

# The Pathological Study of Amputated Limbs Infected by *Pythium insidiosum*: To Propose Adequacy of Surgical Margins

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## ABSTRACT

**Objective:** Arterial pythiosis, caused by *Pythium insidiosum*, is a life threatening condition. The lesions usually involve main arteries of the lower extremities, which extend to the abdominal aorta. Early amputation is required to save patient's life. The adequacy of amputation needs to ensure that the resection margins are devoid of the organisms. The aim of this study is to suggest the level that should be devoid of organisms for surgical amputation.

**Methods:** We studied amputated limbs of patients diagnosed as arterial pythiosis. The major arteries of the limbs were dissected, then serial cross cut at 0.3-0.5 cm intervals, and consecutively submitted from proximal to distal segments for routine tissue processing and staining. Segments of non-inflamed vessels continuing above or below the level of arterial occlusion were identified. To demonstrate fungal-like hyphae, all corresponding blocks of non-inflamed arteries were stained with Grocott's methenamine silver staining (GMS) and thoroughly examined under a microscope.

**Results:** From June 2010 to June 2011, there were 4 cases of arterial pythiosis. Chronic arteritis obliterans, acute necrotizing arteritis with thrombosis, or ruptured aneurysm was demonstrated from distal to proximal segments without skip lesions. At the level of 5 cm. from the occlusion, neither arteritis nor fungal-like hyphae was identified.

**Conclusion:** Based on the evidence from only 4 cases, we have proposed that at 5 cm. or more above the occlusion, the non-inflamed arteries were free of *P. insidiosum*, while fungal hyphae might be observed in inflamed soft tissue.

**Keywords:** Arterial pythiosis, soft tissue pythiosis, infectious arteritis, *P. insidiosum*

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## INTRODUCTION

*Pythium insidiosum* is a fungus-like organism in the Kingdom Chromista or Straminopila causing pythiosis in humans and animals.<sup>1,2</sup> In humans, the disease occurs in four forms: ocular pythiosis, cutaneous or subcutaneous pythiosis, arterial pythiosis, and disseminated form.<sup>3-5</sup> Arterial pythiosis is a life threatening con-

dition with high morbidity and mortality rates.<sup>6,7</sup> Most arterial pythiosis are associated with thalassemia.<sup>8,9</sup> The lesions usually involve the main arteries of the lower extremities, which extend to the abdominal aorta.<sup>7</sup> The effective treatment of vascular pythiosis is amputation at the level above the infected part.<sup>8</sup> Early amputation in combination with medical treatment and immunotherapy are required to save the patient's life.<sup>10-11</sup> The adequacy of amputation needs to ensure that the resection margins are devoid of the organism. Therefore, precise histopathology of arteritis caused by *P. insidiosum* has to be studied. There has so far been no report which recommends the resection margin for the amputation site. Therefore, this study has two main objectives.

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1. To describe the histopathology of amputated limbs infected by *P. insidiosum*
2. To propose the level of arterial resection margins devoid of the organism.

## MATERIALS AND METHODS

The study was performed at the Department of Pathology, Siriraj Hospital during June 2010 to June 2011. The inclusion criteria of studied materials were obtained from patients with conditions as shown below.

1. Patients presented with acute or chronic arterial occlusion and / or chronic ulcers.
2. Patients had risk factors such as thalassemia, hemoglobinopathies, or chronic anemia.
3. Patients had angiographic studies prior to amputation.
4. Patients had positive serodiagnosis for anti-*P. insidiosum* by immunodiffusion and immunochromatography test (ICT).<sup>4,12</sup>
5. Patients had tissue diagnosis demonstrating non-septate hyphae by Grocott's Methenamine silver (GMS) stain prior to amputation or by potassium hydroxide test (KOH) intra-operatively.<sup>13</sup>

For pathological examination, the specimens were processed under the following protocol.

1. The amputated limbs were photographed and measured.
2. The major arteries and surrounding neurovascular bundles were dissected. They were femoral artery (FA), popliteal artery (PA), anterior tibial artery (ATA), posterior tibial artery (PTA), peroneal artery (PNA), and dorsalis pedis artery (DP). The specimens were fixed for at least 24 hours, then serial cross cut at 0.3-0.5 cm intervals, photographed, and consecutively submitted from proximal to distal segments for routine tissue processing and staining.

