washed 3 times in the DMEM. The spleen was then placed onto a fresh Petri dish containing 5 mL of DMEM. The spleen was cut at both ends and passed through with the medium to remove the spleen cells. The spleenocyte suspension was placed in a universal and allowed to stand for 1 min. Then collected supernatant into a new universal and made the volume up to 50 mL of warmed serum-free DMEM.

1.3.4 Fusion

Both type of cells, myeloma and spleenocytes, were removed from their respective tissue culture flasks. They were washed twice with warm DMEM (serum-free) at 150 x g for 10 min. After the second centrifugation the supernatants were removed and 10 mL of fresh warm DMEM (serum-free) was added to resuspend the cells. The myeloma and spleen cells were counted using a haemocytometer. The volumes of the myeloma and spleen suspensions were adjusted using warm DMEM (serum-free) to give a ratio of 1:10 myeloma to spleen cells. Myeloma and spleen suspensions were mixed and centrifuged at 100 x g for 10 min. The supernatant was aspirated and then gently added 2 mL of Polyethylene Glycol (PEG, 50% w/v in DMEM warmed to 37^{o}C) to ease the pellet off the bottom. Allow the cells to stand for 2 min. The gently added 50 mL of serum-free DMEM into the

mixed cells. The cell suspension was centrifuged at 100 x g for 5 min and discarded the supernatant. The pellet was then resuspended very gently in 10 mL of DMEM with additives and hypoxanthine (0.1 mM)-thymidine (0.016 mM). The cells were placed at 37 ^{o}C in a CO_2 incubator for 2 h and 30 min. Then they were centrifuged at 100 x g for 5 min and resuspended in 200 ml of the mouse red blood cell feeder layer as described in section 1.3.2. The cell suspension was placed 180 μL well^{-1} onto the 96-well-plates using a multichannel pipetter. The plates were incubated in the 37 ^{o}C incubator.

The plates were checked five days after fusion for hybridoma growth. Hybridoma growth was determined by the presence of aggregations of tiny cells in the wells. Ten days after the fusion the supernatants from the wells with hybridomas were screened for antibodies specific to *P. fluorescens* (AAHRI 01419), using indirect ELISA.

1.3.5 Screening hybridomas ELISA procedure

Supernatants from the fusion were screened by an indirect ELISA using a modification of the method of Adams *et al*. (1992).

The 96 well plate ELISA plates (NUNC^{TM}, Apogent) were coated with 50 μL of 0.01% (w/v) poly-L-lysine in 0.125 M carbonate-bicarbonate buffer pH 9.5. Plates were incubated for 1 h at RT before washing twice with low salt wash buffer (LSW; 0.02 M phosphate, 0.38 M NaCl, 0.05% Tween-20, pH 7.3). *P. fluorescense* and other bacteria (whole cell bacteria) were adjusted to 1.0 x 10^{7} cells mL^{-1} with sterile saline and were added to each well at 1.0 x 10^{6} cells mL^{-1} final concentration and incubated at 4 ^{o}C overnight. A solution of 0.05% glutaraldehyde in PBS was added into each well with a 20 min further incubation. The plates were then washed three times with LSW.

Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) for 2 h at RT. After washing the plates with LSW, supernatants or anti-sera (100 μL $well^{-1}$) was added and incubated at RT for 2 h. The plates were then washed three times with high salt wash buffer (HSW; 0.02M phosphate, 0.5M NaCl, 0.1% Tween-20, pH 7.7) with a 5-min soaking on the last wash. Goat anti-mouse immunoglubulin-G (IgG) labeled with horseradish peroxidase (Sigma), diluted 1:5,000 in PBS was added 100 μL $well^{-1}$ and incubated at RT for 1 h. Plates were then washed again as described above. The assay was developed with chromogen in substrate buffer (0.1M citric acid, 0.1M sodium acetate (pH 5.4), and 0.33% (v/v) H_2O_2) at 100 μL $well^{-1}$. Finally, the reaction was stopped with 2M H_2SO_4 and measured at 450 nm (BIO-TEK instruments, CERES UV900 HDi).

1.3.6 Second screening prior to first expansion

Supernatants from the wells containing hybridomas were rescreened by indirect ELISA, 10 days after the first screening, to ensure the positive clone and be ready for expansion.

1.3.7 Expansion positive clones

The positive clones were transferred into 24-well-plates with additional fresh medium. They were incubated for a further 10 days and supernatant was again tested. Then the remaining positive clones were expanded into three wells of the 24-well-plate. After a further 10 days incubation, they are ready for the next step.

1.3.8 Cloning of positive clones

Positive clones were removed from the wells and added into the new fresh medium in the 96-well-plate. Place each clone into the first left top well of each plate, then withdrew 100 μL of the first well and added to the next wells all the way down the lane. Then with the same manner did it across the entire plate. When

the plates were seeded with the positive clone, they were again incubated for another 10 days. Then the supernatant of the new clone was screened for a positive reaction to the *P. fluorescens* by indirect ELISA. These cloning were done for three time of each clone to ensure the positive MAb.

1.3.9 Expansion into flasks

When all of the wells tested were positive the cell line was confirmed to be producing monoclonal antibodies. Cells were then expanded into flasks for the production of large quantities of monoclonal antibody production. The presence of any negative wells (by indirect ELISA) containing clones suggested the presence of more than one cell line and therefore further cloning would be necessary.

A positive well, containing a single clone was selected and the cells were dispersed using a pipette. 100 μL was used to seed 5 wells on a 24-well plate each containing 1.5 ml of blood feeder cells in medium. After 10 days the contents of the wells were put into suspension and pooled. This cell suspension was added to two 25 cm^3 tissue culture flasks (Corning) each containing 10 mL of DMEM with additives.

Cell growth and survival in the flasks was checked using an inverted microscope. When cell density reached an optimum, (judged by the color of the medium and the visible condition of cells), 5 mL of cell suspension were removed and added to 45 mL of DMEM with additives in a 200 mL tissue culture flasks (Corning).

1.3.10 Antibody harvesting

Antibodies were harvested by carefully pipetting the medium from the flasks and placing it into universals. The flask medium was replaced immediately with 5 mL of fresh DMEM with additives. The universals, containing

antibody in medium, were centrifuged for 10 min at 150 x g. The supernatant was collected, placed into fresh universals, and stored at -70 ^{o}C.

1.3.11 Cryopreservation of cell lines

Cell lines were routinely cryopreserved throughout the procedure as back-up cells to prevent possible loss of lines through contamination.The suspension was centrifuged for 7 min at 150 x g and resuspended in DMEM containing 10% dimethyl sodium oxide (Sigma) to give an adjusted cell volume of 1 x 10^{6} cells ml^{-1}. The 2 mL of this suspension was transferred to cryovials (Cryogenic vials, Nalge company) placed in a CryoCane, Aluminum (Nalge Nunc International) surrounded by cotton and place overnight at -70 ^{o}C. The next day the vials were transferred to liquid nitrogen for permanent storage.

1.3.12 Thawing cell lines

Frozen vials were transferred from liquid nitrogen storage and immediately placed in a 37 ^{o}C water bath. When the contents of the vials had nearly completely thawed, the tops of the vials were wiped with 70% ethanol and the cell suspension pipetted into 9 mL of DMEM layered upon 1 mL of FCS. The suspension was centrifuged at 150 x g for 7 min, resuspended in 10 mL of DMEM with additives, placed in a 25 cm^{3} tissue culture flask and incubated for 5 days. After this period of time antibody production was determined using indirect ELISA.

1.4 Characterization of MAbs

Specificity of the MAbs was tested by indirect ELISA. The MAbs was further characterized by Western blot. Cross-reactivity was tested by indirect ELISA to different bacteria. The MAbs isotyping was tested with a Mouse-Typer® Isotyping Panel kit (BIO-RAD) as the manufacture described.

1.4.1 SDS-PAGE and Western blot (WB) analysis

Bacterial whole cell were diluted with sample buffer (0.5M Tris-HCl pH 6.8, glycerol, 10% SDS, 0.05% bromphenol blue, β-mercaptoethanol) based on protein concentration (50 μg protein well^{-1}) and boiled for 4 min. They were then centrifuged at 3,500 x g for 5 min before applying into the 12% gels. The gels were then subjected to electrophoresis under constant voltage of 120V for 60 min. A half of the gel was cut and stained with Coomassie brilliant blue R-250, while another was blotted onto nitrocellulose membrane using semi-dry Transblot apparatus (BioRad). Then the membrane was removed and incubated with blocking solution [1% BSA in TBS (10mM TriHCl pH 7.5, 0.5M NaCl)] for 1 h at 37 ^{o}C. Subsequently, it was washed three times, each 5 min with TTBS (0.5% Tween-20 in TBS) and incubated with neat hybridoma supernatants overnight at 4 ^{o}C. After incubation, the blot was washed as previously described and incubated with goat-anti-mouse IgG horseradish peroxidase conjugate for 1 h at RT. The membrane was washed again and incubated with substrate solution (4-chloro-1-naphthol; BioRad) until bands visible. The reaction was finally stopped with distilled water.

1.4.2 Cross reactivity of the MAb

Cross reactivity of each MAb was analyzed by indirect ELISA as described in section 1.3.5. The bacteria were selected including non-pseudomonad and pseudomonad reference strains.

1.5 Conjugate MAb-HRP

Peroxidase (HRP) (Sigma-Aldrich) (4 mg) was dissolved in 4 mL of nanopure water. To this was added 0.8 mL of freshly prepared 0.1M Sodium periodate (Sigma-Aldrich) (0.123g in 10 mL) and stirred 20 min in the dark at RT. Six drops of ethylene glycol was added to the resultant mixture and then was stirred for a further 5 min at RT in the dark. The activated HRP was dialysed at 4 ^{o}C, overnight in PBS. Prepared 7 mg antibody (MAb) (Ammonium sulphate precipitation) and added a

half of activated HRP. Then added carbonate buffer (1 M Carbonate, pH 9.5) 15 μL to get pH 9.5 and stirred for 2 h. Added 200 μL of 4 mg mL^{-1} sodium tetraborate and stirred for another 2 h. Dialysed overnight at 4 ^{o}C in PBS. The MAb-HRP conjugated was tested by indirect ELISA.

2. Study on detection of *Pseudomonas* 16S-23S ribosomal DNA intergenic spacer by PCR

2.1 Bacterial strains and culture conditions

All bacteria used in this study were grown on TSA (Oxoid) at 30^{o}C for 18 h. They were characterized by API 20E and API 20NE with additional tests as described by Cowan (1973).

