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Detection of Antibody to *Burkholderia pseudomallei* in Captive and Wild Macaques in West Java and Bali, Indonesia

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Melioidosis is an emerging zoonotic disease caused by the saprophytic *Burkholderia pseudomallei,* which infects human and a wide range of animal species. Melioidosis may lead to septicemia and pneumonia in human patients, which can be fatal if the patient is not treated accordingly. The disease is spread in tropical areas and is highly endemic to Southeast Asia and Northern Australia. However, melioidosis is poorly reported in Indonesia, especially in the veterinary field. This research provides serological evidence of antibodies to *B. pseudomallei* in both captive and wild nonhuman primates. Plasma samples were taken from a total of 390 monkeys in captivities and wild habitats in West Java and Bali, Indonesia. Enzyme-linked immunosorbent assay (ELISA) showed that the seroprevalence was 42.21% for *Macaca fascicularis* and 43.59% for *Macaca nemestrina.* Furthermore, the seroprevalence was 53.41% for captive macaques and 17.83% for wild macaques. The findings showed that exposure to *B. pseudomallei* happened in both captive and wild macaques. Based on this serosurveillance results, further studies such as comprehensive culture and clinical study are required to discover the clinical burden of the disease in nonhuman primates.

Key words: *Burkholderia pseudomallei*, melioidosis, nonhuman primate

Melioidosis adalah penyakit zoonotik yang disebabkan oleh bakteri saprofit *Burkholderia pseudomallei,* yang dapat menginfeksi manusia dan berbagai jenis hewan. Melioidosis dapat berujung pada septikemia dan pneumonia yang berpotensi fatal jika pasien tidak ditangani dengan tepat. Penyakit ini tersebar di daerah tropis, terutama di daerah Asia Tenggara dan bagian utara Australia, namun sangat sedikit didokumentasikan di Indonesia. Penelitian ini memaparkan data temuan serologis berupa antibodi terhadap *B. pseudomallei* pada satwa primata, baik yang berasal dari penangkaran maupun habitat liar. Spesimen plasma darah diambil dari total 390 satwa primata di penangkaran dan habitat liar di Jawa Barat, Bali, dan Labuan. Hasil *enzyme-linked immunosorbent assay* (ELISA) menunjukkan seroprevalensi sebesar 42,21% untuk *Macaca fascicularis* dan 43,59% untuk *Macaca nemestrina.* Seroprevalensi pada monyet di penangkaran dan di habitat liar berturut-turut adalah sebesar 53,41% dan 17,83%. Temuan ini mengindikasikan bahwa paparan terhadap *B. pseudomallei* terjadi baik pada monyet di penangkaran maupun di habitat liar. Penelitian lebih lanjut yang melibatkan kultur dan studi klinis secara komprehensif perlu dilakukan untuk mengungkap kejadian penyakit melioidosis pada satwa primata.

Kata kunci: *Burkholderia pseudomallei*, melioidosis, satwa primata

Melioidosis is a potentially fatal disease caused by Gram-negative bacilli, *Burkholderia pseudomallei,* which can be found in water and soil in endemic areas (Cheng and Currie 2005). The disease spreads in tropical areas, and is highly endemic in Southeast Asia and Northern Australia (Cheng and Currie 2005). The latest global assessment by Limmathurotsakul *et al*. (2016) indicates that melioidosis is also endemic but underreported in a few countries in Southern America and Africa. Furthermore, there is a list of tropical and subtropical countries where melioidosis is predicted to be endemic, but cases have not been documented (Limmathurotsakul *et al*. 2016).

Indonesia has been recognized as endemic area since early of the 20th century. But for the past few decades, melioidosis cases remain underreported in the country (Tauran *et al.* 2015). Recent case reports in human medicine include cases of four tsunami survivors in Banda Aceh, Sumatra (Athan *et al.* 2005),

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and three cases from a hospital in Makassar, Sulawesi (Tauran *et al.* 2015). However, in a retrospective study focusing on unpublished culture-confirmed melioidosis cases, 45 unpublished cases have been identified from hospitals in Jakarta, Banda Aceh, Medan, Banjarmasin, Bandung, Samarinda, Makassar, Kupang, and Pekanbaru (Tauran *et al.* 2018). Reports of melioidosis in veterinary medicine in Indonesia are limited, including cases in 3 cynomolgus monkeys imported to the Britain (Dance *et al.* 1992), 1 pig-tailed monkey imported to the US (Ritter *et al.* 2013; Johnson *et al.* 2013), 1 cynomolgus monkey in a primate research center (Prabandari 2013), and 1 orangutan in a rehabilitation center (Lestari 2017).

