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# Phylogenetic Diversity of *Burkholderia pseudomallei* isolated from veterinary cases and the environments in Peninsular Malaysia

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ABSTRACT

This study was designed to determine the genotype and the phylogeny of *Burkholderia pseudomallei* isolated from veterinary cases and from the animal environments in Peninsular Malaysia. The Malaysian *B. pseudomallei* population were then compared to those found elsewhere. A total of 113 isolates from veterinary cases (35) and the environment (56 from soil and 22 from water) were characterized using multilocus sequence typing (MLST). Two novel alleles, allele 97 and 69 of the gene locus *ace* and *lepA* respectively were recovered. Isolates were resolved into 12 distinct sequence types (STs) out of which five were novel, namely ST1130, ST1131, ST1338, ST1339 and ST1367. The isolates from veterinary cases co-clustered with those from the environment. *B. pseudomallei* isolates in this study were highly clonal and have descended from a common ancestor clonal complex (CC) 48 found in Southeast Asia. This study shows that veterinary case isolates are often caused by similar STs, with similar populations found in the direct animal environment and those previously reported to cause human infections in Malaysia and elsewhere. Isolates of *B. pseudomallei* from human infections have been given more attention, with a comparatively lower focus on isolates from animals and the farm environment. This study highlighted the genotype and phylogeny of *B. pseudomallei* isolated from animals and the environment and their relations to the isolates from human cases reported in Malaysia and elsewhere. Most STs reported in this study, from veterinary cases and animal environment are similar to those previously reported as causing human infections in Malaysia and elsewhere. Therefore, even though direct zoonosis is uncommon, monitoring melioidosis occurrences in animals can provide insights on the bacterial strains infecting humans.

## 1. Introduction

*Burkholderia pseudomallei* is a saprophytic Gram-negative bacterium, ubiquitous in water and soil of Southeast Asia and the northern tropical part of Australia (Cheng & Currie, 2005). This bacterium is the causal agent of melioidosis, a disease of humans and animals that has a wide range of clinical presentations ranging from asymptomatic to fulminant septicaemia and death (Dance, 2009; Neliyathodi, Thazhathethil, Pallivalappil, & Balakrishnan, 2015; Sprague & Neubauer, 2004). Infection in both humans and animals may occur following skin inoculation, ingestion or inhalation of contaminated soil and water (White, 2003). Animals that are infected may shed the organism via bodily discharges which may increase the risk of direct transmission of the agent between animals, and albeit rare, from animals to human (Choy, Mayo, Janmaat, & Currie, 2000; Dance, 2000; Idris, Rachmat, & Ali, 1998). Importation of infected animals into areas previously unknown to have the disease have been reported to result in an outbreak and subsequent persistence of *B. pseudomallei* in soil (Galimand & Dodin, 1982). It is uncertain if the same strains or sequence types (STS) of *B. pseudomallei* can equally cause disease in humans and in animals. Previous studies have alluded that there was no difference between strains obtained from the environments to those found in human clinical cases (McRobb et al., 2014). Thus, it is likely that similar *B. pseudomallei* strains can infect both humans and animals since soil is the major reservoir of the organism (McRobb et al., 2014; Vongphayloth et al., 2012).

Studies on melioidosis in Malaysia and elsewhere have mainly focused on those that affect humans (How, Ng, Jamalludin, Shah, &

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Rathor, 2005; Pagalavan, 2005; Puthucheary & Vadivelu, 2002). Among local animals, a study by Musa et al. (2012) reported high percentages of serological detection among wide range of species of livestock indicating infection or exposure to the agent. The molecular characteristics and phylogeny of *B. pseudomallei* causing infection in animals and from the environments of Peninsular Malaysia have not been described. In this study, we examined the phylogenetic variability and diversity of a collection of local isolates of *B. pseudomallei* isolated from veterinary melioidosis cases and from the environments (soil and water) using multi locus sequence typing (MLST). We then compared these isolates to those deposited in the MLST database to establish the relationship of our isolates with others found globally. This paper describes the molecular characteristics and distribution of the STs of *B. pseudomallei* isolated from veterinary cases and from the environments, and their relationship with those found elsewhere. We hypothesized that monitoring the phylogeny of this organism in animals and their environment can provide insights on the *B. pseudomallei* strains infecting humans, even though direct zoonotic transmission is uncommon.