3. The skin, soft tissue, vascular resection margins and other abnormal lesions were examined and submitted for routine tissue processing and staining.

4. Paraffin embedded sections were stained with Hematoxylin and Eosin (H&E).

5. To identify hyphae, selected slides showing arteritis, inflammation, eosinophilic abscesses or granuloma were stained with GMS.

6. To identify elastic tissue and smooth muscle of the arterial wall, selected slides were stained with Verhoeff's elastic stain and Masson trichrome.<sup>13</sup>

7. To determine the arterial level devoid of hyphae, segments of non-inflamed vessels 5 cm long continuing above or below the level of arterial occlusion were identified. All corresponding slides were stained with GMS.

8. Immunohistochemistry for *P. insidiosum* 14 was performed on at least one slide from each case.

### Ethical consideration

The study was approved by the Siriraj Institutional Review Board (protocol number 266/2553).

## RESULTS

There were 4 patients whose amputated limbs were infected by *P. insidiosum*. The clinical summary of the cases has been shown in Table 1. The pathological findings have been described below and displayed in an illustration (Fig 1).

### Case 1

Both below knee amputation (BKA) specimens were swollen and firm. The skin was diffusely covered with dark brown and black scaly plaques.

The right BKA: The PNA and DP were obliterated by chronic inflammation. The PA, ATA, PTA were not inflamed and GMS was negative for hyphae. The distal PTA showed perivascular fibrosis without arteritis. There

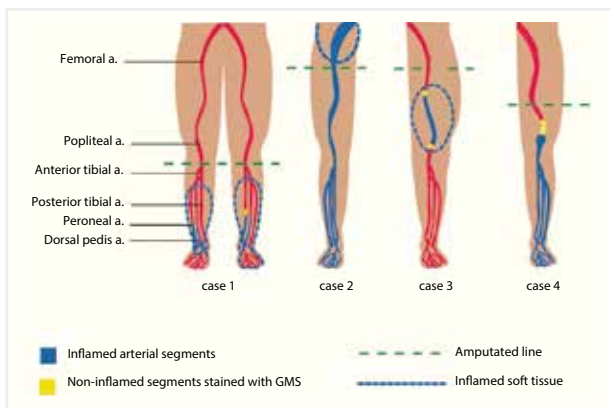
**TABLE 1.** Clinical summary of 4 cases.

Case No.	Age	Sex	Underlying diseases	Symptoms & Duration	Occluded site on CT angiogram	Type of Amputation	Treatment	Outcome (1 year)
1	35	M	Thalassemia Hb H	Chronic dermatitis with indurate ulcers both legs, 10 m.	Rt distal PNA, DP Lt distal ATA to DP, mid PTA	Rt & Lt BK,	Vaccine Itraconazole Terbenafine	Survived
2	35	M	Beta Thalassemia trait	A chronic ulcer, Rt ankle. A mass Rt groin, 1 m.	False aneurysm Rt ext. iliac a. FA, PA, ATA, PNA, prox. PTA, DP	Rt AK	Vaccine Itraconazole Terbenafine	Survived
3	21	M	Thalassemia Hb E	Chronic abscesses Lt thigh, S/P I&D 7 m.	Lt mid. FA. to mid PA	Lt AK	Vaccine Itraconazole Terbenafine	Survived
4	37	M	Thalassemia HB H	Calf pain Lt leg, 4m. Gangrene Lt big toe, 1 wk.	PA, ATA, PTA, PNA, DP, plantar a.	Lt AK	Vaccine Itraconazole Terbenafine	Survived

AK: above knee, BK: below knee, I&D = incision and drainage,

Rt = right, Lt = left, m. = month, wk. = week, FA= femoral a., PA = popliteal a.,

ATA = anterior tibial a., PTA = posterior tibial a., PNA = peroneal a., D = dorsalis pedis a.



**Fig 1.** An illustration demonstrated infected arteries and soft tissue. The yellow segments represented the artery of which consecutive sections were stained with GMS.

was severe myofasciitis with numerous hyphae in the soft tissue. The popliteal nodes showed reactive hyperplasia. The resection margin had no inflammation and no hyphae on GMS.