2.2 Bacterial DNA isolation and amplification of spacer region

Genomic DNAs of four species including *Pseudomonas fluorescens* (TISTR 358), *P. putida* (DMST 10603), *P. aeruginosa* (ATCC 27853), and *P. diminuta* (AAHRI 96144) were extracted as previously described by Boom *et al.* (1990), with modifications. Bacteria were resuspended in 500 μL TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Proteinase K was added at a final concentration of 0.1 mg mL^{-1} and the bacteria were incubated for a further 1 h at 65^{o}C. The mixture was added to 900 μL lysis buffer L1 (5M GuSCN (Sigma), 1% Triton X-100, 50 mM Tris-HCl pH 6.4, 20 mM EDTA) and 20 μL diatom (10 g Colite (Acras), 500 μL HCl, 50 mL H_2O). The tube was mixed on a rotator shaker for 10 min and centrifuged (15 sec) in a microfuge (fixed angle, 12,000 x g), and the supernatant was discarded. The diatom nucleic acid pellet was subsequently washed twice with 900 μL washing buffer L2 (5M GuSCN in 0.1M Tris-HCl, pH 6.4), twice with 1 mL ethanol 70% (v/v) and once with 1 mL acetone. After disposal of the acetone, the pellets were dried at 56^{o}C with open lids for 10 min. Eighty μL TE buffer was added into the tubes and

incubated at 56^{o}C for 10 min. The tube was briefly vortexed again and centrifuged for 2 min at 12,000 x g, and the supernatant containing DNA was used for PCR reaction.

The DNAs of each isolates were then amplified in a DNA thermal cycler (OmniGene, Hybaid Ltd., UK). A typical reaction mixture (25 μL) consisted of reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 3.0 mM $MgCl_2$), 200 μM (each) deoxynucleotide triphosphate, 1 U *Taq* DNA polymerase (Promega), 5 ng DNA sample, and 10 pmol of each primer (P16sf and P23sr, previously described by Sawada *et al.*, 1997). The reaction mixture was cycled 35 times as follows: 30 sec denaturation at 94^{o}C, 30 sec annealing at 55^{o}C and 1 min extension at 72^{o}C. The vials were held at 4^{o}C until the PCR product was detected by 1.5% agarose gel electrophoresis.

2.3 Nucleotide sequencing

The PCR products were purified with phenol-chloroform and precipitated with ethanol. The DNA pellet was dissolved in 50 μL TE buffer. The fragment was ligated into pGEM T-Easy vector (Promega), and the recombinant plasmid was transformed into *Escherichia coli* by standard methods (Sambrook *et al.*, 1989). Plasmid were extracted from positive transformants by the alkaline lysis method (Sambrook *et al.*, 1989). Inserts were amplified with M13 primers using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit. The products were then analyzed by the ABI Prism 377 automatic sequencer (Applied Biosystems) following the manufacturer's instructions.

2.4 Primer design

The sequences of 16S-23S ribosomal intergenic spacer were aligned by using alignment with GENETYX V. 7 Program. They were compared with 16S-23S rRNA spacer regions of other prokaryotes available in the GenBank database (http://ncbi.nlm.nih.gov) including *Streptococcus pyogenes* (AF489597), *Staphylococcus aureus* (U11780), *Mycobacterium bovis* (AJ315569), *Escherichia coli*

(J01702) and *Bacillus subtilis* (J01551). Primers specific to genus were then designed and coded as PsGrF and PsGrR.

2.5 Determination of primer specificity

DNA was extracted from various non-pseudomonad and pseudomonad reference strains. Five ng DNA of each sample was amplified by PCR and examined by agarose gel electrophoresis.

2.6 Determination of primer sensitivity

DNAs were extracted from reference strains including *Pseudomonas fluorescens* (TISTR 358), *P. putida* (DMST 10603), *P. aeruginosa* (ATCC 27853) and *P. diminuta* (AAHRI 96144). Their concentrations were determined by spectrophotometer (Thermo Spectronic, Biomate 3), then they were diluted at 10-fold dilutions ranging from 50 ng to 0.5 fg (which correspond to *Pseudomonas* cultures diluted to 1.0×10^8 cells mL^{-1} to 1 cells mL^{-1}, respectively). Each sample was added to 24 μL PCR mixture and amplified as described above. The amplified DNAs were then analyzed by agarose gel electrophoresis.

2.7 Assessment of PCR

Pseudomonas fluorescens (AAHRI 03575), *P. putida* (AAHRI 00127), and *P. aeruginosa* (AAHRI 02007) were isolated and made up into dilution at 1.0×10^6, 1.0×10^7, and 1.0×10^8 cells mL^{-1}. Twelve frogs (*Rana tigerina*) were intramuscularly injected with each bacterial dilution at 100 μL 100 g^{-1} of body weight, while negative control groups were injected with 0.85 % NaCl at the same route. The frogs were kept in a tank with regular feeding and three frogs were sampled at day 3, 7 and 11. Liver, spleen and kidney were collected separately for bacterial isolation and PCR assay.

3. Study on detection and identification of *Pseudomonas* spp. by polymerase chain reaction-reverse cross blot hybridization (PCR-RCBH) with 16S-23S rDNA intergenic spacer probes

3.1 Oligonucleotide primers

Oligonucleotide primers used for amplifying the 16S-23S rRNA intergenic spacer region were selected from the conserved regions at 3' end of the 16S rRNA and the 5' end of the 23S rRNA genes. The primers were previously described by Sawada *et al.* (1997) namely P16sf (5'-TGAAGTCGTAACAAGGTAGC-3') and P23sr (5'-ATCGCCTCTGACTGCCAAGG-3'). Both primers were labeled with biotin at the 5' end and named as P16sf-Bio and P23sr-Bio.

3.2 Amplification of spacer region

A PCR was performed in a DNA thermal cycler (OmniGene, Hybaid Ltd., UK). The reaction mixture (50 μL) consisted of reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 3.0 mM $MgCl_2$) 200 μM (each) deoxynucleotide triphosphate, 10 pmol of each primer, 2 U of *Taq* DNA polymerase (Promega), and 5 μL of DNA sample. The reaction mixture was cycled 40 times as follows: 1 min denaturation at 94 ^{o}C, 1 min annealing at 52 ^{o}C and 1min 30 sec extension at 72 ^{o}C. The vials were held at 25 ^{o}C until the PCR product was detected by RCBH (Puttinaowarat *et al.*, 2002a).

3.3 Sequencing methods

The method was the same as previously described in section 2.3.

3.4 Reverse cross blot hybridization assay (RCBH)

3.4.1 Tailing of oligonucleotide probes with dTTP

The oligonucleotide probes used in the RCBH assay were homologous to internal sequences of the PCR products. The probes were tailed with dTTP. This facilitated hybridization of the probes by adding a spacer sequence. Two hundred pmol of each oligonucleotide was added to 8.8 μL of tailing solution, which contained 1.6 μL of 5x tailing buffer, 1.6 μL of 2.5 mM $CoCl_2$, 2 μL of 10 mM dTTP (Amersham Pharmacia) and 0.2 μL of 25 U TdT (Roche Diagnostics Ltd, Lewes, UK). The mixture was incubated at 37 ^{o}C for 2 h and 4 μL of 0.2 M EDTA (pH 8.0) was added to stop the reaction. The volume of dTTP-tailed oligonucleotide was made up to 400 μL with nanopure water giving a final concentration of 0.5 μM of dTTP-tailed oligonucleotide. The tailed probes were stored at –20 ^{o}C until required.

3.4.2 Hybridization assay

The hybridization assay followed the method previously described by Puttinaowarat *et al.* (2002a). Basically, a nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schuell) was placed in the hybridization apparatus (Schleicher & Schuell). Fifty pmol of each dTTP-tailed probe was diluted in 0.5 mL of 10x saline-sodium citrate buffer (SSC: 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Each of the diluted oligonucleotide probes were added to one of the slots of the mould and incubated overnight at 28 ^{o}C on a rotary shaker. The membrane was taken out of the apparatus and wrapped in a piece of cling film. The probes were fixed to the membrane by exposing them to UV light (BDH) at 312 nm until 1.5 J cm^{-2} was reached. The membrane was washed twice with 10x SSC and then incubated in hybridization solution [5x SSC, 1% blocking agent (Roche Diagnostics Ltd.), 0.1% *N*-laurylsarcosine, 0.02% SDS] for 5 min. The membrane was allowed to air-dry and kept at 4 ^{o}C until the next step of the process.

The Accutran cross unit was again assembled with the membrane and 200 μL hybridization solution was added to each slot. The membrane was then incubated on a rotator mixer at 20 ^{o}C for 5 min. Thirty μL of PCR product was placed into 1.5 mL screwcap vials and boiled at 100 ^{o}C for 5 min. The vials were placed on ice immediately after boiling and 200 μL of hybridization solution was added to each vial. The hybridization solution was removed from each slot and replaced with the DNA mixture. The unit was incubated at 50 ^{o}C for 1 h. The DNA mixture was discarded from each slot using a vacuum pump and the membrane was then removed from the unit. It was briefly rinsed with 0.1% SDS in 2x SSC and then incubated at 50 ^{o}C for 5 min in fresh 0.1% SDS in 2x SSC. The membrane was washed briefly with 100 mL of washing buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) and then incubated in 100 mL of a blocking buffer [0.5% (w:v) blocking reagent (Roche Diagnostics Ltd.) in washing buffer] at 28 ^{o}C for 30 min on a rotary shaker. The membrane was washed as described above then incubated with 10 mL of streptavidin conjugated alkaline phosphatase (0.1 U mL^{-1}) (Roche Diagnostics Ltd.) in washing buffer for 30 min at 28 ^{o}C. Unbound conjugate was removed by incubating the membrane in 100 mL washing buffer for 30 min. The membrane was equilibrated with 20 mL of a substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 2 min. Finally, it was incubated in 10 mL of substrate solution [45 μL of 4-nitroblue tetrazolium chloride (NBT, Roche Diagnostics Ltd.), 35 μL of 5-bromo-4-choloro-3-indyl phosphate (X-phosphate, Roche Diagnostics Ltd.), 10 mL substrate buffer] until the color completely developed. Rinsing the membrane with distilled water stopped the reaction.

3.5 Determination of probes sensitivity

DNA was extracted from reference strain including *P. aeruginosa* (ATCC 27853), *P. putida* (DMST 10603), *P. fluorescens* (TISTR 358), and *P. diminuta* (AAHRI 96144), which were diluted to 2 x 10^{6}, 2 x 10^{5}, 2 x 10^{4}, 2 x 10^{3}, 2 x 10^{2}, 2 x 10^{1} and 2 x 10^{0} cells mL^{-1}. Five μL of each DNA solution was added to

45 μL PCR mixture and amplified as described in section 3.2. The amplified DNA was then analyzed with RCBH.

