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Cytochrome Oxidase 2 (*COX2*), β-Tubulin (*TUB*) and Chitin Synthase Subunit 2 (*CHS2*) Expression in *Pythium insidiosum* Thai Strains

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Abstract

This study aimed to investigate the real-time fluctuation of temperature-sensitive gene expression in a *Pythium insidiosum* growing at human body temperature (37 ºC) when it can cause life-threatening disease, whereas its natural habitat is in agricultural water sources with lower temperature. Genes encoding cytochrome oxidase 2 (*COX*2), β-tubulin (*TUB*), and chitin synthase subunit 2 (*CHS*2) were tested for their expression at the infection temperature (37 ºC) and natural habitat temperature (27 ºC) using real-time RT-PCR. Sixteen strains of *P. insidiosum*, representing 3 phylogeographic preferences, were analyzed. The subculturing process at 27 ºC was repeated at least 2 - 3 cycles. The results showed that at 37 ºC, the *COX*2 was expressed 2.5-fold higher than that of 27 ºC (p = 0.0347) while the level of *TUB* and *CHS*2 mRNA disclosed indistinguishable profiles. These results suggested that these genes were suitable to be used as housekeeping genes for temperature-susceptible gene expression studies. This work was likely to be the 1st study examining the gene expression levels of the Thai strain of *P. insidiosum* under thermal stress conditions. Follow-up studies for the *COX*2 genes may useful to provide valuable insight into the pathogenesis, diagnostic, or therapeutic targets for further investigation.

Keywords: Chitin synthase 2, Cytochrome oxidase 2, Expression, *Pythium insidiosum*, Tubulin

Introduction

Pythium insidiosum (*P. insidiosum*) has been reported as a human and animals' pathogen [1-3]. The disease caused by this pathogen, called "pythiosis", is found in the tropical, subtropical, and temperate regions of the world [4,5]. The phylogeographic preferences of *P. insidiosum* isolates from different geographic regions were revealed the existence of three phylogenetic using an internal transcribed spacer (ITS), intergenic spacer (IGS) sequences, and cytochrome oxidase (*COX*2) gene [6]. Recently, phylogenetic analysis of the environmental strains of *P. insidiosum* using the ITS regions and revealed that these strains have phylogenetic features in common with the clinical isolate recovered from humans and animals [6,7]. Clinical manifestations of pythiosis include cutaneous/subcutaneous, ocular, vascular, and disseminated forms. Human pythiosis cases are frequently detected in Thailand. Vascular pythiosis is the most common manifestation of human pythiosis, which almost always occurs in patients with underlying hemoglobinopathy complicated by hemochromatosis [8,9].

The life cycle of *P. insidiosum* consists of 2 phases, each with a unique range of temperature: A saprophytic phase in the aquatic reservoir with a temperature range of 25 - 30 ºC, and a parasitic phase in mammals at approximately 37 ºC [10]. It is already known that the ability of pathogenic microorganisms to grow at 37 ºC is a prerequisite significant factor to cause disease in hosts, whereas the failure to grow at 37 ºC is a predictive indicator of attenuated virulence. Our preliminary experiment showed that the growth of the 1st isolates of *P. insidiosum* from human tissue biopsy at 37 ºC was significantly faster than incubation at 27 ºC. If the virulence is related to the ability to grow at 37 ºC, then all clinical and some potentially virulent environmental strains of *P. insidiosum* will have the ability to survive at the expected higher temperature to express possible virulence characteristics.

Based on recently available data, with an emphasis on the ability to survive at different temperatures (27 and 37 ºC), 3 distinct clades based on geographical areas were reported but no data has been published on studies at the genetic level. In this study, we determined candidate genes Cytochrome oxidase 2 (*COX*2), β-tubulin (*TUB*), and chitin synthase 2 (*CHS*2) which may be temperature-sensitive gene expression levels at 37 ºC from each clade of *P. insidiosum*, using real-time RT-PCR. The results of this study present the fundamental information that could be used to support future research, leading to a better understanding of the mechanisms of this organism to cause disease which may lead to better treatment.

Materials and methods

Strains

Sixteen isolates of *P. insidiosum* from clinical (human and animals) and environmental sources were used in this study. All of these aquatic fungus-like organisms were isolated from the proven cases of pythiosis, confirmed by western blot analysis. All strains were tested for their ability of zoospore production and maintained on Sabouraud dextrose agar (SDA) (Bio-Rad) at 37 ºC. To prepare the isolate for the quantitative gene expression study at 27 ºC, the submerged fungal-like colonies growing from SDA at 37 ºC were subcultured on SDA and incubated for 7 days at 27 ºC. The subculturing process at 27 ºC was repeated at least 2 - 3 cycles.