## 2. Materials and methods

### 2.1. Collection of B. pseudomallei isolates

Thirty-five isolates from diagnostic cases of animal melioidosis were obtained from cultures stored at the Veterinary Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) and Regional Diagnostic Laboratories of the Department of Veterinary Services (DVS) Malaysia. These cases originated from several states of Peninsular Malaysia that include Selangor, Kuala Lumpur, Perak, Pulau Pinang, Kedah, Kelantan and Terengganu.

Seventy-eight environmental isolates (56 from soil and 22 from water) were obtained from an earlier study by Musa et al. (2015). These isolates were stored at the Veterinary Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM. The description of the source and origins of the environmental isolates is available in the study conducted by Musa et al. (2015). Briefly, soil and water samples from 60 small ruminant farms in Negeri Sembilan, Pahang, Perak and Selangor were sampled. Study farms from each state were selected using simple random sampling from the list of livestock farms obtained from the Department of Veterinary Services (DVS) (Musa et al., 2015). The bacteria were isolated from soil using the methods published by Limmathurotsakul et al. (2012) and water using the methods published by Mayo et al. (2011).

Stock cultures of *B. pseudomallei* had been stored at −20 °C in 20% glycerol brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, United Kingdom) in the respective laboratories until use in this study.

### 2.2. Bacterial resuscitation procedure

Stock cultures were plated onto Trypticase Soy Agar (TSA) (Oxoid Ltd., Basingstoke, United Kingdom) with 5% horse blood and incubated for 48 h at 37 °C. A single colony was picked and sub-cultured onto TSA with 5% horse blood and incubated at 37 °C for 48 h to produce pure growth for subsequent identification tests. Cultures were initially screened morphologically and biochemically using the methods described by Chantratita et al. (2007) and API 20NE™ (Biomerieux, France) according to manufacturer's instructions respectively. All procedures were conducted in BSL 2 facility at the Veterinary Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM. Additional precautionary PPE such as double gloving and N95 mask were used throughout the procedures.

### 2.3. Nucleic acid extraction

The extraction of DNA from the bacterial cultures was carried out using Qiagen DNeasy™ (Qiagen, Germany) according to the manufacturer's instructions. Sterile PCR grade RNase-free water (Qiagen, Germany) was used to dilute the DNA template to a final concentration of 10 ng $\mu L^{-1}$. Quantification and a test of purity was done on the extracted DNA with a spectrophotometer (BIO-RAD®, California) by measuring absorbance at 260 nm and 280 nm. The extracted DNA of each isolate was labelled with a unique identification code and stored at −20 °C.