3.6 Determination of probes specificity

DNA was extracted from a variety of both non-pseudomonad and pseudomonad reference strains cultures diluted to 2 x 10^8 cells mL^{-1}. Then DNA of each sample was amplified by PCR and examined by RCBH.

3.7 Detection and identification of *Pseudomonas* spp. by PCR-RCBH

DNA was prepared from the pseudomonad isolates and added to 45 μL of PCR mixture and amplified accordingly. The amplified DNA products were then analyzed by RCBH.

RESULTS

In this study, the experiments were divided into 3 parts including study on monoclonal antibody production, study on detection of *Pseudomonas* 16S-23S ribosomal DNA intergenic spacer by PCR and study on detection and identification of *Pseudomonas* spp. by polymerase chain reaction-reverse cross blot hybridization (PCR-RCBH) with 16S-23S rDNA intergenic spacer probes.

1. Study on monoclonal antibody production

1.1 Results of fusion between myeloma and splenocytes for monoclonal antibody production

Two fusions experiments between myeloma and splenocytes for monoclonal antibody production against *Pseudomonas fluorescens* were conducted. The result of the first fusion experiment, the mice were immunized with UV-killed *P. fluorescens* obtains two MAb named MAb 4E10 and MAb 12D2. For the second fusion experiment, the mice were immunized with sonicated *P. fluorescens* and yield 8 MAbs named MAb 2E7, MAb 3H2, MAb 4H7, MAb 6H5, MAb 7C6, MAb 9A1, MAb 10A4 and MAb 12E8.

1.2 Results of the first screening of the positive hybridoma cells (myeloma and splenocytes)

Supernatants from one hundred and sixty two wells from the first fusion between myeloma and splenocytes and five hundred and twelve wells from the second fusion containing growing hybridomas (Fig. 2) were screened positive to *P. fluorescens* for the first time using an indirect ELISA.

The results of the first fusion showed nine wells containing growing hybridomas were positive to *P. fluorescens*. The second fusion showed thirty wells containing growing hybridomas were recognized to *P. fluorescens*.

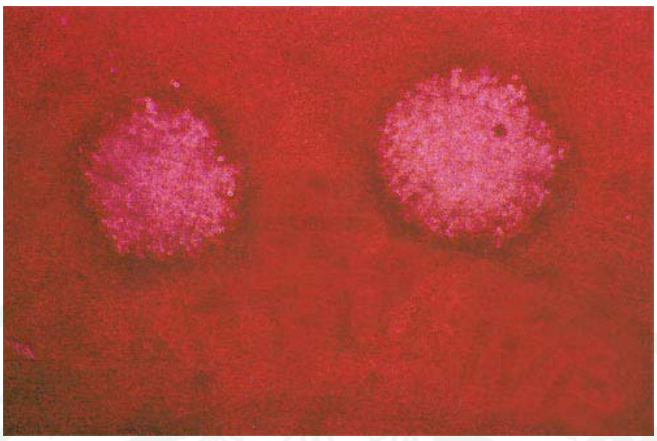

Figure 2 Hybridoma clone eight days following the fusion.

1.3 Results of the second screening of the positive hybridoma cells, following the first expansion

The result of the second screening showed two of nine clones were found to be still positive to *P. fluorescens* for the first fusion, while eight of thirty clones were found to be still positive to the same antigen for the second of the second fusion (Table 3). All positive clones were selected to perform for the second cloning using the limiting dilution method.

1.4 Results of hybridoma line characterization

The result of the first fusion, MAb 4E10 and MAb 12D2 were isotyped as subclass IgG_3 with Kappa light chains. The result of the second fusion, 8 MAbs were isotype as subclass IgG, IgA and IgM with all Kappa light chain (Table 4).

Table 3 Positive monoclonal antibodies tested by indirect ELISA.

Antigen preparation	**MAb name**	**Reaction to *P. fluorescens***
UV-treated *P. fluorescens*	MAb 4E10	+
	MAb 12D2	+
Sonicated *P. fluorescens*	MAb 2E7	+
	MAb 3H2	+
	MAb 4H7	+
	MAb 6H5	+
	MAb 7C6	+
	MAb 9A1	+
	MAb 10A4	+
	MAb 12E8	+

Note: The cut-off point was three times of negative control (0.049)

Table 4 Isotyping of MAbs using a Mouse Typer® Isotyping Panel kit.

Antigen preparation	**MAb name**	**Subclass**	**Light chain type**
UV-treated *P. fluorescens*	MAb 4E10	IgG_3	Kappa
	MAb 12D2	IgG_3	Kappa
Sonicated *P. fluorescens*	MAb 2E7	IgG_{2b}	Kappa
	MAb 3H2	IgG_1	Kappa
	MAb 4H7	IgA	Kappa
	MAb 6H5	IgM	Kappa
	MAb 7C6	IgM	Kappa
	MAb 9A1	IgG_3	Kappa
	MAb 10A4	IgM	Kappa
	MAb 12E8	IgM	Kappa

1.5 Results of cross-reactivity of the MAbs

The result of the first fusion, both MAb 4E10 and 12D2 recognized the immunized antigen strain, *P. fluorescens* (AAHRI 01419) but they did not recognize other *P. fluorescens* as shown in Table 5. The result of the second fusion, MAb 4H7 and MAb 7C6 recognized *P. fluorescens* (AAHRI 01419), MAb 3H2, MAb 6H5, MAb 9A1, MAb 10A4, MAb 12E8 recognized *P. fluorescens* (AAHRI 01419, AAHRI 01213 and AAHRI 03418), but they did not react to other *P. fluorescens*. The MAb 2E7 recognized *P. fluorescens* (AAHRI 01419, AAHRI 03418 and AAHRI 03575), but it did not react to other *P. fluorescens* as shown in Table 5).

Cross reactivity was tested to different non-pseudomonas, the result revealed that there was no cross activity to other bacteria (Table 5).

1.6 Results of SDS-PAGE and Western blot analysis

With the SDS-PAGE analysis of *P. fluorescens*, the result showed major bands of protein profile of the whole cell at 95, 70, 56, 44, 41, 33, 18 and 16 kDa (Fig. 3). With WB analysis, MAb 2E7 and MAb 9A1 reacted to one band of the whole cell *P. fluorescens* (AAHRI 01419) at 41 kDa and 95 kDa respectively (Fig. 3).

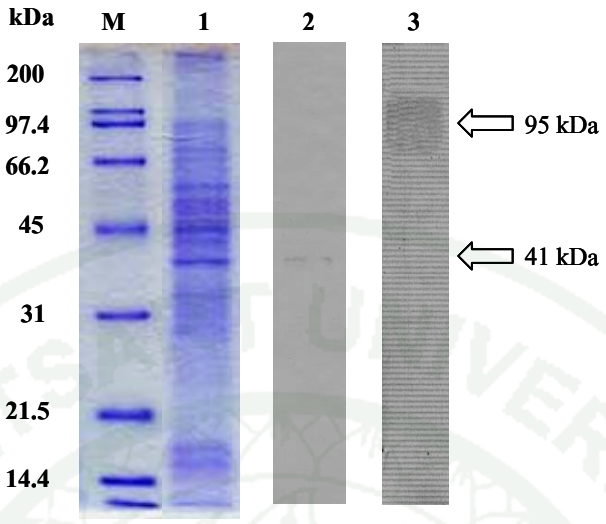

Figure 3 Whole cell were analysis by SDS-PAGE at 12% gel showing a wide range of protein profile of *P. fluorescens* (AAHRI 01419) (1). WB analysis was tested to whole cell with MAb 2E7 (2) and MAb 9A1 (3). Lane M was standard molecular weight marker (kDa).

Table 5 Cross-reactivity (absorbance, 450 nm) of MAbs against *P. fluorescens* (AAHRI 01419) determined by an indirect ELISA with the cutting off point at three time of negative control.

Bacteria	Source[a]	Host	MAb 4E10		MAb 12D2	
			reaction	Response (%)[b]	reaction	Response (%)[b]
P. fluorescens	AAHRI 01419	Flame gourami	+	100.00	+	100.00
Pseudomonas sp.	AAHRI 01031	Guppy	-	8.60	-	10.06
P. fluorescens	AAHRI 01213	Oscar	-	8.45	-	9.87
P. fluorescens	AAHRI 01342	Guppy	-	9.06	-	10.43
P. fluorescens	AAHRI 03418	Gold fish	-	9.37	-	10.80
P. fluorescens	AAHRI 03575	Zebra danio	-	8.76	-	10.43
P. fluorescens	TISTR 358	Unknown	-	8.45	-	9.87
Proteus morganii	AAHRI 98095	Soft shell turtle	-	9.22	-	11.17
Staphylococcus sp.	AAHRI 00126	Catfish	-	8.76	-	10.80
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	8.14	-	9.50
Edwardsiella tarda	AAHRI 01041	Tilapia	-	9.06	-	10.99
Vibrio cholerae	AAHRI 01260	Tilapia	-	9.22	-	11.17
Aeromonas sorbia	AAHRI 03570	Gold fish	-	8.91	-	10.99
A. hydrophila	AAHRI 04031	Siamense fighting fish	-	8.91	-	10.80
Plesiomonas shigelloides	AAHRI 04040	Carp	-	8.14	-	9.31

Table 5 (Continued)

Bacteria	Source[a]	Host	MAb 2E7		MAb 3H2	
			reaction	Response (%)[b]	reaction	Response (%)[b]
P. fluorescens	AAHRI 01419	Flame gourami	+	100.00	+	100.00
Pseudomonas sp.	AAHRI 01031	Guppy	-	5.93	-	2.56
P. fluorescens	AAHRI 01213	Oscar	-	5.38	+	42.13
P. fluorescens	AAHRI 01342	Guppy	-	6.57	-	2.42
P. fluorescens	AAHRI 03418	Gold fish	+	98.02	+	34.32
P. fluorescens	AAHRI 03575	Zebra danio	+	43.75	-	2.69
P. fluorescens	TISTR 358	Unknown	-	4.75	-	2.69
Proteus morganii	AAHRI 98095	Soft shell turtle	-	6.17	-	2.42
Staphylococcus sp.	AAHRI 00126	Catfish	-	5.85	-	2.29
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	5.93	-	2.56
Edwardsiella tarda	AAHRI 01041	Tilapia	-	5.93	-	2.56
Vibrio cholerae	AAHRI 01260	Tilapia	-	5.54	-	2.29
Aeromonas sorbia	AAHRI 03570	Gold fish	-	5.85	-	2.42
A. hydrophila	AAHRI 04031	Siamense fighting fish	-	5.46	-	2.42
Plesiomonas shigelloides	AAHRI 04040	Carp	-	5.38	-	2.69

Table 5 (Continued)