RNA extraction

The 7-day-old colonies of each active strain were inoculated into 300 mL. Sabouraud dextrose broth (SDB) (Difco) and incubated at 37 or 27 ºC depending on the original incubation temperature and shook at 155 round/min (rpm) for 5 days in a shaking incubator. Prior to RNA extraction, the mycelial masses were filtered using a 0.22 µm pore size PES membrane filter (Corning) and ground in liquid nitrogen. To extract RNA, a hot phenol extraction method [11] was used. The precipitation step was performed twice as well, at −20 and −70 ºC using RNA precipitation solution of cold isopropanol. Finally, the pellets were suspended in sterile RNase–free water. The quality and quantity of this total RNA were measured by using denatured gel electrophoresis and NanoDrop spectrophotometer (Thermocycler). RNA concentration was calculated based on the optical density (OD) at A260 using Beer-Lambert law [12].

First-strand cDNA synthesis and real-time reverse-transcriptase PCR (real-time RT-PCR)

The DNase digestion reaction using RQ1 RNase-Free DNase (Promega) was set up following the manufacturer's protocol. The cDNA was synthesized from an mRNA template using The RevertAidTM 1st Strand cDNA Synthesis kit (Fermentas). The 20 µL reaction mixture contained 3µg DNA-free RNA and 0.5 µg Oilgo (dT)18 primer, 1x Reaction Buffer, 20 U RiboLockTM Ribonuclease Inhibitor, 1 mM dNTPs and 10 U RevertAidTM A-MuLV Reverse Transcriptase. The reaction was incubated at 42 ºC for 1 h and followed by incubating at 70 ºC for 5 min. To quantify the level of mRNA expression, the LightCycler FastStart DNA Master SYBR Green I kit (Roche) was used for this experiment. The PCR mixture for a 20 µL standard reaction contained 1x LightCycler FastStart DNA Master SYBR Green I, 0.2 µM of each primer **(Table 1)**, 0.2 µM of $MgCl_2$ and 2 µg of cDNA templates. The program consisted of an initial pre-incubation step (10 min at 95 ºC), followed by 45 PCR cycles (10 s at 95 ºC, 10 s at 55 ºC and 25 s at 72 ºC) and a melting curve step (55 - 95 ºC), then cooling to 40 ºC.

Table 1 List of primers used for real-time PCR of the *COX* 2, ITS regions, *TUB*, and *CHS*2.

Primer	Sequence (5'-3')	PCR product (bp)
***COX* 2 region**		
Forward: FM58	5'- CCACAAATTTCACTACATTGA- 3'	600
Reverse: FM66	5'- TAGGATTTCAAGATCCTGC- 3'	
ITS region		
Forward: ITS-1	5'- TCCGTAGGTGAACCTGCGG- 3'	550
Reverse: ITS-4	5'- TCCTCCGCTTATTGATATGC- 3'	
TUB		
Forward: TubuF	5'- AACGCCGAAGAGGTCATG-3'	500
Reverse: TubuR	5'- CCAGGAGATGTTCAAGCG-3'	
***CHS*2**		
Forward: ChiF	5'- GAACACTCACAGGCATCGCAAG-3'	900
Reverse: ChiR	5'- GGGTTACGATCCATGCTTC-3'	

Data analysis

The most common analysis of the Comparative threshold (CT) method is ΔΔ CT [13], where ΔΔ CT = Δ CT 37 ºC - Δ CT 27 ºC and Δ CT = CT target gene - CT reference gene. The comparative expression level was calculated using 2 - ΔΔCT. Graphpad Prism® software was used for reporting data and performing statistical analyses. Data were expressed as mean + standard deviation (SD). The statistical difference of each pair was evaluated by Student's t-test using a generic approach. The level of statistical significance was set at $p < 0.05$.