The left BKA: Dissected arteries showed severe necrotizing arteritis of mid ATA, DP, distal segment of PTA with recent thrombus in PTA. Hyphae were identified in the arterial lumen and necrotic wall. There were severe myofasciitis and numerous hyphae. The popliteal nodes showed reactive hyperplasia. The resection margin had no inflammation and no hyphae.

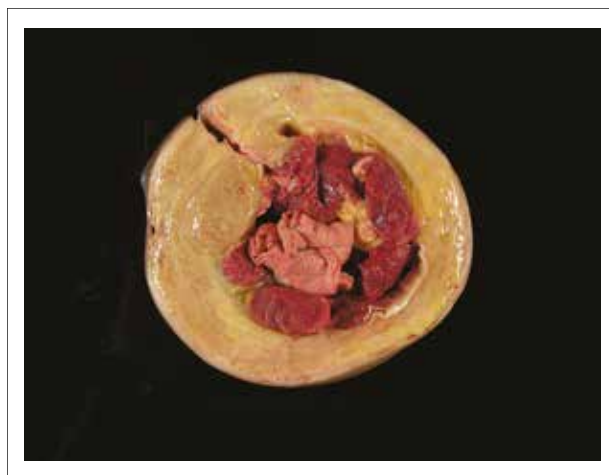
Consecutive sections superior to the thrombus in the left PTA demonstrated arteritis up to 1 cm. Consecutive sections with GMS stain at the level of 1 cm up to 5 cm proximal to the thrombus in the left PTA were devoid of hyphae. No hyphae were identified in the non-inflamed segments proximal to the thrombus.

### Case 2

The right above knee amputation (AKA) showed an oval chronic ulcer, 8x7 cms, at the medial malleolus.

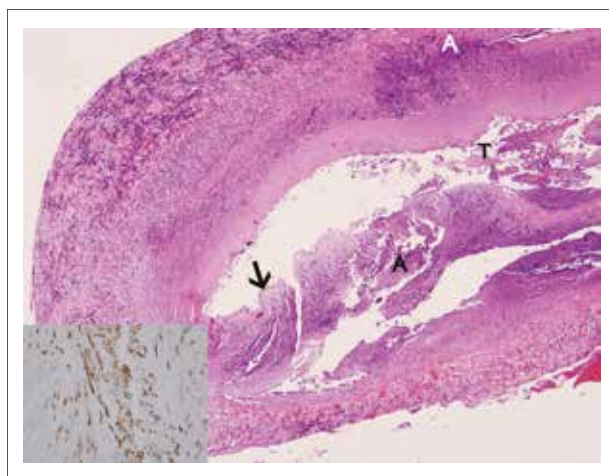


**Fig 2.** Ruptured infected arteritis (Case 2). Serial cross sections of the infected femoral artery demonstrated a part of false aneurysm at the most proximal end (➡). The distal segments showed active arteritis, followed by circular fibrosis and became yellow spots (▶). Sections were aligned from bottom to top, left to right.

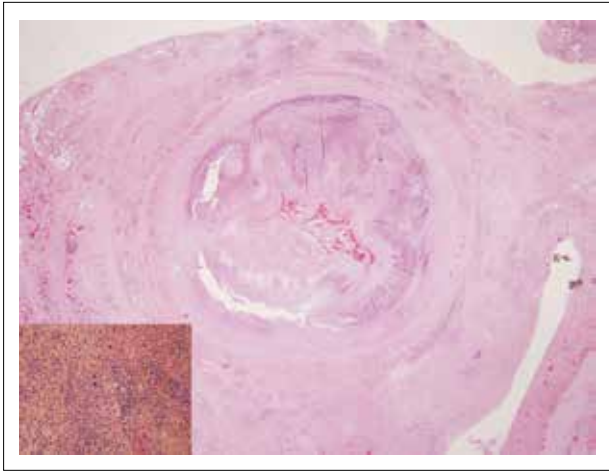


**Fig 3.** Chronic myofasciitis. Cross section of the left thigh after removed neurovascular bundle and bone. The subcutaneous tissue and infected muscle fascicles had pale yellow opaque necrosis.