Bacteria	Source[a]	Host	MAb 4H7		MAb 6H5	
			reaction	Response (%)[b]	reaction	Response (%)[b]
P. fluorescens	AAHRI 01419	Flame gourami	+	100.00	+	100.00
Pseudomonas sp.	AAHRI 01031	Guppy	-	6.33	-	6.33
P. fluorescens	AAHRI 01213	Oscar	-	6.84	+	52.94
P. fluorescens	AAHRI 01342	Guppy	-	6.46	-	6.56
P. fluorescens	AAHRI 03418	Gold fish	-	5.57	+	50.00
P. fluorescens	AAHRI 03575	Zebra danio	-	5.70	-	7.81
P. fluorescens	TISTR 358	Unknown	-	7.22	-	6.45
Proteus morganii	AAHRI 98095	Soft shell turtle	-	7.34	-	8.26
Staphylococcus sp.	AAHRI 00126	Catfish	-	8.86	-	5.32
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	7.22	-	7.13
Edwardsiella tarda	AAHRI 01041	Tilapia	-	8.61	-	6.45
Vibrio cholerae	AAHRI 01260	Tilapia	-	8.35	-	6.45
Aeromonas sorbia	AAHRI 03570	Gold fish	-	8.23	-	6.45
A. hydrophila	AAHRI 04031	Siamense fighting fish	-	7.09	-	6.22
Plesiomonas shigelloides	AAHRI 04040	Carp	-	8.10	-	6.45

Table 5 (Continued)

Bacteria	Source[a]	Host	MAb 7C6		MAb 9A1	
			reaction	Response (%)[b]	reaction	Response (%)[b]
P. fluorescens	AAHRI 01419	Flame gourami	+	100.00	+	100.00
Pseudomonas sp.	AAHRI 01031	Guppy	-	6.20	-	6.46
P. fluorescens	AAHRI 01213	Oscar	-	6.49	+	32.45
P. fluorescens	AAHRI 01342	Guppy	-	6.87	-	7.10
P. fluorescens	AAHRI 03418	Gold fish	-	6.39	+	29.78
P. fluorescens	AAHRI 03575	Zebra danio	-	9.83	-	8.49
P. fluorescens	TISTR 358	Unknown	-	7.82	-	6.21
Proteus morganii	AAHRI 98095	Soft shell turtle	-	8.97	-	10.27
Staphylococcus sp.	AAHRI 00126	Catfish	-	7.35	-	6.72
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	7.82	-	8.37
Edwardsiella tarda	AAHRI 01041	Tilapia	-	7.44	-	3.80
Vibrio cholerae	AAHRI 01260	Tilapia	-	7.82	-	8.24
Aeromonas sorbia	AAHRI 03570	Gold fish	-	8.30	-	8.75
A. hydrophila	AAHRI 04031	Siamense fighting fish	-	7.06	-	7.73
Plesiomonas shigelloides	AAHRI 04040	Carp	-	6.77	-	6.34

Table 5 (Continued)

Bacteria	Source[a]	Host	MAb 10A4		MAb 12E8	
			reaction	Response (%)[b]	reaction	Response (%)[b]
P. fluorescens	AAHRI 01419	Flame gourami	+	100.00	+	100.00
Pseudomonas sp.	AAHRI 01031	Guppy	-	6.43	-	6.26
P. fluorescens	AAHRI 01213	Oscar	+	64.01	+	65.15
P. fluorescens	AAHRI 01342	Guppy	-	7.07	-	6.99
P. fluorescens	AAHRI 03418	Gold fish	+	65.68	+	66.75
P. fluorescens	AAHRI 03575	Zebra danio	-	6.56	-	7.61
P. fluorescens	TISTR 358	Unknown	-	6.30	-	4.91
Proteus morganii	AAHRI 98095	Soft shell turtle	-	10.28	-	9.57
Staphylococcus sp.	AAHRI 00126	Catfish	-	6.17	-	6.13
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	7.97	-	7.85
Edwardsiella tarda	AAHRI 01041	Tilapia	-	5.27	-	4.66
Vibrio cholerae	AAHRI 01260	Tilapia	-	8.23	-	8.10
Aeromonas sorbia	AAHRI 03570	Gold fish	-	7.71	-	7.85
A. hydrophila	AAHRI 04031	Siamense fighting fish	-	7.46	-	6.87
Plesiomonas shigelloides	AAHRI 04040	Carp	-	6.17	-	7.24

a = **ATCC**: American Type Culture Collection, **DMST**: Department of Medical Sciences Thailand, **TISTR**: Thailand Institute of Scientific and Technological Research, **AAHRI**: Aquatic Animal Health Research Institute

b = Response was the mean of triplicate wells, expressed as a percentage of the optical density obtained by strain AAHRI 01419 (OD 1.264); OD450 of negative control (PBS) was 0.050

1.7 Results of HRP Conjugation

Two of MAbs (MAb 2E7 and MAb 9A1) were chosen for a trial of HRP conjugation. The results showed that only MAb 9A1 was successfully conjugated to the HRP. The conjugated MAb 9A1 was then tested against a various Pseudomonad and non-pseudomonas by indirect ELISA. The result revealed the same trend as it was tested with non-conjugated MAb 9A1 (Table 6).

Table 6 Results of HRP-conjugate test (absorbance, 450 nm) of MAb 9A1 against *P. fluorescens* (AAHRI 01419) determined by an indirect ELISA with the cutting off point at three time of negative control.

Bacteria	Source	Reaction	Response (%)
P. fluorescens	AAHRI 01419	+	100.00
Pseudomonas sp.	AAHRI 01031	-	11.43
P. fluorescens	AAHRI 01213	+	37.86
P. fluorescens	AAHRI 01342	-	17.50
P. fluorescens	AAHRI 03418	+	35.36
P. fluorescens	AAHRI 03575	-	17.50
P. fluorescens	TISTR 358	-	16.43
Staphylococcus sp.	AAHRI 00126	-	20.71
Citrobacter freundii	AAHRI 01013	-	19.64
Edwardsiella tarda	AAHRI 01041	-	21.43
Vibrio cholerae	AAHRI 01260	-	16.07
Aeromonas sorbia	AAHRI 03570	-	21.79
A. hydrophila	AAHRI 04031	-	18.21
Plesiomonas shigelloides	AAHRI 04040	-	16.79
negative control	PBS	-	10.36

2. Study on detection of *Pseudomonas* 16S-23S ribosomal DNA intergenic spacer by PCR

2.1 Results of sensitivity and specificity of PCR assay

The primers specific to genus were designed and coded as PsGrF and PsGrR (Table 7). The sensitivity of the primers was examined by agarose gel electrophoresis. The detection limit of the primers were 0.5 pg, 5 pg, 50 pg and 5 ng respectively to *P. putida*, *P. fluorescens*, *P. aeruginosa* and *P. diminuta.* These correspond to *Pseudomonas* cultures diluted to 1.0×10^3 cells mL^{-1}, 1.0×10^4 cells mL^{-1}, 1.0×10^5 cells mL^{-1} and 1.0×10^7 cells mL^{-1} respectively (Fig. 4). Specificity of the PCR with primers PsGrF and PsGrR were tested against 13 bacterial isolates including reference strains and non-pseudomonad (Table 8) and was also tested to various bacterial isolates recovered from different aquatic animals as showed in Table 9. The positive bands of *P. fluorescens*, *P. putida* and *P. diminuta* were 150 bp, while it was 130 bp for *P. aeruginosa.* The positive bands either 150 bp or 130 bp were only detected among pseudomonad isolates, while no band was detected among those non-pseudomonad isolates (Fig. 5).

Table 7 16S-23S rDNA intergenic spacer-based primer set.

Primer	**Sequence (5'-3')**
PsGrF	TGTCAGATCGTTCTTTAA
PsGrR	ACTGTGAAGACGACATTCG

Table 8 Specificity of PCR with primer PsGrF and PsGrR against DNA from *Pseudomonas* reference strains and non-pseudomonad.

Bacteria	**Source**	**Specificity**
Pseudomonas aeruginosa	ATCC 27853	+
P. putida	DMST 10603	+
P. fluorescens	TISTR 358	+
P. diminuta	AAHRI 96144	+
Staphylococcus aureus	ATCC 25923	-
Escherichia coli	ATCC 25922	-
Aeromonas hydrophilla	LMG 2844	-
Yersinia ruckeri	LMG 3280	-
Vibrio harveyi	LMG 4044	-
V. alginolyticus	LMG 4409	-
E. coli	LMG 8223	-
Photobacterium damsela	LMG 7892	-
Salmonella enteritidis	LMG 10395	-

ATCC: American Type Culture Collection

LMG: BCCM/LMG Bacteria Collection

DMST: Department of Medical Sciences Thailand

TISTR: Thailand Institute of Scientific and Technological Research

AAHRI: Aquatic Animal Health Research Institute

Table 9 Assessment of PCR assay with primers PsGrF and PsGrR to various bacterial isolates recovered from different aquatic animals during outbreaks in Thailand.

Bacteria	Source	Host	Specificity
Pseudomonas putida	AAHRI 95033	Frog	+
P. diminuta	AAHRI 96163	Frog	+
P. aeruginosa	AAHRI 00086	Guppy	+
P. fluorescens	AAHRI 00107	Gold fish	+
P. fluorescens	AAHRI 00121	Guppy	+
P. putida	AAHRI 00127	Frog	+
P. aeruginosa	AAHRI 01027	Guppy	+
Pseudomonas sp.	AAHRI 01031	Guppy	+
P. diminuta	AAHRI 01158	Frog	+
P. fluorescens	AAHRI 01213	Oscar	+
P. fluorescens	AAHRI 01342	Guppy	+
P. fluorescens	AAHRI 01419	Flame gourami	+
P. aeruginosa	AAHRI 02007	Striped catfish	+
P. diminuta	AAHRI 02022	Frog	+
P. putida	AAHRI 02276	Frog	+
P. fluorescens	AAHRI 02285	Oscar	+
P. fluorescens	AAHRI 02353	Tilapia	+
P. fluorescens	AAHRI 02375	Cichild	+
P. fluorescens	AAHRI 02378	Oscar	+
P. fluorescens	AAHRI 02403	Gold fish	+
P. putida	AAHRI 03409	Folwer horn	+
P. fluorescens	AAHRI 03418	Gold fish	+
P. fluorescens	AAHRI 03575	Zebra danio	+
Pseudomonas sp.	AAHRI 06053	Cichild	+
Pseudomonas sp.	AAHRI 06060	Snakeskin gouramy	+
P. aeruginosa	AAHRI 06069	Giant gouramy	+
Pseudomonas sp.	AAHRI 07006	Frog	+
Pseudomonas sp.	AAHRI 07020	Giant gouramy	+

