การตอบสนองทางภูมิคุ้มกันต่อเชื้อพิเทียม อินซิดิโอซุมในผู้ป่วยคนไทยที่ได้รับการรักษาด้วยอิมมูโนเทอราปี

นางสาวทวิภัทร์ ไพศาลชัชวาล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

IMMUNE RESPONSE AGAINST *PYTHIUM INSIDIOSUM* IN THAI PATIENTS WITH IMMUNOTHERAPY

Miss Thawipat Phaisanchatchawan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2012

Thesis	IMMUNE RESPONSE AGAINST *PYTHIUM INSIDIOSUM* IN THAI PATIENTS WITH IMMUNOTHERAPY
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ทวิภัทร์ ไพศาลชัชวาล : การตอบสนองทางภูมิคุ้มกันต่อเชื้อพิเทียม อินซิดิโอซุมในผู้ป่วยคนไทยที่ได้รับการรักษาด้วยอิมมูโนเทอราปี. (IMMUNE RESPONSE AGAINST *PYTHIUM INSIDIOSUM* IN THAI PATIENTS WITH IMMUNOTHERAPY) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อริยา จินดามพร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.สพ.ญ.ดร.สันนิภา สุรทัตต์, 127 หน้า.

ประเทศไทยมีรายงานอุบัติการณ์ของโรค pythiosis ในคนมากที่สุดในโลก ปัจจุบันการรักษาด้วยวิธีอิมมูนโนเทอราปีนิยมทำร่วมกับการผ่าตัด และการให้ยาต้านเชื้อรา มีรายงานพบว่ามีการเปลี่ยนแปลงการตอบสนองจาก T_H2 ไปเป็น T_H1 ภายหลังการได้รับแอนติเจน แต่ไม่พบรายงานเกี่ยวกับ isotypes ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์ คือดูการตอบสนองในระดับ IgG โดยที่ IgG_1(แสดงถึงการตอบสนองแบบT_H1) และ IgG_4 (แสดงถึงการตอบสนองแบบT_H2) ของผู้ป่วยที่ได้รับการรักษาด้วยวิธีอิมมูนโนเทอราปี จำนวน 13 รายซึ่งแบ่งออกเป็น 3 กลุ่ม ตามลักษณะอาการและช่วงเวลาของการรักษาดังนี้ กลุ่มผู้ป่วยที่มีการตอบสนองระยะยาว (LRg, ≥14-34 mo.; n=4), กลุ่มผู้ป่วยที่มีการตอบสนองระยะสั้น (SRg, <14 mo.; n=6) และกลุ่มผู้ป่วยที่ไม่มีการตอบสนอง(NRg; n=3) การหาปริมาณของ IgG isotype จากซีรั่มของผู้ป่วยก่อนการรับแอนติเจนต่อโปรตีนที่เตรียมจากเชื้อพิเทียม อินซิดิโอซุม 3 สายพันธุ์จากกลุ่มย่อย 3 กลุ่มคือ MTPI19 (A_{TH}), PMS (B_{TH}) และ PC (C_{TH}) ด้วยวิธี ELISA ผลการศึกษาพบว่าผู้ป่วยส่วนใหญ่มีปริมาณของ total IgG แอนติบอดีกับแอนติเจน C-PC ($\bar{x}$=283.2, SD=302.2) สูงกว่ากับแอนติเจน C-MTPI19 ($\bar{x}$=31.15, SD=19.81) และ C-PMS ($\bar{x}$=46.85, SD=25.50) อย่างมีนัยสำคัญ (P<0.001) สำหรับปริมาณ IgG_1แอนติบอดีต่อแอนติเจน C-PC ($\bar{x}$=458.0, SD=735.7) พบว่ามีปริมาณสูงกว่า C-MTPI19 ($\bar{x}$=54.23, SD=43.48) อย่างมีนัยสำคัญ (P<0.05) แต่ไม่พบความแตกต่างกับ C-PMS ($\bar{x}$=119.0, SD=162.6) ในขณะที่ปริมาณของ IgG_4 ต่อแอนติเจน C-PC ($\bar{x}$=37.38, SD=50.0) และ C-PMS ($\bar{x}$=27.23, SD=27.87) มีปริมาณที่สูงกว่า C-MTPI19 ($\bar{x}$=18.15, SD=28.03) อย่างไม่มีนัยสำคัญ (P>0.05) ในการศึกษาปริมาณ IgGs isotype ในผู้ป่วยแต่ละคนต่อแอนติเจน C-MTPI19 (เตรียมจากสายพันธุ์ที่เตรียม PIA) จะเปรียบเทียบกับปริมาณแอนติบอดีก่อนการรักษา ระดับแอนติบอดีที่แตกต่าง 2 เท่าแสดงถึงการเกิดเปลี่ยนแปลง ผลการทดลองพบว่า 2/4 ราย (PY22, PY51) ของผู้ป่วยในกลุ่ม LRg มีการเพิ่มขึ้นของ IgG_1 แอนติบอดี แต่ทว่าผู้ป่วยอีก 2 ราย (PY5, PY30) มีการเปลี่ยนแปลงเล็กน้อยซึ่งอาจจะแสดงถึงการรักษาที่ได้ผล นอกจากนี้ไม่พบการเปลี่ยนแปลงแอนติบอดีในกลุ่ม SRg และ NRg การศึกษา antigenic pattern จากซีรั่มต่อโปรตีน C-MTPI19 โดยวิธี western blot พบแถบที่พบร่วมกันจากรูปแบบของ total IgG ที่ขนาดประมาณ120, 55 และ ประมาณ 40ถึง34 kDa ในขณะที่แถบที่พบร่วมกันจากรูปแบบของ IgG_1 และ IgG_4 พบที่ขนาดประมาณ120 kDa และที่ขนาดประมาณ32 ถึง28 และ 24 kDa ตามลำดับ นอกจากนี้จำนวนและความเข้มของแถบโปรตีนแสดงถึงความหลากหลายของรูปแบบของ IgG (11 รูปแบบ), IgG_1 (9 รูปแบบ) และ IgG_4 (10 รูปแบบ) ซึ่งพบมากในโปรตีนขนาดเล็กที่ขนาดประมาณ 55, 40 ถึง34และ28 kDa ด้วยข้อจำกัดของจำนวนผู้ป่วย และลักษณะอาการที่แตกต่างกัน ทำให้ยากต่อการสรุปการเปลี่ยนแปลงการตอบสนอง T_H2 เป็น T_H1 จำเป็นต้องมีการศึกษาทางด้านไซโตไคน์เพิ่มเติม อย่างไรก็ตามจากการศึกษานี้พบว่า C-PC เป็นแอนติเจนที่เหมาะสำหรับวิธี ELISA ในขณะที่ C-MTPI19 เป็นแอนติเจนที่เหมาะสำหรับวิธี western blot

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5287165920 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS : *Pythium insidiosum* / Immunotherapy/ Immune response

THAWIPAT PHAISANCHATCHAWAN : IMMUNE RESPONSE AGAINST *PYTHIUM INSIDIOSUM* IN THAI PATIENTS WITH IMMUNOTHERAPY. ADVISOR : ASSOC. PROF. ARIYA CHINDAMPORN, Ph.D., CO-ADVISOR : ASSOC. PROF. SANIPA SURADHAT, Ph.D., 127 pp.

The highest incidence of human pythiosis, the life threatening disease, has been found in Thailand. In the present, immunotherapy has been commonly combined with surgery and antifungal drug for treatment. It has been reported that after vaccination, the immune response switches from T_H2 to T_H1, however, no clear evidence of the isotypes were demonstrated. Thus, here, the immune responses, IgG, IgG_1 (represent of T_H1 type) and IgG_4 (represent of T_H2 type) isotype approach, were analyzed. Three groups of 13 patients were divided, base on the clinical, manifestation and the period of treatment. There were long term response (LRg, $\geq$14-34 mo.; n = 4), short term response (SRg, <14 mo.; n = 6), and non response (NRg, n = 3) group. First, the IgGs level from pre-vaccination serum against crude protein, prepared from each of *P. insidiosum* clade, A_{TH} (MTPI19), B_{TH} (PMS), and C_{TH} (PC), were determined using ELISA. The result showed that total IgG antibody level against C-PC ($\bar{x}$=283.2, SD=302.2) were significantly higher than those against other two antigens C-MTPI19 ($\bar{x}$=31.15, SD=19.81) and C-PMS ($\bar{x}$=46.85, SD=25.50) (P<0.001). For the specific IgG_1 antibody level recognized C-PC ($\bar{x}$=458.0, SD=735.7) was significantly higher (P<0.05) than C-MTPI19 ($\bar{x}$=54.23, SD=43.48) but not C-PMS ($\bar{x}$=119.0, SD=162.6). In terms of specific IgG_4 antibody, the levels against C-PC ($\bar{x}$=37.38, SD=50.0) and C-PMS ($\bar{x}$=27.23, SD=27.87) were not significantly higher (P>0.05) than C-MTPI19 ($\bar{x}$=18.15, SD=28.03). After that, all the IgGs levels in PIA-treated patient sera against C-MTPI19 (prepared from the PIA strain) were compared with pre-vaccination sera in each individual. Two times difference in level was the criteria as the level changing. The IgG_1 level increasing was found in only 2/4 case (PY22, PY51) in LRg whereas very slightly change was found in the other 2 cases (PY5, PY30). This might implied that these patients were recovered. In addition, no typical change was observed in both SRg and NRg. Finally, all the sera were exposed to C-MTPI19 antigen to study their antigenic profiles by western blot. Four bands, ~120, ~55 and ~40-~34 kDa, only one band, ~120 kDa, and three bands, ~32-~28 and ~24 kDa, were commonly found against total IgG, IgG_1 and IgG_4 antibody, in order, in all 13 pre-vaccination sera. In contrast, the variation of profile against IgG (11 profiles), IgG_1 (9 profiles) and IgG_4 (10 profiles), antibody in post-vaccination sera in both number and the density were revealed. These were found mostly in the low molecular weight antigen, ~55, ~40-~34 and~28 kDa. Since the limitation of the case number and their different clinical status, it was hardly to conclude the switching T_H2 to T_H1 response. More study of their cytokine is required. However, this study demonstrated the optimum antigen for the ELISA technique (C-PC) and western blot analysis (C-MTPI19).

Field of Study : Medical Microbiology Student's Signature .....

Academic Year : 2012 Advisor's Signature .....

Co-advisor's Signature.....

ACKNOWNLEDGEMENTS

I would like to express my deepest appreciation and sincere gratitude to Associate Professor Dr. Ariya Chindamporn, my thesis advisor and Associate Professor Dr. Sanipa Suradhat, my co-advisor who devoted their especial efforts, valuable advice, memorable support, excellent guidance, great encouragement and endurance throughout my graduate works.

My special thanks go to the Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of his majesty king Bhumibol Adulyadej, the 90th Anniversary of Chulalongkorn University Fund, Graduate School, Chulalongkorn University and the National Research Universities Fund, Office of the Higher Education Commission, Grant No.HR1164A3 for the main research fund.

I also appreciate to Associate Professor Phisit Prapunwattana, Department of Biochemistry, Chulalongkorn who always gave their comments on my work.

Additionally, I am thankful to all members both in Mycology Unit, Department of Microbiology, Chulalongkorn Memorial Hospital and mycology research unit, Faculty of medicine, Chulalongkorn University for stimulating and fun environmental in which to learn and grow

Finally, but most significantly, I am especially grateful to my parents and my brother for their love, attention, understanding, encouragement, and endless support throughout my life.

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ABBREVIATIONS

A_{TH}	=	Clade I
B_{TH}	=	Clade II
C_{TH}	=	Clade III
%	=	Percent
µg	=	Microgram (10^{-6})
µl	=	Microliter (10^{-6} liter)
µm	=	Micrometer (10^{-6} meter)
APC	=	Antigen presenting cell
ATCC	=	American Type Culture Collection
BCIP	=	5-bromo-4-chloro-3-indolyl phosphate
BSA	=	Bovine serum albumin
Cat.	=	Catalog
CBS	=	Centraalbureau voor Schimmelcultures
CMI	=	Cell-mediated immunity
COX II	=	Cytochrome oxidase II gene
CTL	=	Cytotoxic T cel
DNA	=	Deoxyribose nucleic acid
DW	=	Distilled water
ELISA	=	Immunosorbent assay
et.al.	=	And others
F	=	Female
FcγR	=	Fc receptor of IgG
h	=	hour (s)
H&E stain	=	Hematoxylin and eosin stain
ICT	=	Immunochromatographic test
ID	=	Immunodiffusion test
IgA	=	Immunoglobulin A
IgD	=	Immunoglobulin D
IgE	=	Immunoglobulin E

IgG	=	Immunoglobulin G
IgG1	=	Immunoglobulin G1
IgG2		Immunoglobulin G2
IgG3	=	Immunoglobulin G3
IgG4	=	Immunoglobulin G4
IgGs	=	IgG and IgG subclass
IgM	=	Immunoglobulin M
IGS	=	ribosomal intergenic spacer
IL-12	=	Interleukin 12
IL-1 β	=	Interleukin 1 beta
IL-2	=	Interleukin 2
IL-23	=	Interleukin 23
IL-4	=	Interleukin 4
IL-5	=	Interleukin 5
INF- γ	=	Interferon gamma
ITS gene	=	Internal transcribed spaer gene
kDa	=	Kilo dalton
LCB	=	Lactophenol Cotton Blue
M	=	Male
mg	=	Milligram(s)
MHC	=	Major histocompatibility complex
min	=	minute
ml	=	Milliliter(s)
MW	=	Molecular weight
MYD88	=	Myeloid differentiation primary response gene
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NBT	=	Nitro blue tetrazolium chloride
ND	=	No data
ng	=	Nanogram (10^{-9} gram)
NK cell	=	Natural killer cell

nm	=	Nanometer (10^{-9} meter)
No.	=	Number
^{o}C	=	Degree Celsius
OD	=	Optical density
OPD	=	O-phenylenediamine dihydrochloride
PAMPs	=	Pathogen associated molecular pattern
PAS	=	periodic acid-Schiff stiain
PBS	=	Phosphat buffer saline
PBS-T	=	Phosphat buffer saline with Tween 20
PCR	=	polymerase chain reaction
PIA	=	*Pythium insidiosum* antigen
PNH	=	Aplastic anemia/paroxysmal nocturnal hemoglobinuria
PRRs	=	Pattern recognition receptor
PV	=	Pre-vaccination
PVDF membrane	=	Polyvinylidene Fluoride membrane
PY	=	Pythium code.
ROIs	=	Reactive oxygen intermediated
rpm	=	Revolutions per minute
SDA	=	Sabouraud dextrose agar
SDB	=	Sabouraud dextrose broth
SSKI	=	Saturated solution of potassium iodide
TBS	=	Tris buffer saline
TBS-T	=	Tris buffer saline with Tween 20
T_H cell	=	T helper cell
T_H0	=	naïve T cell
T_H1	=	T helper 1
T_H2	=	T helper 2
TLRs	=	Toll-like receptors
TNF	=	Tumor necrosis factor
TNF-α	=	Tumor necrosis factor alpha

wk	=	Week(s)
β	=	Beta
κ	=	Kappa
λ	=	Wavelenght

CHAPTER I

INTRODUCTION

Pythium insidiosum, an organism in genus *Oomycete*, causes pythiosis in human and both livestocks [1] and small animals including horses [2-4], sheep [5], dogs [6, 7], cats and cattle [1]. Pythiosis is emerging in tropical, subtropical and temperate regions of the world and the tendency of the disease is increasing [1, 8, 9]. It was divided in three clades by internal transcribed spacer (ITS) gene which is clade I, II, and III. Clade I was isolated from America. Clade II was isolated from Australia and Asia while clade III was isolated from Thailand and USA [10]. However, the recent study found that cytochrome oxidase II gene (*COX* II) could classify and even provide more discriminatory power of classification than ITS gene [11]. Human pythiosis was first reported from Thailand in 1985 and up to the present, most of human pythiosis has been revealed from Thailand [12, 13]. From the clinical review of more than 100 human cases during the year of 1985-2003, three major of clinical manifestation were found. There are (i) cutaneous/subcutaneous pythiosis (ii) vascular/disseminated pythosis and (iii) ocular pythiosis. Cutaneous/subcutaneous pythiosis was the major form in animals while vascular/disseminated pythosis and ocular pythiosis were found in human [14]. Furthermore, most pythiosis patients had the abnormality of red blood cells as underlying disease such as thalassemia [15], leukemia and aplastic anemia/paroxysmal nocturnal hemoglobinuria (PNH) [12-14, 16]. Due to the limitation of knowledge, diagnosis tools and the efficiency of treatment, this infection caused life-threatening, resulting high mortality and morbidity rate. More than that, the information of this human pythiosis has been recognized mainly, in infectious man but the patients often treated by surgeon and hematology specialist due to their symptoms. Faster diagnosis and faster treatment will

relieve the patients' suffering. Since this organism is not a fungus, then antifungal drugs were doubt whether it is useful to treat or not [17-19]. In 1981, immunotherapy using *Pythium insidosum* antigen (PIA) was started to treat the infected horse. Later, the application of this PIA was expanded to treat infected bovine and other animals [1]. Until the year of 1998, this PIA treatment was a successful therapy for a 14 year old Thai boy suffering with arteritic infection [20]. From the previous study, the successful rate of PIA were shown in horse (60%) more than in human (56%) and dogs (33%), respectively [18]. Currently, this immunotherapeutic treatment is still one of the choices to safe the patients' life when combinds with the amputation and some antifungal drug regimen [19].

The previous study reported that the PIA-treated horse showed the switching of immune response from T_H2 response to T_H1 response [18]. In horse, prior to the injection of PIA vaccine, naive T-cell was drove into T_H2 subset, released IL-5 and IL-4. B-lymphocytes were stimulated by IL-4, making the secretion of IgE, IgM and IgG which is useful for detection of the precipitin line in immunodiffusion test [21, 22]. Furthermore, IgE and IL-5 induce eosinophils and mast cells migration into the infection site, causing the Splendore-Hopplei phenomenon. After PIA immunotherapy, antigen presenting cells secrete INF-γ which function as the stimulators of cytotoxic T cells (CTL). These authors proposed that these CTL should be clearance the hyphae in the host. Similarly, in PIA-treated vascular human pythiosis, the increasing of INF-γ and IL-2 and the decreasing of IL-4 and IL-5 were found during the first month after injection [19, 20]. In principle, two pathways of the fungal immune response, innate immunity as non-specific response and adaptive immunity as specific one were existed. This indicated that the CTL [23-26] and B-lymphocytes functions are the significant mechanisms in clearance of the germ. Furthermore the pro-inflammatory cytokine such as INF-γ and IL-4 can induce IgG isotype switching. INF-γ stimulated plasma cell to produce IgG_1 and IgG_3 antibody whereas IL-4 stimulated IgE and IgG_4 secretion [27, 28]. The cytokine

secretions performed the indirect mechanism of the immune response. In the vaccine retrospective study, it was shown that the life-threatening condition was found in PIA-poor responders than well responding group [19]. To safe the patients' life, the early of efficiency treatment are indispensable apart from the rapid, accurate with high sensitivity and specificity diagnostic. As we know that antibody will play an important role in the clearance of the organism. In the year 2009, it was shown that the diversity of antibody recognized antigens were proved. This result can explain why the antigenic pattern of each individual or in other words, the specific immune response of each individual is different. The selection of strong immunogen is one of the significance role for immunotherapy [29]. One year later, one of *P. insidiosum* immunodominant antigen was identified as exo-1,3 - β -glucanase [30, 31]. However, the few information of the specific antibody productions has been confined. To reveal the immune response, the specific IgG isotypes against *P. insidiosum* in serum before and after PIA injections will be studied.

CHAPTER II

OBJECTIVE

Hypothesis

The T_H1 response (IgG_1 antibody) will predominate in well immune response of immunotherapeutic patients more than T_H2 response (IgG_4 antibody).

Objective

To examine the IgG isotypes to *P. insidiosum* in human pythiosis treated with PIA-immunotherapy.

CHATER III

LITERATURE REVIEWS

1. Background History of *Pythium insidiosum*

Oomycetes are eukaryotic organisms also known as water molds because they inhabit in primarily aquatic and moist soil. They have fungal-like characteristic but lack affinity with true fungi [32]. The phylogenetic analysis found that they are relatively with diatom and brown algae in the Kingdom Straminipila than true fungi because the oomycetes structure composes of the mixture of cellulose and glycan while the cell wall of fungi constructs of chitin [1, 33]. The sexual stage produced biflagellate zoospore in wet environment. Furthermore, they have diploid nuclei in the filament as the fungus has haploid nuclei. Phylum Oomycetes have been divided to seven orders as Saproleginales, Rhipidiales, Leptromitales, Salilagenidiales, Pythiales, Peronosporales and Sclerosporales. In the order Pythiales have two important genera as *Phytophthora* and *Pythium* [32]. They infect and damage plants such as root rot in numerous plants, late blight of potato or tomato *etc*. However, *P. insidiosum* is the only member of genus causing in animals and humans.

P. insidiosum is the pathogenic agents in animals and humans called Pythiosis. *P. insidiosum* was first reported by Bridges and Emmons in 1961 and named as *Hyphomyces dertruens* which caused Hyphomycosis destruens disease [1]. In 1974, the organism which was isolated from horses in Papua New Guinea, developed biflagellate zoospore in aqueous medium and concluded that *H. dertruens* was relative in genus *Pythium* [34, 35]. In 1980, Ichitani and Amemiya isolated *P. insidiosum* from horse disease and found similar the morphology of *P.gracile* [36]. In 1987, De Cock *et.al* reported the sexual reproduction of *P. insidiosum* [34]. In the same time, Shipton

described the organism *P. dertruens* isolated from Australian horse with pythiosis which considered the same species as *P. insidiosum* [35]. In the present, *P. insidiosum* was classified in the kingdom Straminipila, class Peronosporomycetes, order Pythiales, family Pythiaceae and genus *Pythium.* However, this classification was not base on DNA sequence data and may be change in the future.

P. insidiosum is the only of member in the genus causing pythiosis in animal such as horses [2, 4, 37-39], cats [34], dogs [7], sheep [5, 40], birds [41] and human [20, 42-44]. Human pythiosis was first reported in 1985 which was found mostly in Thailand [13]. The clinical manifestation of this disease has three forms as i) cutaneous/subcutaneous form ii) ocular form and iii) vascular/disseminated form. Cutaneous/subcutaneous form was found in animal while the other forms were found in human [1, 18]. The pathogenesis of disease was not clear but the scientists expected that the susceptible hosts had skin wound and contacted zoospores in water. Furthermore, susceptible hosts with thalassemia underlying disease were considered to be risk factor for *P. insidiosum* infection [45].

2. Biology of *P. insidiosum*

2.1.Morphology features [1, 32]

The cultivation of *P. insidiosum* on various artificial media affect to the growth rate and characteristic of colony. On Sabouraud dextrose agar (SDA) and corn meal agar, *P. insidiosum* colony is colorless to white (Figure 1A, 1B). The short aerial mycelium with finely radiate pattern as hair-like is shown in Sabouraud dextrose broth (SDB) (Figure 1C). *P. insidiosum* is able to grow in the various temperatures. They grow well at 25°C or 37°C on SDA but the growth of this organism is inhibited at low (4°C) and high (42°C) temperatures. Microscopic characteristics show that the coenocytic hyphae range between 4 and 10 µm diameter with 90° perpendicular lateral branches by Lactophenol Cotton Blue (LCB) staining (Figure 1D). The septate hyphae only are occasionally observed in early hyphae. The vesicle of sporangial in zoosporogenesis is approximately 20-120 µm in diameter. The encysted zoospores are 8-10 µm in diameter. On the laboratory, the zoospore induction is the basic diagnosis in *P. insidiosum*.

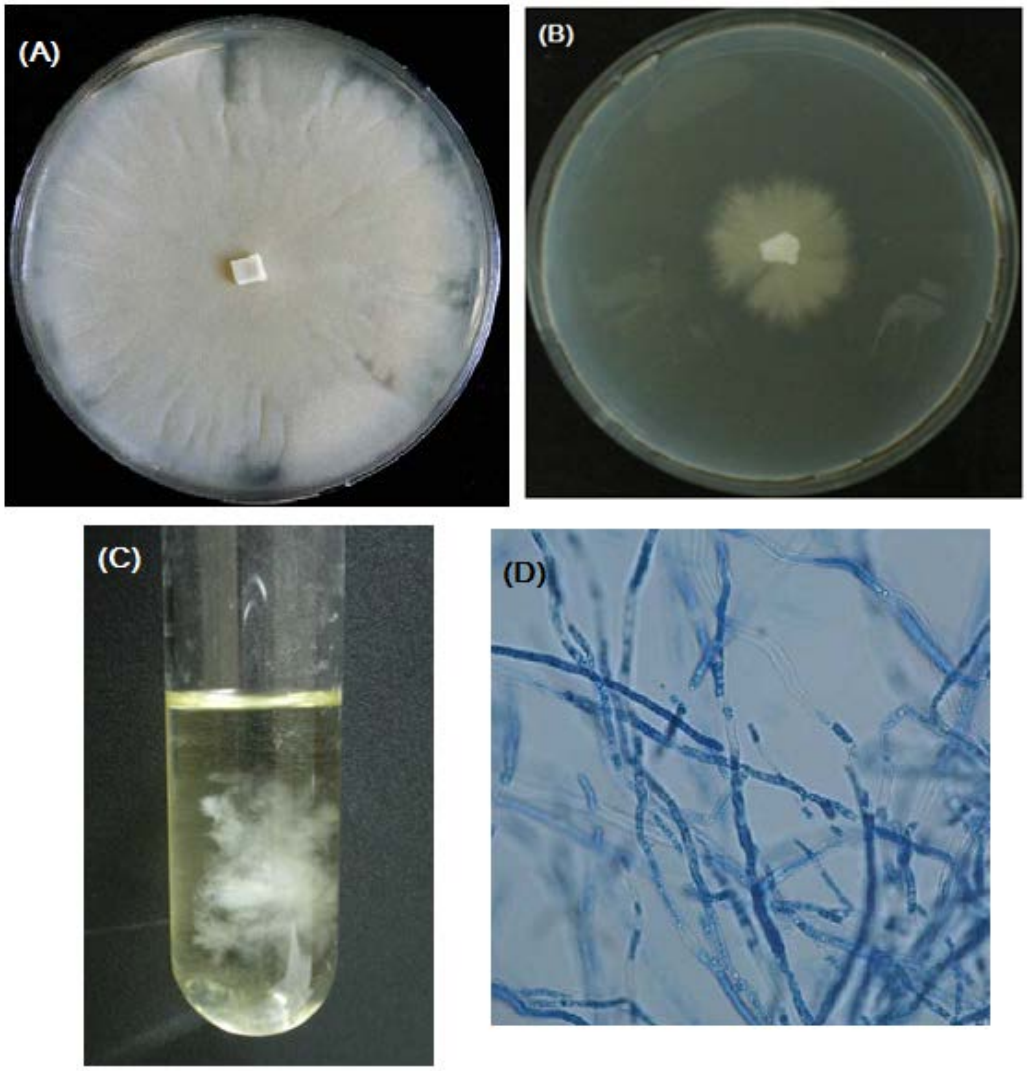

Figure 1 *P. insidiosum* colony (A) five days old at 37°C on SDA (B) three days old at 35°C on corn meal agar (C) Five days on SB at 35°C (D) *P. insidiosum* showing non-septate and sparsely hyphae in lactophenol blue (20x)

2.2. Life cycle of *P. insidiosum* [45]

In nature, *P. insidiosum* produces sexual or asexual spores depending on the environmental condition.

2.2.1. Sexual reproduction

Sexual reproduction of genus *Pythium* produce specialized reproductive structure containing oogonium as known as female reproductive cells and antheridium as known as male reproductive cells (Figure 2A). The location of antheridium can divide this organism two types; homothallic and heterothallic. In homothallic species, fertilization occurs in a single stain whereas heterothallic species use two strains for fertilization. The process of fertilization occur meiosis and haploid nuclei transportation from antheridium into the oogonium via a fertilization tube. After fertilization, the oogoniums develop a thick wall, becoming mature oospores. The oospores can resistant to harsh environment condition which need to wait the suitable condition for growth [32].

2.2.2. Asexual reproduction

P. insidiosum is the organism in aquatic environment. In the nature, *P. insidiosum* produced and released zoospores in the water. The zoospores have two flagella containing the anterior shorter flagellum and the posterior flagellum. The anterior shorter flagellum is the tinsel type for direction control whereas the other flagellum is the whiplash type for direct movement. The released zoospores can move by two flagella and the endogenous energy for 10 to 15 min before encystment. After encystment, zoospores detached flagella and developed germ tube that became elongated the filament after 24 hour at 37^{o}C. In 1993, Mendoza *et.al.* indicated the attachment and penetration of zoospores on plant and animal tissue for pathogenesis formation (Figure 3) [45].

