*J. Vet. Malaysia (2019) 31 (1): 34-37*

*Case Reports*

# LESSON LEARNT: FIRST REPORTED CASE OF COMPLICATED CUTANEOUS PYTHIOSIS IN A DOG IN MALAYSIA

**S. Omar.[1]*, S.F. Lau[1], N.A.I. Mohd Zainudin[2], N. Azman[2] and J.X. Jessie-Bay[3]**

*[1]Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia.*
*[2]Faculty of Science, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia.*
*[3]Universiti Veterinary Hospital, Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia.*

## SUMMARY

This report documents a case of 5-month old intact male German Shepherd dog diagnosed with pythiosis on its left forelimb. This is the first ever reported case of pythiosis presented at the University Veterinary Teaching Hospital (UVH), Universiti Putra Malaysia and may be the first ever reported incidence in Malaysia with a complaint of a chronic non-healing wound. The case became complicated as the dog was concurrently infected with a mixed bacterial infection and the identified bacteria were resistant towards a number of antibiotics tested. The antibiotic that was determined to be sensitive was only able to act on certain bacteria and not to the others. The journey of getting to the final diagnosis was almost impossible if we had not tried different media preparation: with and without Dermasel supplement; and through molecular approach using amplification at ITS region followed by DNA sequence analysis. The unwarranted lack during the diagnosis process of this incidence has made us more aware of the presence of *Pythium insidiosum* in Malaysia and plan for a more strategize ways of diagnosing the suspected fungus at laboratory setting in future. The objective of this paper is to share our experience and reflection on the diagnosis of the rare incidence of pythiosis present in Malaysia.

*Keywords: cutaneous, dog, Malaysia, pythiosis, zoonosis*

## INTRODUCTION

Pythiosis is an emerging infectious disease caused by a water-borne fungal-like infection, *Pythium insidiosum*, which is of veterinary and human mycology importance. The disease is widely distributed and is found in swampy areas in tropical, subtropical as well as during summertime in temperate countries that can affect several animal species (Konradt *et al.,* 2016; Grooters, 2003; Mendoza *et al.,* 1996; Rakich *et al.,* 2005; Santurio *et al.,* 2008) including wildlife (Buergelt *et al.,* 2006; Camus *et al.,* 2004; Grooters, 2003; Wellehan *et al.,* 2004) and birds (Pesavento *et al.,* 2008) in which canine being second mostly affected animal species. The organism is an aquatic oomycete *P. insidiosum* and the disease is known as 'swamp cancer' among veterinary pathologist. The infective stage is the motile zoospores that can be found in the contaminated and invade the host via open wounds or mucosal surfaces where the organism then germinates at body temperature of host (De Cock *et al.,* 1987) which later infiltrate in the blood circulation and spread to body tissues that were observed in human (Krajaejun *et al.,* 2006).

The disease is commonly characterised by the development of cutaneous, respiratory or gastrointestinal lesions depending on the route of infection the animal encountered. Nonetheless, a concurrent infection is very rare although it has been reported before in a young Labrador Retriever (Pereira *et al.,* 2010). Although pythiosis is more commonly seen in dogs and horses, an unusual manifestation of feline pythiosis in the oral cavity has also been reported (Fortin *et al*., 2017). Human infections are often reported in Thailand but recently, human cases were also observed in the North America (Hilton *et al.,* 2016) where patients were known to often expose to stagnant water or involve in agriculture. The prognosis for pythiosis both in human and animals are usually poor and may end up with fatality due to a combination of late confirmative diagnosis followed by unsuccessful treatment. Therefore, this paper aims at creating an awareness of the disease in veterinary medicine in Malaysia as many are presumed not aware of the disease present in the country. Although uncommonly affecting animal species in Malaysia, it is still important to monitor the disease as it may potentially become an emerging fungal-like disease in the region due to its high morbidity and mortality rate (Hilton *et al.,* 2016).