Results and discussion

Reference (housekeeping) genes have been described in the literature and many different genes are currently in use [14,15]. For the real-time RT-PCR, the average cycle threshold (C_T) values of ITS at 27 and 37 ºC conditions were 26.68 ± 3.95 and 26.27 ± 3.6, respectively. The Student's t-test indicated that the cycle threshold of ITS between 27 and 37 ºC conditions were not statistically different (p = 0.3201). Thus, these data suggested that ITS rRNA is suitable for a reference control for quantitative gene expression analysis. Sixteen strains of *P. insidiosum* were representative of 3 phylogeographic preferences clades, based on ITS1/2 and *COX*2. The transcriptional quantifications of three genes (*COX*2, *TUB*, and *CHS*2) were performed using quantitative real-time RT-PCR. Furthermore, the melting curve analysis of post-amplification revealed that *COX*2 and *TUB* specific primer pairs produced a single peak in the dissociation curve, indicating that no nonspecific products were amplified. Hence, it was established that *TUB*-specific primers specifically amplified the β-tubulin gene of *P. insidiosum* without producing any significant number of nonspecific products. Two peaks were generated from ITS and *CHS*2 specific primers in melting curve analysis however the left peak indicated a lower melting temperature. These might suggest a primer-dimer artifact because a single band of each primer pairs was revealed on 1.5 % agarose gel electrophoresis, whereas the negative control did not appear. In addition to confirming the PCR products of real-time PCR, they were also sequenced and analyzed with GenBank databases. Fragments of rRNA, *COX*2, *TUB*, and *CHS*2 products showed the *T*m values, of approximately 88.4, 78.7, 90.2, and 88.9 ºC, respectively.

Transcription profiling of 3 candidate genes (*COX*2, *TUB,* and *CHS*2) was performed using real-time RT-PCR in various culturing temperatures. The transcripts of the 3 genes were analyzed in individual *P. insidiosum* strain samples by direct comparison of their cycle threshold (C_T), assuming equal C_T for equal transcript number, since all real-time RT-PCR reactions were performed with an equal quantity of total RNA. To ensure precision and reproducibility, PCR was repeated three times for each sample. The results showed that *TUB* and *CHS*2 genes ranked according to their gene expression stability measured in both temperature conditions. Regarding the *TUB* gene, the C_T value at 27 ºC ranged from 14 - 32 (21.6 ± 4.4), while the C_T value at 37 ºC spanned from 14 - 27 (19.4 ± 3.4). The *CHS*2 gene exhibited similar results at both temperatures, with the value of 24.6 ± 7.1 and 22.3 ± 5.9, respectively. In contrast, only the *COX*2 gene was highly expressed as indicated by C_T values at 37 ºC condition that ranged from 11 - 19 (14.8 ± 2) cycles, but it exhibited rather high dispersion over the growth at 27 ºC, as indicated by a span from 10 - 35 (19.7 ± 8) cycles. The mean value, standard derivation (SD), and coefficient of variation (CV) were then determined from the triplicate experiments. Only the *COX*2 mRNA was significantly up-regulated during the growth of *P. insidiosum* at high temperature (37 ºC) (p = 0.0347), whereas the expression level of the *TUB* gene seemed to be down-regulated at 37 ºC. However, no statistical significance was found between the 27 ºC condition and the 37 ºC condition for *TUB* (p = 0.4103) and *CHS*2 mRNA expression between 37 ºC condition and 27 ºC condition (p = 0.4752) (**Table 2**). The average fold change of *COX*2, *TUB,* and *CHS*2 genes at 27 ºC and 37 ºC conditions are shown in **Figure 1**.

Table 2 The fold change of the candidate temperature-sensitive genes (COX2, TUB, and CHS2 gene) relative to the reference control gene (Internal transcribed spacer, ITS) at 2 different temperature conditions (27 and 37 °C).

Gene	Mean fold change in gene expression		SD of C_T		*p*-value
	27 °C	37 °C	27 °C	37 °C	
COX 2	0.004375	2.52	5.581	4.41	0.0347*
TUB	0.000625	-0.21	4.278	2.9	0.4103
CHS2	0.000625	0.05813	4.877	4.56	0.4752

*The data was significantly at $p < 0.05$.