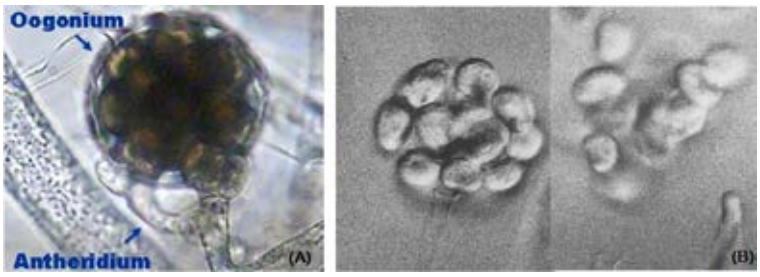

Figure 2 Sexual and asexual reproduction of *P. insidiosum* (A) (http://website.nbm-mnb.ca/mycologywebpages/) oogonium as female reproductive cell contain oospore and antheridium is male reproductive cell. (B) Mature zoospores in sporangia and the motile zoospore

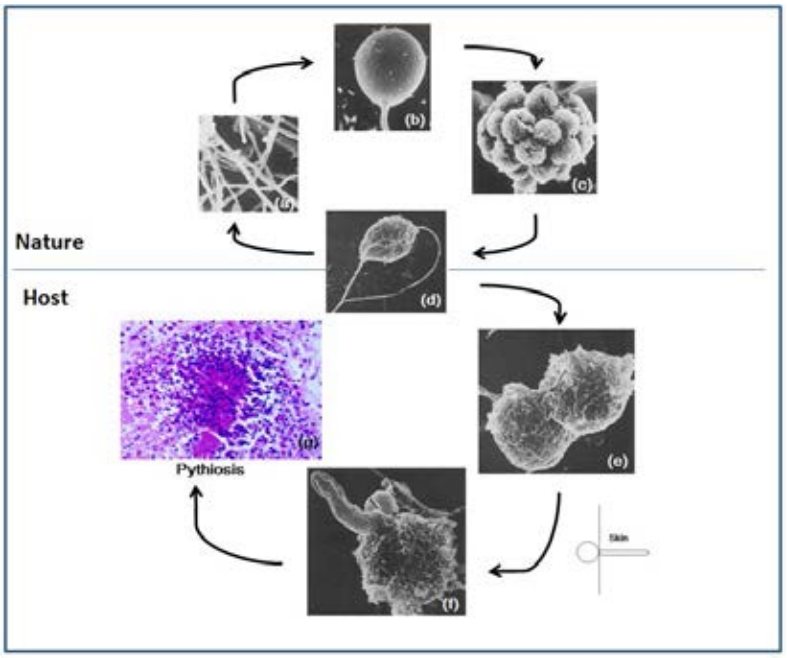

Figure 3 Life cycle of *P. insidiosum* in nature and infectious stage. In the nature, the hyphal tip will differentiate sporangium into mature stage (a-c) and then the biflagellate zoospores release and disperse in the environment (d). The encysted zoospore will attract the injured tissue of plants or animals (e). Then the encysted zoospores germinate and invade into the lesion and cause pythiosis (f). Pathogenesis of pythiosis in horse called splendore-hoeppli phenomenon (g)

3. Epidermalogy

Pythiosis is the infectious disease in animals and humans caused by *P. insidiosum*. Pythiosis can be found in the tropical, subtropical and temperate region of the world (Figure 4) [1]. The both animal and human pythiosis have been reported in India [35, 46], Brazil [5, 9], Australia [43], United State [4] and Thailand [14]. Seven equine pythiosis were recently reported in Venezuela [2, 47]. Furthermore, internal transcribed spacer (ITS) classification can divide this organism into three clades which the isolates are different geographic region. The isolated organism of clade I can be mostly found in Costa Rica, Haiti, Brazil and the USA. Clade II contained *P. insidiosum* isolation from Australia and Thailand. The clade III were isolated from Thailand and USA [10]. In 2011, Kammarnjesadakul *et.al.* demonstrated the phylogenic tree using cytochrome oxidase II (*COX* II) gene comparing ITS gene. *COX* II gene showed higher resolution than ITS gene which revealed three group, Clade I: A_{TH}, Clade II: B_{TH} and Clade III: C_{TH} (Figure 5) [11]. Due to this classification, *P. insidiosum* isolated from clinical and environmental specimens in Thailand were grouped in clade II and clade III.

Figure 4 Epidemiology of human pythiosis

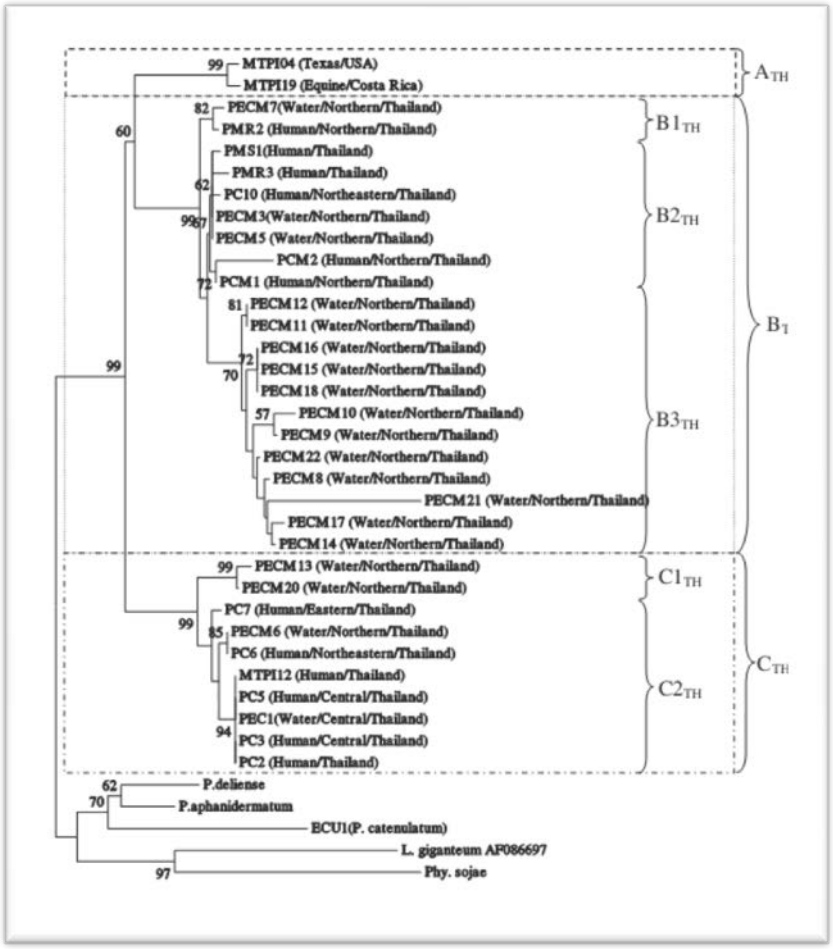

Figure 5 Phylogenetic tree of *P. insidiosum* based on *COX* II gene [11]

In 1985, the first case of human pythiosis was found in Thailand which is the highest incidence in the world. In Thailand, human pythiosis were mostly found in central region (46%) and northern region (27%) (Figure 6). The clinical manifestation in human has three forms as disseminated form, cutaneous/subcutaneous form and ocular form. Disseminated pythiosis form mostly occurs in Thai patients who have underlying hematologic disorder such as thalassemia, leukemia and aplastic anemia/paroxysmal nocturnal hemoglobinuria (PNH), while ocular pythiosis can be found in healthy patients [14, 19]. In 2006, Krajaejun et.al. accumulate the data from 9 tertiary care hospitals in Thailand. In total 102 cases, almost of patients were vascular pythiosis (59%) (Figure 7) and eighty-five percent of all patients have underlying disease. Most of the patients were

male (71%) with the age ranging from 20 to 60 years (86%) (Figure 8) [14]. Seventy-five percent of patients work in agriculture (grew rice, corn or vegetable crops).

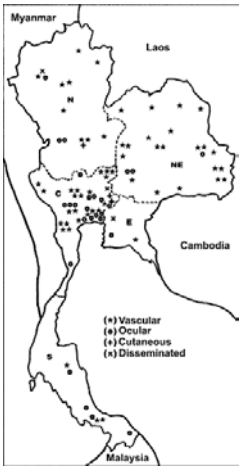

Figure 6 Geographic distributions of pythiosis patients in Thailand. Data were available for 88 patients (86%) [14]

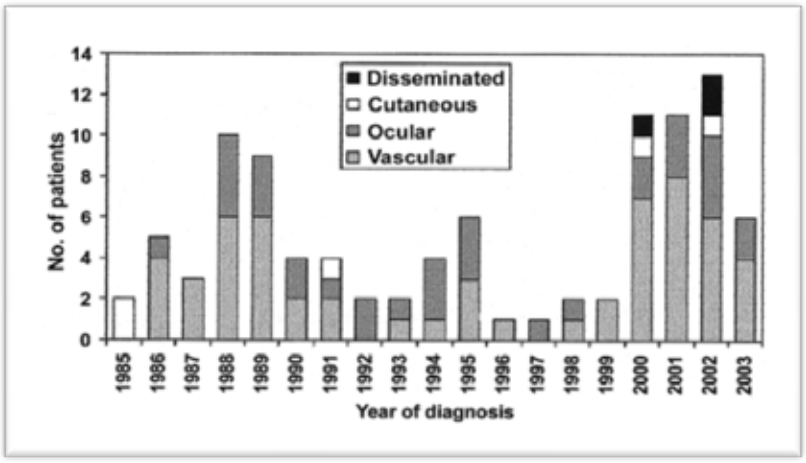

Figure 7 The numbers of Thai patients with various forms were diagnosed during January 1985 to June 2003 [14]

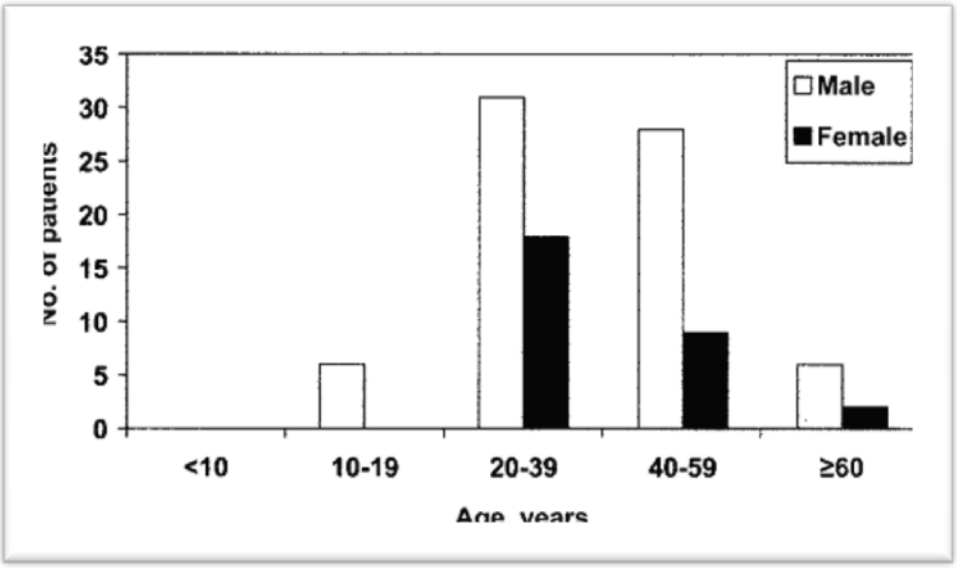

Figure 8 Summary sex of pythiosis patients in each age group [14]

4. Clinical pathogenesis

P. insidiosum is parafungus that cause in animals and human. This organism can grow in host at 37°C which is essential factor for virulence. Scientists predict the zoospore as the infectious stage of *P. insidiosum*. Zoospores will swim toward the animal hair, wounds and other damaged skin. Biflagellate zoospores attract and encyst on the exposed tissue. On the electromicroscopic observation, found that zoospores secreted sticky substance as glycoprotein which maintain tightly contact with the host in initial invasion stages. Temperature of host stimulates zoospores becoming encystment and germ tube development. Germ tube penetrated directly injured tissue which later can infiltrate blood vessel in human. In 2001, Ravishankar and Davis reported the mechanically the tissue penetration that *P. insidiosum* cannot penetrate healthy skin but has the potential to successful penetrate injured tissue. These experiments confirm that zoospores are form of infectious stage [1, 45].

5. Clinical manifestation

Clinical manifestation of pythiosis can be divided to three forms. Subcutaneous form found mostly in animal whereas vascular form and ocular form found mostly in human.

5.1. Subcutaneous/cutaneous pythiosis

All patients have same lesions that were characterized by chronic swelling and a painful, subcutaneous, granulomatous, infiltrative lump and ulcer on limbs. This granulomatous lesion can be found in many kinds of animals such as equine (Figure 9A), sheep [40], dog [9], cat and cattle [48]. For human subcutaneous pythiosis has been reported with necrotizing cellulitis of both legs that he exposed with the standing water in rice fields (Figure 9B) [49]. He was 15-year-old with β-thalassemia/hemoglobin E disease. The lesion demonstrated necrosis of skin and subcutaneous tissue but intact fasciae and muscles furthermore fungal culture grew *P. insidiosum*. He was treated SSKI, itraconazole, and terbinafine which he has not the progression of disease [16].

Histoplathological examination showed many inflammatory cells including eosinophils, lymphocytes, neutrophils, macrophages and mast cells. The *P. insidiosum* hyphae were surrounded a lot of eosinophils which is related the clinical manifestation of zygomycosis caused by *Conidiobolus* and *Basidiobolous*. Cell-mediated immune response of host was stimulated by *P. insidiosum*. The eosinophils and neutrophils migrated at infectious area surrounding the organism which was specific form of cutaneous/subcutaneous pyhtiosis called “Splendore-Hoeppli-like phenomenon” (Figure 10). In addition, small coral-like necrotic masses of tissue containing hyphae as known as “kunker” were found only in tissue of horse but not in the other species. However, clinical manifestation of pythiosis is similar with habronemiasis and subcutaneous zygomycosis that can lead to misdiagnosis of the disease [1, 18].

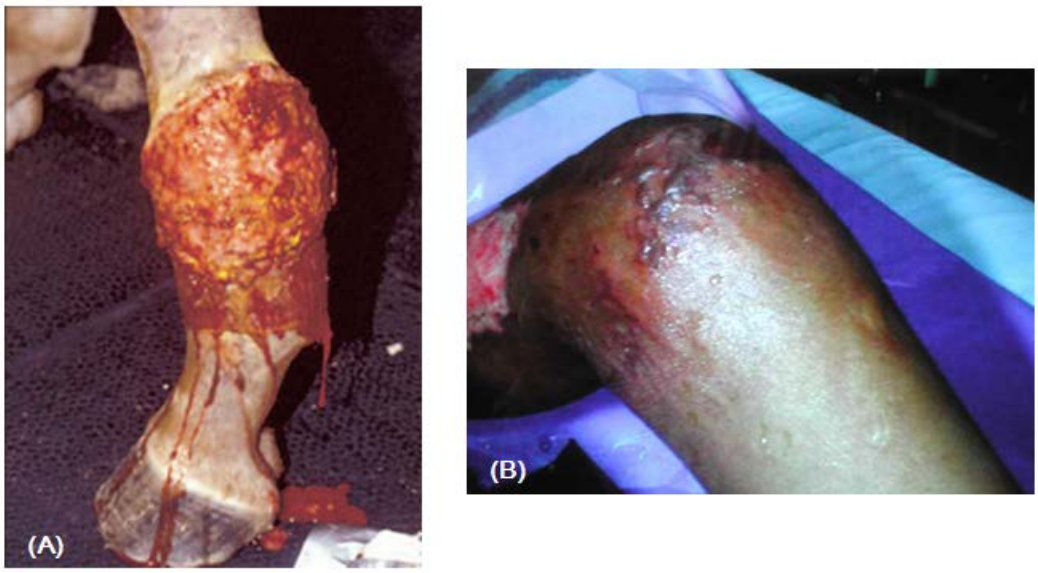

Figure 9 Cutaneous/subcutaneous pythiosis (A) lesion in horse[50]. (b) lesion in human [18]

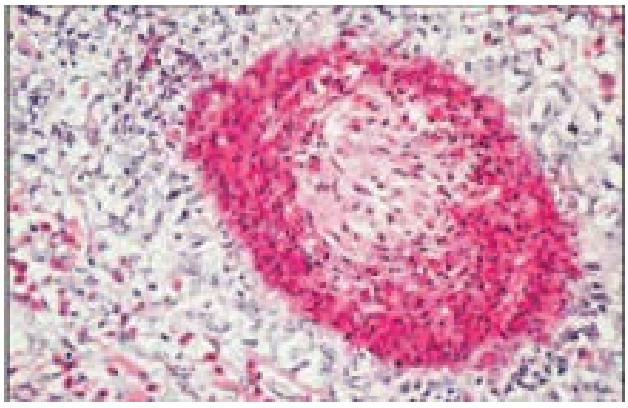

Figure 10 Histopathological examination from cutaneous pythiosis lesion [50]

5.2. Ocular pythiosis

The patients usually presented with corneal ulcer or keratitis (Figure 11) [8]. Pain, irritation, visual cavity decreasing, eyelid swelling, conjunctival injection, corneal infiltrates and hypopyon can observe in some cases. Most patients had not underlying disease. The duration time of treatment is shorter than other vascular and cutaneous/subcutaneous form. Ocular pythiosis patients were not response from treatment with conventional antifungal agents such as amphotericin B, ketoconazole and itraconazole [16]. Most ocular patients lost their eyes for controlling infection.

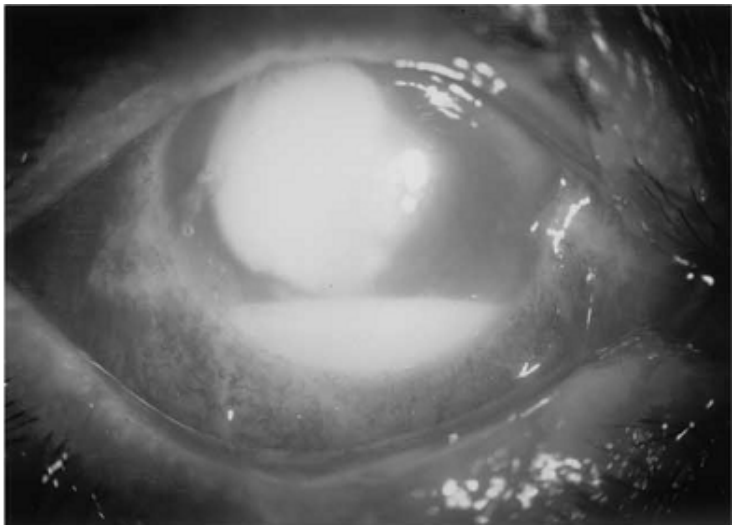

Figure 11 Clinical presentation of ocular pythiosis [8]

5.3. Vascular pythiosis

Vascular pythiosis is the most common form among in patients with thalassemia and anemia-paroxymal nocturmal hemoglobiruria (PNH) [15]. This infection was specific with arterial tissue as resulting in arterial occlusion or aneurysm. Clinical features varied symptom from intermittent claudicating to gangrenous ulceration. Other symptoms and signs were presented such as fever, paresthesia, itching, skin ulcer (Figure 12A), cellulitis, necrotizing fasciitis, leg swelling, arterial pulse absence, goin mass and aortic aneurysm. The duration time of symptom was 3 month before seeking medical care. Almost patients come to medical care late in the treatment course. Angiographic finding presented an occlusion in blood vessel of infected medium-to-large-sized arteries of the lower extremities (Figure 12B). The pathological finding revealed hyphae invading the arterial, eosinophil migration and infiltration, focal suppurative granuloma and giant cells surrounding the hyphae, as well as a blood clot in arterial lumen. Progression of infection caused aneurysm or arterial occlusion from a thrombus or fibrosis. Most patients get to the surgery by removal infectious area but a major cause of death is from relapse of the diseases [51].

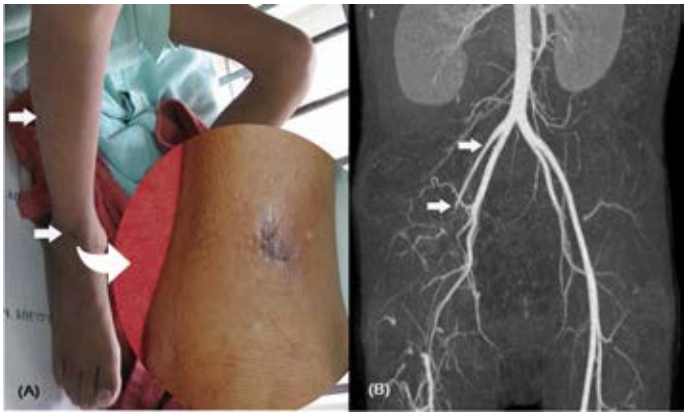

Figure 12 The pathological feature of Thai patients with vascular pythiosis (A) Ulcerated skin on the right ankle. (B) Occlusion of the right iliac artery and artery branching by tomography angiography [51]

6. Laboratory diagnosis

6.1. Specimen collection

The collection of specimens depends on the pythiosis forms of patients. Skin biopsy was collected for cutaneous/subcutaneous pythiosis. For ocular pythiosis, corneal scraping should be collected while serum samples were needed for vascular pythiosis. The biopsy tissue could be transported in sterile distilled water or saline solution. The clinical specimens should be kept at room temperature (not kept at 4°C) as this temperature is optimal for cultivation [1].

6.2.Cultivation and direct examination

The clinical specimens will be cut to small pieces which inoculate on Sabouraud dextrose agar (SDA) or broth (SDB) and incubated at 37°C for 24-48 hours. After incubation, the white/colorless submerged colony and no aerial mycelium appears on media. Direct examination by wet mount 10% KOH shows sparsely septate hyphae and perpendicular lateral branches (Figure 13).

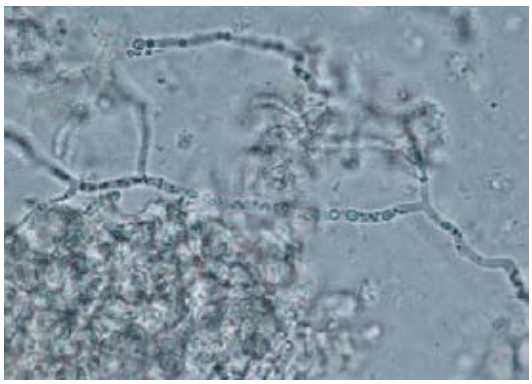

Figure 13 10%KOH wet mount preparation of kunker form horse [2]

6.3.Zoospore production

Zoospore induction is the standard method for pythiosis diagnosis. Zoospores are induced by induction medium containing calcium and magnesium ions. After incubation, zoosporangia containing zoospores are observed on grass pieces under light microscope. Zoosporangia has characterize hyaline and globose which its inside found biflagellate zoospores. *P. insidiosum.* The biflagellate zoospores were recognized the only oomycete pathogenic in mammals for a long time so zoospores induction was considered enough for presumptive diagnosis of pythiosis [1]. However, the other methods of identification were developed for increasing of specificity and sensitivity such as serological tests and molecular tools.

6.4. Histopathology

Tissue biopsy from granulomatous lesion was stained with periodic acid-Schiff (PAS), hematoxylin-eosin (H&E) or Gomori methenamind silver staning. The sparsely short septate hyphae with perpendicular branching were observed by surrounded with many eosinophils, giant cells, mast cell and other immune cells (Figure 14) [6, 9].

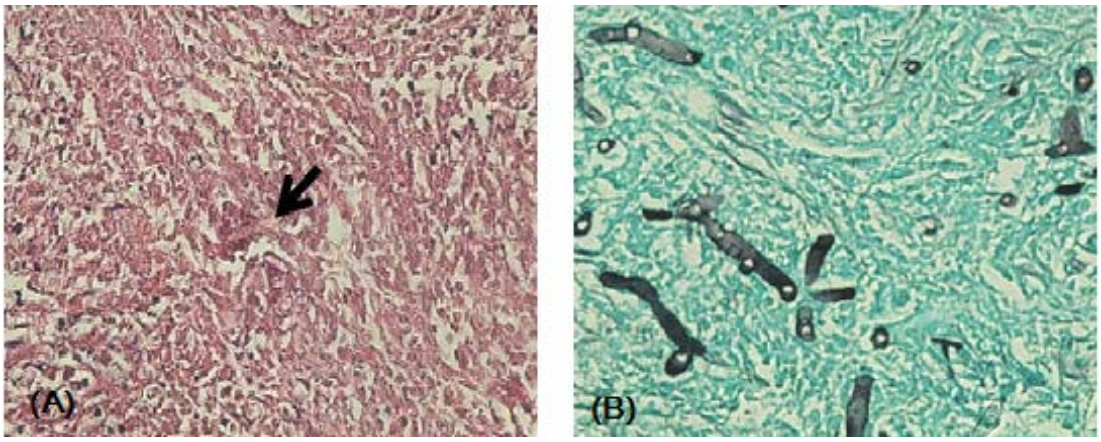

Figure 14 Granulomatous lesion from intestinal case of dog pythiosis (A) H&E staining and (B) Silver staining [9]

6.5. Molecular diagnosis-DNA based

Molecular analysis was developed for *P. insidiosum* detection in clinical laboratory. The methods of *P. insidiosum* DNA used for diagnosis have been developed, based on polymerase chain reaction (PCR) [10], nested PCR [52], DNA sequencing [53] and hybridization technique [54]. In 2002, Badenoch *et.al.* showed that the first to use DNA sequencing from internal transcribed spacer (ITS) region in keratitis patient. In the next times, Grooters and Gee developed nested PCR by using specific primers to amplify 105 base pairs from *P. insidiosum* genomic DNA. Schurko and his colleage in 2004, developed species-specific DNA probe from ribosomal intergenic spacer (IGS) and furthermore they used IGS1 region for classification of *P. insidiosum*. However, molecular analysis is ideal to identify *P. insidiosum* from clinical specimens and cultures.

6.6. Serological method

Serology assay were developed in the beginning of the century. Immunodiffusion (ID) was the first method used for diagnosis of pythiosis [21]. Moreover, several methods were developed such as enzyme-linked immunosorbant assay (ELISA) [55-57], western blot [30, 58] and immunohistochemical assay [59]. Each technique has been different specificity and sensitivity.

Immunodiffusion test was used to diagnose in horse pythiosis in early stage of disease, but it gave false negative in chonic infection. So this test is high specific but poor sensitivity [21, 22].

Immunohistochemical tests were developed for detection of *P. insidiosum* in tissue biopsy which consist of two tests as immunofluorescence test and immunoperoxidase test.

ELISA method was developed to detect pythiosis in humans and animals which showed 100% of both sensitivity and specificity. This method requires serum specimens from healthy individuals, other infections, and serum of patients diagnosed with pythiosis. However, this test showed very high sensitivity and specificity in diagnosis that is suitable to monitor the response to the treatment [55-57].

Western blot was developed later to increase in sensitivity and specificity. This method gave benefits in finding dominant antigen and diagnosis. This test can show antigenic pattern of immunoglobulin G in serum against *P. insidiosum* antigen, and immune stains of patients after treatment [30, 60].

7. Treatment.

The treatment of *P. insidiosum* infection is difficult. However, three therapeutic methods, as surgery, antifungal drug and immunotherapy, were often used with success.

Surgery is a removal of the lesion and their kunkers. This therapeutic method has been highly success in equines and human pythiosis. But surgical debridement of skin lesion in dogs and cats gave the opportunity to recurrent of the disease. Almost surgery treatment will be combined with antifungal drug and immunotherapy [19].

The conventional antifungal drugs were not effective in the treatment because *P. insidiosum* lacked ergosterol in the cytoplasmic membrane. However, some agents such as itraconazole, ketoconazole, fluconazole, interfere ergosterol biosysthesis, and some, for example terbinafine and amphotericin B, affect on permeability of cell membrane, causing cell lysis [1]. In patients with cutaneous/subcutaneous pythiosis were good responsders when were treated with saturated solution of potassium iodide (SSKI) and amphotericin B [17]. In vascular pythiosis, the antifungal had no effective except combination of itraconazole and terbinafine were reported the successful of treatment in 2-year-old child [20].