### 2.4. Polymerase Chain Reaction (PCR) Confirmation of Isolates

The *B. pseudomallei* isolates were re-confirmed by PCR amplification of 550 bp gene fragment using *B. pseudomallei* specific 16S rRNA region primers (PPM3- forward primer) 5′-AATCATTCTGGCTAATACCCG-3′ and (PPM4- reverse primer) 5′-CGGTTCTCTTTCGAGCTCG-3′ described in previous study by Brook, Currie, and Desmarchelier (1997). A PCR (50 µl volume) mixture comprised of 25 µl of Top Taq Master Mix® (Qiagen, Germany), (containing 10x TopTaq PCR buffer, 25 mmol $MgCl_2$, 400 µmol of each dNTP 10x and Taq DNA polymerase 5 units/µL), CoralLoad 5 µl 1x concentrate, forward primer 0.5 µmol; reverse primer 0.5 µmol, 0.25 µg of DNA template and 13 µl PCR grade RNase-free water. The PCR protocol consisted of 30 cycles of 1 minute at 94 °C, 30 s at 54 °C and 2 min at 72 °C, with a final extension step of 10 min at 72 °C (Brook et al., 1997). Gel electrophoresis of the PCR products was done using 1.5% agarose gel in 1.5 times TBE at 80 V for one hour and viewed with Gel Viewer (BioRad, USA). Two veterinary isolates MBTP_A1 and MBTP_B3 and a soil isolate, PRK/02/01S were selected and their 16S rRNA region was amplified and sequenced. The DNA sequences obtained were analysed by using the default blastn search against the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST) to confirm their identities. The 16S rRNA sequences were annotated and submitted to GenBank under the accession number KP844638, KP844639 and KT258752 for MBTP_A1, MBTP_B3 and PRK/02/01S respectively. Reference isolates of *B. pseudomallei* for use as positive controls are no longer available in the market due to the strict regulations on this select agent (Clark, 2008). For this reason, ATCC has removed this organism from its catalogue of microorganisms since 1998. Consequently, the three above-named isolates were used as positive controls in all subsequent tests performed in this study.

### 2.5. Multilocus Sequence Typing (MLST)

The primer oligonucleotides used for the amplification of about 400-550 bp of *B. pseudomallei* seven housekeeping genes were obtained from MLST website (http://bpseudomallei.mlst.net). PCR amplification of the seven housekeeping gene loci on the chromosomal DNA was carried out according to the protocol described on the MLST website (http://bpseudomallei.mlst.net). Briefly, 50 µl volumes mixture was prepared comprising 25 µl of Top Taq Master Mix™ (Qiagen, Germany), 10x TopTaq PCR buffer, 25 mmol $MgCl_2$, 400 µmol of each dNTP 10x and TopTaq DNA Polymerase 5 units $\mu l^{-1}$; CoralLoad 5 µl 1x concentrate, forward primer 0.5 µmol; reverse primer 0.5 µmol, 0.25 µg of DNA template and 13 µl RNase-free water. The PCR amplification condition was set at an initial denaturation time of 4 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s. Samples were then maintained at 72 °C for a further 10 min for final extension, cooled to 4 °C and stored at −20 °C. Gel electrophoresis of the PCR product was done using 1.5% agarose gel in 1.5 times TBE at 80 V for one hour and viewed with Gel Viewer (BioRad, USA).

The PCR products were purified using purification kits (MEGAquickspin,™ iNtRON Biotechnology, Korea) according to manufacturer's instructions. The sequencing was carried out using Sanger sequencing method utilizing same primers used for the initial PCR amplification.