## CASE REPORT

Two months before being presented at the University Veterinary Hospital (UVH), Universiti Putra Malaysia, the dog was initially brought to a private veterinary clinic with a complaint of a chronic wound on its left radius and ulnar region and was treated as bacteria-infected wound. The lesion worsened albeit completing the course of the treatment. Therefore, the owner decided to seek for a second opinion at UVH after noticing the affected forelimb was enlarged. According to the owner, the dog had a habit of digging soil. Upon physical examination, the dog was slightly hypothermic, there were multiple fresh, ulcerated wound with various sizes of nodules palpable growing beneath the skin and the limb including the left carpal joint appeared to be swollen and warm on palpation. A radiograph was

**Corresponding author: Dr Sharina Omar (Omar S);*
*Phone No.: +603-8609 3410; E-mail:* sharina@upm.edu.my

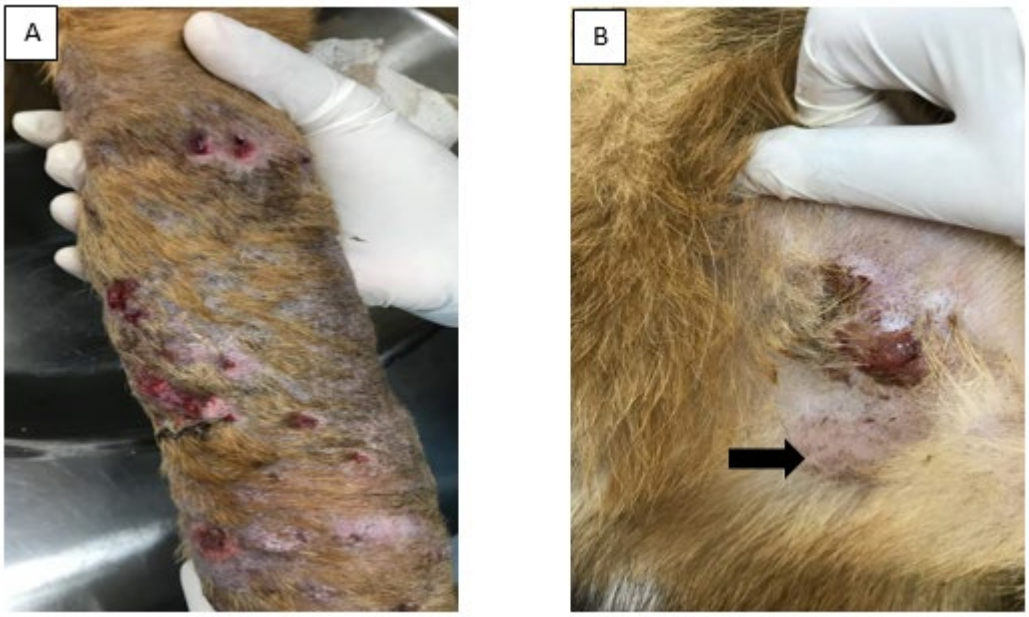


**Figure 1. Clinical manifestation of the affected limb in the dog. (A) Note the swollen forelimb with presence of ulcerated wounds. (B) Nodular (arrow) and ulcerated wound was also seen on the shoulder region**

performed on the affected site but there was no significant finding (Figure 1).

Sample from the lesion was also taken for bacterial identification and antimicrobial sensitivity testing. *Staphylococcus intermedius* was isolated from the lesion but no fungal growth was seen on Sabouraud dextrose agar (SDA) supplemented with Dermasel Selective medium. Two weeks later, the dog was brought again since the symptoms did not resolve post-antibiotic treatment, which was doxycycline 10 mg/kg. The second sample from the wound was taken for both histological and microbiological examination. Several different media and incubation conditions were set to isolate the suspected case of fungal infection. Histologically, sections from wounded tissue showed necrotizing granulomas with central suppurative abscess surrounded by loose granulation tissue, abscess, and skin appendages, which indicating of myositis with a secondary bacterial infection. No evidence of malignancy can be seen. From the fungal culture, a fluffy, white to the cream colour colony was observed on non-supplemented SDA and potato dextrose agar (PDA) incubated at 25ºC. A yeast-like colony was seen on the SDA and PDA media and on blood agar when allowed to grow at 37ºC. The fungal culture was pursued molecular identification where the result indicated the infection was due to *P. insidiosum*.