In the present, immunotherapy is a choice for treatment diseases. Immunotherapy has two types of activities. First, active immunotherapy attempts to stimulate the host's intrinsic immune response to diseases such as cancer and vaccine. Second, suppression immunotherapy dampens an abnormal immune response in autoimmune diseases or reduces a normal immune response to prevent rejection of transplanted organs or cells. In the case of pythiosis, immunotherapy is activation type. Humans and animals pythiosis were treated with *P. insidiosum* antigen which activated the switching of T_H2 to T_H1 response. *P. insidiosum* vaccine has been recently developed by preparing from culture-derived *Pythium* immunogen by Miller *et.al.* in 1981. The efficacy of vaccine treatment was around fifty percent and no 100% in chronic cases [61]. In 2003, Medoza

et.al. represented a new immunotherapeutic immunogen of *P. insidiosum* antigen as PIA containing exoantigen and endo-antigen. This new immunogen gave 100% efficiency to early cases of the disease, showing symptom at least 20 days, but only 20-40% to the chronic cases with more than 2 months old lesion [62]. Furthermore, the efficacy of new immunotherapeutic antigen in other animals was reported. It gave 60% of effectiveness in horse, 97% in cattle and 33% in dogs [18]. Because of high mortality in human pythiosis, *P. insidiosum* antigen was used for treatment. The first successful case of immunotherapy in human was a young boy with terminal pythiosis in artery treated with PIA. After treatment, he had a mild inflammatory response at the site of injection and no side effect. This successful case suggested that immunotherapy may be an effective and safe method for vascular pythiosis treatment [42]. Among 43 patients with vascular pythiosis as final stage, 40% died, whereas 60% were cured the disease but later undergoing amputation and immunotherapy [18]. After vaccination, five cases were cured, two patients died, two patients had persistent infection and three patients were lost follow-up. The duration time of infection had effect on success in treatment [19]. Early diagnosis and treatment were important for successful treatment.

8. Immunology

The knowledge of immune response to *P. insidiosum* in humans and animals are not well understood. However, Mendoza *et.al.,* in 2005, reported the immune response of successful immunotherapy in horses. In the nature, *P. insidiosum* zoospores attached to the injured skin of a host, the encysted zoospore will develop a germ tube to penetrate the host tissue. After infection process, *P. insidiosum* hyphae will secrete exoantigen around their hyphae. The antigens were presented to antigen presenting cells (APC) that release interleukin 4 (IL-4) activated naïve T helper cells (T_H0). The T_H0 cell differentiated into T helper 2 (T_H2). The stimulated T_H2 subset will release IL-4, IL-5 and other T_H2 cytokines. These released cytokines stimulated B cell to produce IgE, IgG and IgM molecule. IgE and IL-5 trigger the mast cells and eosinophils migration to the infected site and degranulation of cells cause tissue damage in the infected host. While PIA injection, the immune system will be activated by antigen presenting of APS to T_H0 that become to T_H1 cell. The T_H1 subset release interferon gramma (IFN- γ) and IL-2 that trigger the cell-mediated immunity (CMI) including cytotoxic lymphocytes (CTL) and macrophages. The cell-mediated immune response can damage the *P. insidiosum* hyphae and B cells are stimulated to produce the protective IgG classes (Figure 15) [18].

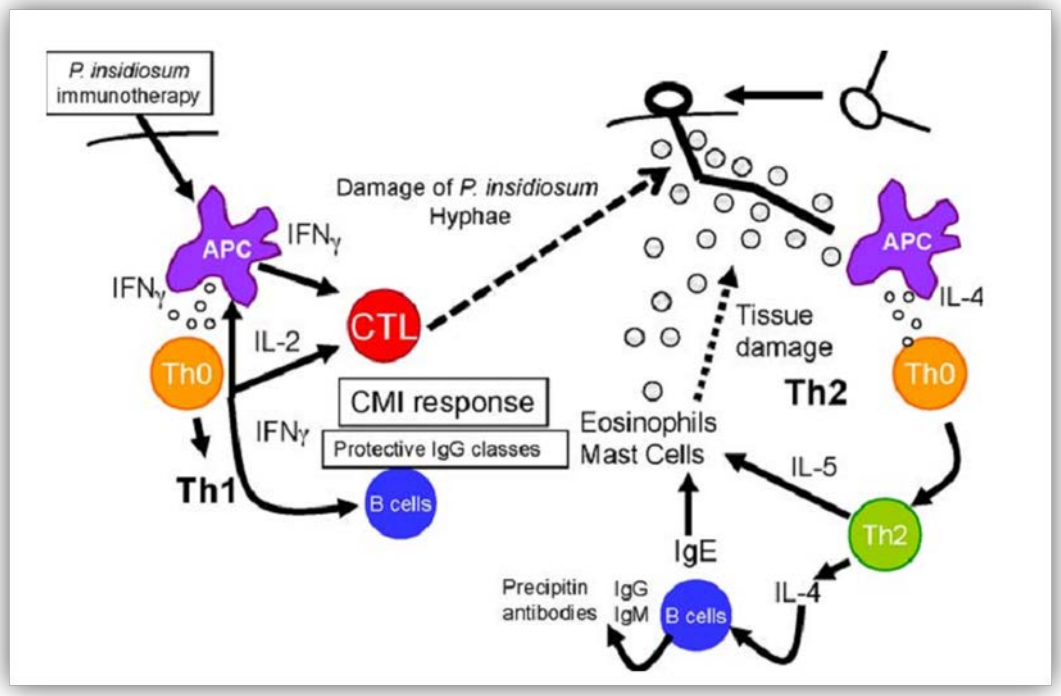

Figure 15 Immune response in horse with immunotherapy to *P. insidiosum* hyphae [18]

In the same way, the pythiosis Thai patients with treated PIA showed good response to immunotherapy. The immunity's pythiosis patients had the switching of immune response from T_H2 to T_H1 response [19, 51]. The immunity of patients is similar to immune response of horse with immunotherapy.

Though, *P. insidiosum* is a parafungal organism but the morphology is hyphae form and their cell wall consists of β-glucan. So the immune response to this organism is like the immunity of fungal

9. Immunity to fungal infection

From the basic knowledge of fungal infection immunity, host defense mechanism against fungus infection includes innate immunity and adaptive immunity.

9.1. Innate immunity

Innate immunity is the first line of defense mechanism during the critical period after the exposure of host with pathogen. Almost, the surface of immune cells, including neutrophils, monocytes, macrophages and natural killer (NK) cells, have a set of pattern recognition receptor (PRRs) which recognize invariant molecular structure of fungal as pathogen associated molecular pattern (PAMPs) such as Toll-like receptors (TLRs). PAMP-PRR interaction leads to the activation of antifungal effectors functions in phagocytes and production of pro-inflammatory cytokines by dendritic cell [24].

Antifungal effectors activities consist of enhance phagocytosis and growth inhibition in dimorphism. The growth inhibition contains defensins, neutrophil cationic peptide and enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The NADPH induced nitric oxidase synthase or the oxidative pathways as known as respiratory burst [63]. The respiratory burst will produce reactive oxygen intermediated (ROIs) as toxic that damages fungal by increasing oxidative killing in phagocytes [25].

Toll-like receptors are a set of conserved cell receptors that mediated cell-signaling systems. Activation of TLRs induces inflammation during the infection and specific expression of gene as myeloid differentiation primary response gene (MYD88) in innate cells. Example, the signaling of TLR2 with zymosan occur together with the β-glucan receptor as known as dectin-1 leads to secretion of inflammatory cytokine such as tumor-necrosis factor (TNF) and

IL-1 β (Figure 16). Furthermore, PAMP-PRR interaction mediates a complex cascade of intracellular signaling that stimulate to release of interleukin 12 (IL-12) and interleukin 23 (IL-23). The IL-12 and IL-23 activate naïve T cell differentiation into antigen-specific CD4+ T helper (T_H cell) or CD8+ T cell (Cytotoxic T cell; CTL) and adaptive immunity expression (Figure 17) [23, 25].

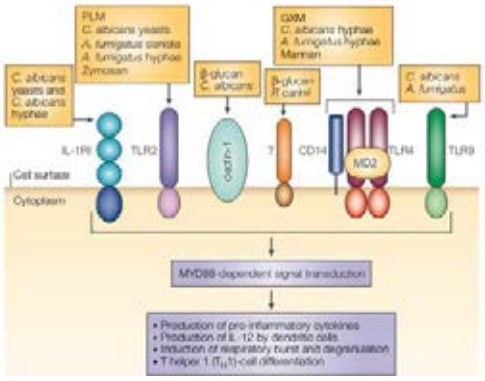

Figure 16 Role of TLR in innate immunity and adaptive immunity against fungal [23]

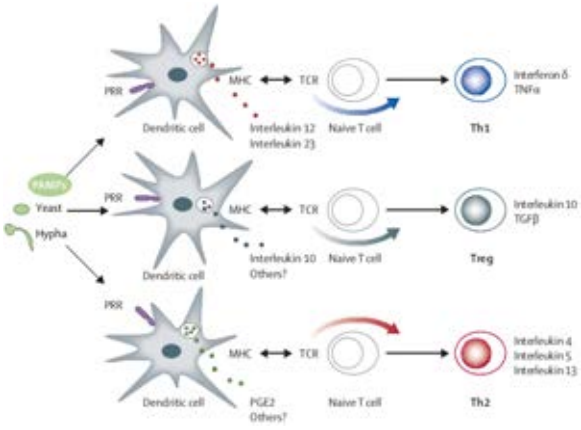

Figure 17 Role of pro-inflammatory cytokines from dendritic cells induced adaptive immunity [23-25]

9.2.Adaptive immunity

Adaptive immunity displays high degree of specificity to immune response in fungal infections. The major functions include:

- The recognition of specific “non-self” antigen by antigen presentation process.
- The development of immunological memory by specific antibody and the memory cells.

The cells in adaptive immunity are type of leukocyte called lymphocyte B cells and lymphocyte T cells. The both are produced from bone marrow. The lymphocyte B cells play a role in the humoral immune response while T cells involve the cell-mediated immune response [25, 26].

9.2.1. Cell mediated immunity

Lymphocyte T cells from bone marrow will migrate to the thymus for maturation. Mature T lymphocytes have cell surface antigen-binding molecule called T cell receptor (TCR). The TCRs recognize a complex ligand including the antigenic peptide bound to a major histocompatibility complex (MHC) and co-stimulatory ligand. Two majors form of MHC are MHC class I and MHC class II which both class interact with different co-receptor on the T cell as CD8 and CD4 respectively. Mature T lymphocytes express either CD4 or CD8 molecules can be identified T helper cell and cytotoxic T cell, respectively [24, 25].

a) Cytotoxic T cell (CD8+)

The cytotoxic T cells have three distinct pathways. Two involve direct cell-cell contacts of CTL and target cells and the third is mediated by cytokines such as IFN- γ and tumor necrosis factor- α (TNF- α). Tumor necrosis factor- α bind tumor necrosis factor receptor on target cell and induce the caspase cascade resulting the apoptosis (Figure 18A).

In one case, the interferon gramma (IFN- γ) induces the antigen presentation pathway of MHC class I and CD95 as Fas receptor on target cell. The binding of Fas ligand with CD95 trigger apoptosis of target cells though the classical caspase cascade (Figure 18B).

In the other case, cytotoxic T cell produce and release perforin and granzyme into target cells. The CTLs have devised the elaborate mechanism to protect neighboring cells from killing (Figure 18C) [23].

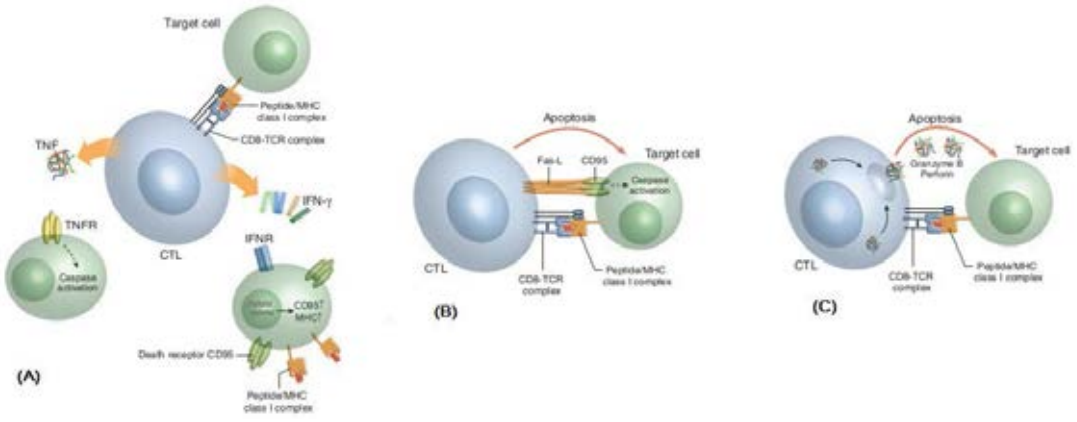

Figure 18 Killing of CTL-mediated cytotoxicity. (A) Indirect killing of target cell by tumor necrosis factor- α . (B) Induction of apoptosis via Fas ligand (C) Direct killing by cytotoxic protein [23]

b) T helper cell (CD4+)

The naïve T cells are activated by presentation of antigen via MHC class II. The naïve T cells are differentiate become T helper cell (T_H). The response of T helper cell is defined by two distinct pathways involving two different subtypes of T helper cell; T_H1 and T_H2 cells which depend on a type of cytokines. The naïve T cells (T_H0) were induced by IL-12 which differentiates toward T helper 1 (T_H1). The targets of T_H1 cell are intracellular pathogens such as bacteria and parasites. T_H1 cells secrete interferon gamma (INF-γ) which activate killing of macrophages by opsoniztion. Furthermore, T_H1 cytokines promote to cell mediated immunity and proliferation of cytotoxic T cells.

Whereas, T_H2 cells release IL-4 which promote humoral immunity as stimulate B cells proliferation, induce B-cell antibody class switching and increase antibody neutralization. Furthermore, IL-10 from T_H2 cells inhibits a variety of cytokine including IL-2 and INF-γ in T_H1 and IL-12 in dendritic cell and macrophages. T helper cell activation leads to the induction of a number of pathways that can result in B-cell antibody production and immunoglobulin class switching [27], and macrophage action via both direct interaction and through the release of soluble factors [25].

9.2.2. Humoral immunity

The humoral immunity is called the antibody-mediated system which the major of cell is B lymphocytes. The initial stage is antigen presentation of macrophages to T helper cells. Interleukin-4 from activated T helper cell as T_H2 activates B cell proliferation and differentiation become toward memory B cells or plasma cells. Memory B cells are long-lived and stimulated B lymphocytes that are primed for rapid response to repeated exposure antigen. While plasma cells produce antibodies called immunoglobulins that will be specific bind with the antigen. The antibody binding help to immune cell such as macrophages for clearance pathogen by enhance phagocytosis and the classical complement pathway activation. Plasma cells produce a various form of antibodies which are divided five classes as IgM, IgA, IgE, IgD and IgG [27, 28].

a) Immunoglobulin M

IgM is the first antibody to appear in the initial immune response to antigen. They are found in the human circulatory system and are particularly effective at complement activation.

b) Immunoglobulin A

IgA plays a critical role in mucosal immunity which is produced in mucosal epithelial cells. IgA prevent colonization of pathogen.

c) Immunoglobulin E

The main function is immunity to parasites such as parasitic worms and activate hypersensitivity type I which manifests various allergic diseases such as allergic asthma, allergic rhinitis and food allergy.

d) Immunoglobulin D

IgD is found to bind to basophils and mast cells which are activated antimicrobial factors production in respiratory immune defense in human.

e) Immunoglobulin G

The human IgG antibodies are glycoproteins composed of two heavy chains and two light chains linked together by interchain disulfide bond as known as hinge region. The light chains have two types which are referred to as lambda (λ) and kappa (κ) chains. Differences in the amino acid content of heavy chains and the ratio of κ to λ in light chain are characteristic of the different subclasses of IgG. Furthermore, the hinge region is the most diverse structure feature of the different in IgG subclass. The IgG antibodies are mostly found in blood and extracellular fluid which compose of four different subtypes of IgG molecules called the IgG subclasses consisting IgG_1, IgG_2, IgG_3 and IgG_4. The concentration of each IgG subclass in serum of individuals depends on the number of plasma cells, the rate of systhesis and catabolism. In adults have the highest concentration of IgG_1, followed by IgG_2, IgG_3 and IgG_4. The IgG subclass has two major effector functions: complement activation and opsonization. These functions are mediated via the interaction of antibody with antigen. Four IgG subclasses differ from each other with respect to the functions which were showed in Figure 19 [28].

Properties	IgG_1	IgG_2	IgG_3	IgG_4
Human myeloma protein frequency (%)	60-70	14-20	4-8	2-6
Proportion of total IgG in normal adult serum (%) (54)	60.3-71.5	19.4-31.0	5.0-8.4	0.7-4.2
Average serum concentration (mg/ml)	8	4	0.8	0.4
Range in normal serum (mg/ml) (2)	5-12	2-6	0.5-1	0.2-1
Subclass distribution on circulating B cells by immunofluorescence (%) (270)	40	48	8	1
Subclass distribution on IgG plasma cells in bone marrow, spleen, tonsil (%) (270)	64	26	8	1
Half-life (days)	21-23	20-23	7-8	21-23
Transport across the placenta	++	+	++	++
Complement fixation (classical pathway)	++	+	++	0
Antibody response to:				
Proteins	++	+/–	++	+/–
Polysaccharides	+	++	–	–
Allergens	+	–	–	++
Cytophilic properties*				
Human monocytes (FcR-I, II & Io)	++	+	++	+/–
Human neutrophils (FcR-II)	++	+/–	++	+
Human platelets (FcR-II)	++	+	++	+
Human lymphocytes (FcR-II or Io)	++	+/–	++	+

Figure 19 Biological Properties of the human IgG subclasses [28]

The difference of their function is related to structure of immunoglobulin. The hinge region of IgG subclasses affect to the ability of complement activation. In initial of complement activation is binding of C1q to C_H2 domain of IgG. The capacity of the four human IgG subclasses to bind C1q increase in the order: $IgG_4 < IgG_2 < IgG_1 < IgG_3$. IgG_4 does not activate complement fixation whereas IgG_3 is the most effective complement fixation because IgG_3 have the longest hinge region than the other IgG subclasses.

In the other function of antibody is the activation of phagocytosis which is stimulated by the interaction of Fc receptor and Fc fragment of immunoglobulin. The Fc receptor for IgG (Fc γ R) is divide three form as Fc γ RI, Fc γ RII and Fc γ RIII which are differentially expressed on leukocyte subsets. Fc γ RI can be found in macrophages, monocytes, myeloid cells and dendritic cells which have a high affinity with IgG_1 and IgG_3. The affinity of receptor for IgG_4 is very low and not binds IgG_2.

Fc γ RII is a low-affinity receptor which is expressed on Langerhans cells, dendritic cells and platelets. Fc γ RII bind IgG_1 and IgG_3 but not bind IgG_2 and IgG_4. Fc γ RIII has two isoforms as Fc γ RIIIa and Fc γ RIIIb which the both are able to bind IgG_1 and IgG_3. Fc γ RIIIa can be found on macrophages, monocytes and natural killer cells whereas Fc γ RIIIb is expressed on neutrophils.

In the nature, T helper cells stimulate and regulate most isotype of IgG subclasses which involve the interaction of T cell ligand (CD40L) with the molecule on B cell surface as CD40. The cytokines from T_H1 and T_H2 stimulate the switching of IgG subclass. Interleukin 4 (IL-4) from T_H2 is able to induce switching to IgE and IgG_4 in human B cells whereas interferon gamma (INF- γ) from T_H1 subsets induce to switching to IgG_1 and IgG_3 in human (Figure 20) [27].

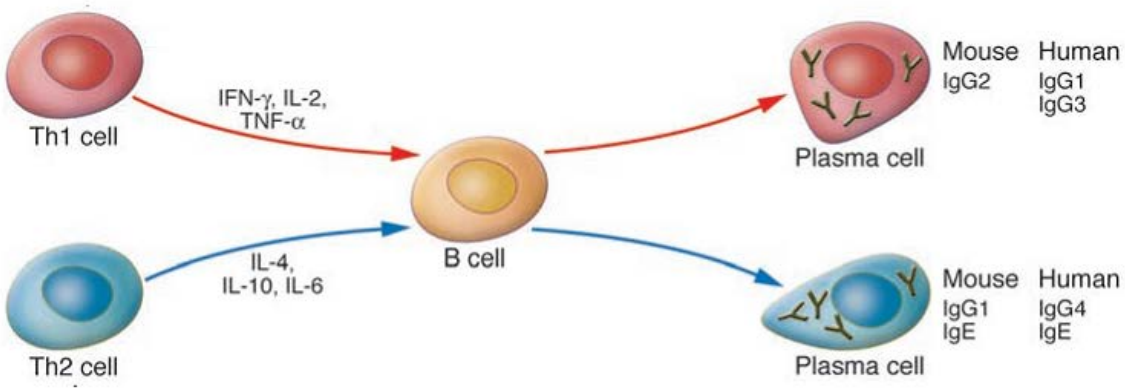

Figure 20 Activation of immunoglobulin switching in B cell by cytokines from T helper cells [64]

Furthermore, each IgG subclass plays role different functions in protecting the body against infection. For example, the IgG_1 and IgG_3 subclasses are rich in antibodies against proteins such as the toxins produced by the diphtheria and tetanus bacteria. However, these studies found that the type or concentration of IgG subclasses can indicate immune response of body to infection.

CHATER IV

MATERIALS AND METHODS

1. Specimen Collection

Sera from thirteen vascular pythiosis patients (Table 1) treated with immunotherapy were recruited in this study. These groups were treated not only with PIA vaccine but also in combination with amputation and antifungal drugs (Table 2). All these patients should reach these criteria:

1. Confirmation by both *P. insidiosum* organism detection and serological method (s)
    a. The detection method: isolation with classical method or histopathology, or molecular tool using PCR technique
    b. Serological method: western blot analysis, or ELISA, immunodiffusion test.
2. PIA treatment: 2 mg/ml of PIA were applied to treat patients every 1 or 2 weeks via subcutaneous route.

Their sera were collected before treated with PIA-immunotherapy and after injection in the period of 0 – 96 weeks (wk) At least the PIA-treated serum should be collected in three periods, 1-3, 4-7, and 8-96 wk. Ten sera from healthy volunteers were used as experimental control. These sera samples were stored in -20^{o}C in Mycology unit, Faculty of Medicine, Chulalongkorn University.

Any patients who did not match these criteria were excluded from this study. Regarding their clinical outcome (s) which observed by the physicians, the patients were divided into 2 groups, good responding and non-responding. The responding group was

composed of patients whom their clinical symptom did not progress whereas the non-responding group was composed of the expired patients or the patients whom their symptom were relapsed. Their demography and clinical information were summarized in Table 2.

(This project had been approved by IRB No. 133/54 at date on June 21, 2011)

Table 1 List of collected serum from 13 vascular pythiosis treated with PIA immunotherapy in 0-96 week (wk) period, of the abbreviation of PV represented pre-vaccination and the number after PV represented the time that the patient was injected

Patient Code.	0 wk	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk	8-24 wk	25-48 wk	49-72 wk	73-96 wk
No.1 (PY5)	PV1			PV4			PV7					
No.2 (PY7)	PV1		PV3						PV9 (10 wk)			
No.3 (PY8)	PV1	PV2			PV5							
No.4 (PY12)	PV1		PV3		PV5							
No.5 (PY15)	PV1	PV2						PV8				
No.6 (PY22)	PV1				PV5				PV9 (8 wk)			
No.7 (PY27)	PV1			PV4				PV8				
No.8 (PY30)	PV1			PV4							PV11 (72 wk)	**PV12 (96 wk)**
No.9 (PY31)	PV1				PV5				PV9 (12 wk)			
No.10 (PY49)	PV1		PV3							PV10 (26 wk)		
No.11 (PY50)	PV1		PV3			PV6						
No.12 (PY51)	PV1		PV3						PV9 (24 wk)	PV10 (44wk)		
No.13 (PY62)	PV1		PV3							PV10 (32 wk)		

Table 2 Summary of the clinical data of 13 vascular pythiosis patients included in this study

Patients Code (Sex/Age)	Vaccine schedule	Underlying disease	Pathological feature	Diagnosis	Treatment	Clinical status
No.1 (PY5; M/38)**	D 0; 100ul. ID D 7, D14, D21, D35; 100ul. SC	Thalassemia	Acute arterial occlusion Lt. foot	Western blot	Itraconazole,terbinafine immunotherapy	Stable (14 mo ,May. 55)
No.2 (PY7; M/49)*	D 0; 100ul. ID D 14, D44; 100ul. SC	Thalassemia	Rt. Leg chronic arterial occlusion	Western blot & ID	AK amputation© immunotherapy	Relapse (2 mo, Jan. 49)
No.3 (PY8; F/38)*	D 0; 100ul. ID D 7, D 14, D24; 100ul. SC	Thalassemia	chronic arterial occlusion	Western blot	Itraconazole, terbinafine AK amputation immunotherapy	Death (1 mo, Nov.48)
No.4 (PY12; M/44)*	D 0; 100ul. ID D 14, D 28; 100ul. SC	PNH	chronic ulcer & arterial occlusion	Isolation & ID	AK amputation immunotherapy	Death (1 mo, Oct.48)
No.5 (PY15; F/74)***	D 0; 100ul. ID D 7, D14, D21, D28, D44, D51; 100ul. SC	ND	chronic arterial occlusion	Western blot & PCR	AK amputation, immunotherapy	Stable (2mo, Jan 49)
No.6 (PY22; M/44)***	D 0; 100ul. ID D12, D26, D44, D51; 100ul. SC	Thalassemia	chronic arterial occlusion	Western blot & histopathology	Immunotherapy	Stable (34 mo, Mar. 51)
No.7 (PY27; M/26)**	D 0; 100ul. ID D7, D23, D37, D51; 100ul. SC	Thalassemia	occlusion of right popliteal artery	Isolation & western blot	Itraconazole, terbinafine, SSKI AK amputation, immunotherapy	Stable (2 mo, Nov. 49)

Patients Code (Sex/Age)	Vaccine schedule	Underlying disease	Pathological feature	Diagnosis	Treatment	Clinical status
No.8 (PY30; M/35)***	D 0; 1ml. ID D7, D14, D21, D111;1ml. SC	Thalassemia	Right and left posterial tibial artery occlusion	ID & ICT	Itraconazole, terbinafine AK amputation, immunotherapy	Stable (24 mo, Feb.55)
No.9 (PY31; M/31)**	D 0; 1ml. ID D7, D21, D28, D35, D42, D49, D56, D63; 1ml. SC	Thalassemia	chronic arterial occlusion	Histopathology & western blot	Itraconazole,terbinafine, SSKI AK amputation,immnotherapy	Stable (4 mo, Dec.52)
No.10(PY49; M/32)**	D 0; 1ml. ID D7, D14, D194; 1ml. SC	Thalassemia	chronic Lt. leg ulcer	Western blot & ELISA	AK amputation, immnotherapy	Stable (7 mo, May 54)
No.11 (PY50; M/54)**	D 0; 1ml. ID D15, D36; 1ml. SC	Thalassemia	chronic arterial occlusion	ELISA	AK amputation, immnotherapy	Stable (2 mo, July 54)
No.12 (PY51; M/42)***	D 0; 1ml. ID D7, D14, D174, D188; 1ml. SC	Thalassemia	Occlusion with intraluminal thombus in right popliteal artery	ELISA	AK amputation, immnotherapy	Stable (18 mo, April 55)
No.13 (PY62; F/52)**	D 0; 1ml. ID D7, D21, D231, D411; 1ml. SC	Thalassemia	Artery margin	Western blot & ELISA	Itraconazole, terbinafine AK amputation, immunotherapy	Stable (8 mo, Sep.55)

© AK amputation: Above knee amputation

*** Long-term response group (≥14 month), ** Short-term response group (< 14 month) and * Non-response group

2. Quantitative Determination of total IgG and IgG Subclass :

To examine the immune response of the PIA-treated vascular pythiosis patients, total IgG and IgG isotypes were focused using direct ELISA technique. The semi-quantitative of IgG level in individual serum from different period of PIA injection was determined and their values from were compared in individual.

To investigate antigen from which clade will be the well immunogen for Thai patients, all the IgGs levels were determined against crude endogenous antigen of *P. insidosum* from three clades, $A_{TH,}$ B_{TH}, and $C_{TH,}$ which were grouped based the nucleotide sequences of ITSs and *COX* II [10, 11]. Actually, as described previously, the PIA vaccine used in the human pythiosis was prepared from *P. insidiosum*, clade A_{TH}. Whereas the etiologic agents were isolated from Thai patients was classified in Clade B_{TH} and $C_{TH,}$ merely.