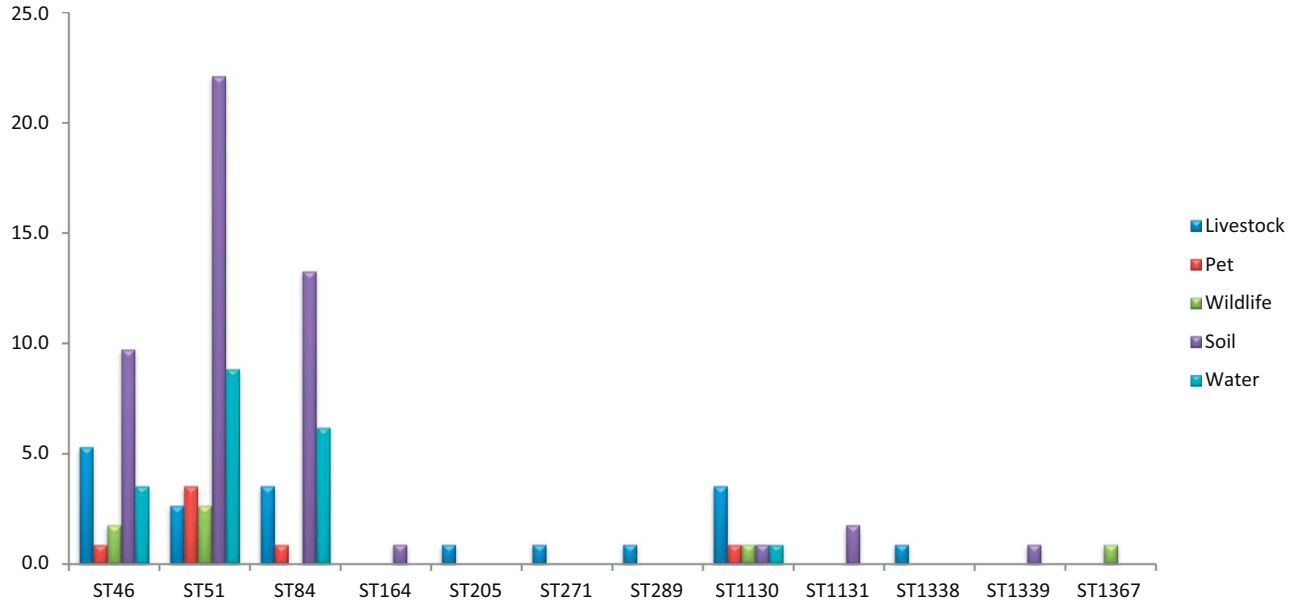


**Fig. 1.** Distribution of STs based on specific source of *B. pseudomallei* isolate**s.**
Fisher's Exact Test = 18.678; $p = 0.056$.
* ST164, ST205, ST271, ST289, ST1130, ST1131, ST1338, ST1339 and ST1367 were merged into a single category 'other STs' in the analysis.

### 2.6. Sequence analysis

For each gene fragment, the sequences obtained were aligned and trimmed to the appropriate size for each locus. The sequences of each locus from the strains were queried in the MLST database to determine the allele number. For each strain, the combination of alleles numbers at each locus of the seven loci, in the order *ace-gltB-gmhD-lepA-lipA-narK-ndh*, defined its allelic profile or genotype referred to as sequence type (ST). When new alleles and STs were encountered, they were submitted to the MLST database curator for verification and assignment of allele numbers and STs.

The phylogenetic relationship and population structure among bacterial isolates from this study to those submitted previously from Malaysia as, well as to those submitted from elsewhere were captured using eBURST v3 (Feil, Li, Aanensen, Hanage, & Spratt, 2004). The sequences of the seven loci were concatenated in the order of loci used to define the allelic profile (http://bpseudomallei.mlst.net) and a rooted neighbour joining (NJ) dendrogram was constructed using Kimura two-parameter method to calculate the pairwise genetic distances. The comparative analysis of molecular sequence data to reconstruct the evolutionary histories of species was performed using Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). MEGA analysis includes collection of maximum likelihood (ML) analyses to enable comparison of the isolates in this study to ascertain their relatedness. Bootstrap method with 1,000 re-sampling was used to evaluate the significance of the nodes on the tree.

### 2.7. Statistical analysis

The data obtained from the MLST analysis were entered into Microsoft Office Excel for Windows 10 (Microsoft Inc. USA) and analysed using SPSS (Version 22.0; SPSS Inc., Chicago, USA). The association between the STs and origin (veterinary cases or the environment) of the isolate was tested using Fischer's Exact test at $\alpha = 0.05$ (Bower, 2003; Sprent, 2011). When data are sparse, they were merged accordingly to allow meaningful analysis be performed.

Clonality, allele diversity and recombination, and the extent of the non-random association of alleles (linkage equilibrium) within *B. pseudomallei* population of this study was statistically measured by quantifying the amount of recombination among a set of sequences and detecting association between alleles at different loci using sequence type analysis and recombination tests software (version 2 or START2) as described by Jolley, Feil, Chan, & Maiden (2001). Index of Association (Smith, Smith, O'Rourke, & Spratt, 1993) and Sawyer's runs test (Sawyer, 1989) were performed at an alpha ($\alpha$) level of significance 0.05.

