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Case Reports

FATAL MELIOIDOSIS IN A CAPTIVE ELEPHANT TRUNK SNAKE (*Acrochordus javanicus*) IN KUALA LUMPUR, MALAYSIA

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SUMMARY

An adult female Elephant Trunk Snake (*Acrochordus javanicus*) was reported to have been weak and inappetent for five days. The following morning the snake found dead, while in the process of shedding its skin. On post mortem examination, there were multiple circumscribed caseous nodules of various sizes distributed all over the liver, along the respiratory tract and on the lungs. Bacteriological analysis of the lungs and liver swab samples yielded *Burkholderia pseudomallei*, which was confirmed by PCR amplification of specific 16S rRNA. The condition was diagnosed as melioidosis and the organism was genotypically characterized as sequence type 51, a genotype that has been previously characterized in humans in Malaysia. Antibiotic susceptibility by both Disc diffusion or Kirby Bauer and E-test minimum inhibitory concentration (MIC) showed that the organism exhibited susceptibility to meropenem, imipenem, ceftazidime, cotrimoxazole and co-amoxyclav; the antibiotics recommended in the treatment of melioidosis.

Keywords: Melioidosis, Elephant Trunk Snake, Burkholderia pseudomallei, sequence type, pathology

INTRODUCTION

Melioidosis, a likely fatal infectious disease of both humans and animals is caused by an environmental (soil and water) dwelling saprophytic bacterium; *Burkholderia pseudomallei* (Inglis and Sousa, 2009; Currie *et al*., 2010). The disease was now known to be hyperendemic in some parts of Southeast Asia and northern Australia (Currie *et al*., 2008). It is believed that the disease is now expanding beyond its traditionally known endemic region to other tropical regions of the world, including the Indian subcontinent, southern China, Hong Kong, Taiwan, Brazil and Malawi (Currie *et al*., 2008; Katangwe *et al*., 2013). However it is still unclear whether the infection has been there but hitherto undetected (Dance, 2000). There are unconfirmed reports of new cases in South Africa and the Middle East, while some imported cases are described in several temperate countries (Dance, 2000). Transmission of the disease, in both humans and animals, are believed to occur most often via traumatic skin inoculation and through ingestion or inhalation of contaminated soil and water (White, 2003). There are evidences of infections acquired following near drowning events and rarely from sexual transmission (McCormick *et al*., 1975; Pruekprasert and Jitsurong, 1991; Cheng and Currie, 2005; Mukhopadhyay *et al*., 2009). Emergences of melioidosis related to travelling and importation of cases have been observed in developed countries of the world (Currie *et al*., 2008). Virtually all organs of the body in both humans and animals can be infected by the disease (Puthucheary and Vadivelu, 2002; Sprague and Neubauer, 2004). The clinical spectrum of melioidosis may range from indolent localised infection to fulminating septicaemia (Currie et al., 2000). Treatment of melioidosis can be done usually by antibiotic chemotherapy to improve patients' condition and disease control (Cheng and Currie, 2005). There are two distinct phases of antibiotic treatments used to treat melioidosis: (a) the acute septicaemic phase of the disease or intensive phase, using the cephalosporin, ceftazidime and carbapenems, meropenem and imipenem and (b) the subsequent eradication phase treatment using trimethoprim-sulfamethoxazole (cotrimoxazole) (Inglis, 2010). Due to the organisms' high level of intrinsic resistance against many common clinically available antibiotics, antibiotic treatment of this infection is proving difficult (Simpson *et al*., 1999). Melioidosis fatality rate may range from 20 to 40% even with expeditious diagnosis and prompt and vigorous antibiotic treatment (Schweizer, 2012). Melioidosis is a disease of public health significance and its public health implications have been previously reviewed (Inglis and Sousa, 2009). Previously it has been shown that importations of animals with melioidosis into areas that have been known to be free from the disease have resulted in outbreaks and subsequent persistence of *B. pseudomallei* in the contaminated soil (Galimand and Dodin, 1982). It has been known that human to humantransmission of this disease is rare; anecdotal evidences of zoonotic transmission of the organism from animal to human do exist. Animals with melioidosis may shed the *B. pseudomallei* via bodily discharges with consequent increase in the risk of direct animal to animal, or animal to human disease transmission (Idris *et al*., 1998; Choy *et al*., 2000; Currie, 2010). Melioidosis has been previously reported in camels (Forbes-Faulkner *et al*., 1992), alpacas (Janmaat *et al*., 2004), swine (Najdenski *et al*., 2004), captive whales and dolphins (Hicks *et al*., 2000), deer (Srikawkheaw and Lawhavinit, 2007), feline (O'Brien *et al*., 2003), canine and wild avian (Ouadah *et al*., 2007) and pet iguana (Iguana iguana) (Zehnder *et al*., 2014). There has been no published report of cases of melioidosis in snakes. Therefore this paper presents a case of fatal melioidosis in a captive Elephant trunk snake (*Acrochordus javanicus*) in Kuala Lumpur, Malaysia and phylogenetic assessment of the aetiological agent.