Table 9 (Continued)

Bacteria	Source	Host	Specificity
Proteus morganii	AAHRI 98095	Soft shell turtle	-
Staphylococcus sp.	AAHRI 00126	Catfish	-
Citrobacter freundii	AAHRI 01013	Giant gouramy	-
Aeromonas sobria	AAHRI 01018	Gold fish	-
Edwardsiella tarda	AAHRI 01041	Tilapia	-
Plesiomonas shigelloides	AAHRI 01230	Catfish	-
Vibrio cholerae	AAHRI 01260	Tilapia	-
A. hydrophila	AAHRI 01277	Discus	-
Streptococcus sp.	AAHRI 01285	Frog	-

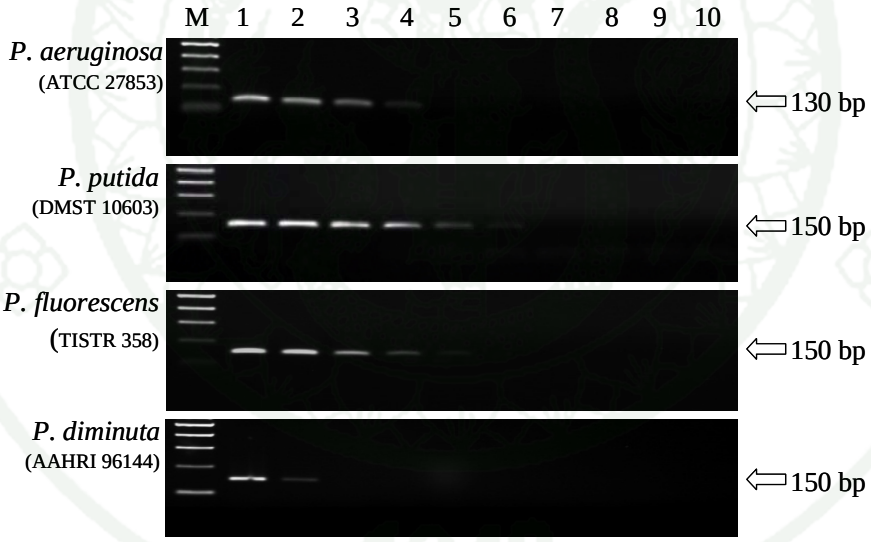

Figure 4 Sensitivity test of primers, PsGrF and PsGrR. Lanes 1-9 represented dilutions of *Pseudomonas* DNA of 50 ng DNA, 5 ng DNA, 0.5 ng DNA, 50 pg DNA, 5 pg DNA, 0.5 pg DNA, 50 fg DNA, 5 fg DNA and 0.5 fg DNA respectively. Lane M was DNA marker (100 bp ladder, BIOLINE). Lane 10 was negative control (dH_2O).

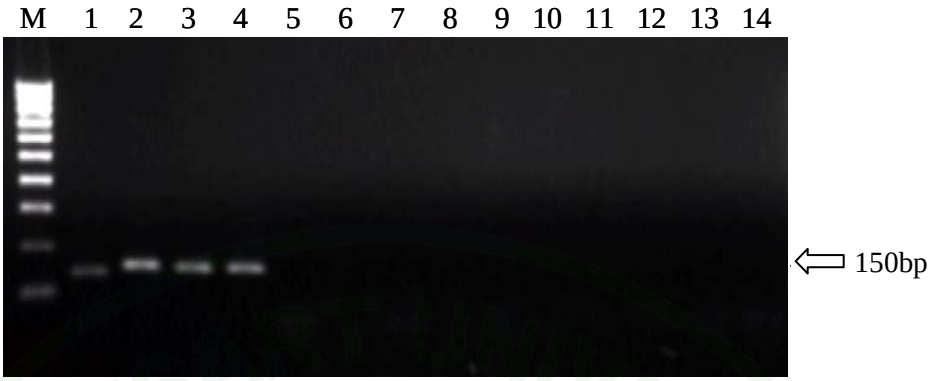

Figure 5 PCR specificity tests of primer, PsGrF and PsGrR. Lanes: (1) *P. aeruginosa* (ATCC 27853), (2) *P. putida* (DMST 10603), (3) *P. fluorescens* (TISTR 358), (4) *P. diminuta* (AAHRI 96144), (5) *Staphylococcus aureus* (ATCC 25923), (6) *Escherichia coli* (ATCC 25922), (7) *Aeromonas hydrophilla* (LMG 2844), (8) *Yersinia ruckeri* (LMG 3280), (9) *Vibrio harveyi* (LMG 4044), (10) *V. alginolyticus* (LMG 4409), (11) *E. coli* (LMG 8223), (12) *Photobacterium damsela* (LMG 7892), (13) *Salmonella enteritidis* (LMG 10395). Lane M was DNA marker (100 bp ladder, BIOLINE). Lane 14 was negative control (dH_2O).

2.2 Results of assessment of PCR

Experimental infected frogs showed the same clinical signs regardless to the bacterial species. At day 2, some frogs (7.4%) showed loss of balance and some (16%) deteriorated to opaque eye at day 10 (Fig. 6). The end of experiment, accumulated mortality was 34%. The PCR showed positive bands in all treatment groups since day 3 of all dilution, while the biochemical test showed positive at day 11 of the lowest dilution (1.0×10^6 cells mL^{-1}) and at day 3 of a higher dilution (over 1.0×10^7 cells mL^{-1}) as illustrated in Table 10.

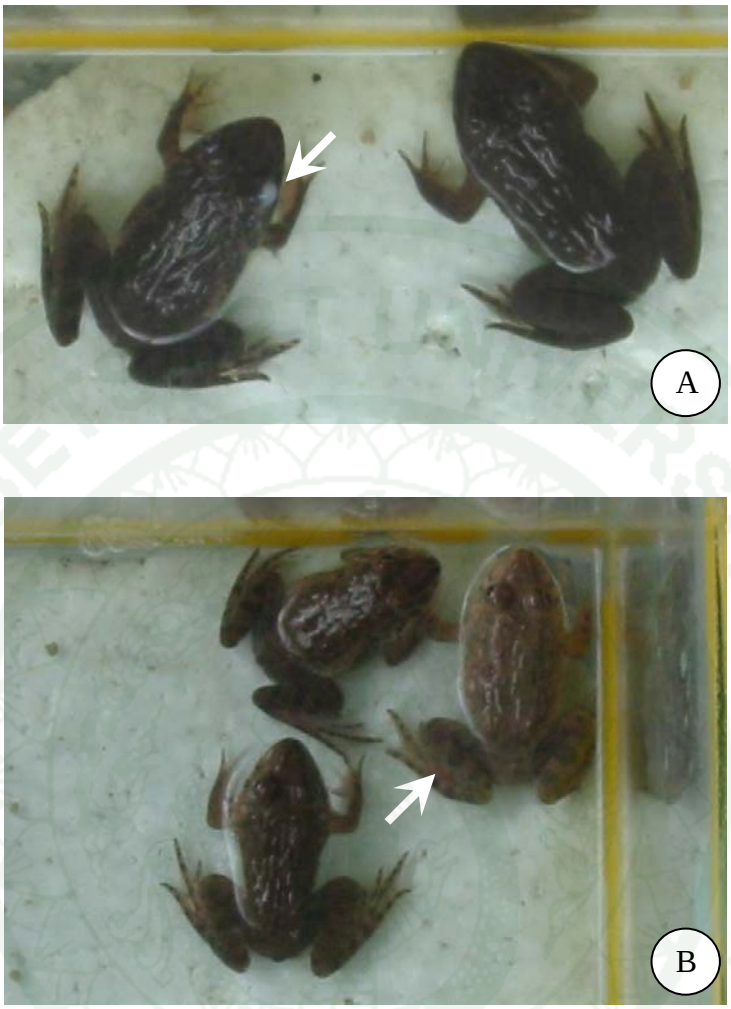

Figure 6 Opaque eye (A) and haemorrhage (B) were observed in experimental infected frogs at day 10 after injection with *Pseudomonas* spp.

Table 10 Assessment of primer designed to *Pseudomonas* spp. in frog.

bacteria	day after injected	Day 3		Day 7		Day 11	
concentration	method	Biochem.	PCR	Biochem.	PCR	Biochem.	PCR
$1x10^6$ cells mL^{-1}	*Pseudomonas putida*	-	+	-	+	+	+
	P. fluorescens	-	+	-	+	-	+
	P. aeruginosa	-	+	-	+	-	+
$1x10^7$ cells mL^{-1}	*Pseudomonas putida*	-	+	-	+	-	+
	P. fluorescens	+	+	+	+	+	+
	P. aeruginosa	-	+	-	+	-	+
$1x10^8$ cells mL^{-1}	*Pseudomonas putida*	-	+	+	+	-	+
	P. fluorescens	-	+	-	+	-	+
	P. aeruginosa	+	+	-	+	-	+

3. Study on detection and identification of *Pseudomonas* spp. by polymerase chain reaction-reverse cross blot hybridization (PCR-RCBH) with 16S-23S rDNA intergenic spacer probes

3.1 Results of PCR amplification and cloning

PCR amplification of genomic DNA's from all four *Pseudomonas* species, *P. aeruginosa* (ATCC 27853), *P. putida* (DMST 10603), *P. fluorescens* (TISTR 358), and *P. diminuta* (AAHRI 96144) with primers P16sf-Bio and P23sr-Bio yielded an amplification product of about 650 bp. However, the amplification of *P. putida* also yielded other two products of 700 bp and 350 bp. The 650 bp. fragment of each isolate was cloned by pGEM T-Easy system (Promega) and the sequences were then analyzed.

3.2 Results of nucleotide sequence analysis

The 650 bp fragment was sequenced and its result were aligned to the Gen Bank DATA base of the 16S-23S rRNA spacer regions of other prokaryotes (http://ncbi.nlm.nih.gov). Those prokaryotes were including *Streptococcus pyogenes* (AF489597), *Staphylococcus aureus* (U11780), *Mycobacterium bovis* (AJ315569), *Escherichia coli* (J01702) and *Bacillus subtilis* (J01551). The 16S-23S rRNA spacer sequences of *P. diminuta* and the fluorescent pseudomonad group were found to be identical. Probes specific to the group and each species were finally designed (Table 11).

Table 11 Oligonucleotide probes using in reverse cross blot hybridization.