## 3. Results

### 3.1. Diversity of bacterial isolates from this study

At the time of this study, the MLST database for *B. pseudomallei* contained 3,649 registered isolates comprising of 1,092 STs. In total, 2,271 (62%) of the isolates were from clinical (human) cases, 1.063 (29.1%) from environment, 221 (6%) from veterinary cases and 94 (2.6%) from unknown sources.

From the collection of 113 isolates examined in the study, 18 distinct allele sequences were encountered among which were two novel alleles, 97 and 69 of the gene locus *ace* and *lepA* (*ace*97 and *lepA*69) respectively. The isolates were resolved to 12 STs, five (41.67%) of which were novel STs designated as ST1130, ST1131, ST1338 ST1339 and ST1367. Two of the novel STs, ST1130 and ST1338 are most similar to ST46 reported previously from Malaysia, Thailand, Indonesia, Vietnam, Cambodia and Bangladesh. The ST46 was previously isolated from humans, monkey, water and soil. The remaining three STs, ST1131, ST1339 and ST1367 were most similar to ST51 isolated from humans and the environment of Thailand, Malaysia, Singapore and Hong Kong. More than half (58%) of isolates from this study were categorized into the existing STs in the MLST database.

The 35 veterinary cases isolates were categorized into nine distinct STs, while the 78 environmental isolates resolved into seven STs. Five of the 12 STs were encountered only from veterinary cases; three STs were exclusively recovered from the animal environment while four STs were recovered from veterinary cases and from the animal environment. The *B. pseudomallei* isolates with ST51, ST46 and ST84 were the most predominant genotypes observed mainly from samples from the environment but were also seen in veterinary cases (Fig. 1). Among the

novel STs, the ST1130 was recovered from veterinary cases and from the environment. Goats appeared to be affected by several *B. pseudomallei* strains, whereby six different STs were observed in cases of caprine melioidosis. The distribution of various STs did not appear to be influenced by the origin of isolates as no significant association was observed between STs and sources of isolates (Fisher's Exact Test = 18.678; $p = 0.056$).

There was a significant clonality and linkage dis-equilibrium between *B. pseudomallei* housekeeping gene alleles obtained in this study ($I_{A;}$ 1.704, $p < 0.0001$). Sawyer runs test did not show any statistically significant recombination among alleles in all the seven housekeeping genes ($p > 0.05$).

### 3.2. Relationship among B. pseudomallei isolates from Malaysia

At the time of this study, the MLST database contained 347 isolates that were previously submitted from Malaysia. These isolates were resolved into 60 STs. Of the total isolates submitted from the country, 194 were from clinical cases, 37 from veterinary cases and 80 were from the environment while 36 were from unknown sources. An eBURSTS analysis was performed including the 113 isolates from this study. The local bacterial population was depicted as two distinctive phylogenetic clonal complexes with ST50 and ST84 as the predicted group founders (Fig. 2). *B. pseudomallei* isolates with ST164, ST205 and the five novel STs; ST1130, ST1131, ST1338, ST1339 and ST1367 obtained in this study were characterized for the first time in Malaysia, while those ST46, ST51, ST84, ST271 and ST289 had previously been reported. Multiple outliers (singletons) STs surround the complexes since they are distinct from the predicted group founding STs and therefore remained unlinked (Fig. 2).