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CASE REPORT

History

An adult female Elephant Trunk Snake (*Acrochordus javanicus*), 159 cm in length, that has been housed in a specially made plastic aquarium in a private facility in Kuala Lumpur, Malaysia was reported to have been weak and inappetent for about four to five days. In the morning, the snake was found dead while in the process of shedding its skin.

Post-mortem and Histopathology findings

Post-mortem examination of the carcass was carried out, the body condition score of the snake was estimated to be 3/5, although the snake has naturally loose skins and folds, there was evidence of exoskeletal shedding. Necropsy findings showed no pathological lesions along the gastrointestinal tract (GIT), however the GIT was found to be empty with presence of yellowish mucus. There were multiple circumscribed caseous nodules of various sizes scattered all over the liver, respiratory tract and lungs. These circumscribed protruding caseous nodules were particularly more pronounced in the lungs and when the surface of the lungs was cut, there was the presence of a cheesy exudate and numerous nodular structures (Figure 1).

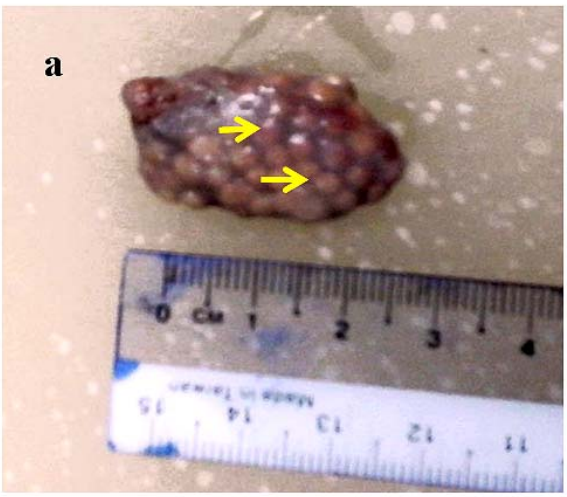

Figure 1: Gross picture of the lung (snake), showing (a) numerous circumscribed protruding nodules. The cut surface of the lungs showing the presence of cheesy exudate and numerous nodular structures (arrows)

Lung biopsy and tissue swabs of both the lungs and liver were taken using sterile swab and sent to histopathology and bacteriology laboratories respectively.

The lung biopsy was sent to the histopathology laboratory of the Faculty of Veterinary Medicine (FPV, UPM) in 10% buffered formalin where it was processed, sectioned and stained with Hematoxylin and Eosin (H&E) stain for light microscopic examination of lesions at various magnifications (100× and 200×).

The histopathological lesions observed varied from an immature to matured garanuloma formation with a centeral area of necrosis containing pus, tissue debris and calcium deposits with calcified wall. Leucocytic infiltration in the interstitium and connective tissue proliferation around the granuloma were also observed. Figures 2 to 5 below showed the varying degrees of lesions observed in the melioidosis positive elephant snake's lungs.

Bacteriological examination

The swab samples were sent to bacteriology laboratory of the Faculty of Veterianary Medicine, Universiti Putra Malaysia (UPM). Following primary culture and subculture on Blood and MacConkey agar, screening was don based on Gram staining reaction, colony morphology, positive culture on Ashdown's agar, and oxidase and catalase tests. Presumptive identification of this organism was based on its appearance as bipolar