*Pythium insidiosum* isolates were grown on Sabouraud Dextrose Broth for 7 days at 28 ± 2ºC. DNA was extracted using UltraClean® Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA), following the manufacturer's protocol. Amplification of Internal transcribed spacer (ITS) region of the isolates was performed using a TProfessional Standard Thermocycler (Biometra Company). The amplification was completed by using 25 µl reaction master mix that contains 5 µl of 5x PCR buffer, 1.25 µl of 0.5 µM primer, 2.5 µl of 0.2 mM deoxynucleotide triphosphate (dNTPs), 2.5 µl of 2.5 mM Magnesium chloride ($MgCl_2$), 0.125 unit of *Taq* Polymerase and 20 ng of DNA template. A set of primer was used: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR cycling for ITS was conducted as follows: initial denaturation at 95ºC for 30 s, followed by 35 cycles of denaturation at 95ºC for 10 s, annealing at 55ºC for 15 s, extension at 72ºC for 30 s, final extension at 72ºC for 5 min and soak until been used at 4ºC (Promega Corporation, 2012) in Biometra Thermocycler. The band of ITS was stained with 0.1% FloroSafe DNA stain electrophoresis and visualised. The amplicon of ITS regions in size between 550-600 bp was determined by 100 bp DNA Ladder (Thermo Fisher Scientific, Carlsbad, California) (Figure X-Gel). PCR products were purified using QIAGEN (QIAquick® Gel Extraction Kit) following manufacturer's instruction. The purified ITS product was sequenced using ABI3730XL sequencer by MyTACG (MyTACG Bioscience Enterprise, Selangor, Malaysia) (Figure 2).

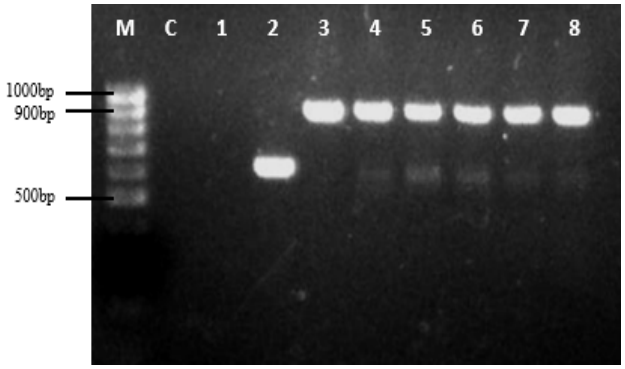


**Figure 2. Gel electrophoresis of PCR products from primer ITS1/ITS4 on 1.5% agarose gel. Lane M: 100bp marker; lane C: control, lane 1 (blank), lane 2 (control isolate): *Trichoderma reesei* S2606, lane 3-8 (*Phytium insidiosum*): B3101, B3102, B3103, B3104, B3105, B3106**

Sequence similarity searches were performed for each of the representative fungal sequences by BLAST and compared to the sequences in GenBank by using the Standard Nucleotide BLAST network services for similarities present in National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) (Geiser *et al.,* 2004). The sequences showed 99% similarity with a deposited sequence of *P. insidiosum* isolate PAC2 (Kammarnjesadakul *et al.,* 2011). ClustalW of MEGA software version 7.0 was used to generate the consensus sequences to align the consensus sequence to each other and to the sequences in GenBank (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). All *P. insidiosum* isolates in this study were grouped in the same cluster of *P. insidiosum* isolate PAC2 (Figure 3).