### 3.3. Relationship between isolates of this study to other isolates deposited in MLST database

In order to determine the phylogenetic nature of the STs from this study in relation to other STs from Southeast Asia (SEA) (Fig. 3, Panel A) as well as STs from the global isolates deposited in the MLST *B. pseudomallei* database (Fig. 3, Panel B) eBURST population snapshot diagrams were generated. The pattern of evolutionary descent of all isolates in this study along with other global strains was captured in Figs. 3A and B. The isolates were resolved into one large complex CC48, with the Southeast Asian (SEA) ST48 as the predicted founding (ancestral) genotype. This complex does not conform to the simple pattern of radial expansion from the predicted group founder. Therefore, it can be inferred that all STs obtained have evolved from ST 48. The figure showed that ST271 was a single locus variant (SLV), while ST 205, ST46, ST51 and ST1339 were double locus variants (DLVs) to the founder. The ST51, ST84, ST271 and ST289 were all subgroup founders of CC48. Within this CC, ST1131, ST1339, ST164, ST51, ST84 and ST289 were predicted to have evolved from a larger subgroup founder ST50, a major CC in Malaysia, which is a SLV to CC48 predicted founder ST48. ST1130 and ST1338 appeared to have evolved from ST46, a DLV to the ST48.

All the ST48 deposited in the MLST database were isolated from both humans and the environments from Thailand. Two closely related STs, ST84 and ST289 although resolved into this CC, have a distant relationship to other STs obtained in this study and both are subgroup founders of CC48. Two novel STs, ST1131 and ST1339 served as the link of the two large subgroups ST84 and ST289 to other members of the CC48 (Figs. 3A and B). Few STs were apparently commonly reported from SEA, Australia and other parts of the world. Several other unrelated minor CCs and singleton STs from SEA and those from Australia and other parts of the world surrounds the SEA CC48 (Figs. 3A and B).