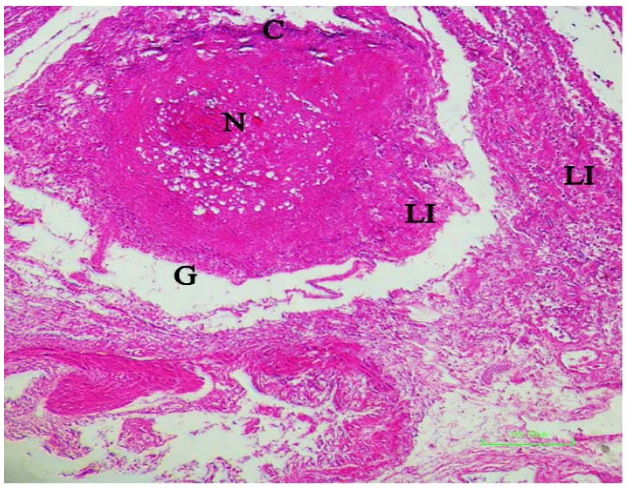

Figure 2: An immature granuloma (G) in the lung of a melioidosis positive snake. Note the necrotic center (N), surrounded by leucocyte infiltration (LI) in the interstitium and partial calcification (C), H&E ×100

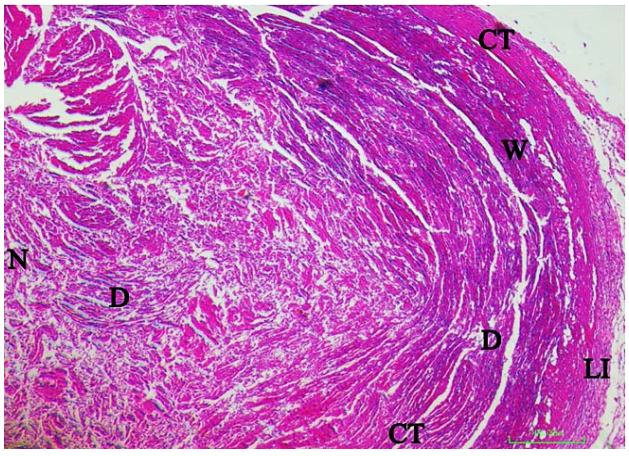

Figure 3: A mature granuloma in the lung of a melioidosis positive snake. Note the necrotic center (N) containing debris and calcium deposits (D), surrounding leucocyte infiltration (LI) around the periphery, a calcified wall (W) and concentric layers of fibrous connective tissue (CT), H&E ×200

organisms, oxidase positive, catalase negative,Gram negative bacilli with characteristic colonies on Ashdown's agar that were purple, flat, dry and wrinkled according to (Chantratita *et al*., 2007).Pure colonies were obtained from the subculture and nucleic acid was extracted using Qiagen DNeasy (Qiagen, Germany) bacterial DNA extraction kit, used according to manufacturer's instructions. Confirmation of the isolate as *B. Pseudomallei*was done by polymerase chain reaction (PCR) amplification of 600bp gene fragment using *B.pseudomallei* specific 16S rRNA region primers (PPM3- forward primer) 5'-AATCATTCTGGCTAATACCCG-3' and (PPM4- reverse primer) 5'-CGGTTCTCTTTCGAGCTCG-3' obtained from a previous study by Brook *et al*. (1997).

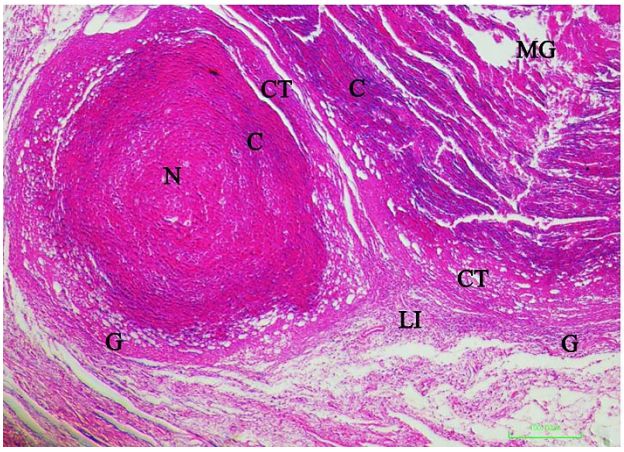

Figure 4: An immature granuloma (G) in the lung of a melioidosis positive snake adjacent to a matured granuloma (MG). Note the necrotic center (N), surrounded by leucocyte infiltration (LI) in the interstitium, partial calcification (C) and connective tissue proliferation around the granuloma (CT), H&E ×100