2.1. Crude endogenous antigen of *P. insidiosum* preparation [29]

One *P. insidiosum* strain from each of three clades was used in this study. There were MTPI19 (ATCC58643) from clade A_{TH}, PMS, and PC from clade B_{TH} and C_{TH}, respectively (Table 3). MTPI19 strain was classified in clade A_{TH} and was isolated from bovine pythiosis Costa Rica. This strain was used for PIA vaccine preparation whereas PMS and PC strains were isolated from Thai vascular pythiosis patients.

To prepare the vaccine, two pieces of three millimeters diameter, approximately, of log phase *P. insidiosum* on SDA were inoculated into flask containing 250 ml. SDB and shaked at 150 rpm. (Forma orbital shaker, Thermo Fisher Scientific Inc.), at 37 ^{o}C for 14 days. After incubation, the culture was killed by adding 0.02% merthiolate and was filtered to separate the hyphae. These dry hyphae were grounded in liquid nitrogen. Then, the obtained powder was suspended in 1XPBS pH 7.2 and centrifuged at 14,000 x

g 4°C for 15 min. The supernatants were aliquoted, put into new tube and kept in -80°C until used.

To determine the concentration of crude antigens, the standard curve using bovine serum albumin (BSA) was prepared and the protein concentration was measured by Pierce® BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA.). The measuring method was followed the protocol of this assay kit. The BSA concentration was varied from 0.025, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml. Their optical density (OD) values were measured by spectrophotometer at the $\lambda_{570\ nm}$. After the standard curved was made, ten-fold dilutions of crude proteins were prepared and were measured with the same method as using in performing BSA standard curve. The protein concentrations were determined by comparing the BSA concentration from BSA standard curve which plotted between ODs and their concentrations.

Table 3 Geographical origins and sources of *P. insidiosum* strains in this study

Code No.	Host	Pathology	Geographical origin	Strain in other collection
C-MTPI19 (A_{TH})	Horse	Abdominal ulcer	Costa Rica	ATCC58643
C-PMS (B_{TH})	Human	Arterial pythiosis	Thailand	℗ ND
C-PC (C_{TH})	Human	Arterial pythiosis	Thailand	ND

ATCC58643 = CBS 574-85

℗ ND : No data

2.2. Semi-quantitative determination of IgG, IgG_1 and IgG_4 antibody by direct ELISA [55, 56]

2.2.1. Antigen titration

To determine the optimum crude antigen concentration against total IgG antibody, the amount of 100 µl. of each antigen ranged from 0.39, 0.78, 1.56 and 3.125 ng/well was coated in each well of 96-well plate (Cat. No.442404 NUNC immuno plate, Denmark) and incubated at 37°C for 1 h. The coated plate was washed five times with phosphate buffered saline with 1% (v/v) tween 20 (PBS-T) and then was blocked with 2% skim milk at 37°C for 1 h. Pooled serum from 10 vascular pythiosis patients were diluted to 1:400, 1:800 and 1:1,600 and another 1:400 of pooled sera from 10 volunteer healthy was prepared as negative control. The amount of 100 µl from each dilution of pooled pythiosis sera and pooled healthy sere was added and then incubated at 37 °C for 1 h. Five times washing with PBS-T were performed again. Then, 100 µl. of 1:2,000 diluted horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G in PBS-T was added and then incubated at 37°C for 1 h. The wells were washed five times again with PBS-T. To detect the reaction, freshly prepared substrate, *O*-phenylenediamine dihydrochloride (OPD) in citric acid with 30% H_2O_2, was added to each well. Twenty minutes after that, the reaction was stopped by 100 µl of 4N H_2SO_4. The OD was measured by spectrophotometer (ELISA plate reader) at 490 nm wavelength. The optimum protein for total IgG antibody assay was derived from the reaction which had OD less than 0.8.

To determine the amount of IgG_1 antibody in pythiosis patients' serum, the optimum of antigen to coat the plate was tittered. The concentration of crude antigen ranged from 2, 4 and 8 ng/well was used to coat the well. The coated plates were washed five times with PBS-T and then were blocked with 2% skim milk at 37°C for 1 h. The dilution of 1:100, 1:200, and 1:400 pooled pythiosis serum and the dilution of 1:200

pooled healthy sera were prepared. The amount of 100 µl of each dilution was added in the well and incubated at 37°C for 1 h. Five times washing with PBS-T were performed again. Then, 100 µl of 1:5,000 diluted mouse anti-human IgG_1 conjugated with biotin was added and incubated at 37°C 1 h. After washing, 100 µl of 1:4000 diluted streptavidin conjugated horseradish peroxidase was added and incubated at 37°C for 1 h. The detection method and the optimum concentration of antigen were the same as described above.

The protocol of the IgG_4 subclass study was similar to that of IgG_1 subclass except the concentration of crude antigen and the dilution of all used sera in the IgG_4 subclass study were difference. The crude antigen ranged from 25, 50 and 75 ng/well for IgG_4 was prepared. The dilution of 1:25, 1:50 and 1:100 of pooled pythiosis serum and 1:50 dilution of pooled healthy sera were used. The amount of 100 µl, 1:4,000 mouse anti-human IgG_4 conjugated with biotin were prepared. The detection method and the optimum concentration of antigen were the same as described above.

2.2.2. Antibody titration

The same ELISA technique was performed. In this experiment, the optimum concentration of antigen was used to titrate the dilution of antibody. Two-fold dilution from 1:200 -1:1,600 of pooled pythiosis serum were prepared for the IgG antibody assay. For the specific IgG_1 and IgG_4 antibody determination, pooled pythiosis serum was diluted at 1:100, 1:200, and 1:400; and 1:25, 1:50, 1:100, and 1:200, respectively. For the experimental control, the pooled volunteer healthy serum was prepared at dilution 1:1400 -1:1,600 for IgG antibody assay. Then, the IgG_1 and IgG_4 antibody determination, the pooled healthy serum was diluted at 1:100, 1:200 and 1:400; and 1:50, 1:100 and 1:200, in order. The criterion of the cut-off value for pythiosis differentiate was 0.2 of the OD value from pooled volunteer healthy serum as negative control.

2.2.3. Secondary antibody titration

For the total IgG antibody determination, anti-human IgG conjugated with horseradish peroxidase (Cat. No.P0214 Dako, Denmark) was used as secondary antibody. The optimization of this antibody was determined by using 2 ng/well crude antigen and the dilution at 1:400, 1:800, 1:1,600 pool pythiosis serum which were the values derived from the results of the previous experiments. All the ELISA condition was the same as explained above. The optimization of secondary antibody for IgG_1 and IgG_4 (Cat. No. ab99775 and 99818 abcam, UK) was performed as described in materials and methods (2.2.1) but the concentration of crude antigen and dilution were difference. The optimal concentration of crude antigens for IgG_1 and IgG_4 was 8 and 50 ng/well, respectively. Pool serum for IgG_1 assay was diluted two-fold, ranged from 1:100 – 1:400 whereas for IgG_4 assay, two-fold dilution of the same serum was prepared from 1:25-1:100. To determine the optimum of the secondary antibody for this study, the dilutions of 1:2,000, 1:5,000 and 1:10,000 were prepared. The optimum dilution of the conjugated

antibody was selected from the well which showed the OD value ranged between 0.2-0.8.

2.2.4. Standard curve of IgG and IgG subclass protein

It is hard to obtain the quantitative amount of IgGs against specific *P. insidiosum* due to it has the limitation of the technique and their small amount. Here, the indirect method was performed using the equation which derived from standard curve plotting between IgGs protein and their OD values.

To prepare IgGs protein standard curve, direct ELISA using IgGs protein against its specific antibody was performed. The concentrations of each IgGs protein were 10, 50, 100, 150, 200 ng/ml. In the detection step, the dilution of secondary antibody for IgG experiment was 1:5,000 whereas for IgG_1 and IgG_4 tests were diluted at 1:5,000 and 1:4,000, respectively. The standard curve of each IgGs protein was plotted between the OD value and the protein concentration.

2.2.5. Quantitative determination of IgG, IgG_1 and IgG_4 antibody against crude proteins using ELISA

To examine the quantitative amount of IgG, IgG_1 and IgG_4 antibody in pythiosis patient serum against crude proteins, OD value at the obtained titer from ELISA was calculated by using the equation, deriving from the IgGs protein standard curve. First, the crude antigen for IgG, IgG_1 and IgG_4 at the concentration of 2, 8 and 50 ng/well, respectively, was coated in each well. Two-fold dilution of each serum for IgG and IgG_1 antibody assay was prepared from 1:400 – 1:1,600 whereas for IgG_4 analysis was from 1:50 – 1:200. The detection step was performed as described previously. In short, the concentration of IgGs antibody was estimated by the standard IgGs protein equation that plotted between the protein concentrations against the OD value.

Linear equation from IgGs protein standard **y = mx + c**

When y = the OD value, m = slope, c = constant term

X= the concentration of IgGs protein (ng/ml)

Thus, the antibody concentration (ug/ml) = $(\frac{y-c}{m}) \times$ dilution factor $\times 10^{-3}$

3. Antigenic pattern of total IgG and IgG subclass:

The direct ELISA as explained above could estimate the quantitative amount of *P. insidiosum* specific IgGs antibody but its limitation was the incapability to demonstrate the recognized antigens. To show the specific antigenic pattern, thus, the western blot assay was performed.

3.1. Crude endogenous protein pattern of *P. insidiosum* by SDS-PAGE

To study the immune response of vascular pythiosis patients with treated immunotherapy, the crude protein of *P. insidiosum* strain MTPI19 (ATCC58643), (C-MTPI19), was prepared as described in materials and methods (2.1). Up This strain has been classified in clade A_{TH} and is the strain used for present PIA vaccine preparation.

The 12.5% acrylamide short SDS-PAGE gel was prepared (Molecular Biology of John Wiley, appendix A). Protein sample was prepared at final concentration 15 ug (containing 4 µl of 15 µg protein in phosphate buffer solution (PBS) and 1 µl of 5x sample buffer) [29]. The protein solution was boiled at 100°C for 5 min and centrifuged at the 14,000 rpm (16,000 xg) at 10°C for 5 min. (Kubota 3740, Kubota corporation; Japan). Then, the supernatant of the protein solution with 4 µl prestained marker (PageRuler™ Prestained Protein Ladder #SM0671, Fermentas) as standard marker was loaded into each well. The proteins were separated by miniVE vertical electrophoresis system (Hoefer®, Inc., USA) at 110 volt. The separating gel was stained by coomassie brilliant blue solution.

3.2 Antigenic pattern of IgG isotypes against crude antigen of *P. insidiosum* by Western blot analysis [29]

3.2.1 Western blot preparation

After the protein separation by SDS-PAGE gel as explained above, one gel was stained and another gel with the same condition was blotted. To obtain the clear antigenic pattern for analysis, the loading crude proteins were decreased from 15 ug/well to 1 µg/well for total IgG, IgG_1 pattern experiment and 10 µg/well for IgG_4 pattern experiment, respectively. The proteins were separated by condition: 110 volt for 3 h. And then, the separated proteins were transferred from SDS-PAGE gel to PVDF membrane (Cat. No. IPVH00010, Immobilon®-P) by miniVE vertical electrophoresis system at 20 volt for 3.30 h. on ice. The membranes were kept at -20^{o}C until used.

3.2.2 Antigenic pattern of *P. insidiosum* strains using western blot

After the transferred membranes preparation derived from material and method (3.2.1), the optimal condition for western blot was required to be tested. The transferred membrane was reacted with twelve pre-vaccination sera from vascular pythiosis patients and ten volunteer healthy sera as experiment control by western blot analysis. To decrease the non-specificity, the membranes were blocked by 10% skim milk in 1xTris-buffered saline with 0.1% tween20 (1xTBS-T) and incubated 37^{o}C for 1 h. The range of serum dilution in IgG_1 and IgG_4 experiments were referred from antibody concentration from the above ELISA experiment (2.2.5). Pre-vaccination serum was diluted in 5% skim milk at 1:4,000 for IgG experiment, 1:500 or 1:1,000 for IgG_1 experiment and 1:25 or 1:50 for IgG_4 experiment. While healthy sera were 2-fold diluted from 1:4,000-1:8,000 for IgG testing, 1:500-1:2,000 for IgG_1 experiment and 1:50-1:1,600 for IgG_4 experiment. After added the serum to the membranes, the membranes were incubated at 37^{o}C for 1 h. Then, the membranes were washed three times with 1xTBS-T.

Then, the dilution of 1:5,000 anti - human IgG (IgG_1 or IgG_4) conjugated with biotin were added and incubated at 37°C for 1 h. Three times washing with 1XTBS-T were performed again. After washing step, streptavidin conjugated alkaline phosphatase that was diluted at 1:1,000 in 1xTBS-T, was added and incubated at 37°C for 1 h. The detection step, 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride (BCIP/NBT) substrate solution was added and kept in dark at room temperature (RT), for 5 min. The reaction was stopped by Distilled water (DW).

3.2.3 The common antigen of *P. insidiosum* strain ATCC58643

From the previous study (materials and methods 3.1 and 3.2.2), the crude protein prepared from *P. insidiosum* ATCC58643 provided the best resolution for both SDS-PAGE and western blot analyses. To investigate the common antigens which could be recognized the specific *P. insidiosum* antibody, twelve sera from vascular pythiosis patients and ten sera from volunteer healthy as negative control were used for this experiment. The western blot protocol was the same as above (3.2.2).

3.2.4. Antigenic pattern recognized by IgGs isotype in PIA-treated patients

To determine the antigenic pattern recognized the IgGs isotype against PIA immunotherapy, the patients sera from the different periods after PIA treatment (Table 1) were recruited. The procedure of western blot was performed as previous described.

CHAPTER V

RESULTS

1. History and demography.

Thirteen sera from vascular pythiosis were used for this study. All patients had red blood cell disorder: eleven of patients with thalassemia, one of patients with aplastic anemia/paroxysmal nocturnal hemoglobinuria (PNH) syndrome; and data not shown in the other case. The PIA injection in these patients followed regimen procedure for the treatment of potential fatal diseases. Ten patients had dramatic and complete remission. Their clinical or radiographic evidence of disease progression were not observed. Two patients as PY8 and PY12 died after their third PIA injections. While patient number PY7 was re-admitted and lost follow-up. Based on their clinical magnification, the patients were divided into 2 groups, response group and non-response group (Table 2). The response group was divided into 2 sub-groups: short-term ($<$14 month) and long-term ($\geq$ 14 month).

- Response groups
    - Short term ($<$14 month) : PY15, PY27, PY31, PY49, PY50 and PY62
    - Long term ($\geq$ 14 -34month) : PY5, PY22, PY30 and PY51
- Non-response groups (Progression or relapse) : PY7, PY8 and PY12

2. Quantitative Determination of total IgG and IgG subclass :

2.1 *P. insidiosum* crude antigen titration

Bovine serum albumin (BSA) was used as standard protein for measuring protein concentration. The set up standard curve was prepared as explained in material and method (2.1). The optimal density (OD) was shown in Table 4 and standard curve of BSA was shown in Figure 21.

Table 4 Showing Optical density (OD) value at $\lambda_{570\ nm}$ of bovine serum albumin as standard curve

BSA protein (µg/ml)	Mean of optimal density (OD) value at $\lambda_{570\ nm}$
0	0.108
25	0.151
125	0.261
250	0.417
500	0.713
750	0.920
1,000	1.243
1,500	1.650
2,000	2.104

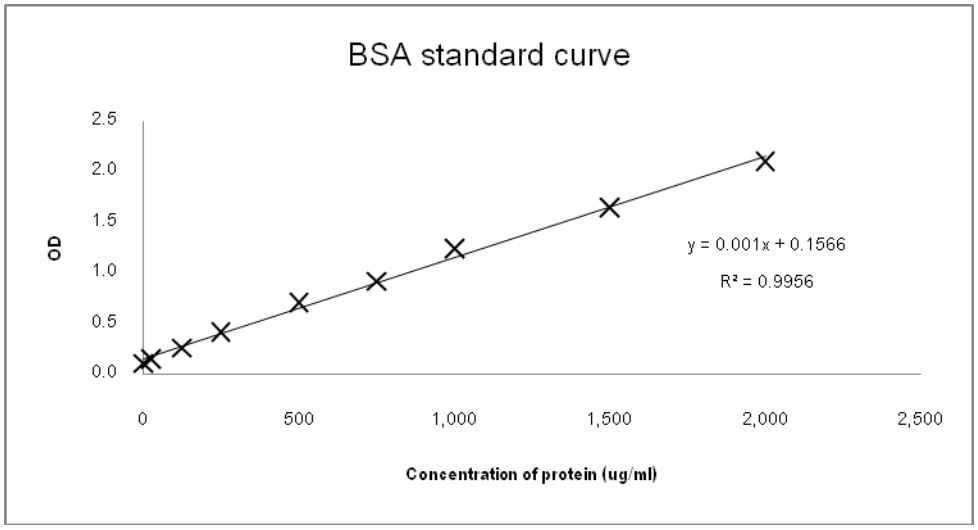

Figure 21 BSA standard curve at OD $\lambda_{570\ nm}$ for protein assay

The endogenous crude proteins of *P. insidiosum* were used in this study. Their concentrations were determined from the equation, derived from the regression line, $R^2 = 0.9956$, of standard curve (Figure 21). The results of the mean OD value at dilution 1:10, from quadruplicate experiment, were shown in Table 5.

Table 5 The concentration of crude protein, C-MTPI19, C-PMS, and C-PC and their optical density (OD) value at $\lambda_{570\ nm}$

Crude Protein	Mean OD at $\lambda_{570\ nm}$.	Final protein concentration (µg/µl)
C-MTPI19	0.521	3.644
C-PMS	0.364	2.074
C-PC	0.286	1.294

2.2 Quantitative Determination of total IgG and IgG subclass

2.2.1 Antigen titration

To assay the quantitative of IgG and IgG subclass antibody, the optimum antigen concentration was titrated first. In the total IgG assay, four concentrations, 0.39, 0.78, 1.56, and 3.125 ng/well crude proteins against three dilutions, 1:400, 1:800, 1:1,600, pooled pythiosis sera and against 1:400 pooled volunteer healthy, were tested. The result showed that 2 ng/well of all three crude antigens was the optimum concentration based on their OD value (0.2-0.8). The OD values obtained from dilution of 1:400, 1:800 of pooled pythiosis in all three crude antigens were two times, at least higher than that from pooled healthy serum. The OD value derived from the reactions in all three crude (Figure 22A, B, & C).

For IgG_1 antibody assay, the optimum concentration of crude antigen was also performed with the same protocol as the total IgG study. This experiment, the concentration of 2, 4, and 8 ng/well crude proteins from all three strains was tested against 1:100, 1:200, and 1:400, of pooled pythiosis sera and 1:200 of pooled healthy volunteer. The result showed that 8 ng/well was the optimum concentration in all three crude antigens at any serum dilutions. This antigen concentration was the point that could separated the pythiosis patient from healthy volunteer (Figure 23A, B, & C).

For IgG_4 antibody level assay by ELISA, the optimal concentration of crude antigen was also performed with the same protocol as the total IgG study. This experiment, three concentrations, 25, 50, and 75 ng/well of crude proteins from all three strains against three dilutions, 1:25, 1:50 and 1:100 of pooled pythiosis sera and 1:50 of pooled healthy volunteer, were tested. Regardless of any dilutionsof serum, the result showed that 50 ng/well of all three crude antigens was the optimum concentration (Figure 24A, B, & C). At dilution of 1:50 and 1:100 of pooled pythiosis against 50 ng/well

of all three crude antigens, it was found that their OD values have clearly different from the OD value obtained from the reaction at dilution of 1:50 pooled healthy volunteer.

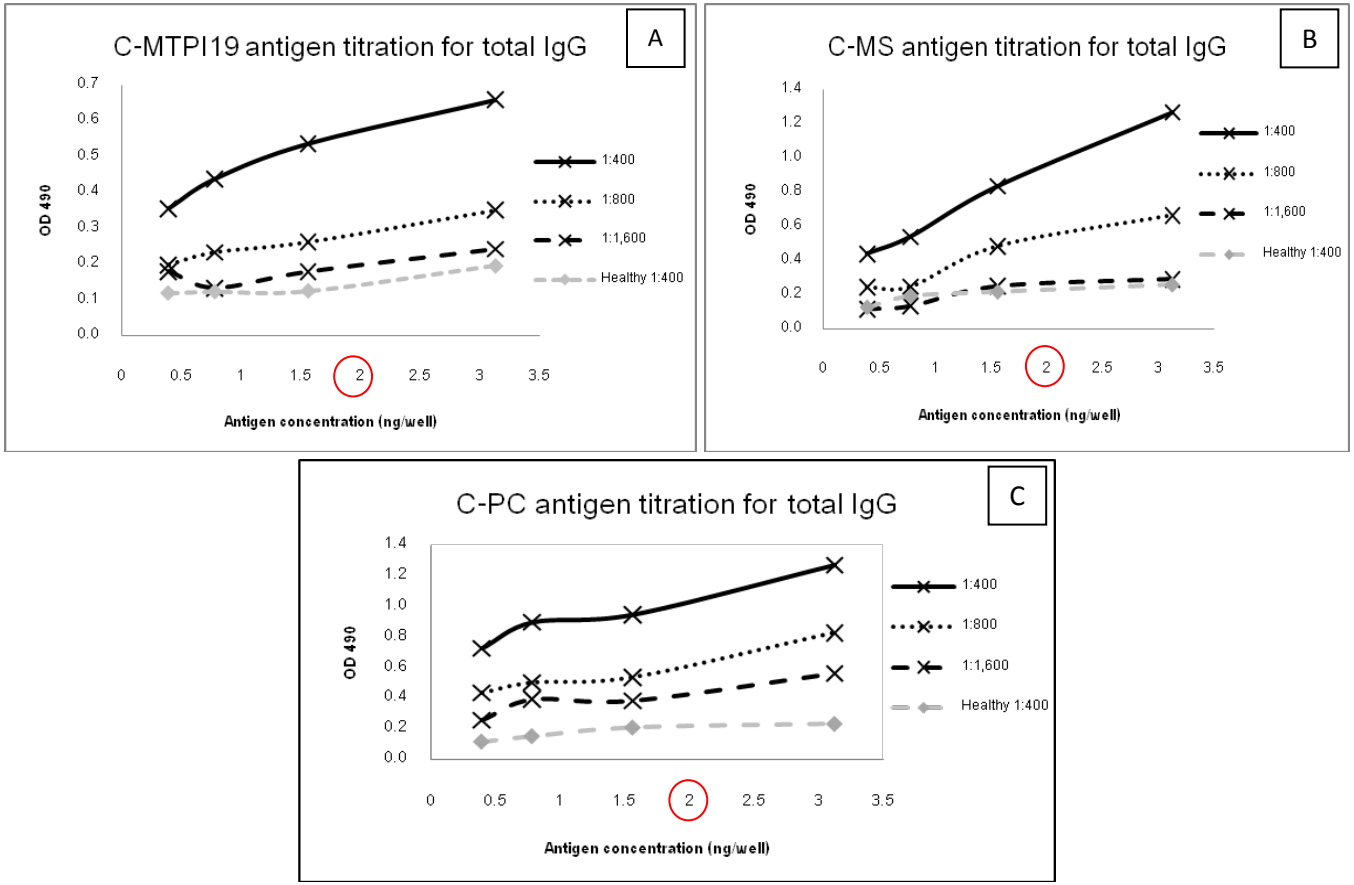

Figure 22 The titration of crude antigen, C-MTPI19 (A), C-PMS (B) and C-PC (C), against pooled pythiosis serum and pooled healthy volunteer. Two-fold dilution of antigen ranged 0.39-3.125 ng/well were coated to determine total IgG antibody concentration in ELISA system

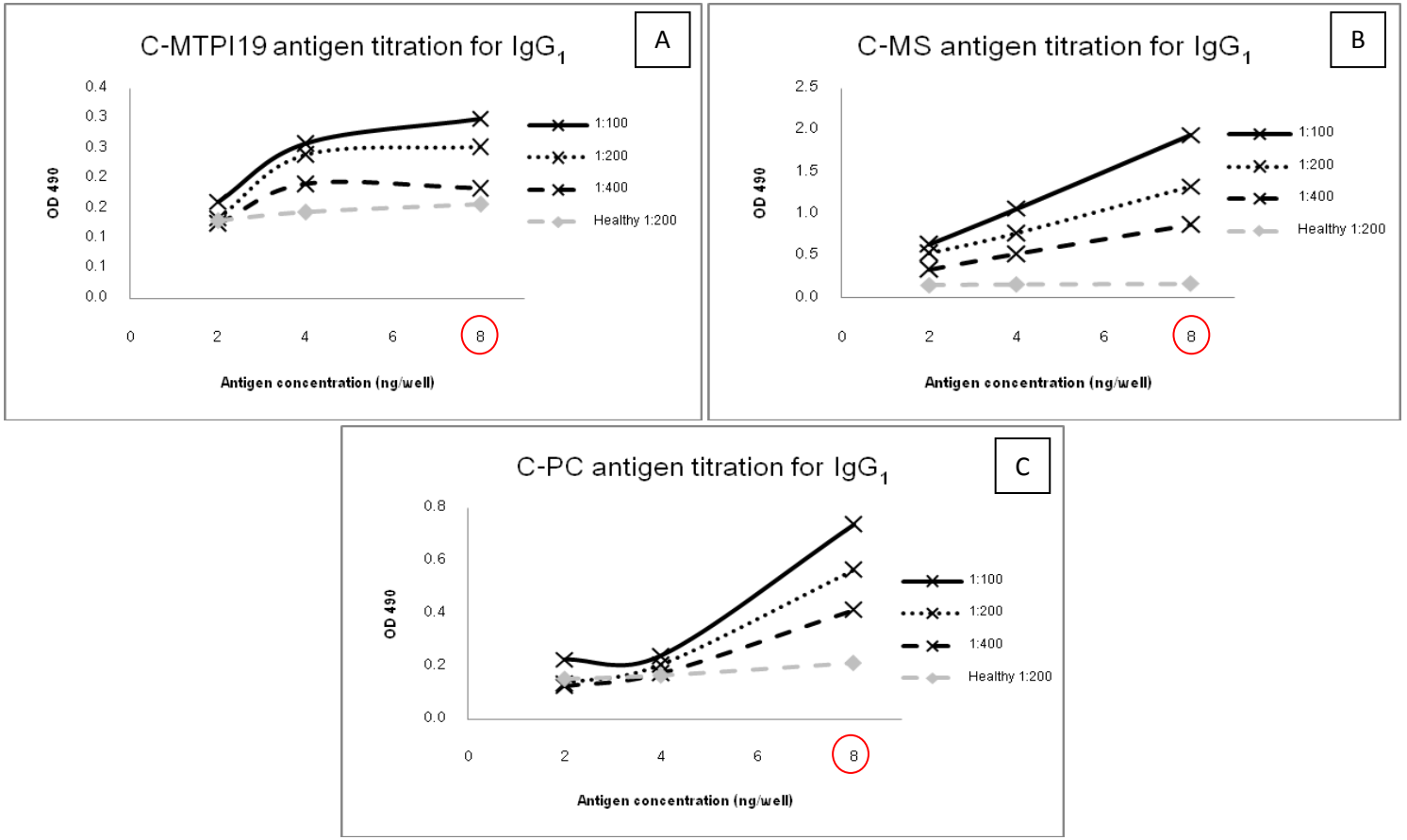

Figure 23 The titration of crude antigen, C-MTPI19 (A), C-PMS (B) and C-PC (C), against pooled pythiosis serum and pooled healthy volunteer. Two-fold dilution of antigen ranged 2-8 ng/well were coated to determine IgG_1 antibody concentration in ELISA system

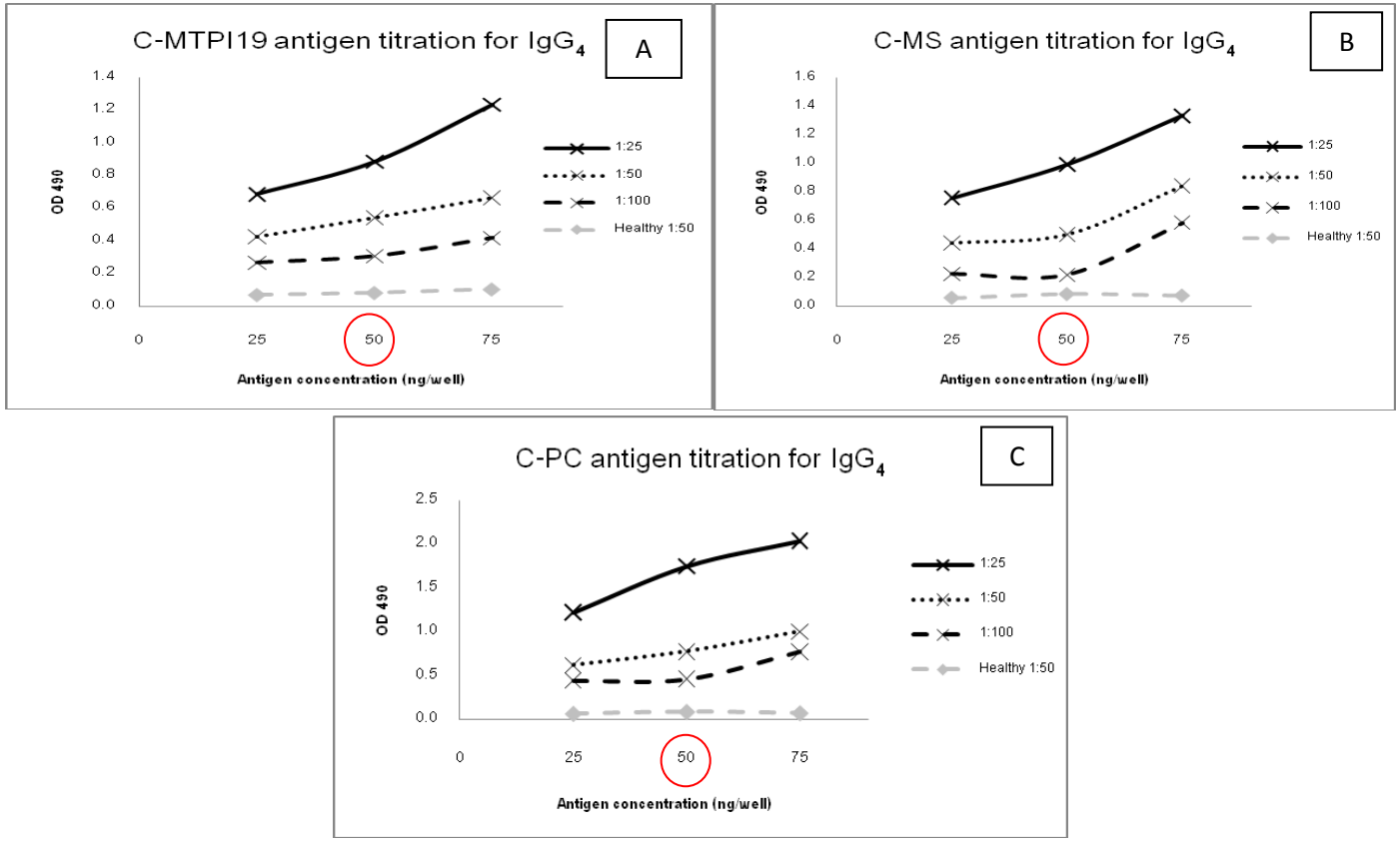

Figure 24 The titration of crude antigen, C-MTPI19 (A), C-PMS (B) and C-PC (C), against pooled pythiosis serum and pooled healthy volunteer. Two-fold dilution of antigen ranged 25-75 ng/well were coated on the well to determine IgG_4 antibody concentration in ELISA system

2.2.2 Antibody titration

To study concentration of IgG subclass, two-fold dilution of pooled pythiosis serum ranged from 1:25 to 1:1,600 and pooled healthy volunteer ranged from 1:50 to 1:1,600 for IgG, IgG_1 and IgG_4 antibody assay were tested against all three crude proteins. From previous study, the cut-off value to indicate the vascular pythiosis in the seroprevalence study of healthy volunteer serum against PIA using ELISA was $\leq$ 0.3 [65]. These results of antibody titration for IgG, IgG_1 and IgG_4 antibody against all crude antigens were shown in Figure 25A, B, & C, respectively. From this experiment, the cut off dilution for IgG and IgG_1 antibody were 1:400 while for IgG_4 antibody was 1:50.