Melioidosis is an emerging threat to human and animal health because the disease causes considerable morbidity and mortality, and is often misdiagnosed. Furthermore, the causative agent is classified as Tier 1 Select Agent by the CDC. Its persistence in the environment, high prevalence of severe sepsis, and three routes of infection are some of the key features contributing to the bioterrorism potential of *B. pseudomallei* (Gilad *et al.* 2007). In a highly endemic area in Thailand, the mortality rate of melioidosis was about 40% in all human patients, and even much higher (90%) in patients with severe sepsis (Wiersinga *et al.* 2012). It is concerning that a disease with considerable case fatality rate and bioweapon potency is not yet classified as a notifiable disease in Indonesia.

In human medicine, melioidosis is often difficult to diagnose due to its non-characteristic, multisystemic lesions, including respiratory infection (Meumann *et al.* 2012), genitourinary infection (Morse *et al.* 2009), skin and soft tissue infection (Gibney *et al.* 2008), bacteremia, neurologic lesions (Saipan 1998), and bone and joint disease (Morse *et al.* 2013). The challenge is even greater in veterinary medicine since clinical manifestations of melioidosis may differ from one animal species to another. Furthermore, even if a physician or veterinarian is suspecting melioidosis based on a nonspecific presentation, collection of the right samples is problematic. In healthcare services where the awareness is low, melioidosis is very likely to be misdiagnosed and misidentified. That is particularly true in healthcare services where lesion or blood culture is not mandatory before treating bacterial infection.

In order to increase the awareness about melioidosis, information regarding the disease prevalence, morbidity and mortality rate, best diagnostic techniques, as well as pathogen characteristics is needed. Increased awareness would lead to improvement in diagnostic capabilities and will support healthcare professionals to perform early identification and proper treatment. Those data will also contribute in showing the magnitude of the disease in Indonesia.

This paper describes the first milestone in revealing melioidosis cases in veterinary medicine in Indonesia through a serosurveillance. This assessment will reveal whether captive and wild macaques in West Java and Bali have been exposed to *B. pseudomallei.* All reported veterinary cases of melioidosis in Indonesia were from nonhuman primates (NHPs), a fact that amplifies the importance of melioidosis surveillance in NHPs. Furthermore, NHPs are beneficial to study as melioidosis in NHPs share many similar clinical manifestations and epidemiological features with human melioidosis.

MATERIALS AND METHODS

Sample Collection. Sample collection in captive macaques was performed from January to April 2016. Captive macaques in this study were 159 *Macaca fascicularis* and 101 *Macaca nemestrina* from both sexes and various ages in breeding facilities in West Java (Table 1). Samples from wild macaques include 52 plasma samples from Tinjil Island, West Java, and 78 archived plasma samples taken from monkeys in various locations in Bali Island and Labuan Island (Table 1). All procedures in animals were carried out using protocol reviewed and approved by the Institutional Animal Care and Use Committee, Primate Research Center, Bogor Agricultural University, number ACUC IPB PRC-15-B0011. All animals were anesthetized using ketamine (10 mg kg^{-1} body weight) prior to physical examination. Heart rate, respiratory rate, body temperature, skin turgor and any abnormality (if presented) were examined and recorded. Approximately 3 mL of blood were taken from the femoral vein of each animal.

ELISA. ELISA was conducted in Microbiology and Immunology Laboratory, IPB Primate Research Center. Plasma separation was performed in BSL-2 in Biosafety Cabinet Class II type A/B3 (Nuaire NU 407-600). ELISA assay was performed using plates coated with *B. pseudomallei* lipopolysaccharide, which were kindly provided by the University of Florida. 300 µL of *blocking buffer* (5% blotto) was added to each well, and the plates were incubated at room temperature for 2 h. Plates were washed 4 times with wash buffer (PBS with

0.05% Tween-20). The heat-inactivated plasma were diluted at 1:200 in blocking buffer and then added to ELISA plates. The plates were incubated at 37 °C for 1 h, and then washed 4 times with wash buffer. 100 µL of secondary antibody (Goat anti-monkey horseradish peroxidase conjugate, diluted at 1:1000 in blocking buffer) was added into each well, and then the plates were incubated at 37 °C for 1 h. After washing the plates with wash buffer, 3,3',5,5'-tetramethylbenzidine (TMB) solution was added into each well. Plates were incubated at room temperature for 15-20 min in dark area. The reaction was stopped by adding 50 µL of 1N sulphuric acid. The color development was measured using ELISA plate reader at 450 nm measurement wavelength and 595 nm reference wavelength. Based on the results of the first plate, two samples were used as positive controls, and two other samples were used as negative controls for the next plates. The positive controls were plasma samples that show similar antibody levels to those of aerosol-induced macaques, whereas the negative controls were plasma samples that show a similar antibody levels to those of non-infected macaques (Michael Norris, personal communication).