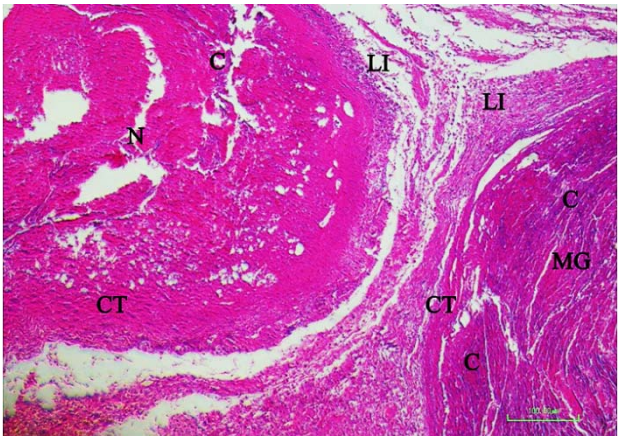

Figure 5: An immature granuloma in the lung of a melioidosis positive snake, adjacent to a matured granuloma (MG). Note the necrotic center (N), surrounded by leucocyte infiltration (LI) , partial calcification (C), and connective tissue proliferation around the granuloma (CT) (C), H&E ×100

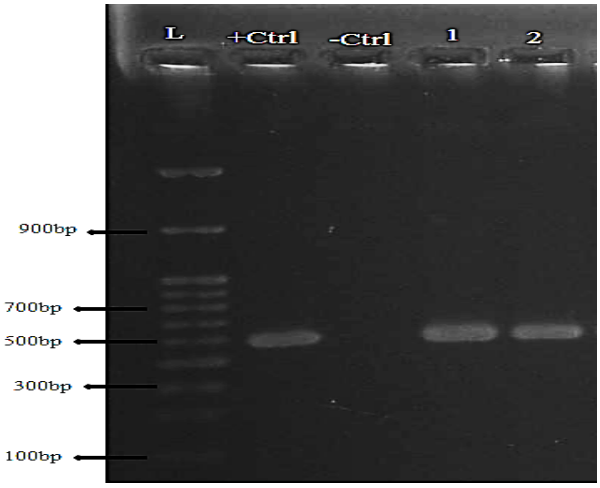

Figure 6: Gel electrophoreses picture of 550bp *B. pseudomallie* 16S rRNA gene fragment. Positive control (+Contrl), Negative control (-Contrl), snake lung isolate (1) and snake liver isolate (2)

PCR amplification was confirmed by gel electrophoresis using 1.5% agarose gel (Promega, USA) electrophoresis in 1X TBE buffer at 90Volts for 1 hour and visualised using ethidium bromide staining under UV illumination (Figure 6).

Molecular characterisation of the B. pseudomallei isolate from the snake

Genetic typing of the isolate was done by multilocus sequence typing (MLST) by PCR amplification of the seven housekeeping genes according to Godoy *et al.* (2003). PCR products were confirmed by gel electrophoresis using 1.5% agarose gel (Promega, USA) electrophoresis in 1X TBE buffer at 90Volts for 1hour and visualised using ethidium bromide staining under UV illumination. The PCR products of each of the seven housekeeping genes were purified using MEGAquick-spin (iNtRON Biotechnology, Korea) purification kits according to manufacturer's instructions.The purified PCR products were sequenced by Sanger sequencing method, using the same primers that were used for the initial PCR amplification.The sequence for each gene fragments were aligned and trimmed to the appropriate size for each locus before queried on the MLST database to get the allele number. The housekeeping genes and their corresponding allele numbers are *ace*3, *gltB*1, *gmhD*2, *lepA*3, *lipA*1, *narK*4 and *ndh*3 these combination of alleles were queried in this order on the MLST profile query to obtain the *B. pseudomallie* isolate's sequence type as 51 (ST51).

Antibiotic susceptibility test (AST)

Antibiotic susceptibility by both Disc diffusion or Kirby Bauer (12 antibiotics) and E-test minimum inhibitory concentration (MIC) (five antibiotics) evaluator strip antibiotics methods were conducted. Disc diffusion test showed that the isolate was susceptible to meropenem, imipenem, ceftazidime, doxycycline,

chloramphenicol, ceftriaxone, tetracycline, ciprofloxacin, and trimethoprim/sulfamethoxazole (cotrimoxazole) while resistant to azteonam, gentamycine and ticarcillin. E-test MIC test showed that the isolate from the snake was suceptible to antibiotics tested; meropenem, imipenem, ceftazidime, cotrimoxazole and amoxycillin/clavulanic acid (co-amoxyclav).

Diagnosis

Based on the gross pathological and histological lesions a tentative diagnosis of granulomatous pneumonia was made. A definitive diagnosis of melioidosis was made following the isolation, identification and characterization of *B. pseudomallei* ST51.