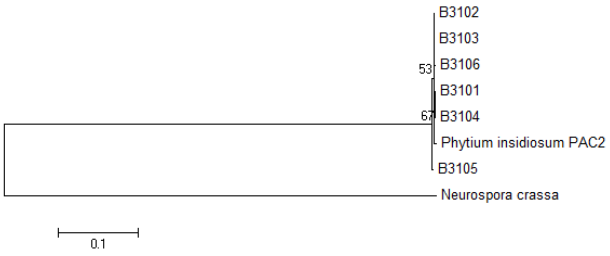


**Figure 3. Maximum-likelihood tree showing the relationship of 6 isolates of *Pythium insidiosum* isolated from 5-month old intact male German Shepherd dog showed pythiosis lesion based on ITS sequences. The tree was generated using Tamura-Nei method with 1000 replicates and bootstrap value more than 50% are shown next to the branches. *Neurospora crassa* (Genbank accession no. DQ235533) is the outgroup**

The dog was immediately prescribed with Itraconazole 10mg/kg once a day for 30 days pre-emptively after fungal growth was found on SDA medium. However, the dog did not show any improvement and a new lesion has developed and extended to the shoulder and ventral thoracic region after two weeks. The dog was concurrently infected with *Klebsiella pneumoniae, Staphylococcus intermedius* and *Pseudomonas aeruginosa*. Unfortunately, the isolated bacteria were resistant to multiple antibiotics tested namely amoxicillin, enrofloxacin, gentamycin, metronidazole, azithromycin. Only ceftriaxone was identified to be effective in combating *K. pneumoniae,* and *P. aeruginosa* but not *S. intermedius.* A weekly revision was advised to assess the dog's condition. Options were given to the owner for ways to treat the dog as the dog's condition had worsened. Unfortunately, the owner refused for any surgical intervention and decided to euthanize the dog. Post-mortem was not conducted at the wish of the owner.

## DISCUSSION

This was the first case report regarding pythiosis incidence in dog in Malaysia. Although it was known that the disease could be treated by antifungal agent, the disease, however, became more severe and complicated to treat following a secondary bacterial infection at the site of the lesion. The isolated bacteria showed resistant to most of the antibiotics tested consequently hindered the recuperation of the host. A single type of antibiotic would not be able to combat the mixed infection whilst *S. intermedius* was resistant to all of the antibiotics tested. Since there was also a delayed in diagnosing the actual agent, we witnessed the rapid spreading of the disease to other parts of the body, at the site where surgical intervention would be a challenge to perform. Majority of the reported cases in animals worldwide had poor to grave prognosis due to the delay in diagnosing pythiosis (Souto *et al.,* 2016; Gaastra *et al.,* 2010; Grooters *et al.,* 2003).

The take-home message for us at the diagnostic laboratory is to have a different media preparation and incubation condition for an unknown of suspected fungal infection especially when the condition was at a chronic phase and lesion was not healed after several attempts of antibiotic treatment. The conventional way of identification of fungi through culture and microscopic examination is time-consuming and requires expertise to avoid misdiagnosis (Wanachiwanawin *et al*., 2014; Krajaejun *et al.,* 2006). Since this was our first time encountering such a case, we finally opted for molecular approach after several attempts to isolate the fungus on culture media. Relying only on conventional isolation and identification method and histopathology would not be enough to diagnose pythiosis (Mendoza *et al.,* 1996; De Cock *et al.,* 1987) although necrotic eosinophilic granulomas observed histologically should prompt the consideration of *P. insidiosum* (Gaastra *et al.,* 2010; Karjaejun *et al.,* 2006). A combination of other techniques such as immunohistochemistry (Krajaejun *et al.,* 2002) and molecular techniques (Znajda *et al.,* 2002) can provide definitive identification of the disease with a high success rate. However, these two techniques still depend on the immunogenicity of the host (Krajaejun *et al.,* 2002) and the amount of oocytes present in the tissues (Grooters *et al.,* 2002; Znajda *et al.,* 2002) to achieve satisfactory results, respectively.