Cutoff value was determined for each plate according to the method by Frey *et al.* (1998) as follows:

$$Cutoff = \bar{X} + SD \times (t\sqrt{1 + (1/n)})$$

Where $\bar{X}$ = mean of optical densities (ODs) of negative controls,

SD = standard deviation of ODs of negative controls,

n = number of negative controls,

t = student's t distribution value with P = 0.0001 and degree of freedom = $n - 1$

RESULTS

Seroprevalence to *B. pseudomallei* in macaques from various locations is shown in Table 2. A remarkably high seropositivity was detected from cynomolgus monkey in Darmaga facility that recently arrived from Tinjil Island in West Java. Monkeys from several locations in West Java showed 48-70% positivity, whereas monkeys from various places in Bali Island and Labuan Island showed relatively low positivity (0-63%). The seroprevalence was 42.21% for all *Macaca fascicularis* and 43.59% for all *Macaca nemestrina.* Furthermore, the seroprevalence was 53.41% for captive macaques and 17.83% for wild macaques.

Some of the seropositive monkeys showed antibody levels similar to those of aerosol-challenged macaques at 14 and 21 days post-infection (Michael Norris, personal communication). This may indicate that the macaques are potentially infected or have been exposed to *B. pseudomallei*.

DISCUSSION

This paper is the first report describing seroprevalence of *B. pseudomallei* infection in nonhuman primates in Indonesia. The seropositivity was considerably high, and some animals had antibody levels similar to those of aerosol-induced macaques (Michael Norris, personal communication). However, seropositive animals in this study did not show any significant clinical manifestations associated with melioidosis. It suggests that exposure to *B. pseudomallei* does not necessarily cause melioidosis. The seropositivity observed in this study may reflect asymptomatic infection, or reflect exposure that happened in the past. The high seroprevalence observed in many sampling locations also indicates that exposure to the bacteria in the environment happen easily.

The macaques from several locations in West Java (Tinjil Island, Jonggol, Darmaga, and Lodaya) showed considerably higher seroprevalence compared to the macaques from Bali Island, Indonesia and Labuan Island, Malaysia. The only exception is the Karangasem area in Bali where 7 of 11 animals were seropositive. This is in line with current reports of melioidosis in the region. To date, melioidosis cases have never reported in Bali and Labuan.

A review by Tauran *et al.* (2015) showed that melioidosis cases have only been officially reported in West Java, Aceh, South Sulawesi, and Central Sulawesi. However, a more recent retrospective study has discovered that there were 45 unpublished cases from hospitals in Jakarta, Banda Aceh, Medan, Banjarmasin, Bandung, Samarinda, Makassar, Kupang, and Pekanbaru (Tauran *et al.* 2018). This amplifies a hypothesis that melioidosis is underreported in Indonesia. Current reports are predicted to be only the tip of an iceberg and does not reflect the actual national burden.

Exposure to *B. pseudomallei* may occur through inoculation, ingestion, and inhalation. The high prevalence in several sampling locations is presumably related to inoculation from soil and ingestion of contaminated, non-chlorinated ground water. Soil and

Table 1 Number of animals from each sampling location

Sampling Location	Habitat	Number of animals	
		M. fascicularis	*M. nemestrina*
Jonggol, West Java*	Captivity	149	0
Darmaga, West Java*	Captivity	-	77
Lodaya, West Java*	Captivity	10	24
Tinjil Island, West Java*	Wild	52	-
Alas Kedaton, Bali	Wild	12	-
Karangasem, Bali	Wild	11	-
Sangeh, Bali	Wild	4	-
Tabanan, Bali	Wild	17	-
Teluk Terima, Bali	Wild	5	-
Ubud, Bali	Wild	9	-
Uluwatu, Bali	Wild	10	-
Labuan Island	Wild	10	-

*Breeding facilities

Table 2 Seropositivity of antibody to *B. pseudomallei* LPS in captive and wild macaques

Species	Sampling Location	Type of habitat	Total number of animals	Seropositive	Seropositivity (%)
M. fascicularis					
	Jonggol	Captivity	149	72	48.32
	Lodaya	Captivity	10	7	70.00
	Tinjil*	Wild	52	32	61.54
	Teluk Terima	Wild	5	0	0.00
	Alas Kedaton	Wild	12	0	0.00
	Uluwatu	Wild	6	0	0.00
	Sangeh	Wild	8	1	12.50
	Karangasem	Wild	11	7	63.64
	Ubud	Wild	9	1	11.11
	Tabanan	Wild	17	2	11.76
	Pulau Labuan	Wild	10	0	0.00
	SUBTOTAL		289	122	42.21
M. nemestrina					
	Darmaga	Captivity	77	32	45.33
	Lodaya	Captivity	24	12	50.00
	SUBTOTAL		101	48	47.52
TOTAL			390	170	43.59

*Sampling was performed in Darmaga on animals that recently arrived from Tinjil Island

water have been proven to be important in melioidosis acquisition (Cheng and Currie 2005). However, *B. pseudomallei* cannot survive exposure of UV light (Tong *et al.* 1996), and therefore is unlikely to present in the surface layer of the soil. Higher incidence of melioidosis has been reported in association with the rainy season (Choy *et al.* 2000). Contact between macaques and the bacteria most likely happen after rainwater exposed the deeper layer of the soil. Water supplies in several captivities in this study are untreated and may also contribute to the exposure. Drinking untreated (non-chlorinated) water is related to increased risk of acquiring melioidosis through ingestion route in human (Limmathurotsakul *et al.* 2013).