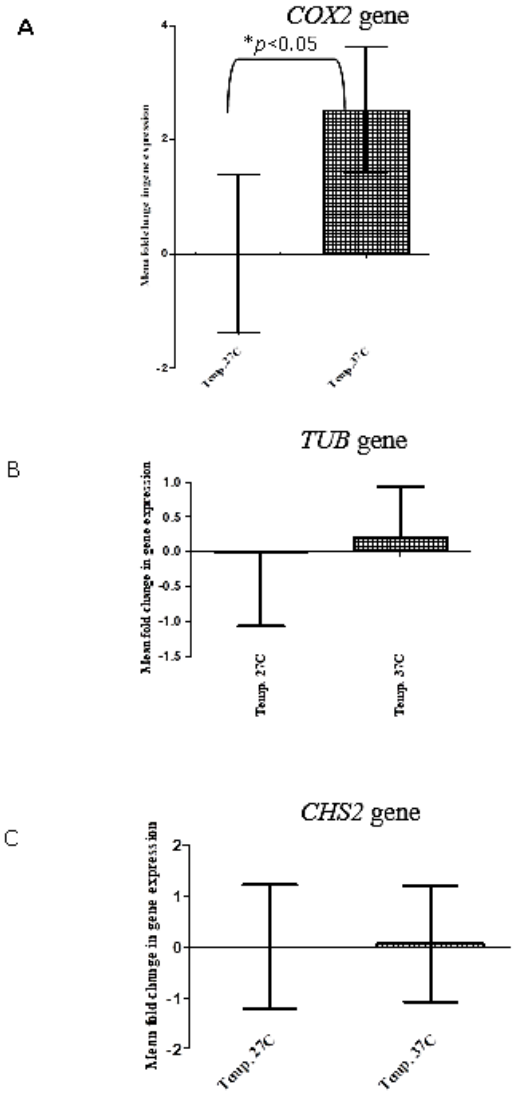

Figure 1 The relative expression of gene-encoded: Cytochrome oxidase 2 (A), β - tubulin (B), and chitin synthase 2 (C) gene, respectively at 27 and 37 °C conditions. Boxes represent the mean of fold change. The lower and upper boundaries of the box indicate the standard error (SEM).

Discussion

It is known that the transition from an environmental reservoir into a human host is associated with temperature-regulated and virulence-related genes that contribute to the pathogenesis of pathogens such as bacteria, fungi, and protozoa. *P. insidiosum* can survive in the environment and body temperature of its mammalian host [13,16,17]. The genetic factors that are required for growth at 37 ºC may be worth considering as one of the various factors for the pathogenesis of *P. insidiosum*.

Many high-throughput techniques can be used to study transcriptomes including quantitative real-time PCR. Based on the information and genetic sequences in GenBank, 3 genes, *COX*2*, TUB,* and *CHS*2 were chosen for this study. Specific primers of the 2 genes; *TUB* and *CHS*2, were designed in this study,

while those of *COX*2 and ITS were referred from Villa *et al*. [18] and White *et al*. [19], respectively. Thus, the expression of *TUB* and *CHS*2, which are located in nuclear DNA, appears to be temperature-independent. This work represents the first study that examined the gene expression levels and used ITS as a reference gene in the Thai strain of *P. insidiosum* under thermal stress conditions. There have been several studies in eukaryotic gene expression using glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*), RNA polymerase 2 (*RNAP*2), ribosomal protein L13 (L13) gene, β-tubulin (*TUB*), 18S and 28S rRNA as housekeeping genes [20-22]. The results of real-time RT-PCR showed, that ITS (28S rRNA) was expressed consistently in both temperature conditions, thus it can be used as a control, there is evidence to support our use of 28S rRNA as a control instead of housekeeping genes [23,24].

P. insidiosum is not a true fungus because its cell walls do not contain chitin but are composed of cellulose and beta-glucan [3,25]. Interestingly, this study found that there is an expression in the chitin synthase 2 gene which is responsible for chitin synthesis. Further analysis provides evidence supporting the occurrence of chitin in the cell wall is important by using more objectives. The result found in the study is a preliminary report which should be further investigated by increasing samples in DNA sequencing to verify the results.

To achieve quantitative analysis, only the *COX*2 gene was expressed higher at 37 ºC than at 27 ºC. The *COX*2 gene is part of the cytochrome oxidase complex, located in the inner mitochondria membrane. Since the structure of this complex contains pores, it facilitates the movement of protons from the mitochondria matrix to the intermembrane space, generating a proton gradient for ATP synthesis in fungi. In lower eukaryotes, this gene participates in both energetic and antioxidant defense of cells and represents an important factor for fungi [26-29]. Another example of the temperature-dependent *COX*2 expression was found in *Saccharomyces cerevisiae* temperature-sensitive strain using mutation by changing Ala189 of the Cox2 protein to proline [30]. Based on our experiment, this is the 1st study to show that *COX*2 is also temperature-dependent, however further study is needed to confirm the relationship between pathogenesis and the *COX*2 gene.

Conclusions

In this study, all 3 candidate genes were subjected to investigate their expression level by real-time RT-PCR. The result showed that *TUB* and *CHS*2, their expressions at both temperatures were difference with non-significant whereas only *COX*2 gene, encoded cytochrome oxidase subunit II, expressed at 37 ºC higher than at 27 ºC condition. Our study suggested that *COX*2 expression in *P. insidiosum* is temperature-dependent.

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