## CONCLUSION

Molecular technique and biopsy of the fungal lesion in addition to fungal culture are required for accurate diagnosis. The success of antifungal therapy has always been a question due to the prolong prescription. The evaluation of antifungal effectiveness is yet to be studied and understood whether the dosage is accurate or the fungus has actually developed resistance towards the antifungal drug. Unfortunately, our patient did not show any improvement and the owner decided to relieve the pain by euthanasia. Therefore, we would not know if a combination of surgical removal and vigorous antifungal and antibiotic therapy would help the animal.

## ACKNOWLEDGEMENTS

We would like to thank Ms Krishnammah d/o Kuppusamy from the Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine,

Universiti Putra Malaysia for her excellent assistance in laboratory work.

## CONFLICT OF INTEREST

There was no conflict of interest to declare among the authors.

## FUNDINGS

This case report was not supported by any research grants.

## REFERENCES

Buergelt, C., Powe, J. and White, T. (2006): Abdominal pythiosis in a Bengal tiger (*Panthera tigris tigris*). Journal of Zoo and Wildlife Medicine. 37(2): 186-189.

Camus, A.C., Grooters, A.M., Aquilar, R.E. (2004): Granulomatous pneumonia caused by *Pythium insidiosum* in a central American jaguar, Panthera onca. Journal of Veterinary Diagnostic Investigation. 16(6): 567-571.

De Cock, A.W., Mendoza, L., Padhye, A.A., Ajello, L. and Kaufman, L. (1987): *Pythium insidiosum* sp. nov., the etiologic agent of pythiosis. Journal of Clinical Microbiology. 25: 344-349.

Fortin, J.S., Calcutt, M.J. and Kim, D.Y. (2017): Sublingual pythiosis in a cat. Acta Veterinaria Scandinavica. 59: 63-67.

Gaastra, W., Lipman, L.J., De Cock, A.W. Exel, T.K., Pegge, R.B.G., Scheurwater, J., Vilela, R and Mendoza, L. (2010): *Pythium insidiosum*: An overview. Veterinary Microbiology. 146:1-16.

Geiser, D.M., Jimenez-Gasco, M.M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A. and O'Donnell, K. (2004): Fusarium-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology. 110: 473-479.

Grooters, A.M., Leise, B.S., Lopez, M.K. (2002): Development and evaluation of an enzyme-linked immunosorbent assay for the serodiagnosis of pythiosis in dogs. Journal of Veterinary Internal Medicine. 16: 142-146.

Grooters, A.M. (2003): Pythiosis, lagenidiosis, and zygomycosis in small animals. Veterinary Clinics Small Animal Practice. 33: 695-720.

Hilton, R.E., Tepedina, K., Glenn, C.J. and Merkel, K.L. (2016): Swamp cancer: a case of human pythiosis and review the literature. British Journal of Dermatology. 175: 394-397.

Kammarnjesadakul, P., Palaga, T., Sritunyalucksana, K., Mendoza, L., Krajaejun, T., Vanittanakom, N., Tongchusak, S., Denduangboripant, J. and Chindamporn, A. (2011): Phylogenetic analysis of *Pythium insidiosum* Thai strains using cytochrome oxidase II (COX II) DNA coding sequences and internal transcribed spacer regions (ITS). Medical Mycology. 49(3): 289-295.

Konradt, G., Bassuino, D.M., Bianchi, M.V., Castro, L., Caprioli, R.A., Pavarini, S.P., Santurio, J.M., Azevedo, M.I., Jesus, F.P. and Driemeier, D. (2016): Cutaneous pythiosis in calves: An epidemiologic, pathologic, serologic and molecular characterization. Medical Mycology Case Reports. 14:24-26.