Even though the mode of exposure in these animals has not been elucidated yet, preventive measures should be taken into consideration. Preventive measures in semi-outdoor NHP captivity may be implemented by refining housing design and chlorinating the water supply. Efforts to minimalize contact with contaminated soil may include replacing soil floor with corals, building more perches, and designing paved floor in the shelter area. This preventive measure would reduce the exposure to the bacteria, especially in rainy season when the incidence is usually higher (Kasantikul *et al.* 2015, Choy *et al.* 2000). Water chlorination is also important to reduce the exposure from a contaminated water source.

Immunoassay is not the primary method in diagnosing melioidosis, but is very useful in preliminary assessment of exposure to *B. pseudomallei*. Furthermore, the whole process of culture and microbiological identification of *B. pseudomallei* from clinical samples may take several days. Less time-consuming and less labor-intensive

immunoassays have been developed, including IHA (Harris *et al.* 2011), ELISA (Nualnoi *et al.* 2017; Suttinsunhakul *et al.* 2016), and rapid latex agglutination test (Suttinsunhakul *et al.* 2015). Those assays rely on the detection of antibody to immunogenic components of *B. pseudomallei,* including lypopolysaccharides (LPS), O-polysaccharide part of LPS (OPS), and capsular polysaccharides (CPS). The sensitivity and specificity of those assays varies. OPS-ELISA performed better with clinical samples from endemic areas, whereas OPS-latex agglutination test performed better with clinical samples from non-endemic areas (Suttinsunhakul *et al.* 2016).

The ELISA plates used in this study were kindly provided by Dr. Apichai Tuanyok in the University of Florida, and utilized *B. pseudomallei* LPS as the coating immunogen. This serosurveillance enabled us to discover whether the macaques have ever been exposed to *B. pseudomallei,* and to decide whether follow-up actions are necessary. For surveillance targeting actual presence of the bacteria, antigen-capture immunoassays will be beneficial. A monoclonal antibody based immunoluorescent assay has been developed for rapid detection and it showed a very high sensitivity and specificity in detecting *B. pseudomallei* in blood culture (Chantratita *et al.* 2013).

Culture remains to be the gold standard; *B. pseudomallei* grows on routine media such as blood agar and MacConkey agar. However, the yield is significantly higher when samples are cultured on selective media such as Ashdown agar and selective broth (Wuthiekanun 1990). A quantitation study by Wuthiekanun *et al.* (2007) showed that median *B. pseudomallei* counts in blood, urine, and respiratory secretion were 1.1 CFU mL^{-1}, 1.5 x 10^4 CFU mL^{-1}, and 1.1 x 10^5 CFU mL^{-1}, respectively.

Molecular assays in detecting melioidosis have also been developed, including conventional PCR targeting 23S rRNA (Shahin and Dorsch 2003), sequencing of 16S rRNA (Gee *et al.* 2003), qPCR targeting type III secretion system (Novak *et al.* 2006), and loop-mediated isothermal amplification (Chantratita *et al.* 2008). To date, qPCR of TTS-1 is considered to be the best performing molecular identification of *B. pseudomallei.*

Even though zoonotic transmission of melioidosis is extremely rare, melioidosis study in NHP may contribute to human medicine in terms of epidemiology model, animal model development and vaccine development. Inhalation of *B. pseudomallei* by African green monkey and rhesus monkey resulted in similar clinical manifestations (fever, leukocytosis, neutrophilia, bacteremia, and severe dyspnea) and pathological lesions (pneumonia, lymphadenitis, myelitis, and splenitis) to those of humans, suggesting that AGM is a valuable model for melioidosis (Yeager *et al.* 2012). A culture-confirmed melioidosis case in a cynomolgus monkey in IPB Primate Research Center also showed similar clinical and pathological lesions to human, including difficulty in breathing due to severe bronchopneumonia, splenic pericapsular abscessation, and suppurative hepatitis (unpublished data).

This research is the first milestone regarding *B. pseudomallei* infection in Indonesian nonhuman primates. Based on the results of this study, we ascertained that more studies focusing on microbiological identification, molecular identification, and environmental aspect of melioidosis in Indonesia are required. Hopefully this study may encourage fellow veterinarians and scientists to discover the burden of melioidosis in Indonesia.

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