The muscle at the resection margin had a dark red friable appearance. Dissection of the FA at the resection margin showed necrotizing arteritis with false aneurysm. There was a recent infected thrombus along the course of the femoral artery. The distal FA was gradually replaced by chronic inflammation to PA. The ATA, PTA, and PNA were obliterated without skip lesions, corresponding to the CT angiogram (Fig 2). A thrombus from a false aneurysm, together weight 255 gm, was examined separately. Numerous hyphae were demonstrated in the thrombus, FA wall from the most proximal resection margin, along the PA, ATA, PTA and PNA and even in the obliterated arteries. There was minimal periarterial inflammation. The nerve fascicle was not inflamed. The popliteal nodes showed reactive hyperplasia. The ulcer at the ankle had chronic gangrenous inflammation with superficial bacterial infection, but no demonstrable hyphae on GMS. Friable soft tissue at the resection margin had



**Fig 4.** Severe necrotizing arteritis. The whole arterial wall showed micro abscesses (A), and necrosis. Residual fragments of the internal elastic membrane lined along the tunica intima. (➡) The lumen was filled with infected thrombus (T) (HE 100x). Inset, Hyphae within the thrombus stained by immunohistochemistry (Ipx 400x).



**Fig 5.** Chronic arteritis. The arterial wall had retracted fibrosis. (HE 100x). Inset, lymphocytes, histiocytes and multinucleated cells in the lumen (H&E 400x).

active necrotizing inflammation with hyphae.

A non-inflamed arterial segment, continuing from the thrombus was not presented in this case.

### Case 3

The left AKA had an enlarged indurated area at the thigh with multiple chronic ulcers and scars. The fifth toe showed dry gangrene. Cut surfaces of the soft tissue and muscle revealed pale muscle fascicles, multifocal yellow opaque necrotic foci and extensive fibrosis (Fig 3). Dissection of the major arteries demonstrated necrotizing arteritis of FA and PA with infected thrombus. The distal PA, ATA, PTA, PNA and DP were not inflamed, but focally lodged by infected thrombo-emboli. GMS demonstrated hyphae within the thrombus, inflamed arterial wall, necrotic soft tissue and muscle. The skin and soft tissue at the resection margins did not have inflammation or hyphae.

Consecutive sections superior to the thrombus demonstrated partial arteritis up to 4 cm. Consecutive sections inferior to the thrombus demonstrated 1 cm of arteritis. Consecutive sections with GMS stain at the level of 5 cm, proximal and distal to the thrombus were devoid of hyphae.

### Case 4

The left AKA had dry gangrene at distal one third of the foot. Dissection of the major arteries showed unremarkable distal FA and PA 10 cm, from the resection margin, and an oval aneurysm, 3x2 cm, containing thrombus. Active arteritis with thrombi continued along the course of the ATA. The PTA and PNA revealed chronic arteritis and obliterated lumens.

GMS demonstrated numerous hyphae within the thrombi, and arterial wall along the course of the ATA, PTA, and PNA. There were no skip lesions of affected arteries. The soft tissue surrounding the aneurysm also showed yellow necrosis, while muscles elsewhere were not inflamed. The skin and soft tissue at resection margins were free of inflammation.

Consecutive sections superior to the aneurysm demonstrated arteritis up to 2 cm. Consecutive sections with GMS stain at the level of 5 cms, proximal to the aneurysm were devoid of hyphae.

The histopathology of 4 amputated limbs have been described in 3 aspects .

1. The arterial inflammation: Active arteritis and thrombosis were observed in all specimens. All layers of the arterial wall had numerous polymorphonuclear cells (PMN), histiocytes and lymphocytes with necrosis. A number of eosinophils were seen, but not as many as PMN. Micro - abscesses within the arterial wall were also found. The internal elastic membrane and muscular layer of the tunica media were destroyed with multinucleated giant cells engulfing elastic fragments. Destruction of muscular layers and fibroblastic proliferation resulted in aneurysmal dilatation or rupture (Fig 4). Hyphae were seen in recent thrombus, and in the arterial wall of inflamed segments in all cases. Chronic arteritis comprised lymphocytes, plasma cells, histiocytes, foam cells and fibroblasts instead of PMN. Fibrosis replaced the muscular layer of the arterial wall. In chronic arteritis obliterans, the artery showed only crenated fibrotic wall. A few foam cells, lymphocytes, siderophages and scanty degenerated hyphae retained in the lumen (Fig 5). Acute and chronic arteritis involved all along the course of the arteries without skip segments. In Case 3, the histopathology clearly demonstrated that inflammation and hyphae extended from the soft tissue to the arterial segment. The artery which was located proximal and distal to this segment had no inflammation.