One of the important factors in ELISA system excludes the optimum antigen and antibody concentration is the concentration of secondary antibody. The optimum concentrations of crude antigen and pooled pythiosis serum dilution for IgG, IgG_1, and IgG_4 was performed separately using secondary antibody at the dilution 1:2,000,1:5,000, and 1:10,000. (Figure 26) The concentration result of the total IgG, IgG_1, and IgG_4 secondary antibody assay was shown in, respectively.

Crude antigen C-MTPI19 related experiment:

For the total IgG study against C-MTPI19 protein, the OD value derived from using 1:2,000 dilution to 1:10,000 dilution was gradually declined whereas for the IgG_1 study, the OD value began to decrease after dilution $\geq$ 1:5,000. The result from IgG_4 experiment was similar to that of IgG_1 (Figure 26A).

Crude antigen C-PMS related experiment:

For the total IgG study against C-PMS protein, the OD value derived from using 1:2,000 dilution to 1:10,000 dilution was gradually declined. Whereas the IgG_1 study, the OD value began to decrease after dilution $\geq$ 1:5,000. The result of the IgG_4 experiment was similar to that of IgG_1 (Figure 26B).

Crude antigen C-PC related experiment:

For the total IgG study against C-PC protein, the OD value derived from using 1:2,000 dilution to 1:10,000 dilution was gradually declined. Whereas the IgG_1 assay, the OD value began to decrease after dilution $\geq$1:5,000. For the IgG4 experiment, the OD value derived from 1:2,000 dilution to 1:10,000 dilution was decreased from 1.7 to 0.4, approximately (Figure 26C).

From these results of secondary antibody titration from all crude antigens, the optimum conjugate dilution for total IgG and IgG_1 determination were used at dilution 1:5,000 while IgG_4 assay was dilution 1:4,000.

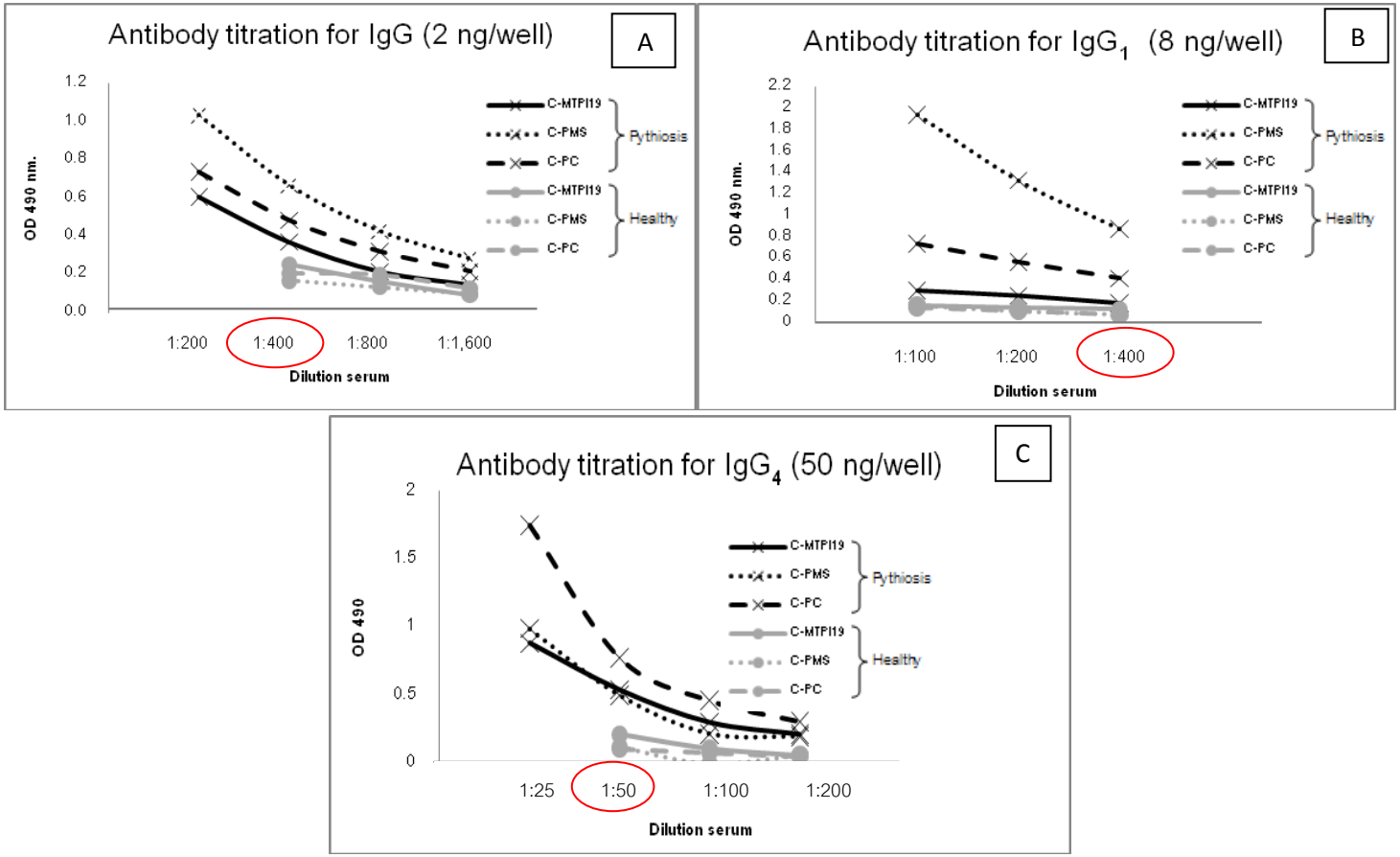

Figure 25 The titration of IgG (A), IgG_1 (B) and IgG_4 (C) antibody against crude antigens at the concentration of 2, 8 and 50 ng/well, respectively. Two-fold dilution of pooled pythiosis serum from 1:25 to 1:1,600 and pooled healthy volunteer from dilution 1:50 to 1:1,600 were tested

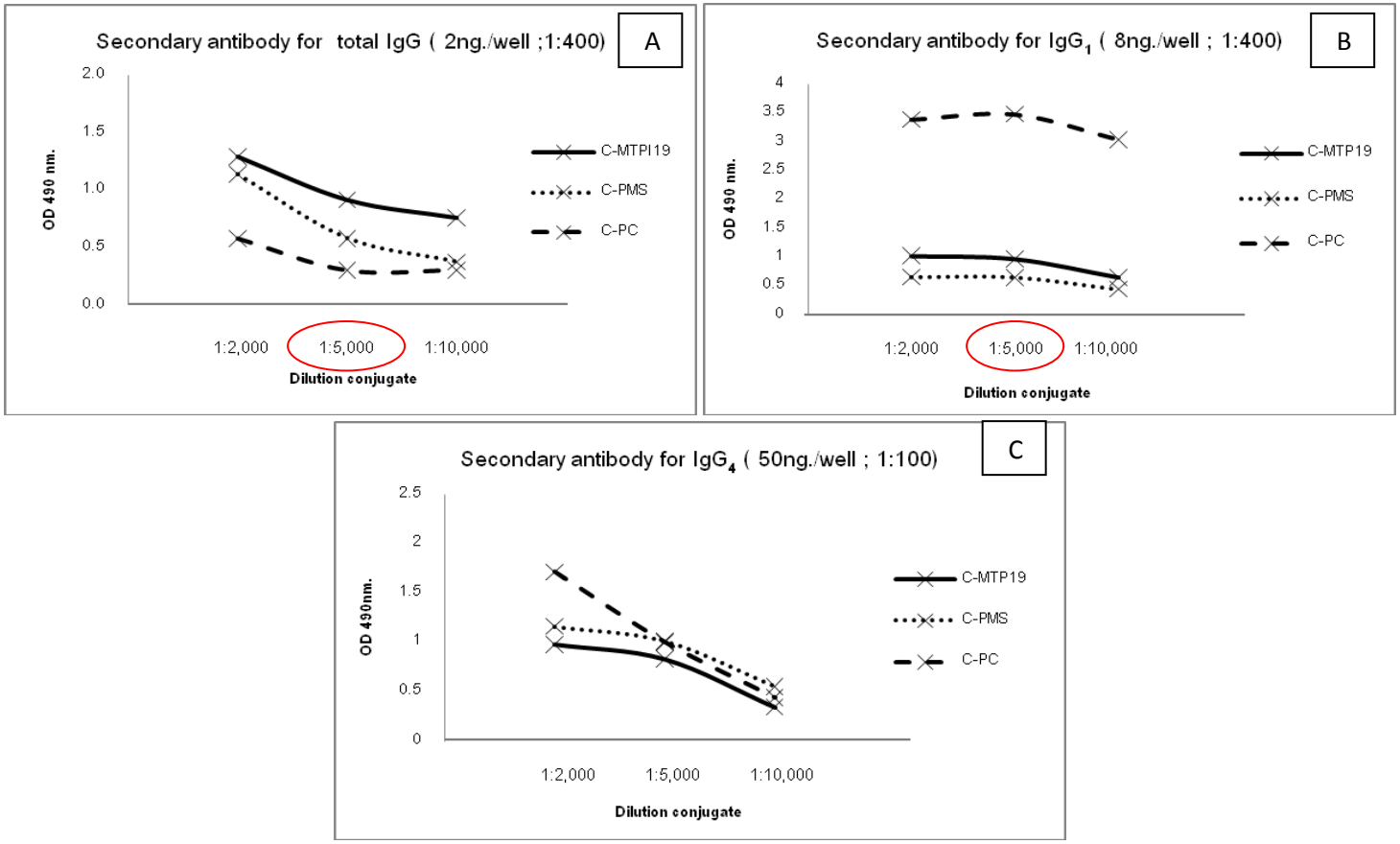

Figure 26 Secondary antibody titration, the concentration of 2, 8 and 50 ng/well each antigen for determination of IgG (A), IgG_1 (B) and IgG_4 (C), in order, and pooled pythiosis serum was used to do the checker board for secondary antibody

2.2.3 IgGs protein standard curve

To determine the quantitative amount of total IgG and IgG subclass, the IgG(Cat. no ab91102), IgG_1 (Cat. No ab90283) and IgG_4 (Cat. No ab90286) standard protein (abcam®, Biomed Diagnostic Co., Ltd) were used. The regression line equation of standard curve, which were plotted between IgGs standard protein concentrations and their OD values, were prepared. The antibody in this experiment was the secondary IgG antibody labeled HRP and the IgG_1 or IgG_4 antibody labeled biotin. The IgGs proteins were recognized the specific IgGs antibody at 1:5,000 dilutions for IgG & IgG_1 assay and 1:4,000 for IgG_4 assay which derived from the previous experiments (from the result 2.2.1 and 2.2.2). Before the detection of IgG subclass, the streptavidin labeled HRP at dilution 1:4,000 was added. The mean OD value of each standard protein ranged from 0-200 ng/ml was shown in Table 6 and their standard curves were shown in Figure 27A, B, & C.

Table 6 The mean OD value $\lambda_{490\ nm}$ of IgG and IgG subclass protein (n=3)

Standard protein concentration (ng/ml)	Mean OD at $\lambda_{490\ nm}$		
	IgG	IgG_1	IgG_4
0	0	0	0
10	0.161	0.090	0.100
50	0.258	0.276	0.550
100	0.501	0.547	0.782
150	0.798	1.106	1.090
200	1.125	1.638	1.482

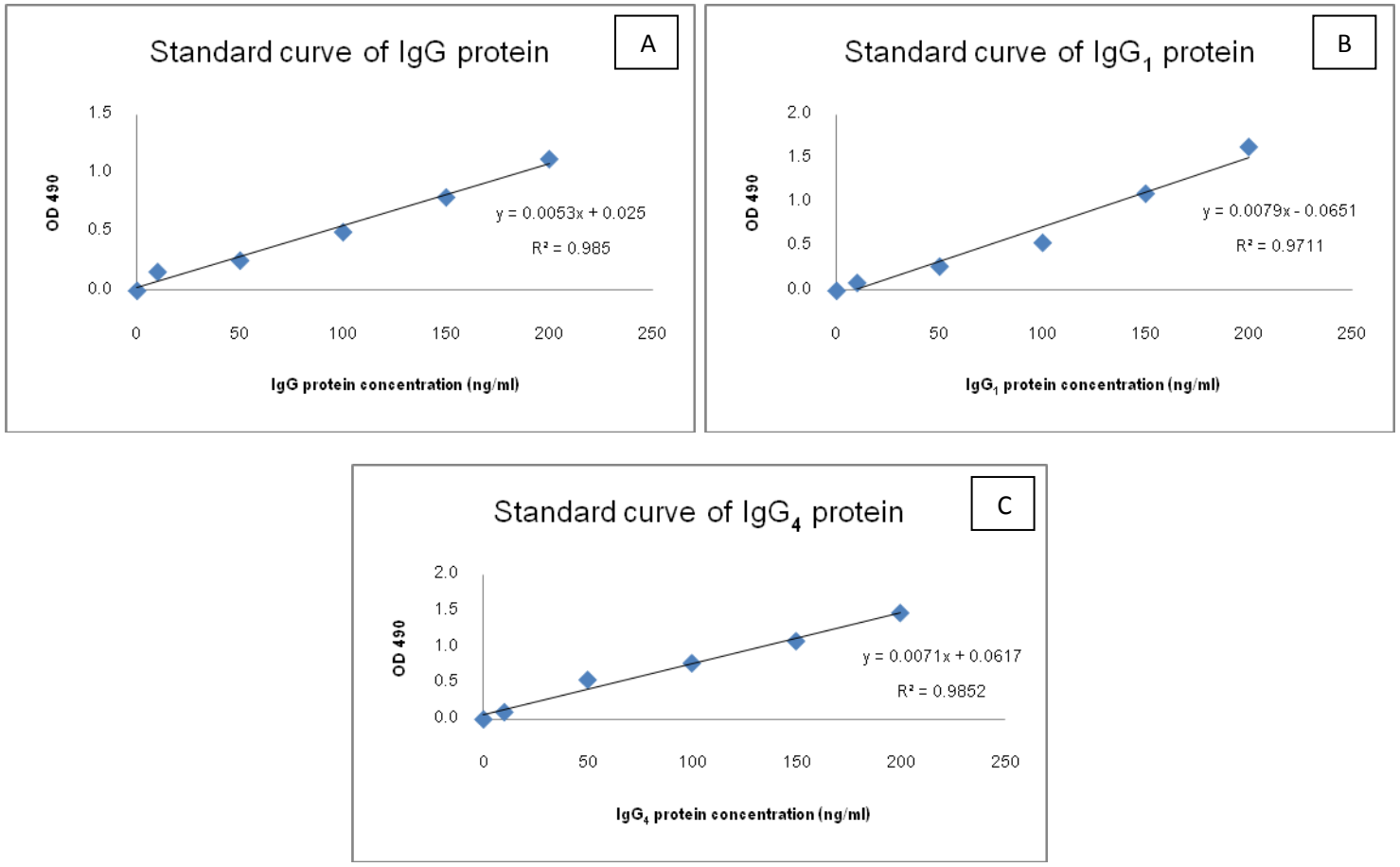

Figure 27 The standard curve of total IgG (A), IgG_1 (B) and IgG_4 (C) protein at concentration 0-200 ng/ml against anti-human IgG (IgG_1 or IgG_4) antibody conjugated HRP

2.2.4 Quantitative determination of IgG, IgG_1 and IgG_4 antibody in pre-vaccination serum against all crude proteins using ELISA

To determine the quantitative amount of specific *P. insidiosum* IgGs antibody response in vascular pythiosis against crude antigen of *P. insidiosum* three strains, the optimum conditions for ELISA (Table 7) and the equation from standard curves from above experiments were used as shown in Figure 27. Furthermore, pre-vaccination serum of patients, was collected from patients before first PIA injection, were tested. The quantitative amount of IgGs antibody showed the immune response of vascular pythiosis patients to *P. insidiosum* each clade prior immunotherapy.

Table 7 Summarize the optimum ELISA condition for specific *P. insidiosum* IgGs antibody determination

Condition	Total IgG antibody/well	IgG_1 antibody/well	IgG_4 antibody/well
Ag concentration (all 3 strains)	2 ng	8 ng	50 ng
Patients' serum dilution	1:400	1:400	1:50
Anti-human IgGs antibody dilution	1:5,000 (IgG conjugated HRP)	1:5,000 (IgG_1 conjugated biotin)	1:4,000 (IgG_4 conjugated biotin)
Streptavidin-HRP dilution	-	1: 4,000	1: 4,000

From the results, it was showed that the IgGs antibody concentration against all three strains, have the variable range as followed:

- Total IgG antibody: C-MTPI19 (8-71 µg/ml), C-PMS (7-88 µg/ml) and C-PC (7-1032 µg/ml)
- IgG_1 antibody : C-MTPI19 (12-147 µg/ml), C-PMS (14-638 µg/ml) and C-PC (12-2690 µg/ml)
- IgG_4 antibody : C-MTPI19 (1-81 µg/ml), C-PMS (1-88 µg/ml) and C-PC (1-155 µg/ml)

To decrease the bias of statistic calculation, the concentration values which showed more than or less than 3 times of median were excluded.

The total IgG antibody:

Since the individual IgG concentration varied in high range, here their mean ($\bar{x}$), standard deviation (SD), minimum value (Min.) and Maximum value (Max) were shown in Table 8A. The mean concentration of total IgG antibody from thirteen vascular pythiosis patients ($\bar{x}$=31.15, SD=19.81, Max=71 and Min=8) against C-MTPI19 crude antigen was higher than that from ten healthy volunteers ($\bar{x}$=0, SD=0) However, the different of the amount of antibody from vascular pythiosis patient with healthy volunteers were not statistical significance at 95% confidence interval ($P>0.05$) due to the high range of data variation. The quantitative amount of IgG antibody against C-PMS was similar to that of C-MTPI19 which the statistic values as follows:

- Vascular pythiosis sera : $\bar{x}$=46.85, SD=25.50, Max=88 and Min=7
- Healthy sera : $\bar{x}$=0, SD=0

When using C-PC as antigen, the amount of IgG antibody from vascular pythiosis patients ($\bar{x}$=283.2, SD=302.2, Max=1032 and Min=7) was significantly higher than that from healthy volunteers ($\bar{x}$=0, SD=0).

In terms of three difference crude antigens in the experiment, the total IgG antibody level of vascular pythiosis patients against C-PC antigen ($\bar{x}$=283.2, SD=302.2) was significantly ($P<0.001$) higher than that using C-MTPI19 ($\bar{x}$=31.15, SD=19.81) and using C-PMS antigen ($\bar{x}$=46.85, SD=25.50) (Figure 28A).

The IgG_1 antibody level experiment:

In the same way, the statistic values of IgG_1 antibody against all three crude antigens were shown in Table 8B.

The mean value of IgG_1 antibody level of vascular pythiosis patients ($\bar{x}$=54.23, SD=43.48, Max=147 and Min=12) against C-MTPI19 crude antigen was higher than that obtained from healthy volunteers ($\bar{x}$=0, SD=0). However, the difference of IgG_1 antibody amount from each serum was not statistically significance ($P>0.05$) due to their means were small different. The results of amount of IgG_1 antibody against C-PMS were similar to that C-MTPI19 which had the statistic value of IgG_1 antibody from vascular pythiosis patients ($\bar{x}$=119.0, SD=162.6, Max=638 and Min=14) and volunteer healthy ($\bar{x}$=0, SD=0). While the quantitative amount of IgG_1 antibody from vascular pythiosis patients ($\bar{x}$=458.0, SD=735.7, Max=2690 and Min=12) against C-PC was significantly higher than that from healthy volunteers ($\bar{x}$=0, SD=0). The difference of IgG_1 antibody between vascular pythiosis patients and volunteer healthy was statistically significance ($P<0.05$)

When the comparison amounts of IgG_1 antibody from vascular pythiosis patients against all three crude antigens shown that the concentration of antibody to C-PC antigen ($\bar{x}$=458.0, SD=735.7) was significantly ($P<0.05$) higher than that C-MTPI19 ($\bar{x}$=54.23, SD=43.48) but not C-PMS ($\bar{x}$=119.0, SD=162.6) (Figure 28B).

The IgG_4 antibody level experiment:

The statistic values of IgG_4 antibody from each type of sera against all three crude antigens were shown in Table 8C.

From the results of IgG_4 antibody were same the IgG_1 antibody. The mean value of IgG_4 antibody from vascular pythiosis patients against C-MTPI19 ($\bar{x}$=18.15, SD=28.03, Max=81 and Min=1) and C-PMS ($\bar{x}$=27.23, SD=27.87, Max=88 and Min=-1) were higher than that from healthy volunteers (C-MTPI19 and C-PMS; $\bar{x}$=0, SD=0). However, the difference of IgG_4 antibody from each sera was not statistically significance ($P>0.05$). Whereas, the quantitative amount of IgG_4 antibody from vascular pythiosis patients against C-PC ($\bar{x}$=37.38, SD=50.0, Max=155 and Min=1) was statistically significance ($P<0.05$) higher than that healthy volunteers ($\bar{x}$=0, SD=0).

The comparison amounts of IgG_4 antibody from vascular pythiosis patients against all three crude antigens shown that the concentration of antibody to C-PC antigen ($\bar{x}$=37.38, SD=50.0) and C-PMS ($\bar{x}$=27.23, SD=27.87) was not significantly higher than that C-MTPI19 ($\bar{x}$=18.15, SD=28.03) (Figure 28C) due to their means data were small different.