Code	Specificity	Nucleotide sequences
pGrou1	*Pseudomonas* spp.	5'-CGGCGAATGTCGTCTTCACAG-3'
pAeru1	*P. aeruginosa*	5'-GGTGTGCTGCGTGATCCG-3'
pPuti1	*P. putida*	5'-GCGGTAGATGTTGCTCCTGC-3'
pFluo1	*P. fluorescens*	5'-GCATTCCATTGTGATGATGGTG-3'
pDim2	*P. diminuta*	5'-GATACAAGTATACGAATAGAGCC-3'

3.3 Results of sensitivity of probes by PCR-RCBH

The sensitivity of the primers P16sf-Bio and P23sr-Bio (for amplification of 16S-23S rDNA intergenic spacer) was determined by RCBH assay. The detection limit of probes pGrou1, pAeru1, pPuti1, pFlou1 and pDim2, which respond with *Pseudomonas* spp., *P. aeruginosa*, *P. putida*, *P. fluorescens* and *P. diminuta*, respectively, had detection limits of 2-20 cells mL^{-1}, 20 cells mL^{-1}, 20 cells mL^{-1}, 2,000 cells mL^{-1} and 200,000 cells mL^{-1} respectively as illustrated in Fig. 7.

3.4 Results of specificity of probes by PCR-RCBH

The specificity of the PCR with primers P16sf-Bio and P23sr-Bio was tested against 11 different other bacteria isolates, mainly to fish pathogens. The primers amplified all bacteria listed in Table 12 with different product sizes. The specificity of all five probes used in RCBH was also tested against the DNA of the 11 different other bacteria as well as the reference strains (Fig. 8). The reference strains reacted specifically with their appropriate probes.

Four specific probes including pAeru1, pPuti1, pFluo1 and pDim2 (Fig. 9) were tested against *Pseudomonas* spp. isolated from fish during the outbreak. The result revealed that, these four probes are specific for fluorescent pseudomonads, *P. aeruginosa*, *P. putida*, and *P. diminuta*, accordingly. All isolates tested positively with the pGrou1 probe (genus-specific in fluorescent pseudomonads) except *P. diminuta*.

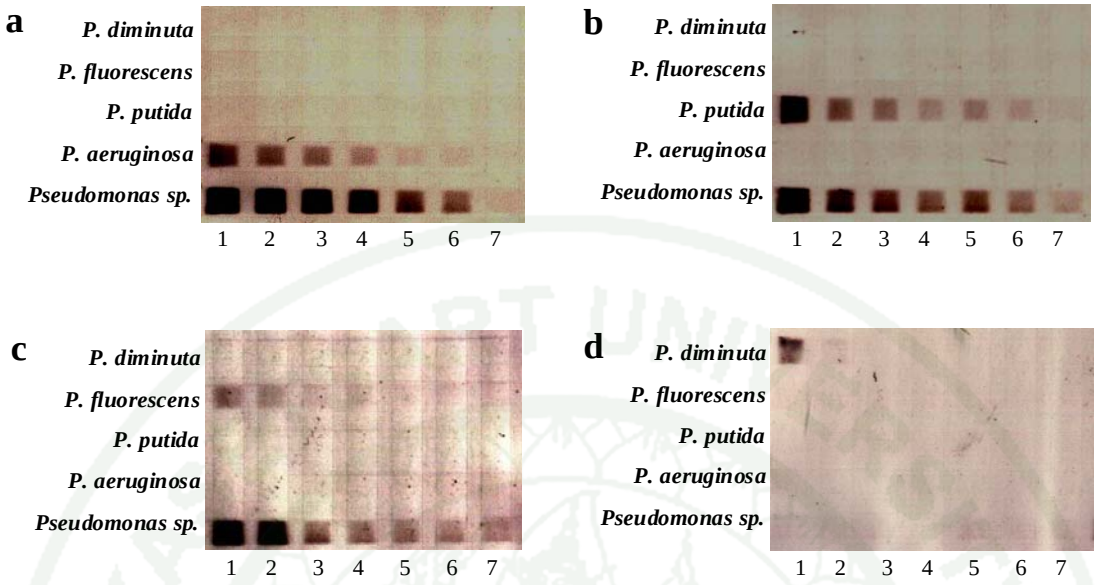

Figure 7 (a) Sensitivity of pAeru1 probe in RCBH with PCR products of *P. aeruginosa* (ATCC 27853). Lane: (1) 2 x 10^6 cells mL^{-1}, (2) 2 x 10^5 cells mL^{-1}, (3) 2 x 10^4 cells mL^{-1}, (4) 2 x 10^3 cells mL^{-1}, (5) 2 x 10^2 cells mL^{-1}, (6) 2 x 10^1 cells mL^{-1}, (7) 2 x 10^0 cells mL^{-1}. (b) Sensitivity of pPuti1 probe in RCBH with PCR products of *P. putida* (DMST 10603). Lane: (1) 2 x 10^6 cells mL^{-1}, (2) 2 x 10^5 cells mL^{-1}, (3) 2 x 10^4 cells mL^{-1}, (4) 2 x 10^3 cells mL^{-1}, (5) 2 x 10^2 cells mL^{-1}, (6) 2 x 10^1 cells mL^{-1}, (7) 2 x 10^0 cells mL^{-1} (c) Sensitivity of pFluo1 probe in RCBH with PCR products of *P. fluorescens* (TISTR 358). Lane: (1) 2 x 10^6 cells mL^{-1}, (2) 2 x 10^5 cells mL^{-1}, (3) 2 x 10^4 cells mL^{-1}, (4) 2 x 10^3 cells mL^{-1}, (5) 2 x 10^2 cells mL^{-1}, (6) 2 x 10^1 cells mL^{-1}, (7) 2 x 10^0 cells mL^{-1} (d) Sensitivity of pDim2 probe in RCBH with PCR products of *P. diminuta* (AAHRI 96144). Lane: (1) 2 x 10^6 cells mL^{-1}, (2) 2 x 10^5 cells mL^{-1}, (3) 2 x 10^4 cells mL^{-1}, (4) 2 x 10^3 cells mL^{-1}, (5) 2 x 10^2 cells mL^{-1}, (6) 2 x 10^1 cells mL^{-1}, (7) 2 x 10^0 cells mL^{-1}.

Table 12 Specificity of PCR with primers P16sf-Bio and P23sr-Bio against DNA from *Pseudomonas* reference strains and other bacteria.

Bacteria	**Source**	**Host**	**Probe**				
			pAeru1	**pPuti1**	**pFluo1**	**pDim2**	**pGrou1**
Pseudomonas aeruginosa	ATCC 27853	Blood culture	+	-	-	-	+
P. putida	DMST 10603	Blood culture	-	+	-	-	+
P. fluorescens	TISTR 358	Unknow	-	-	+	-	+
P. aeruginosa	AAHRI 01024	Guppy	+	-	-	-	+
P. putida	AAHRI 95033	Frog	-	+	-	-	+
P. diminuta	AAHRI 96144	Frog	-	-	-	+	-
P. diminuta	AAHRI 01158	Frog	-	-	-	+	-
P. diminuta	AAHRI 02022	Frog	-	-	-	+	-
Staphylococcus aureus	ATCC 25923	Clinical isolate	-	-	-	+	-
Escherichia coli	ATCC 25922	Clinical isolate	-	-	-	-	-
Proteus morganii	AAHRI 98095	Soft shell turtle	-	-	-	-	-
Staphylococcus sp.	AAHRI 00126	Catfish	-	-	-	-	-
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	-	-	-	-
Aeromonas sobria	AAHRI 01018	Gold fish	-	-	-	-	-
Edwardsiella tarda	AAHRI 01041	Tilapia	-	-	-	-	-
Plesiomonas shigelloides	AAHRI 01230	Catfish	-	-	-	-	-
Vibrio cholerae	AAHRI 01260	Tilapia	-	-	-	-	-
Aermonas hydrophilla	AAHRI 01277	Discus	-	-	-	-	-
Streptococcus sp.	AAHRI 01285	Frog	-	-	-	-	-

ATCC: American Type Culture Collection

DMST: Department of Medical Sciences Thailand

TISTR: Thailand Institute of Scientific and Technological Research

AAHRI: Aquatic Animal Health Research Institute

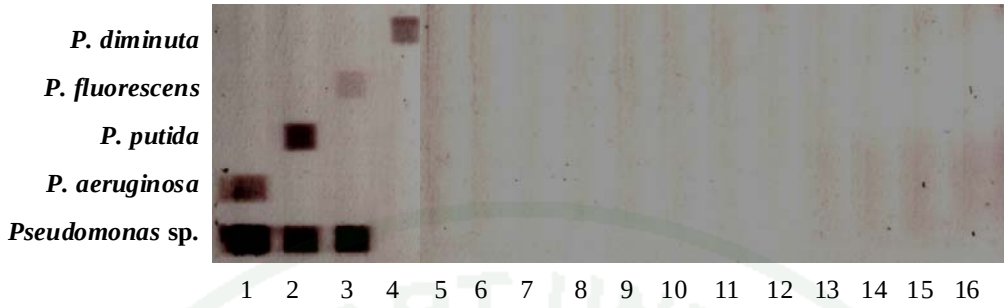

Figure 8 PCR-RCBH of other bacteria and reference strain *Pseudomonas*. Lanes: (1) *P. aeruginosa* (ATCC 27853), (2) *P. putida* (DMST 10603), (3) *P. fluorescens* (TISTR 358), (4) *P. diminuta* (AAHRI 96144), (5) *Staphylococcus aureus* (ATCC 25923), (6) *Escherichia coli* (ATCC 25922), (7) *Proteus morganii* (AAHRI 98095), (8) *Staphylococcus* sp. (AAHRI 00126), (9) *Citrobacter freundii* (AAHRI 01013), (10) *Aeromonas sobria* (AAHRI 01018), (11) *Edwardsiella tarda* (AAHRI 01041), (12) *Plesiomonas shigelloides* (AAHRI 01230), (13) *Vibrio cholerae* (AAHRI 01260), (14) *Aeromonas hydrophila* (AAHRI 01277), (15) *Streptococcus* sp. (AAHRI 01285), (16) TE buffer.

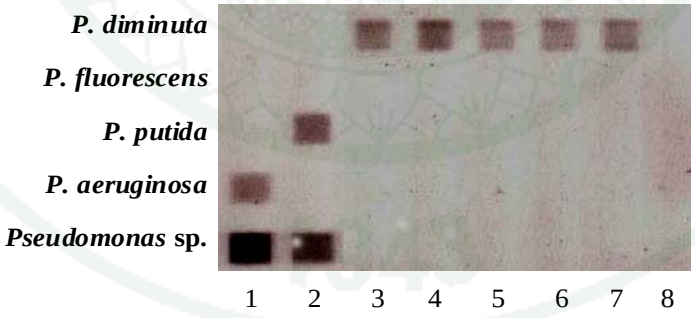

Figure 9 Characterization of *Pseudomonas* spp. isolated from fish in Thailand by PCR-RCBH. Lanes: (1) AAHRI 01024, (2) AAHRI 95033, (3) AAHRI 96163, (4) AAHRI 96144, (5) AAHRI 01158, (6) AAHRI 02022, (7) AAHRI 96174, (8) TE buffer.