Krajaejun, T., Kunakorn, M., Niemhom, S., Chongtrakool, P. and Pracharktam, R. (2002): Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. Clinical and Diagnostic Laboratory Immmunology. 9(2): 378-382.

Krajaejun, T., Kunakorn, M., Pracharktam, R., Chongtrakool, P., Sathapatayavongs, B., Chaipraset, A., Vanittanakom, N., Chindamporn, A. and Mootsikapun, P. (2006): Identification of novel 74-kiloDalton immunodominant antigen of *Pythium insidiosum* recognized by sera from human patients with pythiosis. Journal of Clinical Microbiology. 44: 1674-1680.

Krajaejun, T., Sathapatavongs, B., Pracharktam, R., Nitiyanant, P., Lee-Iachaikul, P., Wanachiwanawin, W., Chaiprasert, A., Assanasen, P., Saipeth, M., Mootsikapun, P., Chetchotisakd, P., Lekhakula, A., Mitarnun, W., Kalnauwakul, S., Supparatpinyo, K., Chaiwarith, R., Chiewchanvit, S., Tananuvat, N., Srisri, S., Suankratay, C., Kulwichit, W., Womgsaisuwan, M. and Somkaew, S. (2006): Clinical and epidemiological analysis of human pythiosis in Thailand. Clinical and Infectious Disease. 43: 569-576.

Mendoza, L., Ajello, L. and Mc Ginnis, M.R. (1996): Infections caused by the oomycetous pathogen *Pythium insidiosum*. Journal of Medical Mycology. 6(4): 151-164.

Pereira, D.I.B., Schild, A.L., Motta, M. A., Fighera, R. A., Sallis, E.S.V. and Marcolongo-Pareira, C. (2010): Cutaneous and gastrointestinal pythiosis in a dog in Brazil. Veterinary Research Communications. 34(3): 301-306.

Pesavento, P.A., Barrs, B., Riggs, S.M., Eigenheer, L., Panma, R. and Walker, R.L. (2008): Cutaneous pythiosis in a nestling white-faced Ibis. Veterinary Pathology. 45: 538-541.

Rakich, P.M., Grooters, A.M. and Tang, K-N. (2005): Gastrointestinal pythiosis in two cats. Journal of Veterinary Investigation. 17: 262-269.

Santurio, J.M., Argenta, J.S., Schwendler, S.E., Cavalheiro, A.S., Pareira, D.I., (2008): Granulomatous rhinitis associated with *Pythium insidiosum* infection in sheep. Veterinary Record. 163: 276-277.

Souto, E.P.F., Maia, L.A., Olinda, R.G., Galiza, G.J.N., Kommers, G.D., Miranda-Neto, E.G., Dantas, A.F.M. and Riet-Correa, F. (2016): Pythiosis in the nasal cavity of horses. Journal of Comparative Pathology. 155: 126-129.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013): MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution. 30(12): 2725-2729.

Wanachiwanawin, W., Mendoza, L., Visuthisakchai, S., Mutsikapan, P., Sathapatayavongs, B., Chaipraset, A., Suwanagool, P., Manuskiatti, W., Ruangsetakit, C. and Ajello, L. (2014): Efficacy of immunotherapy using antigens of *Pythium insidiosum* in the treatment of vascular pythiosis in humans. Vaccine. 22:3613-3621.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR ProtocolsL: A Guide to Methods and Applications. 18(6): 315-322.

Wellehan, J.F., Farina, L.L., Keoughan, C.G., Lafortune, M., Grooters, A.M., Mendoza, L., Brown, M., Terrell, S.P., Jacobson, E.R. and Heard, D.J. (2004): Pythiosis in a dromedary camel (*Camelus dromedaries*). Journal of Zoo and Wildlife Medicine. 35(4): 564-568.

Znajda, N., Grooters, A.M. and Marsella, R. (2002). PCR-based detection of *Pythium* and *Lagenidium* DNA in frozen and ethanol-fixed animal tissues. Veterinary Dermatology. 13: 187-194.