Table 8A Statistical data of total IgG antibody concentration in thirteen pre-vaccination from vascular pythiosis patients and ten volunteer healthy sera to *P. insidiosum* three strains

Antigen	Vascular pythiosis			Healthy		
	C-MTPI19	C-PMS	C-PC	C-MTPI19	C-PMS	C-PC
Number of values	13	13	13	10	10	10
Minimum	8.0	7.0	7.0	0.0	0.0	0.0
25% Percentile	18.00	28.00	50.00	0.0	0.0	0.0
Median	27.00	46.00	178.0	0.0	0.0	0.0
75% Percentile	41.00	63.50	427.0	0.0	0.0	0.0
Maximum	71.00	88.00	1032	0.0	0.0	0.0
Mean	31.15	46.85	283.2	0.0	0.0	0.0
Std. Deviation	19.81	25.50	302.2	0.0	0.0	0.0
Std. Error	5.495	7.072	83.81	0.0	0.0	0.0

Table 8B Statistical data of IgG_1 antibody concentration in thirteen pre-vaccination from vascular pythiosis patients and ten volunteer healthy sera to *P. insidiosum* three strains

Antigen	Vascular pythiosis			Healthy		
	C-MTPI19	C-PMS	C-PC	C-MTPI19	C-PMS	C-PC
Number of values	13	13	13	10	10	10
Minimum	12.0	14.0	12.0	0.0	0.0	0.0
25% Percentile	15.50	40.50	47.00	0.0	0.0	0.0
Median	37.00	63.00	153.0	0.0	0.0	0.0
75% Percentile	93.50	129.5	580.5	0.0	0.0	0.0
Maximum	147.0	638.0	2690	0.0	0.0	0.0
Mean	54.23	119.0	458.0	0.0	0.0	0.0
Std. Deviation	43.48	162.6	735.7	0.0	0.0	0.0
Std. Error	12.06	45.09	204.0	0.0	0.0	0.0

Table 8C Statistical data of IgG_4 antibody concentration in thirteen pre-vaccination from vascular pythiosis patients and ten volunteer healthy sera to *P. insidiosum three* strains

	Vascular pythiosis			Healthy		
Antigen	C-MTPI19	C-PMS	C-PC	C-MTPI19	C-PMS	C-PC
Number of values	13	13	13	10	10	10
Minimum	1.0	1.0	1.0	0.0	0.0	0.0
25% Percentile	1.000	8.000	7.000	0.0	0.0	0.0
Median	3.000	15.00	14.00	0.0	0.0	0.0
75% Percentile	28.00	56.50	52.50	0.0	0.0	0.0
Maximum	81.00	88.00	155.0	0.0	0.0	0.0
Mean	18.15	27.23	37.38	0.0	0.0	0.0
Std. Deviation	28.03	27.87	50.00	0.0	0.0	0.0
Std. Error	7.775	7.729	13.87	0.0	0.0	0.0

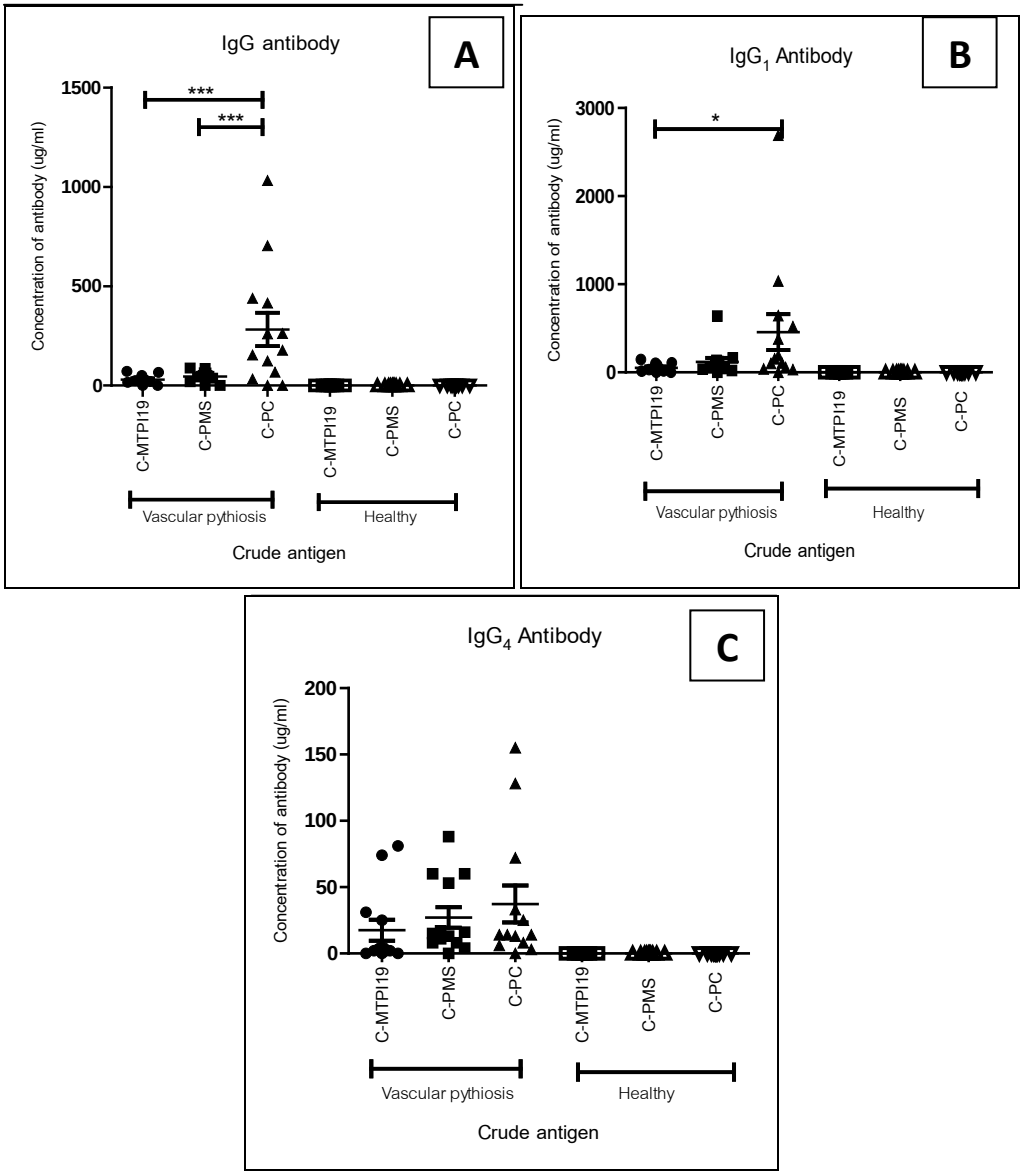

Figure 28 Comparison of total IgG (A), IgG_1(B) and IgG_4(C) antibody concentration in patients with vascular pythiosis and healthy against each clade of *P. insidiosum* ($P<0.05$; * and $P<0.001$; ***)

2.2.5 Quantitative total IgG, IgG_1 and IgG_4 antibody to *P. insidiosum* strain C-MTPI19.

In the present, the 50-60%successful of pythiosis treatment derives from the combination of surgery, antifungal drug and PIA injection [19]. Regarding the immunotherapy, the patient was injected with *P. insidiosum* antigen (PIA) which was prepared from endo- and exo-antigen of *P. insidiosum* strain MTPI19. Immune response in horse and human pythiosis which was treated with PIA-immunotherapy was studied in the form of some cytokines production. The switching of T_H2 to T_H1 response was reported [18-20, 42]. To determine amount of IgGs antibody response in vascular pythiosis patients with immunotherapy against C-MTPI19 antigen, patient serum in each individual prior immunotherapy as shown in Table 1 in Materials and Methods was tested as the IgGs baseline in this study.

All vascular pythiosis patients were divided into two groups as response group and non-response group as listed. Response groups can be divided into 2 subgroups: short-term ($<$14 month) and long-term ($\geq$ 14 month).

- Response group
    - Short term ($<$14 month) : PY15, PY27, PY31, PY49, PY50 and PY62
    - Long term ($\geq$ 14-34 month) : PY5, PY22, PY30 and PY51
- Non-response group (Progression or relapse) : PY7, PY8 and PY12

The result of the antibody level changing whether increasing or decreasing is two times difference, based on the criteria. The quantitative data of IgGs antibody against C-MTPI19 antigen from 13 patients before starting immunotherapy were shown in Table 9.

Non-response group:

Based on the criteria, the tendency of total IgG antibody in this group has two forms as a leveling off and decreasing. No significant change of the *P. insidiosum* specific IgG was observed in PY7 and PY8 after 4-10 week period of 4-8 vaccination while 2-fold decreasing of the IgG level was found after 2 weeks and the level remained until 4^{th} week with total 4 vaccinations in PY12.

For the results ofIgG_1 antibody level, the whole profile was similar to that of IgG amount. That was no significant variation of the level were found in two cases (PY7 and PY12) whereas almost 2 times over was shown in PY8 within the same period of IgG study. In terms of the IgG_4 level, after 10 weeks with 8 vaccinations, the amount of antibody trended to be decreased after a bit increasing at 2^{nd} week with 2 vaccinations. For the other 2 cases, PY8 and PY12, the IgG_4 amount was very less, resulting the difficulty in interpretation.

Short-term response group (6 cases: PY15, 27, 31, 49, 50, 62):

The increasing level of specific *P. insidiosum* IgG, IgG_1, and IgG_4 antibody of two from six cases (PY27, PY31) was shown (Table 9). This short-term response group, the result all IgGs antibody level obtained from other 4 cases showed no significant difference followed the set up criteria. However, it was unable to determine the level of IgG antibody in PY15 and PY62 because their mean OD values have less than cut-off value ($OD < 0.3$).

Long-term response group (4 cases: PY5, 22, 30, 51):

Three forms of antibody level changing were demonstrated. There were increasing (PY22), decreasing (PY30), and no change (PY5, PY51) criteria. The 2.5 and 5 times increasing amount of both IgG and IgG_1 antibody, in order, were found in

PY22 in 8^{th} week after 8 vaccinations. The increasing level of IgG_4 antibody was also shown but the amount was only from 2-8 ug/ml. The clinical manifestation of this case was stable uo to 34 months. Whereas the 3.5 and 2.5 times decreasing level of IgG and IgG_1, respectively, were observed in case PY30. The IgG_4 amount was in small amount with no change followed the criteria. The level of all IgGs antibodies of the other 2 cases, PY5 and PY51, showed no significant difference.

Table 9 The concentration of total IgG, IgG_1 and IgG_4 antibody against *P. insidiosum* strain MTPI19 in 13 vascular pythiosis before immunotherapy

Patients No.	Concentration of IgGs antibody (μg/ml)									Clinic status
	Total IgG			IgG_1			IgG_4			
No.1 (PY5)	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV7 (6 wk)*	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV7 (6 wk)*	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV7 (6 wk)*	Stable (14 mo)
	66	72	78	84	80	92	74	55	81	
No.2 (PY7)	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV9 (10 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV9 (10 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV9 (10 wk)*	Relapse (2 mo)
	30	34	37	147	138	150	81	105	63	
No.3 (PY8)	*PV1 (0 wk)*	*PV2 (1 wk)*	*PV5 (4 wk)*	*PV1 (0 wk)*	*PV2 (1 wk)*	*PV5 (4 wk)*	*PV1 (0 wk)*	*PV2 (1 wk)*	*PV5 (4 wk)*	Die (2 mo)
	29	42	29	108	122	62	2	2	2	
No.4 (PY12)	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV5 (4 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV5 (4 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV5 (4 wk)*	Die (2 mo)
	27	15	15	17	18	12	2	1	1	

Patients No.	Concentration of IgGs antibody (μg/ml)									Clinic status
	Total IgG			IgG_1			IgG_4			
No.5 (PY15)	*PV1 (0 wk)*	*PV2 (1 wk)*	*PV8 (7 wk)*	*PV1 (0 wk)*	*PV2 (1 wk)*	*PV8 (7 wk)*	*PV1 (0 wk)*	*PV2 (1 wk)*	*PV8 (7 wk)*	Stable (2 mo)
	8	10	8	12	16	12	1	1	1	
No.6 (PY22)	*PV1 (0 wk)*	*PV5 (4 wk)*	*PV9 (8 wk)*	*PV1 (0 wk)*	*PV5 (4 wk)*	*PV9 (8 wk)*	*PV1 (0 wk)*	*PV5 (4 wk)*	*PV9 (8 wk)*	Stable (34 mo)
	24	31	60	14	15	79	2	1	8	
No.7 (PY27)	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV8 (7 wk)*	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV8 (7 wk)*	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV8 (7 wk)*	Stable (2 mo)
	20	32	62	37	14	105	1	1	5	
No.8 (PY30)	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV11 (72 wk)*	*PV12 (96 wk)*	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV11 (72 wk)*	*PV12 (96 wk)*	*PV1 (0 wk)*	*PV4 (3 wk)*	*PV11 (72 wk)*	*PV12 (96 wk)*	Stable (24 mo)
	71	39	17	20	103	38	43	44	4	3	3	6	
No.9 (PY31)	*PV1 (0 wk)*	*PV5 (4 wk)*	*PV9 (12 wk)*	*PV1 (0 wk)*	*PV5 (4 wk)*	*PV9 (12 wk)*	*PV1 (0 wk)*	*PV5 (4 wk)*	*PV9 (12 wk)*	Stable (4 mo)
	24	14	48	40	28	66	31	20	57	
No.10 (PY49)	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV10 (26 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV10 (26 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV10 (26 wk)*	Stable (7 mo)
	16	22	21	12	22	15	6	14	9	

3.

Patients No.	Concentration of IgGs antibody (μg/ml)											Clinic status
	Total IgG				IgG_1				IgG_4			
No.11 (PY50)	*PV1 (0 wk)*	*PV3 (2 wk)*		*PV6 (5 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*		*PV6 (5 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*		*PV6 (5 wk)*	Stable (2 mo)
	32	41		47	32	28		39	25	21		27	
No.12 (PY51)	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV9 (24 wk)*	*PV10 (44 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV9 (24 wk)*	*PV10 (44 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*	*PV9 (24 wk)*	*PV10 (44 wk)*	Stable (18 mo)
	50	57	41	38	62	51	14	94	3	3	1	9	
No.13 (PY62)	*PV1 (0 wk)*	*PV3 (2 wk)*		*PV10 (32 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*		*PV10 (32 wk)*	*PV1 (0 wk)*	*PV3 (2 wk)*		*PV10 (32 wk)*	Stable (8 mo)
	8	12		8	37	36		22	4	4		2	

4. Antigenic pattern of total IgG and IgG isotype:

4.1 Crude endogenous protein pattern of *P. insidiosum* strain C-MTPI19 using SDS-PAGE

To study the protein pattern of *P. insidiosum* strain C-MTPI19, the crude protein was prepared at concentration of 15 ng/well and loaded into the 12.5% SDS-PAGE gel. In the protein preparation step, the protein structures were denatured by heat and its covalent bonds were separated by adding of SDS and DTT in sample buffer. The protein molecular weight of crude protein was calculated from equation of standard curve of protein maker which plotted between log MW and relative migration distance (R_f). The equation and relationship value (R^2= 0.9305) of standard curve of protein was shown in Figure 29A. The R^2 value demonstrated. The condition of 12.5%SDS-PAGE was able to show protein profile of molecular weight (MW) ranging from $\geq$ 170 to 17 kDa. From the coomassie blue staining gel, the several bands of molecular weight ranged from 120 to ~16 kDa were found in C-MTPI19 crude protein as shown in Figure 29B. The predominance protein at MW ~120, ~65, ~55, ~48, ~43-~41, ~32-28 ~24 and ~20 kDa were expressed in C-MTPI19 crude antigen as marked.

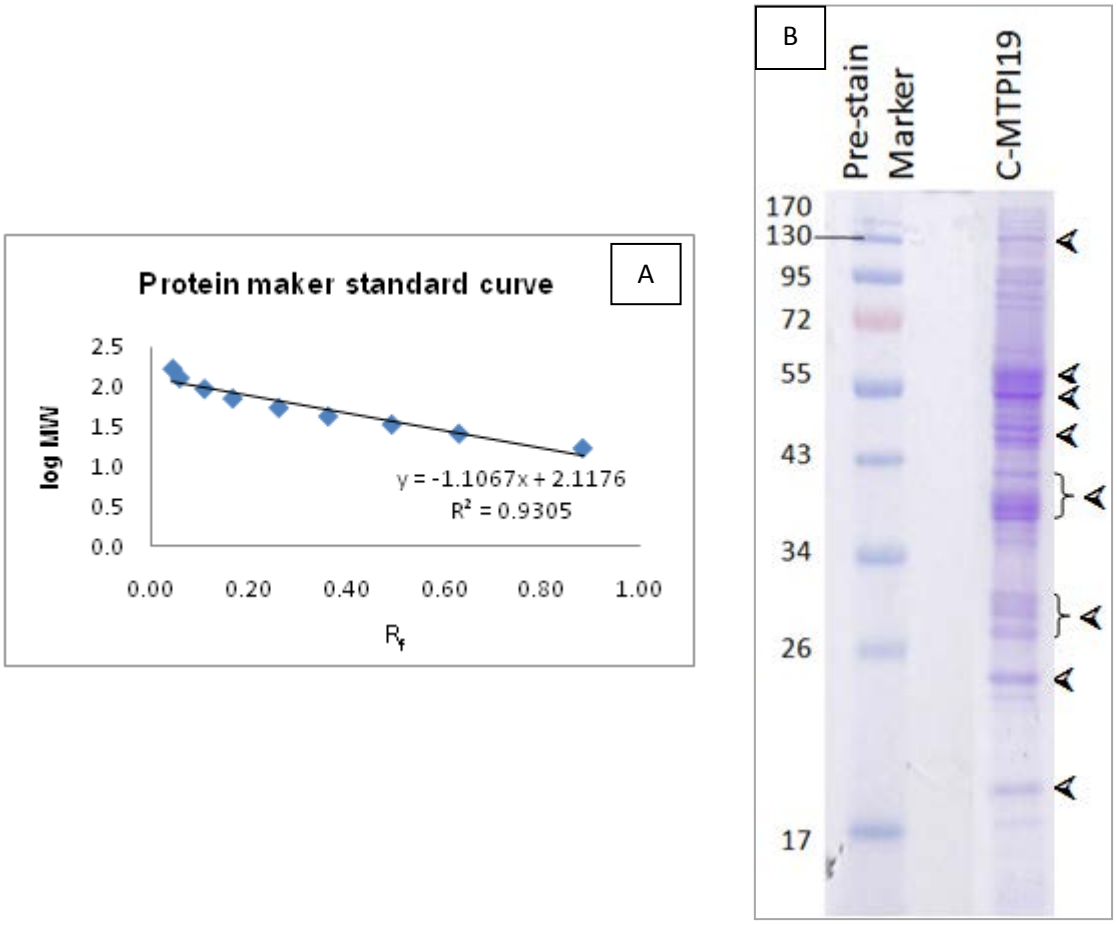

Figure 29 (A) Standard curve of protein maker from SDS-PAGE gel. (B) SDS-PAGE profile of the C-MTPI19 proteins, ranged from 170 – 17 and, the predominant protein band at size ~120, ~65, ~55, ~48, ~43-~41, ~32-28, ~24 and ~20 kDa (➤)

4.2 Antigenic pattern of IgG isotypes against crude antigen of *P. insidiosum* by western blot

4.2.1 Establish condition for western blot analysis

From the experiment 3.1.2, the optimum concentration of C-MTPI19 crude protein for IgG and IgG_1 pattern assay was 1 µg/well while IgG_4 pattern assay was 10 µg/well. From the ELISA study, it was found that the quantitative of IgGs antibody in each pythiosis patients were varied so the investigation of each optimum dilution serum for each individual was important for the well resolution of western blot analysis. Five pre-vaccination sera, PY5, PY7, PY8, PY12 and PY49, were tested. These patients had the different level of IgGs antibody against C-MTPI19 crude antigen from ELISA study (Table 9) for purpose of reducing bias in this research. After the establishment, the optimum conditions were summarized in Table 10.

Table 10 Summarize the optimum condition for western blot analysis

Condition	Total IgG pattern	IgG_1 pattern	IgG_4 pattern
Ag concentration	1 µg/well	1 µg/well	10 µg/well
Patients' serum dilution	1:4,000	1:500 (≤ 50 µg/ml) 1:1,000 (>50 µg/ml)	1:25 (≤ 20 µg/ml) 1:50 (> 20 µg/ml)
Anti-human IgGs antibody conjugated biotin	1:5,000 (IgG conjugated biotin)	1:5,000 (IgG_1 conjugated biotin)	1:5,000 (IgG_4 conjugated biotin)
Streptavidin-AP dilution	1:1,000	1: 1,000	1: 1,000

4.2.2 The common antigen of *P. insidiosum* strain C-MTPI19

To determine the common antigens which were recognized by specific *P. insisiosum* antibody, twelve pre-vaccination sera from vascular pythiosis patients were tested. At the same time, ten sera from volunteer healthy as the negative control were used.

Healthy sera:

From the total IgG and IgG_1 antibody pattern at dilution serum 1:8,000 and 1:2,000, in order, demonstrated the common bands which cross reaction with *P. insidiosum* crude antigen, at size of double band~95 and single band~43 kDa (➤). While IgG_4 antibody pattern at dilution 1:1,600 show the common bands at MW ~120, ~95, ~55 and~43 kDa(➤) (Figure 30A, B, &C). These common bands were used to subtract from the IgGs pattern of vascular pythiosis sera.

Vascular pythiosis sera:

From the subtraction of IgGs pattern from healthy sera, the specific total IgG antibody pattern of sera from vascular pythiosis patients, show the common bands at MW of single band ~120, ~55 and ~40-~34 kDa (➤). The high molecular mass at size of the single band~120 kDa (➤) was recognized by specific IgG_1 antibody while the specific IgG_4 antibody reacted the common bands at MW ~40-~34, ~32-~28 and ~24 kDa (➤) of crude antigen (Figure 31A, B, &C).

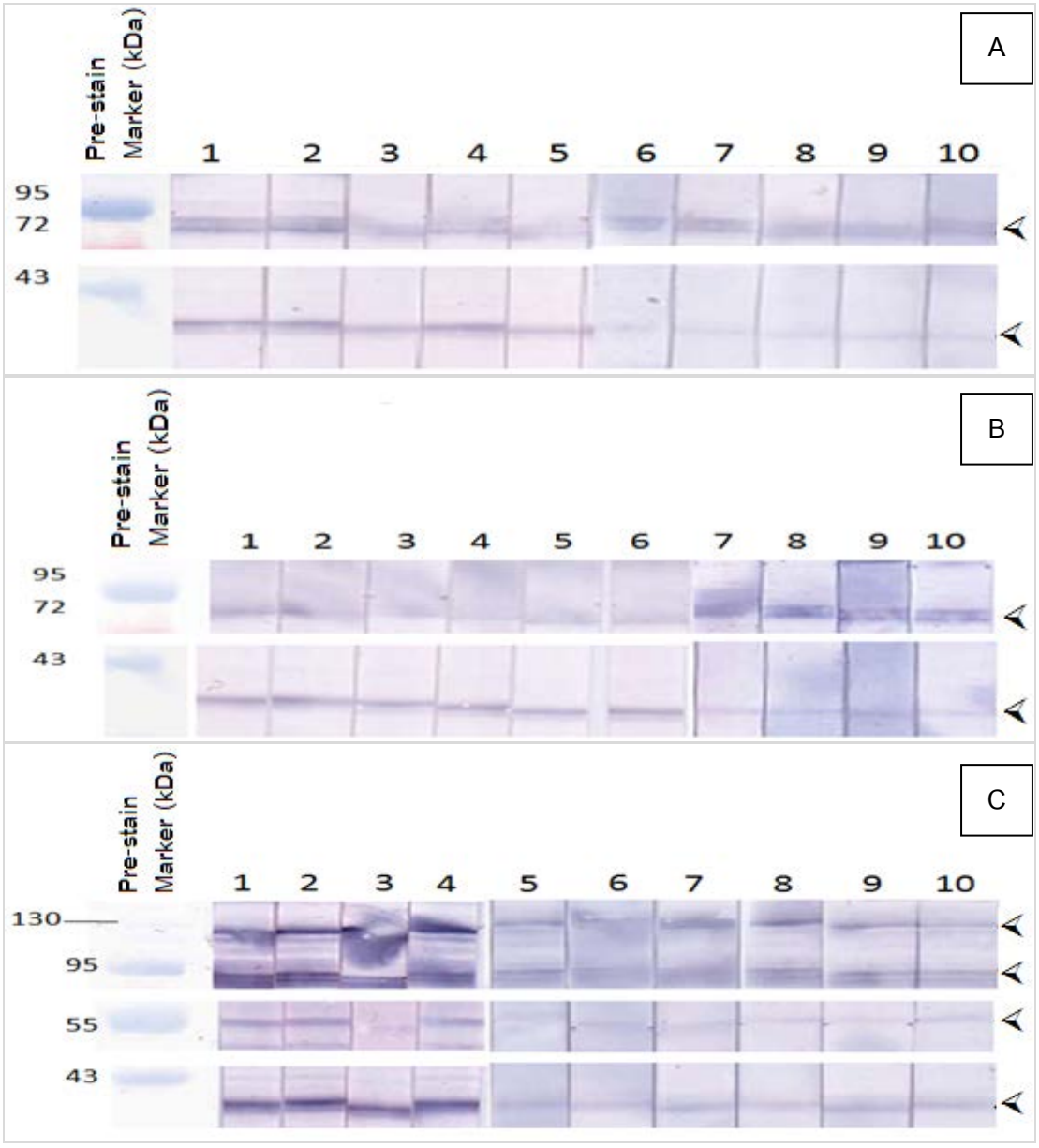

Figure 30 Western blot analysis of IgG (A), IgG_1(B) and IgG_4(C) antibody against *P. insidiosum* strain C-MTPI19 of ten sera from healthy volunteer (Lane 1; H11, lane 2; H21, lane 3; H25, lane 4; H31, lane 5; H41, lane 6; H49, lane 7; H51, lane 8; H61, lane 9; H75 and lane10; PY87)

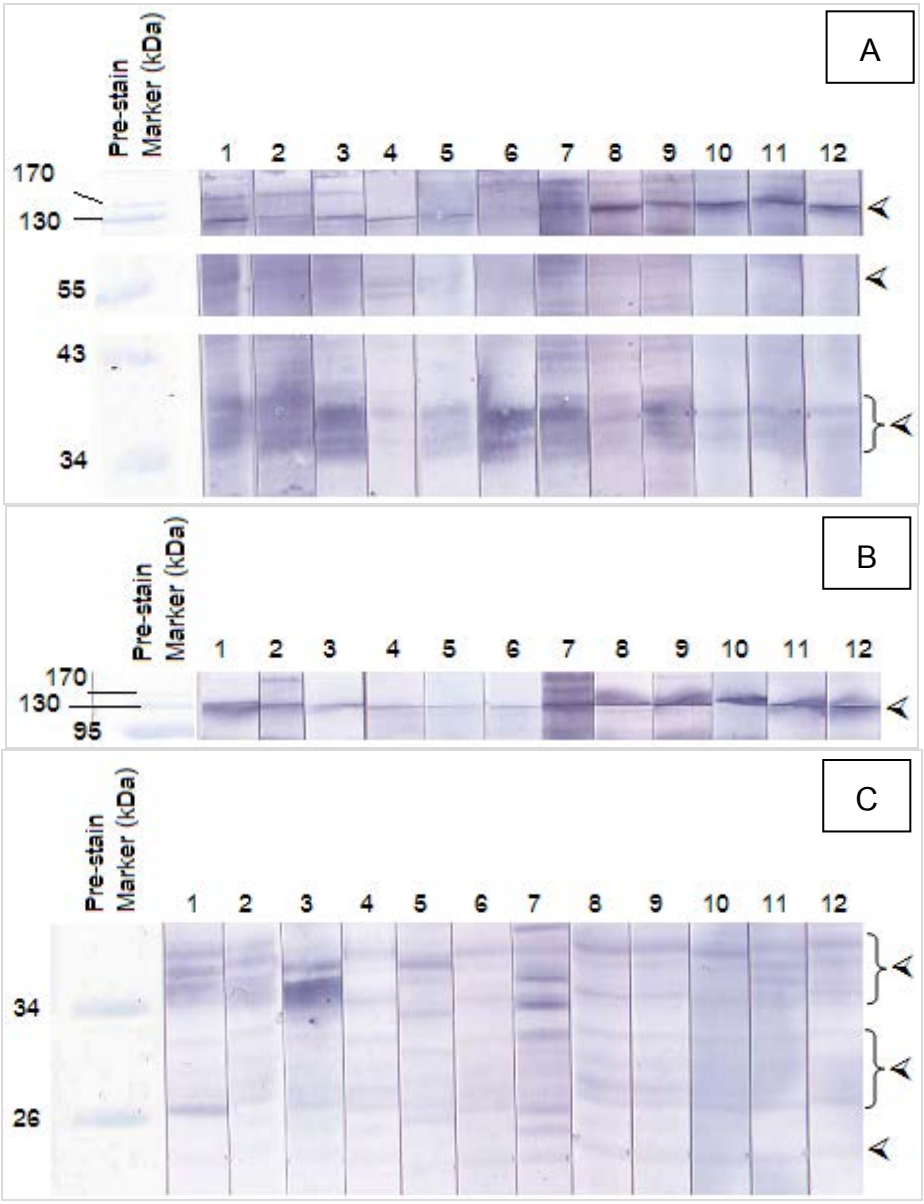

Figure 31 Western blot analysis of IgG (A), IgG_1(B) and IgG_4(C) antibody against C-MTPI19.of twelve pre-vaccination sera from vascular pythiosis patients (Lane1; PY5, lane2; PY7, lane3; PY8, lane4; PY12, lane5; PY22, lane6; PY27, lane7; PY30, lane8; PY31, lane9; PY15, lane10; PY49, lane11; PY50 and lane12; PY51)

4.2.3 Antigenic pattern recognized by IgGs isotype in PIA-treated patients

To determine the specific *P. insidiosum* IgGs antibody response pattern in PIA-treated patients, serum from different periods from thirteen vascular pythiosis was used for western blot analysis (Table1 in materials and methods).

The immune responses against *P. insidiosum* infection have two mechanisms as cell-mediated immunity and humoral immunity. The humoral immunity is mechanism about antibody production which IgG antibodies were produced by plasma cell after the IgM secretion. From the previous study show the patients before vaccination, have the T_H2 response. After PIA injection, the immune response of patients shifted from T_H2 response to T_H1 response. From the basic immunology knowledge, IgG subclasses can demonstrate the host's immune response. IgG_1 and IgG_3 antibody were produced by plasma cells which were activated via T helper 1 cell while IgG_4 and IgE were secreted by plasma cell activated from T helper 2 cells. In this study was antigenic pattern of specific IgGs antibody in vascular pythiosis patients with immunotherapy.

From the results, IgGs antibodies from thirteen patients have the variation of antigen recognition. From the analysis antigenic pattern, thirteen vascular pythiosis patients have eleven different IgG pattern, nine different IgG_1 pattern and ten different IgG_4 pattern as shown in Figure 32A, B and C, respectively. The recognized antigens in each IgGs pattern were shown in Table 11.