DISCUSSION

The Elephant Trunk Snake (*Acrochordus javanicus*), a non-venomous aquatic and nocturnal snake, is found in Southeast Asia, Papua New Guinea, India and Northern Australia and it is most common in Malaysia (Sanders *et al*., 2012). Melioidosis, an infection with *B. pseudomallei* affects a wide range of animal species,horses (Ladds *et al*., 1981), sheep and goats (Fatimah *et al*., 1984; Barbour *et al*., 1997),monkeys (Dance *et al*., 1992; Yap *et al*., 1995), deer (Babjee and Nor Aidah, 1994; Choy et al., 2000), cats and dogs (Yap *et al*., 1995; O'Brien *et al*., 2003) and pet iguana (Zehnder *et al*., 2014). The incubation period of this disease in naturally infected animal is still not known (Sprague and Neubauer, 2004), however, the clinical presentation varies in both humans and animals (Puthucheary and Vadivelu, 2002; Sprague and Neubauer, 2004). In animals, the signs may range from acute fulminant septicaemia, localised infection, subacute disease to chronic infection and subclinical (inapperant) disease (Sprague and Neubauer, 2004). In this case, the time of onset of the disease in the snake was not known and vague signs of inappetence and weakness were the only signs observed prior to its death. These non-specific signs are similar to reluctance or refusal to perform, lethargy and dullness, dyspnoea and partial to complete anorexia as described among marine mammals with melioidosis (Kinoshita, 2008). In this case, the necropsy findings in the snake showed multiple circumscribed protruding purulent nodules all over the lungs and the liver. These finding are consistent with those described by Omar (1963), who reported that the typical gross pathological features of melioidosisis the formation of multiple abscesses in most of the organs. Such multiple nodules or abcesses, especially in the lungs and associated lymph nodes, but also in the liver and the spleen were observed to be typical characteristics of subacute melioidosis (Sprague and Neubauer, 2004). In this case, multiple abscesses of different sizes were found on the liver and clustering along the respiratory tract and particularly more pronounced in the lungs. This finding seem to be in tandem with the observation of Choy *et al*. (2000), that the lungs, spleen, liver and associated lymph nodes appear to be the most commonly affected organs. Histopathologically, the snake lungs revealed the presence of immature to matured garanuloma formation, central area of necrosis containing pus, tissue debris and calcium deposits around the granuloma wall, with leucocytic infiltration in the interstitium. Hicks *et al*. (2000), has decribed the typical histopathological lesions of melioidosis regardless of the tissue type as a focal necrosis, hemorrhage, fibrin exudation, microabscesses with variable accumulation of polymorphonuclear neutrophils mainly distributed in the lungs, liver and spleen.

The or definitive diagnosis of meloidosis is isolation and identification of the causative agent (Leelarasamee and Bovornkitti, 1989; Limmathurotsakul *et al*., 2010). In this case, both the lung and liver swabs yielded an organism that was phenotypically, biochemically and molecularly identified as *B. pseudomallei*. Dance *et al*. (1989) and Walsh and Wuthiekanun (1996), have described that this organism can simply be identified by its colonial morphology on Ashdown's medium, biochemical profile and antibiotic susceptibility patterns. Several genetic identification techniques are now employed as an alternative or complementary identification method to established phenotypic methods. In this case, identification of *B. pseudomallei* targeting the 16SrRNA gene fragment specific for this organism was used. This PCR based test was proven to be a more sensitive method than culture and it is a useful confirmatory test in determining the identity of isolates where conventional biochemical tests gave ambiguous results (Brook *et al*., 1997).