To ascertain the phylogenetic relationship of the isolates from

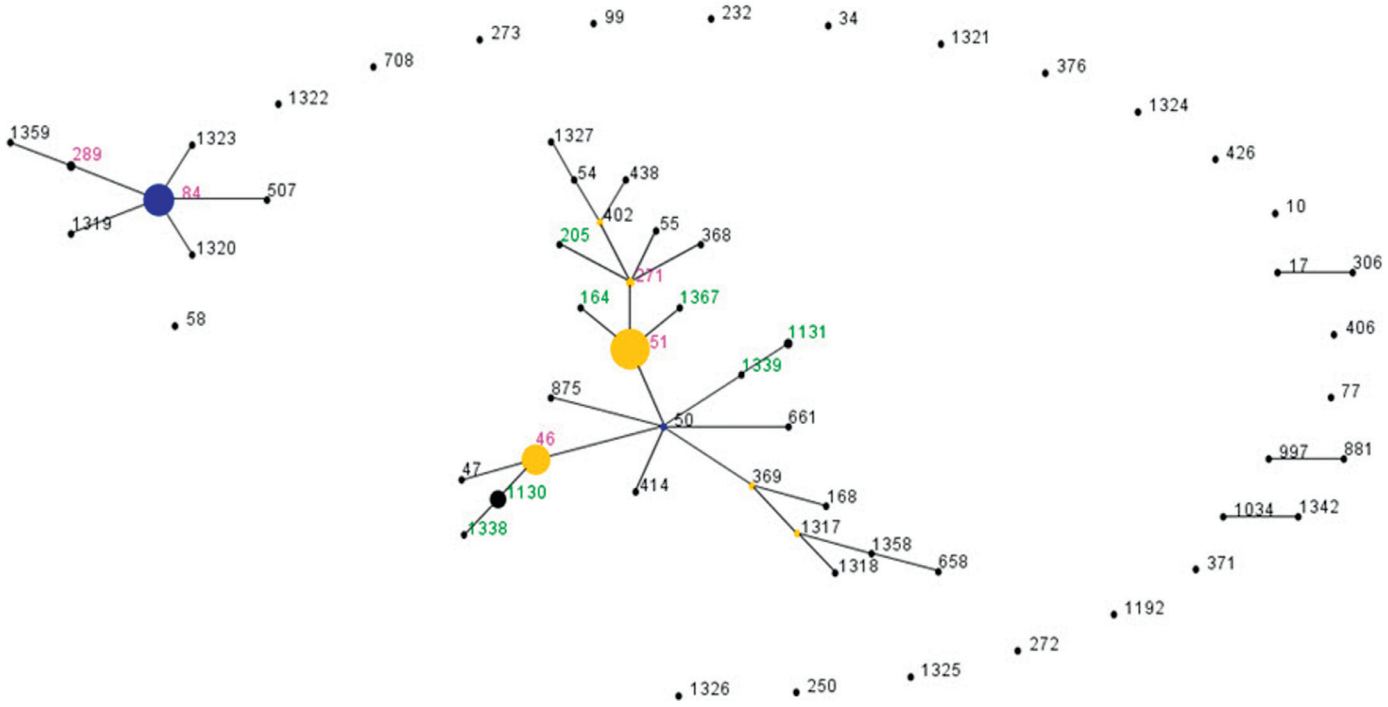


**Fig. 2.** eBURST snapshot of STs from veterinary cases and the environments in Peninsular Malaysia and those catalogued on the MLST database. The blue nodes are the clonal complex founders (ST50 and ST84); the yellow nodes represent subgroup founders. The five novel STs in the present study and two other STs, ST164 and ST205 that were reported for the first time in Malaysia are labelled green. Previously reported STs in Malaysia and reported also in the present study are labelled magenta, other STs that were reported in Malaysia are labelled black. Surrounding STs are singletons and thus unrelated to either CCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