2. The soft tissue inflammation: In the soft tissue, active lesions showed multifocal necrosis. Eosinophilic granuloma with degranulated eosinophils and Splendor Hoeppli were identified in two cases of soft tissue pythiosis. In chronic lesions, only some granuloma, lymphocytes, plasma cells, and extensive fibrosis were seen. The veins, nerves and lymph nodes were not the preferential sites of *Pythium* infection. Short segments of hemi-phlebitis only on the side adjacent to the inflamed artery were identified.

3. The organism: In the tissue sections, *P. insidiosum* displayed thin wall, slightly curved, irregular branching hyphae, with infrequent septation, varying from 5-10  $\mu$ m. in width. The hyphae encircled or penetrated perpendicular to the circumference of the arterial wall. The hyphae were hardly detected on routine H&E stain, but were highlighted by GMS. Most of the hyphae were degenerated. In the soft tissue, the hyphae were often seen in the center of granuloma or necrosis. There were no demonstrable hyphae by GMS in the veins, lymph nodes, or nerve fascicles. In non-inflamed arterial segments, no hyphae were identified although consecutive sections from the inflamed segments were stained with GMS. Immunohistochemical examination using the rabbit anti-*P.insidiosum* antibody<sup>14</sup> were positive in all 4 cases.

## DISCUSSION

Patients who have *P. insidiosum* infection of the limbs can present as acute & chronic arterial occlusion,



chronic ulcers or myofasciitis. Here we demonstrated 2 cases of arterial pythiosis (cases 2 and 4) and 2 cases of myofasciitis, which progressed to infect arteries (cases 1 and 3). These two conditions may occur together, but the pathological process and the distribution of inflammation are quite different. Our study showed that arterial pythiosis is a peculiar infectious arteritis. It can involve more than one artery at the same time. Progression from distal to proximal segments was clearly seen in Case 2 and Case 4. Infected emboli may cause arteritis of other segments distally. The causative agent was demonstrated in all stages of arteritis, but not in the non-inflamed segments. Since the inflammation usually involved all layers of the arterial wall, the following complications developed, infected thrombosis with arterial occlusion, infected thrombo-embolism, and aneurysmal dilatation with or without rupture. If the healing process overcame destructive inflammation, arterial fibrosis occurred, leaving only fibrous streaks. Non-inflamed segment between the inflammation segment of the same artery (skip lesion) was not presented.

For surgical purposes, we identified the inflamed segments occluded by thrombi, which corresponded to the angiography. We carefully searched for hyphae on every GMS stained section of non-inflamed segment continuing from the occlusion. At the level of 5 cm, no arteritis or hyphae were seen on H & E, and GMS. This observation may help surgeons to make decisions for the appropriate level of amputation in which vascular spreading is quite predictable.

Unlike arterial pythiosis, soft tissue pythiosis produces widespread inflammation of the skin, subcutaneous tissue, muscle and deep fascia with unpredictable routes. Grossly, necrotic areas and yellow opaque lesions mimicking caseation still contain hyphae. Regarding pathological findings of the 4 cases, if the objective of amputation is to remove dead tissue, to minimize numbers of *P. insidiosum*, and to preserve patients' limbs for prosthesis, surgeons should consider the resection margins of both artery and soft tissue pythiosis. Adjuvant therapy including antifungal agents and in particular, immunotherapy are our suggestions.

## CONCLUSION

Clinical presentation of infected limbs caused by *P. insidiosum*, either arteritis or soft tissue myofasciitis, includes acute or chronic ischemia, ulcers or myofasciitis.

For amputation, an angiogram can be used as a guideline. Based on limited case studies<sup>4</sup> we have proposed that at the level of 5 cm, above the occlusion, there are no demonstrable hyphae. However, inflamed periarterial soft tissue and myofasciitis should be of concern and might be the source of organisms at the resection margin.

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