4. Comparative methods to detection *Pseudomonas* spp. in aquatic animals

Capacity of each detection method developed here were summerized as illustrated in Table 13.

Table 13 Results of comparative methods to detection *Pseudomonas* spp.

Bacteria	**Source**	**Host**	**Methodology**		
			MAb-ELISA	**PCR**	**PCR-RCBH**
Pseudomonas aeruginosa	ATCC 27853	Blood culture	-	+	+
P. putida	DMST 10603	Blood culture	-	+	+
P. fluorescens	TISTR 358	Unknow	-	+	+
P. diminuta	AAHRI 96144	Frog	-	+	+
Pseudomonas sp.	AAHRI 01031	Guppy	-	+	+
P. fluorescens	AAHRI 01213	Oscar	+	+	+
P. fluorescens	AAHRI 01419	Flame gourami	+	+	+
Staphylococcus aureus	ATCC 25923	Clinical isolate	-	-	-
Escherichia coli	ATCC 25922	Clinical isolate	-	-	-
Proteus morganii	AAHRI 98095	Soft shell turtle	-	-	-
Staphylococcus sp.	AAHRI 00126	Catfish	-	-	-
Citrobacter freundii	AAHRI 01013	Giant gouramy	-	-	-
Edwardsiella tarda	AAHRI 01041	Tilapia	-	-	-

Note: MAb-ELISA tested with MAb 9A1

DISCUSSION

1. Study on monoclonal antibody production

Antibodies are symmetrical molecules made up to two identical glycosylated heavy chains and two identical nongylcosylated light chains. The class of an immunoglobulin molecule is determined by its heavy chains while the light chains are two types, κ and λ. There are not only the heavy chains are played functions of antibodies but also the light chains that play an important part in determining the specificity of antibodies. The isotype of MAbs produced in this study was evaluated and the result showed different immunoglobulin group, IgM, IgA, IgG_1, IgG_{2b} and IgG_3. In contrast, the light chain showed all κ light chain, which correspond to the mouse that its light chain was on identified as Kappa chain (Goding, 1996).

Monoclonal antibodies (MAbs), following the development of this technology in 1975, first described by Köhler and Milstein, have become an extraordinarily improtant resource for medical research, diagnosis, therapy, and basic science. MAb probes have made a significant impact on the rapid diagnosis of numerous fish diseases (Adam *et al.*, 1995).

Some problems with MAbs are that they may be so specific that too few strains of a species express the target antigen. This study, with the UV-treated antigen the resulted MAbs recognized only *P. fluorescens*. Thus, sonicated antigen was prepared to break up the cell with an attempt to increase antigenic determinant. The result showed the MAbs recognized the strains of most *P. fluorescens*. Based on their reaction, the MAb 2E7 and MAb 9A1 were chosed to undergo a further analysis. With ELISA analysis, MAb 2E7 reacted to most of *P. fluorescens* (AAHRI 01419, AAHRI 03418 and AAHRI 03575) and MAb 9A1 reacted to most of *P. fluorescens* (AAHRI 01419, AAHRI 01213 and AAHRI 03418). As Swain *et al*. (2003) reported an existing of a number of serotypes and strains found in *P. fluorescens*, it may suggest that MAb 2E7 and MAb 9A1 recognize in some serotype. However, the MAb 2E7 and MAb 9A1 did not cross-react to the other tested bacteria. Moreover class of

immunoglobulin reflect its affinity, which the IgG is stronger than those of the IgM antibody. This may suggest that the MAb could be used widely for serological tests (Shi *et al*., 2003). The MAb 2E7 and MAb 9A1 possed IgG_{2b} and IgG_3 respectively were considered to a further analysis.

In addition, the MAb 9A1 was successfully conjugated with peroxidase but MAb 2E7. Considering isotype between MAb 2E7 and MAb 9A1 showed IgG_{2b} and IgG_3 respectively, mouse IgG_3 has some properties that set them apart from the other IgG subclass (Goding, 1996). This may cause affect MAb 2E7 difficult to conjugate with peroxidase. However, some lose sensitivity of MAb 9A1-HRP was observed as in the result.

Recently, production of MAb to *Pseudomonas* sp. in refrigerated meat was reported by Gutierrez *et al*. (1997). The MAb namely AH12 was produced against live cells of mixture of *P. fluorescens* strains AH-70, AR-11, B-52, DC-5, DC-7 and NT-19. It was reported that the MAb AH12 only recognized *P. fluorescens* DC-5, DC-7, NT-19, *P. putida* and *P. fragi*, suggesting that the MAb AH12 may be genus specific. Thus, monoclonal antibody production against whole cell of *P. fluorescens* difficult to successfully perform, while specific antigen with known function and presented all *Pseudomonas* spp. has higher opportunity to success. In contrast, polyclonal antibodies against the protein F of *Pseudomonas fluorescens* for the detection of psychrotrophic bacteria in meat using an indirect ELISA were reported (González *et al*., 1996), while Kragelund *et al*. (1996) reported the PAb against the outer membrane protein *opr*F as a probe for Group I Pseudomonads in rhizosphere soil using dot immunobinding. However, studies have shown that polyclonal antibodies have limitations to produced maintain standardization between production running over the period of time.

Considering the bacteria detection, the MAb 9A1 has a good character for a further development of a rapid diagnosis kit to screen *P. fluorescens* infection in aquatic animals. This would envisage the disease situation of the fish ponds as well as provide relevant information for farm management.

2. Study on detection of *Pseudomonas* 16S-23S ribosomal DNA intergenic spacer by PCR

Molecular-based methods have been rapidly developed to detect and identify pathogens in aquatic animals since these techniques have advantage on accuracy, sensitivity, and less time consuming. Furthermore, these techniques allow direct detection without the necessity of culture (Chun *et al.*, 1999). Among these, PCR is one of the most popular methods, which can be applied in conjunction with other methods such as sequencing (De Vos *et al.*, 1997; Xian-Yu *et al*., 2006; Hassan *et al*., 2008), random amplified polymorphic DNA (RAPD) (Campbell *et al.*, 2000), single-strand conformational polymorphism (SSCP) (Mora *et al*., 2003) and restriction enzyme treatment (Widmer *et al.*, 1998; Laganowska and Kaznowski, 2004).

Over decades, many studies have focused on the genus *Pseudomonas*; identification of *Pseudomonas* in environment and plants has been described. Among the various methods used to identify *Pseudomonas*, Kragelund *et al*. (1996) reported to use of an *opr*F, raising a polyclonal antibody against outer membrane protein F from *P. fluorescens* DF57 as a probe for Group I Pseudomonads from barley rhizosphere soil. In contrast, De Vos *et al*. (1998) has been sequenced major outer membrane protein I (*opr*I) that conserved among the fluorescens pseudomonads and suggested *opr*I gene was a suitable additional marker for the molecular detect Group I Pseudomonads. Moreover Widmer *et al*. (1998) reported the PCR protocol for detecting 16S rRNA genes, a highly selective of the genus *Pseuomonas* (Sensu strico) in environment samples. In addition, the *gac*A gene of *Pseudomonas* species was used as a phylogenetic marker for the identification. De Souza *et al* (2003) determined the *gac*A sequences of 10 *Pseudomonas* strains isolated from plant-associated environments. The results showed *gac*A gene conserved within the genus *Pseudomonas*. It is worthy to note that none of these PCR assays are applied to detect *Pseudomonas* spp. in aquatic animals.

Palleroni presented *Pseudomonas* into five groups based on the RNA sequences (Palleroni, 1992). Among these, *P. aeruginosa, P. putida, P. fluorescens*

belong to Group I, while *P. diminuta* belongs to Group IV. Up to date, no other species are reported in aquatic animals in Thailand (Chinabut and Somsiri, 1998; Somsiri and Soontronvit, 2002). This disease cause the problem for many species of aquarium fish such as guppy (*Poecilia recticulata*), zebra danio (*Brachydanio rerio*), flame gourami (*Colisa laria*) and in food fish like nile tilapia (*Oreochromis niloticus*) and also affect amphibian like frog (*Rana tigerinaa*).

This current study was focused on amplification of the 16S-23S rDNA intergenic spacer as it has been proved to be under less evolutionary pressure (Chun *et al*., 1999; Berridge *et al*., 1998; Sawada *et al*., 1997; Rijpens *et al*., 1996; Smart *et al*., 1996;). Thus the 16S-23S rDNA intergenic spacer has received increased attention as a suitable target for molecular bacterial identification and detection techniques (Berridge *et al*., 1998).

The designed primers (PsGrF/PsGrR) amplified *P. aeruginosa* differently from *P. fluorescens* and *P. putida* which they are all belonged to Group1 as the 16S-23S rDNA intergenic spacer of *P. aeruginosa* has nucleotide sequence shorter than other *Pseudomonas*. It is worthy to note that *P. diminuta*, which belonged to Group IV, produced positive band as same as those Group I. This happen may reflect some polymorphisms within the 16S-23S ITS among these four species, which supported the finding of Tyler *et al.* (1995). However, either band 150 bp or 130 bp were not detected in any non-pseudomonad isolates. Taking advantage on this phenomenon, this set of primers could be used to identify *P. aeruginosa* separately from the other three pseudomonads.

The fluorescens pseudomonas group, *P. putida*, *P. fluorescens* and *P. aeruginosa*, was experimentally injected into frogs with the aim to validate the diagnostic methods. It showed that the PCR method can detect the bacteria at the sub-clinical infection level since day 3 of infection, while the conventional bacteriology method can detect at day 11. This has proved that the PCR is useful to detect the *Pseudomonas* spp. in frogs at the early infection stage.

This study demonstrates a rapid detection of *Pseudomonas*, which can be applied to diagnose the sub-clinical infection since the limit detection was as small as 0.5 pg of DNA or 1.0 x 10^3 cells mL^{-1} and identification at the genus level is sufficient because all species, as up to date has reported are considered potentially pathogenic. Rapid detection and identification method of bacterial from diseased or carrier aquatic animals have advantage over the evaluation of colony morphology and biochemical characterization in term of farm management. Since it can be used as an early warning system.

3. Study on detection and identification of *Pseudomonas* spp. by PCR-RCBH

PCR-RCBH has been described to detect different bacteria. For instance, Rijpens *et al*. (1996) described the assay for detection of *Brucella* sp. in raw milk. Kox *et al*. (1995) described the PCR followed reverse cross blot hybridization based on 16S rRNA for detection and identification of *Mycobacterium* sp. in clinical samples up to 7 species of the most important pathogenic and opportunistic mycobacteria. Recently, the PCR-RCBH was developed to identification of *Mycobacterium* spp. isolated from diseased fish up to 3 species, *M. marinum* & *M. ulcerans*, *M. chelonae* and *M. fortuitum* & *M. senegalense* (Puttinaowarat *et al*., 2002a) and used for detection the pathogen in fish farm workers and environment (Puttinaowarat *et al*., 2002b).