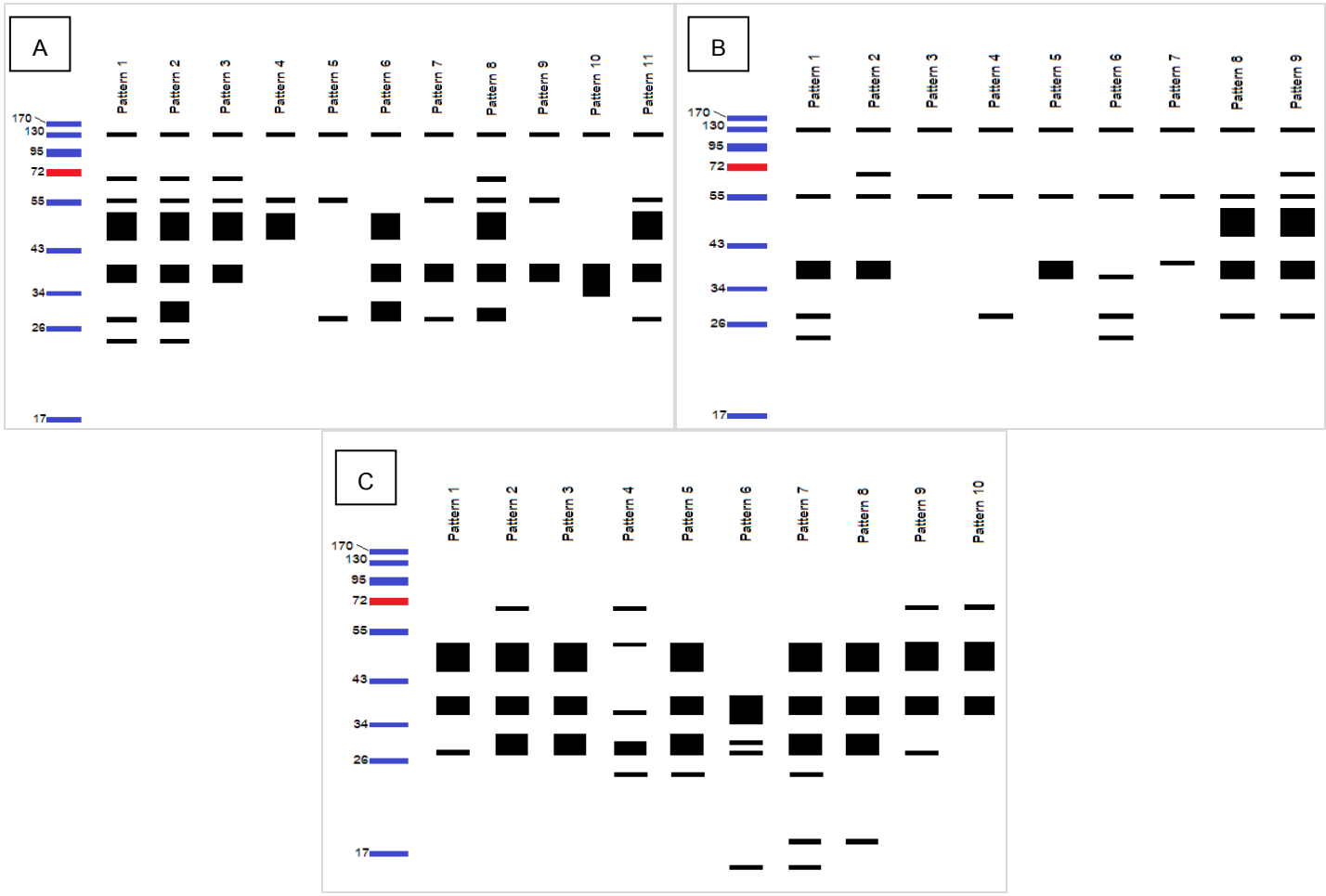

Figure 32 IgG, IgG_1 and IgG_4 pattern against C-MTPI19 crude antigen of thirteen vascular pythiosis patients before and after immunotherapy

Table 11 Summarize of the molecular weight of antigen were recognized by IgG, IgG_1 and IgG_4 antibody from thirteen vascular pythiosis patients

IgG pattern			IgG_1 pattern			IgG_4 pattern		
Pattern No.	Immunogen (kDa)	Patient No.	Pattern No.	Immungen (kDa)	Patient No.	Pattern No.	Immunogen (kDa)	Patient No.
1	120, 65, 55, 50-48 ,40-38, 28, 24	PY5	1	120, 55, 40-38, 28, 24	PY5	1	50-48, 40-38, 28	PY5
2	120, 65, 55, 50-48, 40-38, 32-28, 24	PY7	2	120, 65, 55, 40-38	PY7, PY8, PY30	2	65, 50-48, 40-38, 32-28, 24	PY7, PY27
3	120, 65, 55, 50-48, 40-38	PY8, PY31, PY62	3	120, 55	PY12, PY31	3	50-48, 40-38, 32-28	PY8, PY12, PY50
4	120, 55, 50-48	PY12	4	120, 55, 28	PY15	4	65, 50, 38, 30-28, 24	PY15
5	120, 55, 28	PY15	5	120, 55, 40-38	PY22, PY50	5	50-48, 40-38, 32-28, 24	PY22
6	120, 50-48, 40-38, 32-28	PY22	6	120, 55, 38, 28, 24	PY27	6	40-34, 30, 28, 14	PY30
7	120, 55, 40-38, 28	PY27	7	120, 55, 40	PY49	7	50-48, 40-38, 32-28, 24, 20, 14	PY31

IgG pattern			IgG_1 pattern			IgG_4 pattern		
Pattern No.	Immunogen (kDa)	Patient No.	Pattern No.	Immungen (kDa)	Patient No.	Pattern No.	Immunogen (kDa)	Patient No.
8	120, 65, 55, 50-48, 40-38, 30-28	PY30	8	120, 55, 50-48, 40-38, 28	PY51	8	50-48, 40-38, 32-28, 20	PY49
9	120, 55, 40-38	PY49	9	120, 65, 55, 50-48, 40-38, 28	PY62	9	65, 50-48, 40-38, 28	PY51
10	120, 40-34, 28	PY50				10	65, 50-48, 40-38	PY62
11	120, 55, 50-48, 40-38, 28	PY51						

From all IgG pattern, Pattern 3 was mostly found in three patients: PY8, PY31 and PY62 while the others IgG patterns were demonstrated in only each individual patient. Pattern 2, 3 and 5 of IgG_1 pattern were mostly found in nine IgG_1 patterns. The pattern 2 was shown in patient PY7, PY8 and PY30 while pattern 3 was presented in patient PY12 and PY31. In addition, the pattern 3 was found in patient PY22 and PY50. Furthermore, the others of IgG_1 pattern were demonstrated in each patient. For the IgG_4 pattern, pattern 2 and 3 were the main profile found in ten IgG_4 patterns. The pattern 3 of IgG_4 pattern was shown in three patients, PY8, PY12 and PY50, while pattern 2 was discovered in two patients as PY7 and PY27. And then, the others of IgG_4 pattern were found in each patient.

After immunotherapy, antigenic pattern of some patients have the change of intensity some bands. From thirteen patients, it was found that eight patients showed the change of intensity band from IgG pattern while the changes of IgG_1 and IgG_4 pattern were found in eleven and five patients, respectively (Table 12A, B &C). To examine the change of antigenic pattern from PIA-treated patients, thirteen vascular pythiosis patients were divided into 2 groups as response groups and non-response groups. The response group could be divided from aftercare of patients into 2 groups as long-term ($\geq$ 14 month) and short-term ($<$ 14 month).

Non-response group:

The change of intensity band of IgGs pattern was found in 1/3 cases, PY7 found .The increasing of band intensity was found at the molecular mass of crude antigen at ~24, ~40-38 and ~40-38 kDa, in the total IgG, IgG_1 and IgG_4 pattern, respectively.

Short-term response group:

Regarding the total IgG pattern, the change of intensity band was observed in 3/6 cases of this group.. The tendency of increasing the band intensity at MW ~55 kDa was demonstrated in PY15 and PY49 after treated with PIA. In contrast to the decreasing band intensity at MW ~40-38 kDa were found in PY49 and PY62. Regarding the IgG_1 the changing of band intensity was seen in. The increasing of band intensity was found at molecular weight ~55 (in PY15, PY49 and PY50), ~40 to 38 (in PY49 and PY50), ~28 and ~24 kDa (in PY27) while the decreasing of the band intensity was found at MW ~55 (in PY31) and ~28 kDa (in PY62). Similarly, the change of the band intensity in IgG_4 pattern was also presented in 2/6 cases of this group. . However, the increasing of band intensity was observed at molecular weight ~50 to 48 and ~40 to 34 kDa (in PY15) while the decreasing of the band intensity was found at MW ~32 to 28 kDa (in PY31).

Long-term response group:

All patients in this group demonstrated the change of the band intensity in total IgG and IgG_1 pattern. Regarding the total IgG pattern, the increasing of band intensity was found at molecular weight ~40 to 38 (in PY5), ~32 to 28 kDa (in PY22) while the decreasing of the band intensity was at MW ~40 to 38 (in PY30 and PY51), ~32 to 28 (in PY30) and ~24 kDa (in PY5). For the IgG_1 pattern, the increasing of band intensity was at molecular weight ~40 to 38 kDa (in PY22) while the decreasing of the band intensity was at MW ~65 (in PY30), ~55 (in PY22 and PY51), ~ 40 to 38 (in PY5 and PY30), ~28 and ~24 kDa (in PY5). The 2 of 4 cases of this group presented the decreasing of band intensity of IgG_4 pattern which was at molecular weight ~50-48 (in PY5), ~40-34 and ~32-28 kDa (in PY5 and PY30).

Table 12A The change of band intensity of IgG pattern from eight patients

Immunogen (kDa)	Trend of intensity band							
	PY5	PY7	PY15	PY22	PY30	PY49	PY51	PY62
55			↑			↑		
40-38	↑				↓	↓	↓	↓
32-28				↑	↓			
24	↓	↑						

Table 12B The change of band intensity of IgG_1 pattern from eleven patients

Immunogen (kDa)	Trend of intensity band										
	PY5	PY7	PY15	PY22	PY27	PY30	PY31	PY49	PY50	PY51	PY62
65						↓					
55			↑	↓			↓	↑	↑	↓	
40-38	↓	↑		↑		↓		↑	↑		
28	↓				↑						↓
24	↓				↑						

Table 12C The change of band intensity of IgG_4 pattern from five patients

Immunogen (kDa)	Trend of intensity band				
	PY5	PY7	PY15	PY30	PY31
50-48	↓		↑		
40-34	↓	↑	↑	↓	
32-28	↓			↓	↓

CHAPTER VI

DISCUSSION

P. insidiosum is the etiologic agent causing pythiosis in mammalian including human [1]. According to the geographical area, this organism was divided by ITS region and *COX* II gene into 3 clades; A_{TH} (I), B_{TH} (II) and C_{TH} (III) [10, 11]. Most of the organisms in clade B_{TH} and C_{TH} were isolated from environment and human pythiosis patients in Thailand. The immunotherapy of these human pythiosis patients was a treatment with endogenous and exogenous PIA which was prepared from type strain in clade A_{TH} which the members in these clades are from United State [14, 18]. The hyphae morphology of this organism is similar to the typical structure of mould. This reason is a cause of misdiagnosis. The serological tests, such as immunodiffusion (ID) test, western blot and ELISA, have been developed to diagnose vascular and systemic pythiosis. The ELISA technique provides higher sensitivity but less specificity result when compares with western blot assay, whereas ID is simple test with less sensitivity and high specificity. To develop the higher specificity diagnosis in ELISA, the better specific antigen which is able to recognize antibody is one chance to be tried. In 2002, Krajaejun et. al. established in-house ELISA for pythiosis diagnosis. Their antigens were prepared from soluble antigen broken hyphae (SABH) of *P. insidiosum* Thai strain with unidentified clade. From their results, in-house ELISA had high sensitivity (100%) and specificity (100%) with 15 pythiosis sera and 120 sera from healthy blood donors. In 2009, Chindamporn et. al. reported the antigenic variation of *P. insidiosum* and antibody diversity of host [29]. Later, Koitio and his coworker investigated the seroprevalence of pythiosis patients against endo-and exo-antigen from three clades of *P. insidiosum*. The antigen from clade A_{TH} was prepared from *P. insidiosum* ATCC58643 which is the same

strain as PIA for immunotherapy. While the antigens from clade B_{TH} and C_{TH} were prepared from clinical isolates. Their results found that the antigen from clade C_{TH} as antigen to detect specific *P. insidiosum* antibody. The sensitivity and ELISA had (94.73%) and specificity (99.32%) [65]. Our goal of this study is to investigate of immune response against endogenous antigen, prepared from each of three clades of *P. insidiosum*, in patients receiving immunotherapy (Table 3). To examine optimum antigen of *P. insidiosum* for quantitative specific IgGs isotype determination using ELISA, three strains of endogenous *P. insidiosum* were prepared. From the results, we found that C-PC antigen (clade C_{TH}) was the optimum antigen for the quantitative study for total IgG and IgG_1 antibody while the quantitative study of IgG_4 antibody was not found the different of three crude antigens. Regarding the etiologic agent which could be isolated from these 13 patients, most of them were classified in clade A_{TH} and clade B_{TH}. However, the sera from most of these patients recognized C-PC antigen because this antigen was classified into clade C_{TH} which was mostly found in Thai patients [66].

From previous studies, most of animals and patients with immunotherapy showed switching of cytokine secretion which indicated the switching of T_H2 to T_H1 response [19, 20, 51]. By theory, the T_H2 or T_H1 response activated the switching IgG subclass [27, 28]. Human plasma cells produced IgG_1 and IgG_3 antibody via T_H1 activation while production of IgG_4 and IgE antibody were activated by T_H2 response. So, to investigate the immune response of Thai patients with immunotherapy, C-MTPI19 antigen was tested. This strain was used to prepare the PIA for treatment. As far as the literatures were reviewed, no report related to the IgG subclass antibody to *P. insidiosum* has been found. The IgGs antibody production was up to the immune response of each individual. In this study, we defined the change of IgGs antibody was more or less than 2 times of the amount of the IgGs antibody from pre-vaccination serum. From the results, the tendency of total IgG and IgG_1 in non-response group has two forms as no change and

decreasing while IgG_4 antibody was not significant variation of the level. In short-term response group demonstrated the increasing level of IgGs antibody (PY27, PY31) whereas the other cases showed no significant difference. For the long-term response group shows three forms as increasing (PY22), decreasing (PY30) and no change (PY5, PY51). From the results of ELISA, could not demonstrate the immunogen of *P. insidiosum* because it is semi-quantitative technique. And then the amounts of specific *P. insidiosum* IgG subclass have a few in the serum pythiosis patients. To study the predominance antigen of this organism against IgG isotype antibodies from patients with PIA-treated, the concentration of IgGs antibody from ELISA were used to predict the titer dilution serum for western blot analysis.

The first *P. insidiosum* antigenic pattern using western blot analysis were demonstrated by Mendoza et.al. They reported that the IgG antibodies from five sera of equine pythiosis reacted with antigen ranged in 68 to 14 kDa. The specific IgG antibody form horses receiving immunotherapy recognized immunodominant antigen at molecular weight 32 kDa, 30 kDa, and 28 kDa [60]. In 2004, Vanittanakom et.al. showed the antigens at sizes 110, 73, 56, 42 to 35, 30 to 28, 26, and 23 kDa were recognized by IgG antibody from pythiosis patients while antigen were strongly reacted with antibody [67]. Krajaejun et.al. in 2006, found that ~74 kDa was an important antigen of *P. insidiosum* which this antigen was strongly reacted with IgG antibody from pythiosis patients and then was identified as exo-1,3-β glucanase [30, 31]. However, immunogen of *P. insidiosum* against IgG isotype antibody from pythiosis patients with immunotherapy has a one report. Thus, to examine the immunogen recognized by specific IgGs antibody, the western blot analysis was preformed. From the result of protein profiles of C-MTPI19 (clade A_{TH}) on the 12.5% SDS-PAGE gel, the predominant proteins wereat size ~120, ~65, ~55, ~48, ~43-~41, ~32-28, ~24 and ~20 kDa. This result was corresponded

to the reported by Chindamporn et al. They also used the same strain and same protocol for protein preparation. [29].

Due to the antigen variation of this organism and the antibody diversity of individual host, the investigation of immune response in patient is hard. However, this study show C-MTPI19 was the optimal antigen for western blot analysis. Nevertheless, this study indicated the important of antigen selection for the difference technique. By the results from ELISA show that the C-PC was the optimum antigen for the determination of amount of total IgG antibody and IgG_1 antibody while the western blot analysis indicated that C-MTPI19 was optimum antigen for specific antigen study. The inconsistency of optimum antigen from ELISA and western blot analysis might due to the difference form of protein. The coated proteins for ELISA were the native forms whereas the proteins for western blot analysis were denatures form because of adding the sample buffer as consist of DTT and SDS and then boiling at 100^{o}C in the preparation step [68]. The different form of protein showed the difference epitope sites which cause the ability of antibody binding.

From the western blot analysis, the non-specific bands revealed from IgG and IgG_1 antibody of ten healthy volunteer sera were at the sizes d ~95 and ~43 kDa while those from IgG_4 pattern were at the sizes of ~120, ~95, ~55 and~43 kDa. These non-specific bands were used to subtract from the IgGs pattern obtained from vascular pythiosis patients. The common bands of C-MTPI19 crude antigen against specific total IgG from twelve pre-vaccination vascular pythiosis were the single band at MW ~120, ~55 and ~40-~34 kDa. The high molecular mass at MW of single band ~120 was recognized by IgG_1 antibody while the low molecular mass at MW ~40-~34, ~32-~28 and ~24 kDa were reacted with specific IgG_4 antibody. The previous study by Krajaejun et.al., found that the immunodominant at MW ~74 kDa in all pythiosis patients as identified to the exo-1,3-β glucanase [30, 31]. From our study, found this

immunodominant was less detected the specific IgGs antibody due to the different of methodology of protein preparation.

The determination of specific IgGs antibody responded pattern of difference period sera from vascular pythiosis patients are hard to analysis these patterns because the base line of immunity each patients were different. Furthermore, this organism has the variation of antigen in each strain of *P. insidiosum*. To avoid the antigen variation of this organism, C-MTPI19 crude antigen was used to this study. For the vascular pythiosis patients, Eleven antigenic patterns recognized by IgG antibody were demonstrated whereas nine and ten profiles recognized by IgG_1 and IgG_4, in order, were found (Figure 33 & Table 11). All the total IgG pattern demonstrated the predominance antigen as high molecular mass at the single band ~120kDa while IgG_1 profile, the strong recognized bands were at ~120 kDa and ~55kDa, respectively. The antigenic patterns from sera of pythiosis patients with immunotherapy were not conserved. However, it is of noted that some variable band was observed in some sera from PIA-treated patients such as ~50 -~48, ~40-~34 and ~30-~28 due to the variation of recognize antibody in each patients (Table 12A, B and C).

From the previous study, the immune response of equine pythiosis [18] and human pythiosis [19, 20, 42] with immunotherapy showed the switching of immune respose from T_H2 to T_H1 which this switching was proved by the secretion of cytokine. However, the recent study of Bach et.al. showed that the determination of Ecto-adenosine deaminase (E-ADA) activity was in lymphocytes from rabbits with pythiosis and controls. The rabbits were induced pythiosis by *P. insidiosum* zoospores inoculation and were cured by immunotherapy. Their result demonstrated the increasing of E-ADA activity as the increasing of ATP, and the decreasing of adenosine after immumotherapy which indicated the shift of immune response from T_H2 to T_H1 in rabbits with immunotherapy [69]. From our results cannot show the switching of T_H2 to T_H1 response

in pythiosis patients with immunotherapy due to the diversity of antibody against antigen from *P. insidiosum* and the different of individual's baseline immune response. However, this study demonstrated the predominant antigens (~120 kDa and ~55 kDa) and the other antigen at low molecular weight which were important antigen for diagnosis or monitoring. Thus, the purification and identification of predominance antigen from this study should be done in the future.

CHAPTER VII

SUMMARY

The objective of this study is the IgGs isotype against endogenous antigen of *P. insidiosum* in pythiosis patients with immunotherapy. ELISA and western blot techniques were used for determination concentration of specific IgGs antibody and their antigenic pattern, respectively. Endogenous antigens of the organism were prepared from three clades, A_{TH}: C-MTPI19, B_{TH}: C-PMS and C_{TH}: C-PC, of *P. insidiosum* isolations. These crude antigens were tested with specific antibody from thirteen vascular pythiosis. The quantitative study of specific total IgG and IgG_1 antibody from all vascular pythiosis recognized the C-PC antigens more than C-MTPI19 and C-PMS while recognition of specific IgG_4 antibody were not different in three crude antigens. On the other hand from western blot analysis found that the antigenic pattern results of total IgG, IgG_1 and IgG_4 antibody from vascular pythiosis patients shown the predominance antigen at MW ~120, ~55 ~40-~34 and ~32-~28 and ~24 kDa. For the study antigenic pattern recognized by IgGs isotype from patients with immunotherapy, the total IgG, IgG_1 and IgG_4 antigenic pattern from individual patients have the variation pattern. Four bands, ~120, ~55 and ~40-~34 kDa, only one band, ~120 kDa, and three bands, ~32-~28 and ~24 kDa, were commonly found against total IgG, IgG_1 and IgG_4 antibody, in order, in all 13 pre-vaccination sera. In contrast, the variation of profile against IgG (11 profiles), IgG_1 (9 profiles) and IgG_4 (10 profiles), antibody in post-vaccination sera in both number and the density were revealed. These were found mostly in the low molecular weight antigen, ~55, ~40-~34 and~28 kDa. Since the limitation of the case number and their different clinical status, it was hardly to conclude the switching T_H2 to T_H1 response. More study of their cytokine is required.

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APPENDICES

APPENDIX A

BUFFER AND REAGENT

Reagents for Protein preparation

1. Sabouraud Dextrose Agar (SDA)
   - Dehydrated SDA agar 65 g.
   - DW 1000 ml.
   - Sterilized by autoclaving 121 °C 15 minutes

2. Sabouraud Dextrose Broth (SDB)
   - Dehydrated SDB 30 g.
   - DW 1000 ml.
   - Sterilized by autoclaving 121 °C 15 minutes

3. PBS buffer (1x) 500 ml.
   - 10x PBS 50 ml.
   - DW 450 ml.
   - Sterilized by autoclaving 121 °C 15 minutes

4. Sample buffer with DTT (6x)

- 4X Tris-HCl pH 8.8 7 ml.
- Glycerol 3 ml.
- SDS 1 g.
- DTT 0.93 g.
- Bromphenol Blue 1.2 mg.
- Dissolve in distilled water and adjust volume to 10 ml. Store at room temperature.

Reagents for ELISA

1. PBS buffer (10x) 1000 ml.

- NaCl 80 g.
- KCl 2 g.
- KH_2PO_4 1.4 g.
- Na_2HPO_4 9.1 g.
- DW 1000 ml.
- Sterilized by autoclaving 121 °C 15 minutes

2. PBS buffer (1x) Tween 20 1000 ml.

- 10x PBS 100 ml.
- DW 900 ml.
- Tween 20 500 µl.
- Sterilized by autoclaving 121 °C 15 minutes

3. Coating Buffer 200 ml.

   - Na_2CO_3 0.318 g.
   - $NaHCO_3$ 0.586 g.
   - NaN_3 0.04 g.
   - DW 200 ml.
   - Sterilized by autoclaving 121 °C 15 minutes

4. IgG_1 protein

   - IgG_1 protein
   - Coating buffer

5. IgG_4 protein

   - IgG_4 protein
   - Coating buffer

6. Citric acid for soluble Substrate : OPD

   - Citric acid 3.63 g.
   - $Na_2 HPO_4$ 4.735 g.
   - DW 500 ml
   - Sterilized by autoclaving 121 °C 15 minutes

7. Blocking Buffer

   - 2% Skim milk
   - PBS buffer 1x Tween 20

8. Dilution serum

   - 0.5% Skim milk
   - PBS buffer 1x Tween 20

9. Conjugate : Anti human IgG (Dako : P0214)

   - Anti- human IgG (Dako : P0214) 1:2000
   - PBS buffer 1x Tween 20

10. Conjugate with biotin : Anti human IgG_1

    - Anti-human IgG_1 1:5000
    - PBS buffer 1x Tween 20

11. Conjugate with biotin : Anti human IgG_4

    - Anti-human IgG_4 1:4000
    - PBS buffer 1x Tween 20

12. Streptavidin conjugated with HRP

    - Streptavidin –HRP 1:4000
    - PBS buffer 1x Tween 20

13. Stop reaction : 4N H_2SO_4

    - Conc. H_2SO_4 12 ml.
    - DW 88 ml.

Reagents for SDS- Polyacrylamide Gel Ectrophoresis (SDS-PAGE)

1. 1 M Tris-HCl pH 8.8
   - Tris base 12.11 g.
   - DW 100 ml.
   - Adjust pH 8.8 with conc.HCl and Sterilize the solution by autoclaving at 121 ºC for 15 minute.

2. 0.5 M Tris –HCL pH 6.8
   - Tris base 6.055 g.
   - DW 100 ml.
   - Adjust pH 6.8 with conc.HCl and Sterilize the solution by autoclaving at 121 ºC for 15 minute

3. 10% Ammonium Persulfate (APS)
   - APS 1 g.
   - DW 10 ml.

4. 10% Sodium laury sacosine (SDS)
   - Sodium laury sacosine (SDS) 1 g.
   - DW 10 ml.

5. 12.5% Separating gel (10 ml.)

- Distilled water	3.15	ml.
- 1.5 M Tris-HCl	2.5	ml.
- 30% Bis-acrylamide	4.2	ml.
- 10% SDS	50	µl.
- 10%APS	50	µl.
- TEMED	5	µl.

6. 4% Stacking gel (10 ml.)

- Distilled water	2.9	ml.
- 0.5 M Tris-HCl	1.25	ml.
- 30% Bis-acrylamide	0.8	ml.
- 10% SDS	50	µl.
- 10%APS	50	µl.
- TEMED	5	µl.

7. Running Buffer (10x) pH 8.5

- Glycine	144	g.
- SDS	30	g.
- DW	1000	ml.

8. Coomassiae Brillian blue R for stain gel

- Coomassiae Brillian Blue R	0.1	g.
- Methanol	40	ml.
- Acetic acid	10	ml.
- DW	50	ml.

9. Destain gel I

- Methanol	40	ml.
- Acetic acid	40	ml.
- DW	320	ml.

10. Destain gel II

- Methanol	80	ml.
- Acetic acid	40	ml.
- DW	280	ml.

Reagents for Western Blot

1. Transfer Buffer (10) pH 8.3

- Tris-base	72.5	g.
- Glycine	36.25	g.
- SDS	4.626	g.
- DW	1000	ml.

2. Transfer buffer (1x)

- 10x Transfer buffer	30	ml.
- DW	270	ml.
- Methanol	75	ml.

3. TBS (1x) pH 7.5

- Tris-base	12.11	g.
- NaCl	8.766	g.
- DW	1000	ml.

4. TBS buffer (1x) Tween 20 : TBS-T

- 1x TBS	100	ml.
- Tween20	100	µl.

5. Blocking solution

- 10% Skim milk
- 1x TBS-T

6. Solution for diluted serum

- 5% Skim milk
- 1x TBS-T

7. Conjugate anti-human IgG and IgG subclasses with Biotin

   - Antihuman IgG and IgG subclass 1:5000
   - 1x TBS-T

8. Streptavidin conjugated Alkaline phosphatase

   - Streptavidin conjugated AP 1:1000
   - 1x TBS-T

9. Substate : BCIT/NBT

   - Buffer for BCIT/NBT 1000 ml.
   - BCIT solution 100 µl.
   - NBT solution 100 µl.

10. Coomassiae Brillian blue R for stain PVDF membrane

    - Coomassiae Brillian Blue R 0.05 g.
    - Methanol 25 ml.
    - DW 25 ml.

11. stain membrane

    - Methanol 50 ml.
    - DW 50 ml.