The environmental saprophyte *B. pseudomallei* have been known to be biogeographically and phylogenetically variable. Several previous studies have suggested biogeographical clustering of *B. pseudomallei* strains and genotypes (Vesaratchavest *et al*., 2006; Currie *et al*., 2007; Pearson *et al*., 2009; Dale *et al*., 2011; McRobb *et al*., 2014). In this case, we characterised the isolate from the snake using MLST which showed it to be *B. pseudomallei* ST51. Currently there are 66 ST51 isolates on the MLST database, whereby over 88% were reported from melioidosis cases in humans (Godoy *et al*., 2003) and from water (McCombie *et al*., 2006), mainly from Thailand, Malaysia, Singapore and Cambodia. The source of the infection cannot be ascertained because we could not get the clients cooperation to get water sample which we thought could be the source of the infection. However this type of snakes are naturally aquatic, the disease might have been acquired before captivity in its natural environment. Being the predominantly found ST in Malaysia, eBURST algorithm of *B. pseudomallei* ST51 has shown that this ST was resolved into the major Malaysian clonal complex 50 (CC50) with ST50 as the complex predicted founder. When compared with the global deposited *B. pseudomallei* isolates, *B. pseudomallei* ST51 was found to belong to the major Southeast Asian CC48 as double locus variant (DLV) to the CC founder ST48.This ST being previously isolated from human cases signifies the public health implications of the *B. pseudomallei* isolates with ST51.This can be attributed to the fact that animals with melioidosis might be shedding the organism via external wound exudates and other bodily secretions such as nasal, milk, faeces and urine, thereby contaminating the environment and increasing the

risk of bacterial transmission to humans and other animals (Idris *et al*., 1998; Choy *et al*., 2000; Currie, 2010). This was evidenced by an outbreak of melioidosis from an importation of infected animals with subsequent environmental contamination and persistence of infection in a zoo in Paris, France (Dodin, 1992; Dodin and Galimand, 1986).

Burkholderia pseudomallei is naturally resistant to a variety of antibiotics that include most penicillins, all narrow-spectrum cephalosporins, all macrolides, all polymyxins, and the aminoglycosides (Livermore, 1987; Moore *et al*., 1999; Sam *et al*., 2009). This intrinsic ability of the organism to resist antimicrobial agents makes the treatment of melioidosis difficult. The treatment of melioidosis is divided into two phases; the acute or intensive and eradication phases (Lipsitz *et al*., 2012). Antibiotic treatment of melioidosis is often prolonged, cost intensive and often unsuccessful if not properly implemented (Choy *et al*., 2000). Due to the risks of contamination of the environment with body secretions and discharges from infected animals, treatment of animals with melioidosis is not usually recommended. Furthermore, the optimum doses and regimen of antibiotics for treatment of melioidosis has not yet been ascertained. Infected animals are usually destroyed by incineration (FAO, 2004).

Because of the natural resistance of this bacterium to antibiotics, antibiotic susceptibility testing is recommended. In this case, antibiotic susceptibility test was done using both disc diffusion and E-test MIC evaluation tests. The disc diffusion test showed that the *B. pseudomallei* isolate was susceptible to meropenem, imipenem, ceftazidime, doxycycline, chloramphenicol, ceftriaxone, tetracycline, ciprofloxacin and cotrimoxazole. The susceptibility of *B. pseudomallei* to these antibiotics was consistent with several previous works (Jenney *et al*., 2001; Ahmad *et al*., 2013; Bandeira *et al*., 2013; Khosravi *et al*., 2014). On the other hand, the isolates in this study showed resistance to azteonam, gentamycin and ticarcillin. In a previous study, Cheng and Currie (2005), described that *B. Pseudomallei*was resistant to first, second, and third-generation cephalosporins; penicillins and polymyxin B, while natural resistance to aminoglycosides was described by Moore *et al*. (1999). The five drugs that are involved in the treatment of melioidosi are ceftazidime or carbapenem (either meropenem or imipenem), used in the intensive phase treatment and cotrimoxazole in the eradication phase treatment with co-amoxyclav as its substitute (Lipsitz *et al*., 2012). In this case, the isolate from the elephant trunk snake was suceptible to meropenem, imipenem, ceftazidime, cotrimoxazole and co-amoxyclav by E-test MIC method. This outcome further upholds the current recommendation of the workshop on antibiotic treatment and post-exposure prophylaxis of *B. pseudomallei* and *B. mallei* in 2010 (Lipsitz *et al*., 2012).

Having diagnosed the condition, the client's attention was drawn on the potential risk of this disease to public health and the need to take personal protective measures. Immediate disinfection of the premises of the snake with 10% solution of sodium hydroxide (NaOH) or 5% formaldehyde was recommended, while the snake carcass was incinerated. It can be concluded that *B. pseudomallei* can cause a fatal melioidosis in elephant trunk snakes. The gross and histopathological lesions showed a circumscribed pus containing granuloma. Bacterial isolation and characterisation identified an organism with an ST51 similar to what was previously reported in human cases of melioidosis. The organism was susceptible to antibiotics recommended for treatment of melioidosis. Personal protective measures and disinfection of the premises have been recommended.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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