As the conventional method is limited, more advanced methods have been developed including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and DNA sequencing (De Vos *et al.*, 1997; Widmer *et al.*, 1998; Campbell *et al.*, 2000). However, these studies inconclusively identified an individual species of *Pseudomonas* spp. In this study, we described the use of a PCR amplification of the intergenic spacer regions (ISRs), between the 16S and 23S rDNAs followed with a reverse hybridization technique to speciate *Pseudomonas* spp. up to species level

Multiple 16S-23S spacer amplicons of various lengths, like those detected in *P. putida*, have been observed in other bacteria. The result of *P. putida* amplification was shown other two bands beside the 650 bp fragment. This heterogeneity among the spacers within the various copies of the rRNA operon is present within the bacterial genome and has made this region useful as a means of differentiating closely related bacterial species (Berridge *et al.*, 1998).

DNA purified from the reference strains was used to determine the level of sensitivity of the method. The species-specific probes were able to identify from 20 up to 2 x 10^5 cells ml^{-1}. For diagnostic purposes, the RCBH has the advantage of not only being more sensitive than other methods, but identification of bacteria to species level was also possible. The sensitivity has been shown to vary in different bacteria, thus 2.8 x 10^4 cells ml^{-1} for *Brucella* spp. (Rijpens *et al.*, 1996), 20 mycobacteria cells for *Mycobacterium* spp. (Puttinaowarat *et al*, 2002a), and 2 mycobacteria cells for *Mycobacterium* spp. (Kox *et al.*, 1995) and could be increased by using a nested PCR. However, when a nested PCR is applied in practice, one has to consider stringent measures to avoid contamination (Rijpens *et al.*, 1996).

The specificity of primers in the PCR was also examined by RCBH. Primer P16sf-Bio and P23sr-Bio, amplifing a gene coding for 16S-23S rRNA, have cross-reactivity to other bacteria but this was eliminated by the specific probes of RCBH assay. The pDim2 probe reacted only with *P. diminuta* because the 16S-23S rRNA sequence differed from fluorescent pseudomonads likewise reported. According to Palleroni (1992) the present five groups of *Pseudomonas* described fluorescent pseudomonads in RNA Group I and *P. diminuta* in RNA Group IV.

Although pseudomonads do not always cause a high mortality, antibiotics treatment is commonly introduced into the farm practice, which consequently may cause drug residue problems. This study has demonstrated that the identification of *Pseudomonads* by PCR-RCBH is highly specific and less time-consuming than the conventional bacterial culture method. This may be useful in preventing disease outbreak as well as limiting the use of antibiotic prophylaxis.

CONCLUSION

Monoclonal antibodies (MAbs) against a *P. fluorescens* were produced by immunization of Balb/c mice with whole cell of the bacteria. The immunization was established into two sets: one was injection with UV-killed *P. fluorescens* and another was injection with sonicated *P. fluorescens*. Two of MAbs (MAb 2E7 and MAb 9A1) recognized the strains of most *P. fluorescens*. Based on their reactions, the MAb 2E7 and MAb 9A1 were chosen for a trial of HRP conjugation. The results showed that only MAb 9A1 was successfully conjugated to the HRP. The conjugated MAb 9A1 was then tested against a various pseudomonad and non-pseudomonad by indirect ELISA. The result revealed the same trend as it was tested with non-conjugated MAb 9A1. Considering the bacteria detection, the MAb 9A1 possesses good character for a further development of a rapid diagnosis kit to screen *P. fluorescens* infection in aquatic animals.

In addition, DNA-based method was also developed for detection and identification of *Pseudomonas* spp. The 16S-23S rDNA intergenic spacer was characterized and subsequently polymerase chain reaction (PCR) was developed. This method was proven to be specific and sensitive to detect *Pseudomonas* spp. to the sub-clinical infection level. Furthermore, PCR followed by reverse cross blot hybridization (RCBH) was adapted in this study to speciate *Pseudomonas*. Five probes specific to genus and species were designed to be used in the RCBH assay. The specificity was tested with 7 *Pseudomonas* spp. and 11 strains of other bacteria. The result revealed that the method was highly specific to *Pseudomonas* spp. with a detection limit of 20 cells mL^{-1}.

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APPENDIX

APPENDIX

Buffers and formulas

1. ELISA Buffers

Coating buffer

Na_2CO_3 0.153 g

$NaHCO_3$ 0.293 g

Dissolved in 100 mL distilled water. Adjusted the pH to 9.6 and freshly make.

Low salt wash buffer (LSW, x10)

Trisma base 24.2 g

NaCl 222.2 g

Merthiolate 1.0 g

Tween 20 5.0 mL

Dissolved in 1,000 mL distilled water. Adjusted the pH to 7.3 with concentrate HCl. Dilute 100 mL x10 stock with 1,000 mL of distilled water.

High salt wash buffer (HSW, x10)

Trisma base 24.2 g

NaCl 292.2 g

Merthiolate 1.0 g

Tween 20 10.0 mL

Dissolved in 1,000 mL distilled water. Adjusted the pH to 7.7 with concentrate HCl. Dilute 100 mL x10 stock with 1,000 mL of distilled water.

0.2 M Phosphate buffered saline (PBS)

$NaH_2PO_4.2H_2O$ 0.438 g

$Na_2HPO_4.2H_2O$ 1.280 g

NaCl 4.385 g

Dissolved in 500 mL distilled water. Adjusted the pH to 7.2.

Substrate buffer

Citric acid 10.5 g

Sodium acetate 4.1 g

Dissolved in 500 mL distilled water. Adjusted the pH to 5.4 with 1M NaOH.

Substrate (Stock chromogen)

42 mM of 3’3’5’5’-Tetramethylbenidine dihydrochloride (TMB) in 1:2 acetic acid : distilled water. The chromogen was kept in dark 4^{o}C.

Stop reagent (2M H_2SO_4)

H_2SO_4 19.16 mL in distilled water 100 mL

2. Electrophoresis buffers and gel

Separating gel buffer

1.5 M Tris 91 g/500 mL

0.4% SDS 2 g/500 mL

Adjust the pH to 8.7 with HCl. Stored at 4^{o}C

Stacking gel buffer

0.5 M Tris 6.05 g/100 mL

0.4% SDS 0.4 g/100 mL

Adjust the pH to 6.8 with HCl. Stored at 4^{o}C

Ammonium persulphate solution (ASP)

Ammonium persulphate 1 g/10 mL

Make up fresh just before use.

Water saturated-n-Butanol

n-Butanol	50 mL
distilled water	5 mL

Sample buffer

0.5 M Tris-HCl, pH 6.8	1.0 mL
Glycerol	0.8 mL
10% (w/v) SDS	1.6 mL
2-β-mercaptoethanol	0.4 mL
0.05% (w/v) Bromophenol blue	0.2 mL

Dissolved in 4.0 mL distilled water. Stored at room temperature.

Reservoir buffer (x5)

Trisma base	0.9 g
Glycine	43.2 g
SDS	3.0 g

Make up to 600 mL with distilled water, adjust the pH to 8.3 with HCl. Stored at 4 ^{o}C. Dilute 60 mL x5 stock with 240 mL of distilled water for one electrophoretic run.

Coomassie blue

0.25% (w/v) Commassie brilliant blue R-250

50% (v/v) methanol

10% (v/v) acetic acid

De-staining solution I

Glacial acetic acid	100 mL
Methanol	100 mL
Distilled water	800 mL

De-staining solution II

Glacial acetic acid 70 mL

Methanol 50 mL

Distilled water 880 mL

Tris buffered saline (TBS)

Trisma base 2.42 g

NaCl 29.24 g

Dissolved in 1,000 mL distilled water. Adjusted the pH to 7.5 with HCl.

Tween-Tris buffered saline (TTBS)

Add 0.5 mL of Tween 20 in 1,000 mL of TBS.

Transbolt buffer, pH 8.3

Glycine 14.40 g

Trisma base 3.03 g

Methanol 200 mL

Dissolved in 1,000 mL distilled water. Do not adjust the pH.

HRP colour development

Stock solution, 0.3 g of 4-chloronaphthol in 100 mL methanol. Add 2 mL of the stock solution and 10 μL of H_2O_2 in 10 mL PBS with pH 7.2.

3. Boom buffer

Lysis buffer (L1)

1. Dissolve 120 g of GuSCN in 100 mL of 0.1 M Tris HCl, pH 6.4
2. Heat the solution at 60 ^{o}C to dissolve the GuSCN
3. Add 22 mL of 0.2 EDTA solution adjusted with NaOH to pH 8.0
4. And add 2.6 mL of Triton X-100
5. Store in dark at room temperature

Lysis buffer (L2)

Dissolve 120 g of GuSCN in 100 mL of 0.1 M Tris HCl, pH 6.4

TE buffer

0.01 M EDTA in 0.01 M Tris HCl, pH 8.0

Diatom suspension

Celite 10 g

Distilled water 50 mL

32% HCl 500 µL

Mix well and aliquote 200 µL each.

Protenase K solution (x10)

Triton 5%

Protenase K 1 mg mL^{-1}

0.2 M Tris-HCl, pH 8.3

0.01 M EDTA

4. Cross blot solutions

Buffer 1 (x10)

1M Tris-HCl, pH 7.5 121.1 g

1.5 M NaCl 87.7 g

Dissolved in 1,000 mL distilled water. Adjusted the pH to 7.5.

Buffer 2

0.5% blocking reagent in buffer 1 (x1)

Heat the solution at 60 ^{o}C in a water bath until it is totally dissolved. Keep the buffer 2 in freezer and it can be re-used 10 times.

Buffer 3 (x10)

1M Tris-HCl, pH 9.5 121.1 g

1.0 M NaCl 58.4 g

Dissolved in 1,000 mL distilled water. Adjusted the pH to 9.5.

Stock $MgCl_2$

1M $MgCl_2$ (20.33 g $MgCl_2.6H_2O$/89.2 g)

20x SSC (stock)

Sodium chloride 87.7 g

Trisodium citrate 44.1 g

Dissolved in 500 mL distilled water.

Hybridization mix

50 mL 20x SSC

0.2 g N-laurylsarcosine

0.4 mL 10% SDS

2 g blocking reagent

150 mL distilled water (sterile)

Dissolve at 66 ^oC in a water bath and then keep the solution in a freezer.

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