APPENDIX B

WESTERN BLOT ANALYSIS

Antigenic pattern of thirteen vascular pythiosis patients

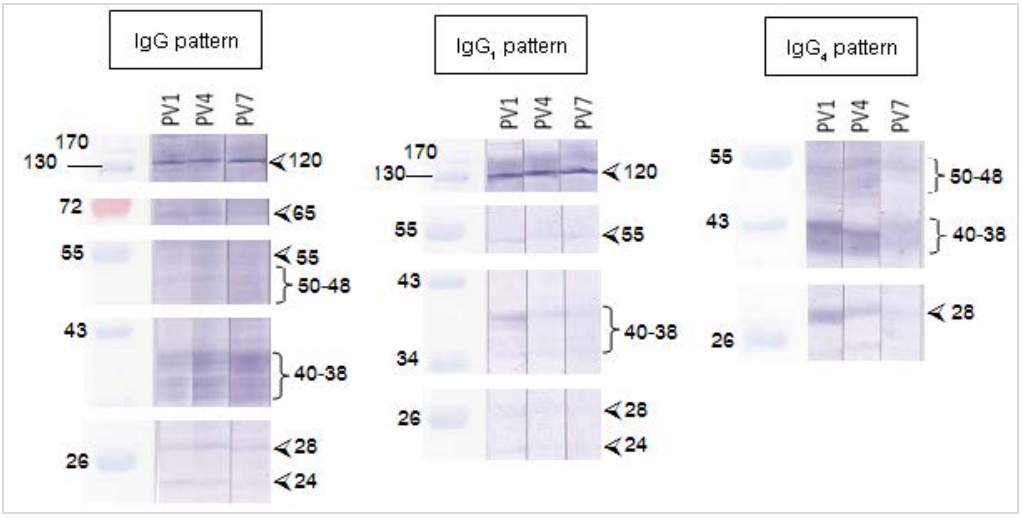

Figure 33 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY5 sera.

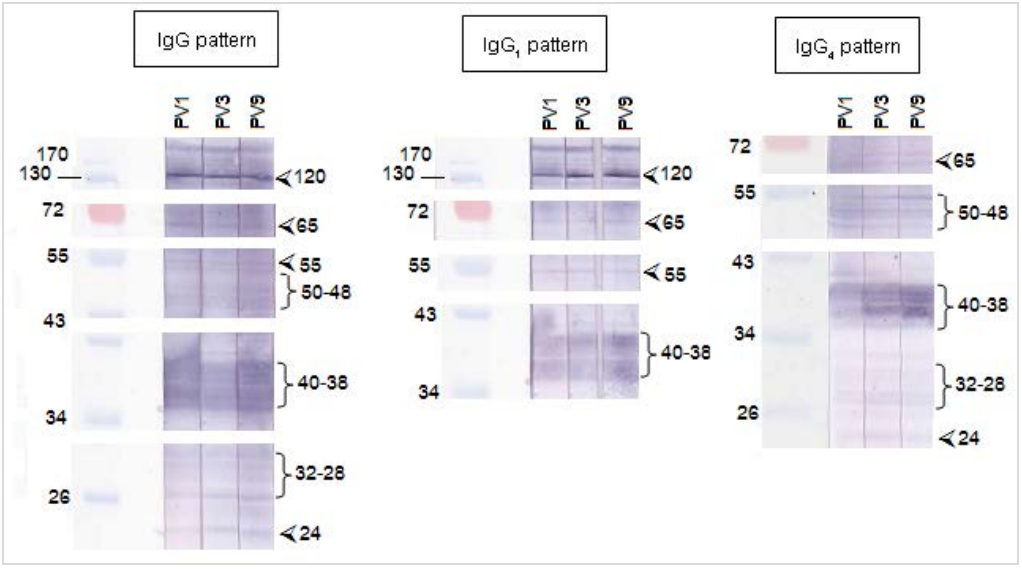

Figure 34 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY7 sera.

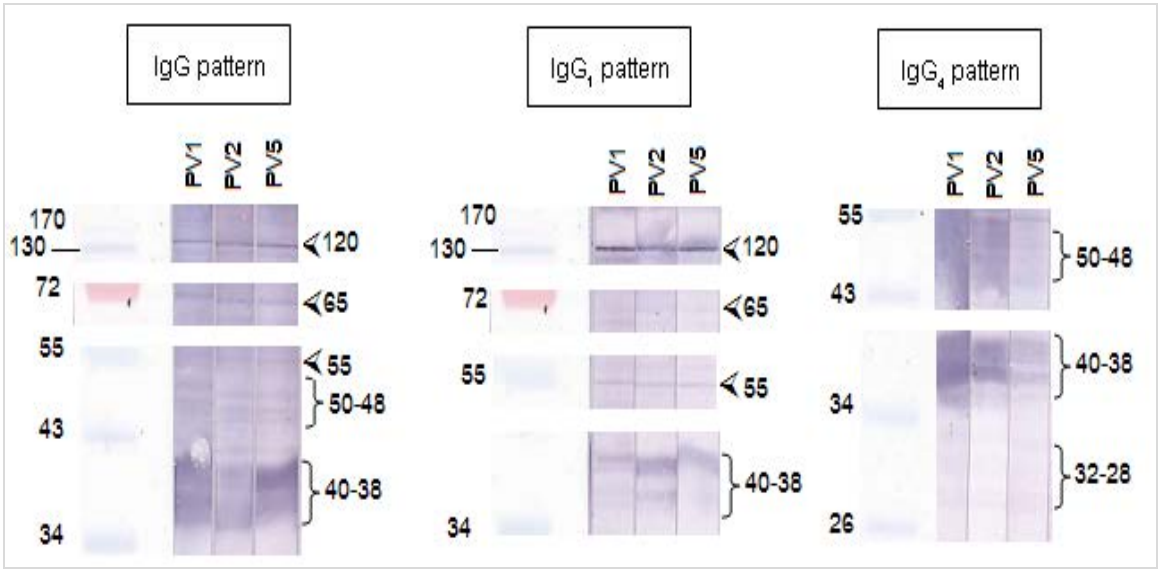

Figure 35 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY8 sera.

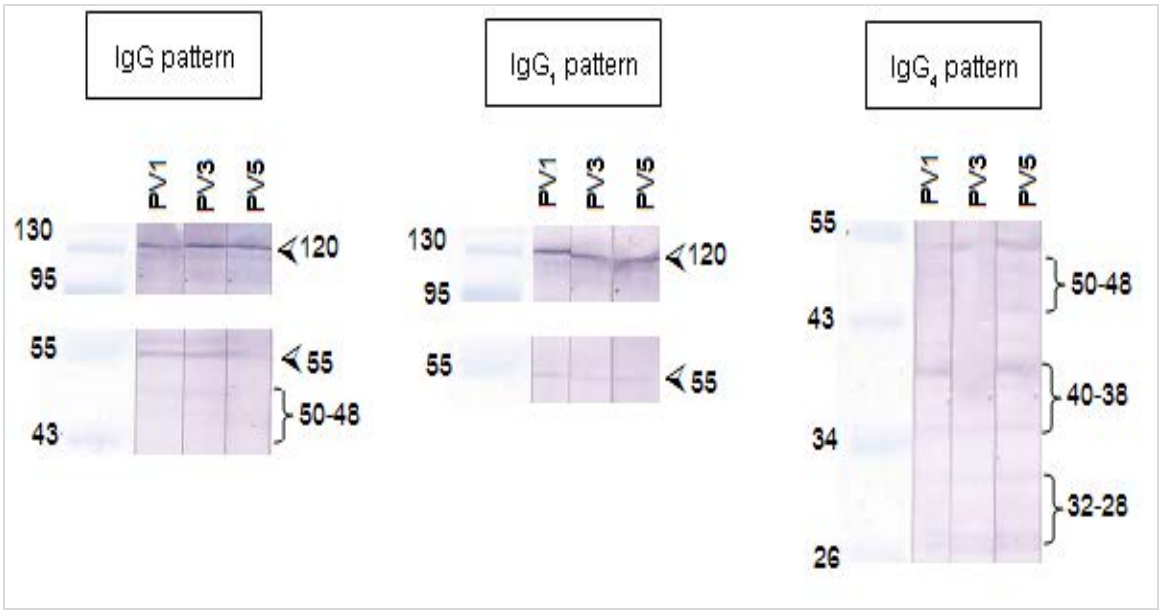

Figure 36 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY12 sera.

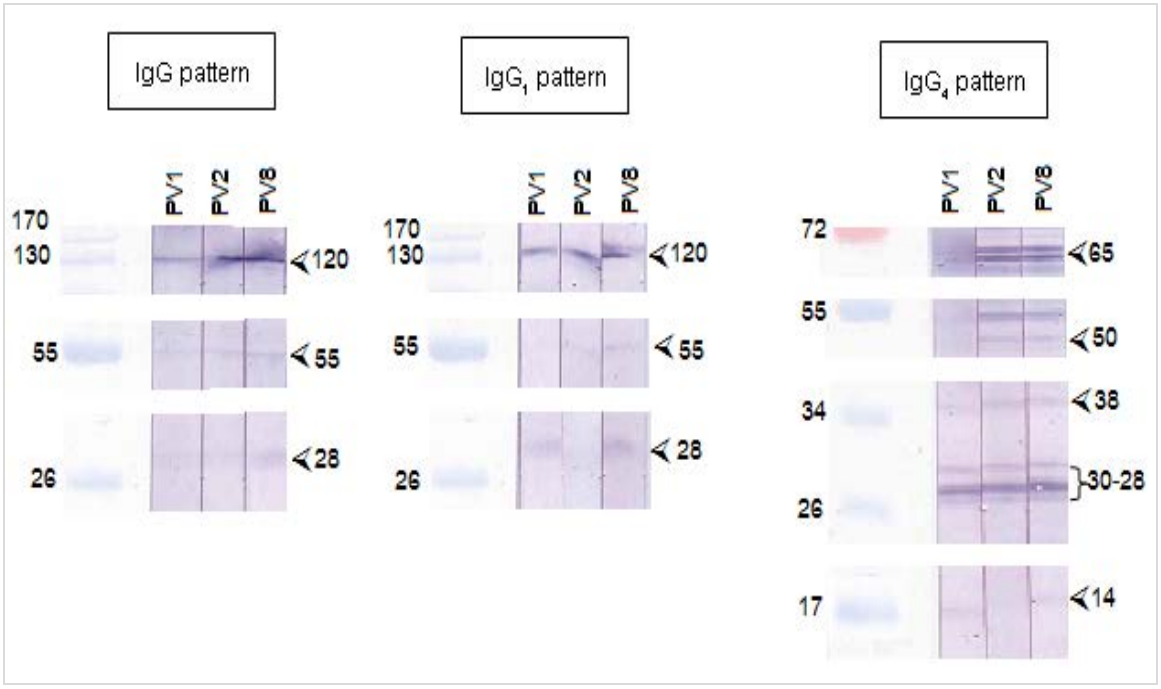

Figure 37 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY15 sera.

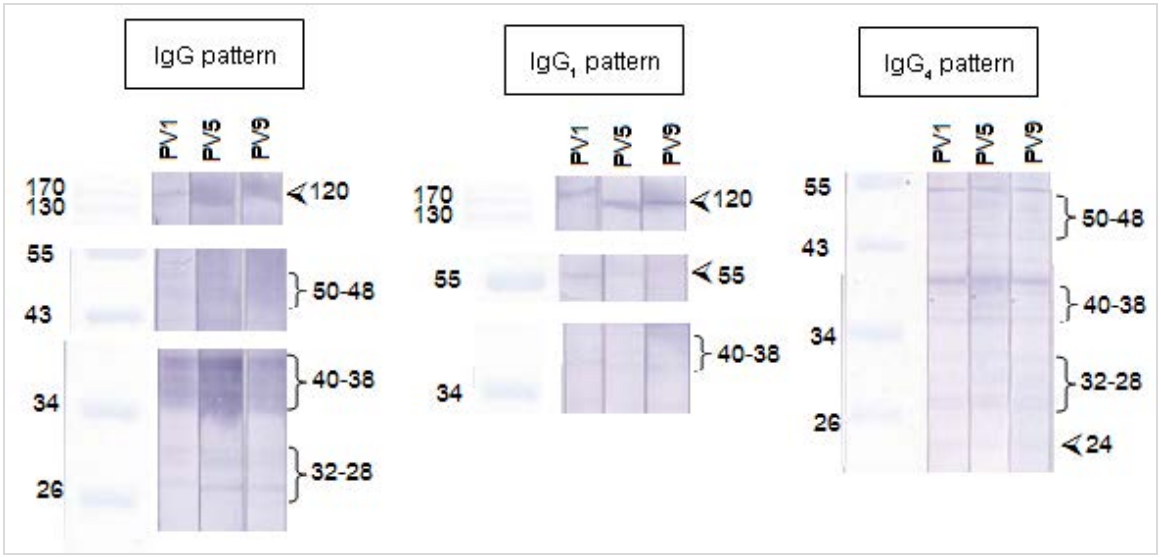

Figure 38 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY22 sera.

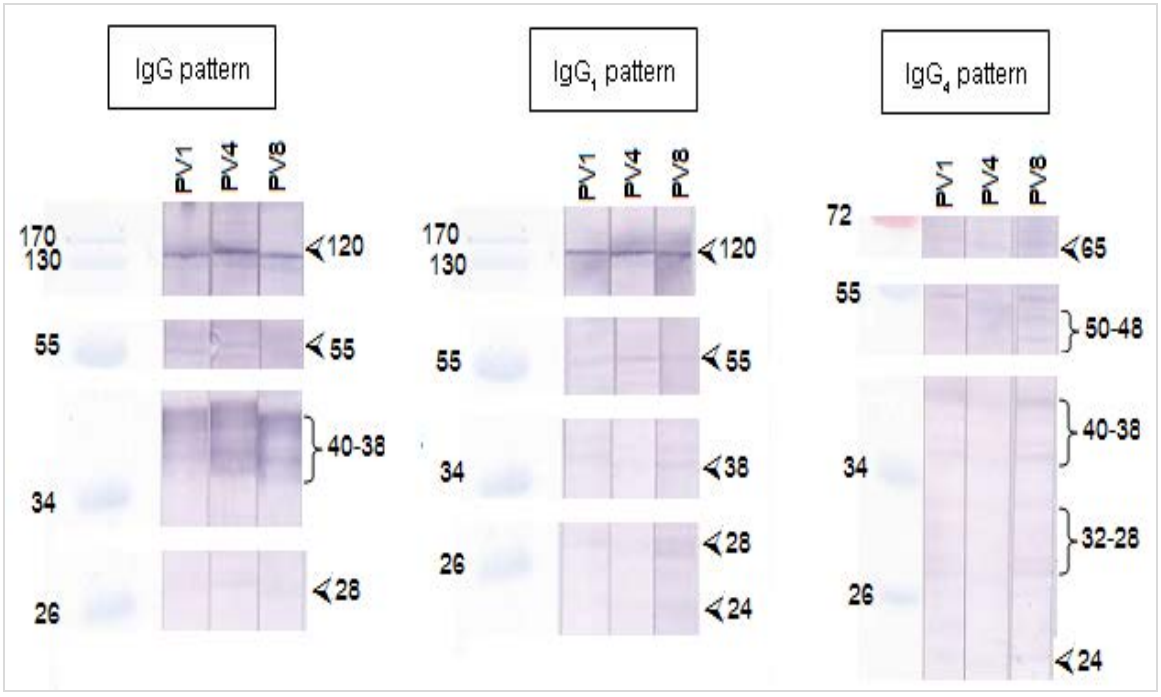

Figure 39 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY27 sera.

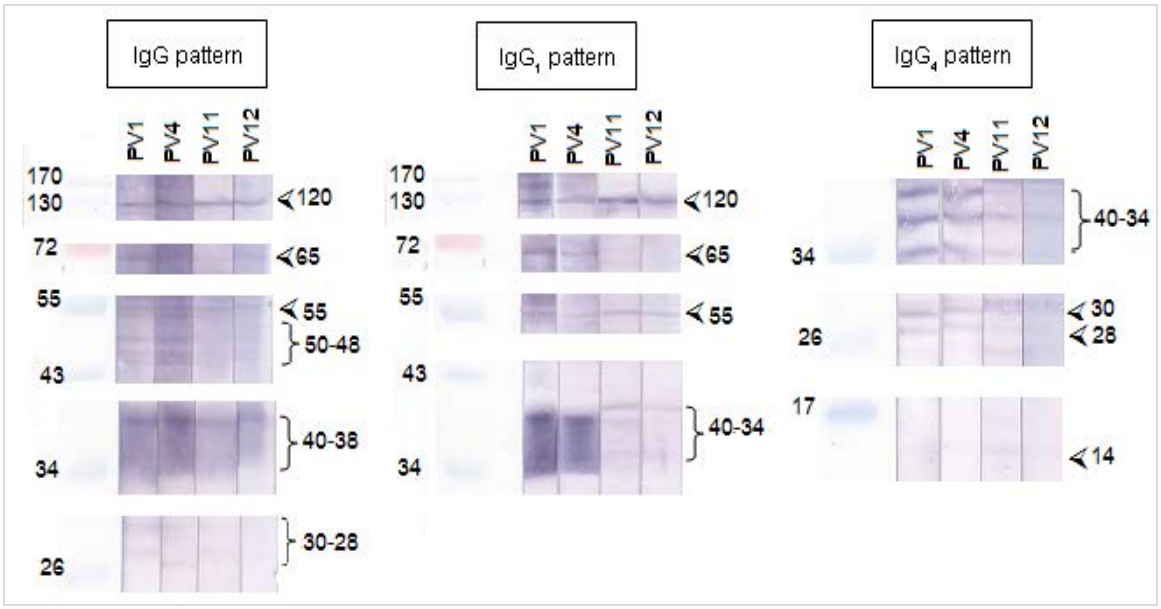

Figure 40 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY30 sera.

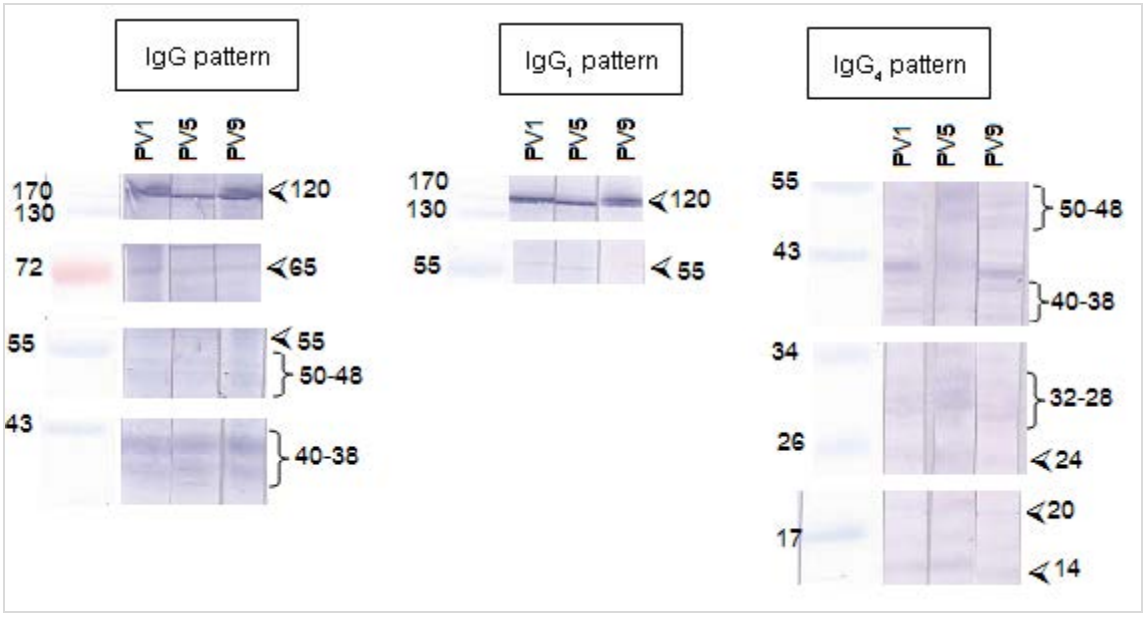

Figure 41 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY31 sera.

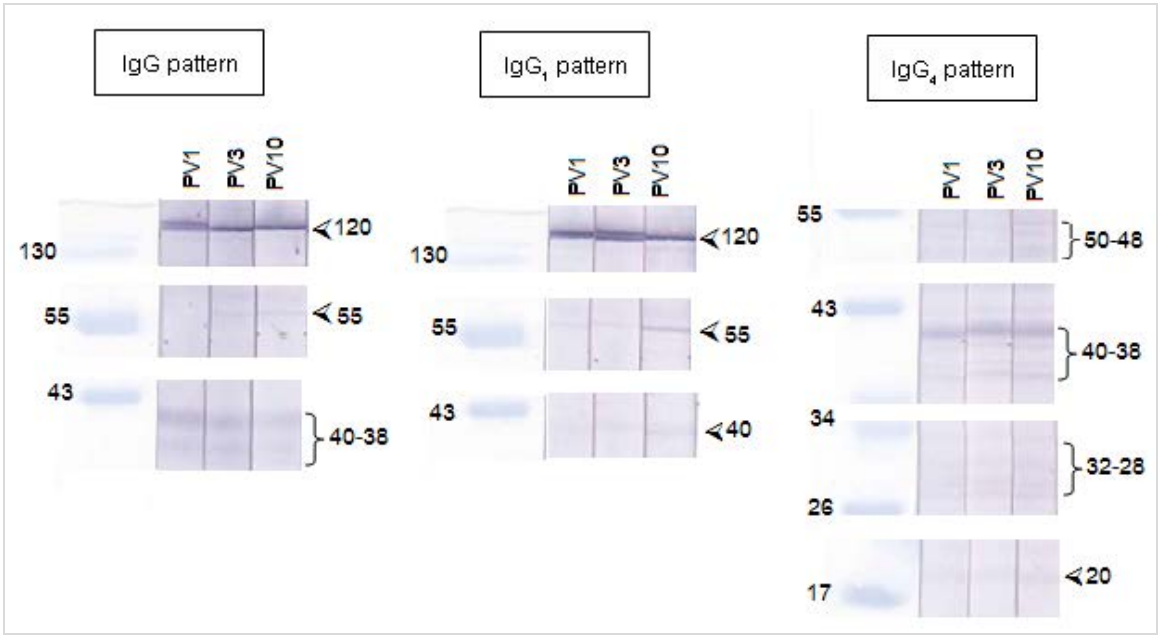

Figure 42 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY49 sera.

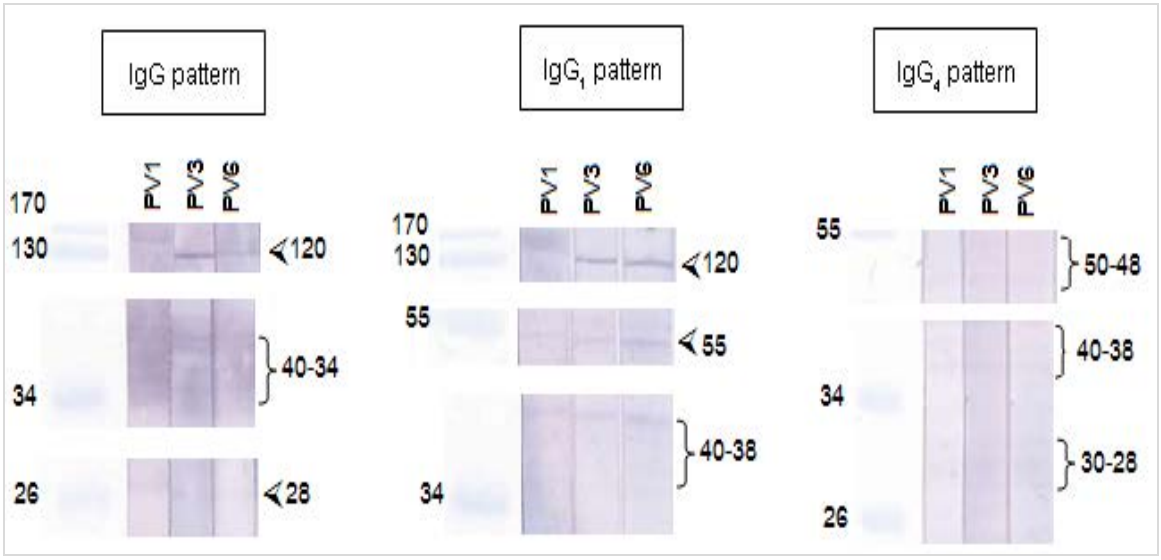

Figure 43 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY50 sera.

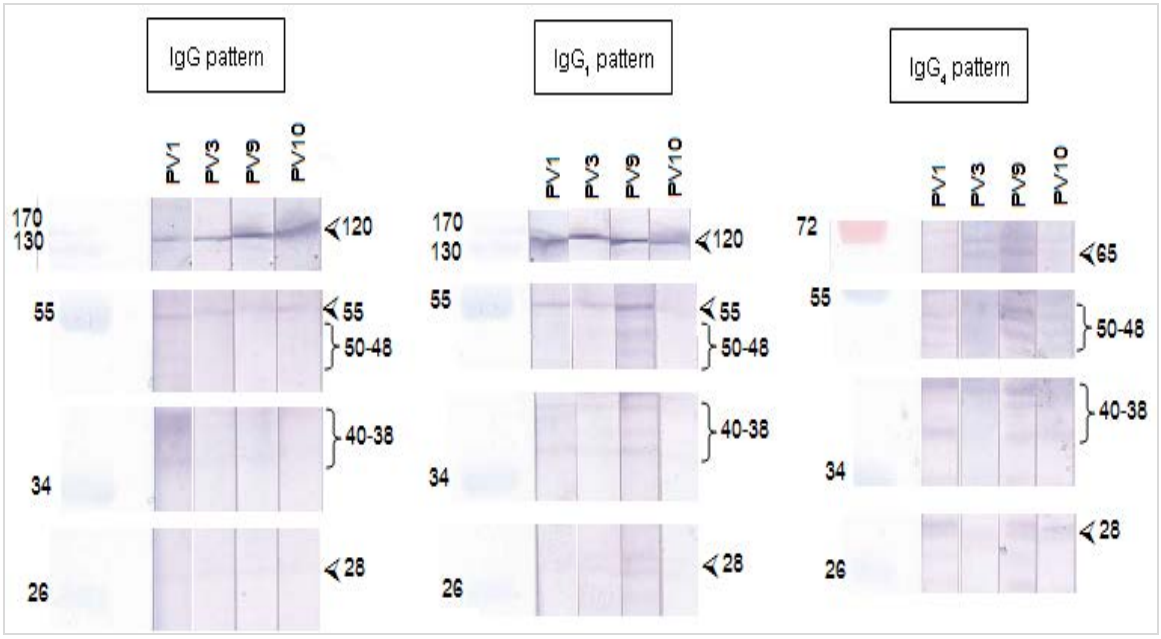

Figure 44 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY51 sera.

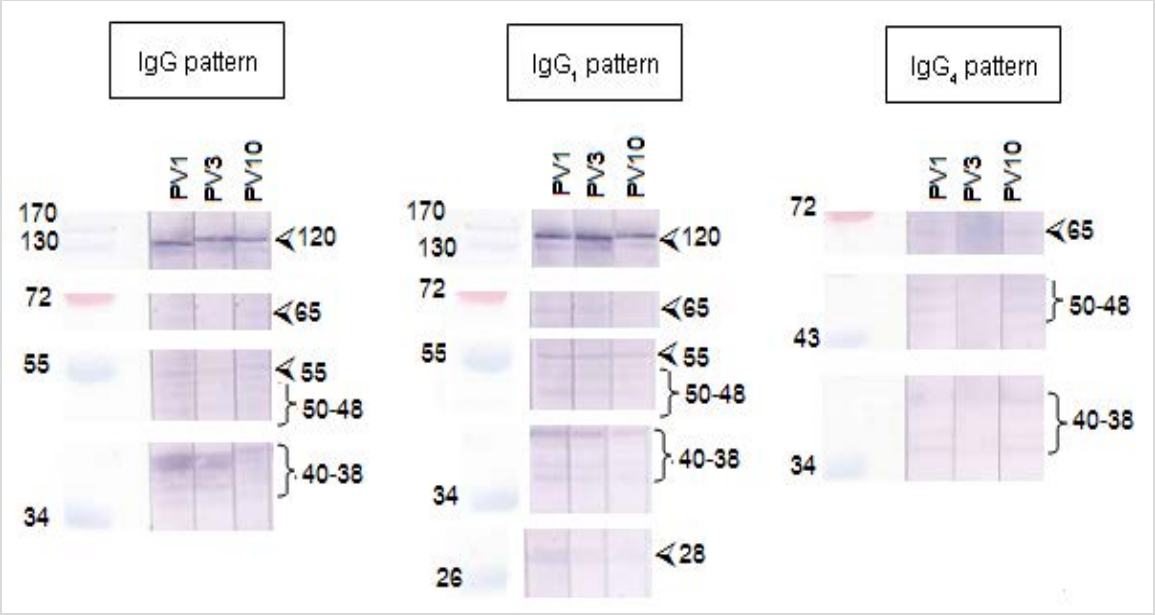

Figure 45 Antigenic patterns of total IgG, IgG_1, and IgG_4 antibody from PY62 sera.

BIOGRAPHY

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PUBLICATION	-