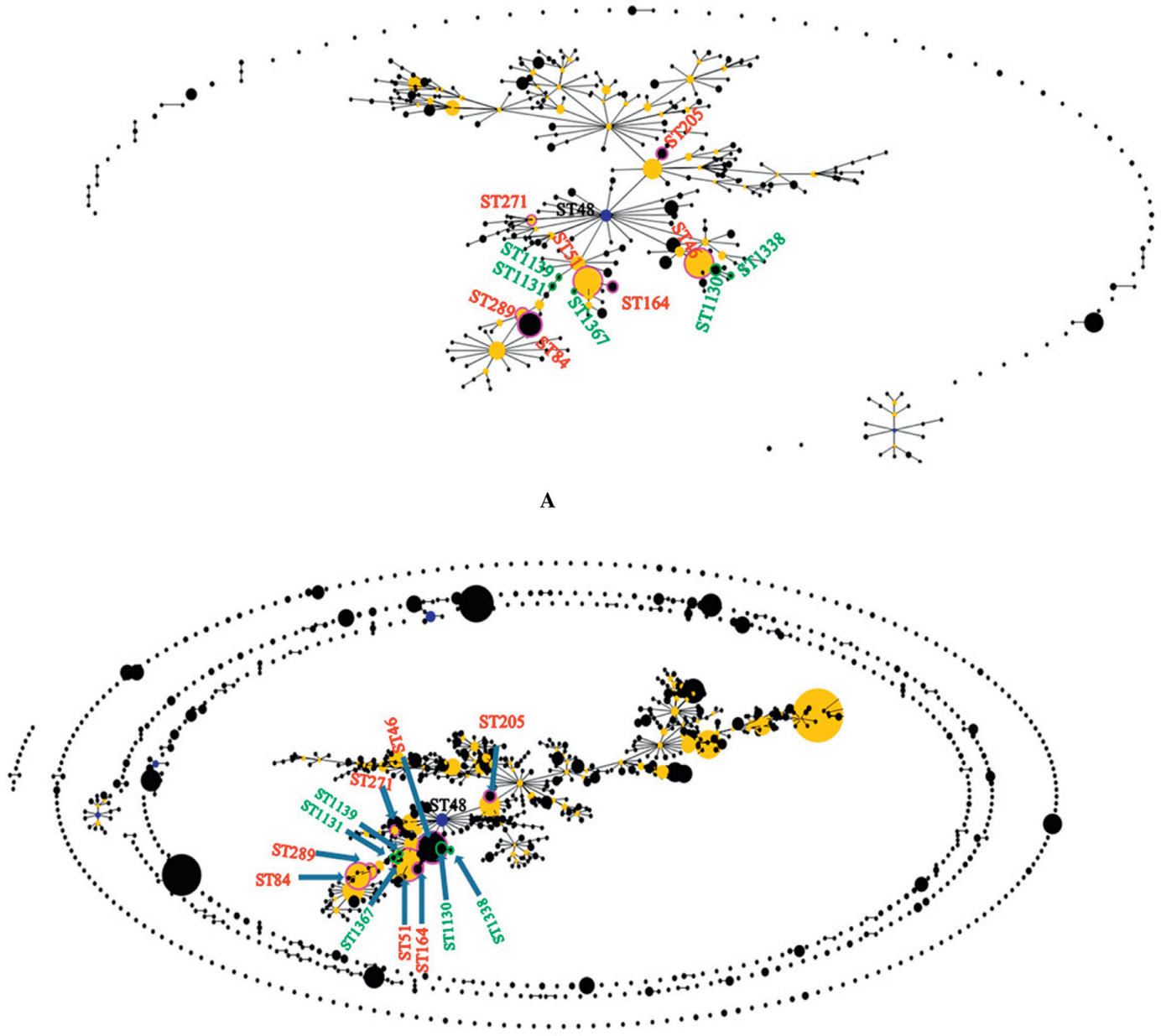


**Fig. 3.** (A) eBURST diagram showing the STs from this study compare to the STs from Southeast Asia (SEA)l isolates deposited in the MLST *B. pseudomallei* database. Black nodes with green outline are the Novel STs (labelled green) obtained from this study. The black and yellow nodes with magenta outline are STs and subgroup founders respectively that are found in this study and previously reported elsewhere (labelled red). Black and yellow nodes without outline are STs and subgroup founders respectively from SEA. Southeast Asian CC48 founder ST48 (labelled black) is represented with a blue node at the centre while all yellow nodes denote subgroup founders. Group with blue node in the surrounding is other CC founder and the surrounding nodes are other unrelated STs (outliers or singletons). (B) eBURST diagram showing the STs from this study compared to the STs from the global isolates deposited in the MLST *B. pseudomallei* database. Black nodes with green outline are the Novel STs (labelled green) obtained from this study. The black and yellow nodes with magenta outline are STs and subgroup founders respectively that are found in this study and previously reported elsewhere (labelled red). Black and yellow nodes without outline are STs and subgroup founders respectively from the global isolates in the MLST database. Southeast Asian CC48 founder ST48 (labelled black) is represented with a blue node at the centre while all yellow nodes denote subgroup founders. Blue nodes surrounding the diagram are other CC founders and the surrounding nodes are other unrelated STs (outliers or singletons). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

veterinary cases and from the environment, a *B. thailandensis* rooted neighbour joining (NJ) tree was generated from concatenated sequences of all seven housekeeping genes (Fig. 4). The 113 *B. pseudomallei* isolates were resolved into three phylogenetically related clades. A clade is a group of isolates that have common recent ancestor. Clade 1 was made up of 33 isolates from veterinary cases (16), soil (12) and water (5). Twenty-nine phylogenetically related isolates formed Clade 2 comprising those from veterinary cases (6), soil (17) and water (6). Three soil isolates S1, S5 and S12 were divergent within Clade 2. Clade 3 was the largest and consisted of 51 isolates from veterinary cases (13), soil (27) and water (11). Clustering of isolates from veterinary cases and from environmental sources is consistent with the knowledge that environment is the reservoir for the agent.


**Fig. 4.** Rooted Neighbour Joining (NJ) tree generated from concatenated sequences of *B. pseudomallei* isolates from veterinary cases, water and soil and the bootstrap values at the nodes. The bar represents differences at 0.005% of nucleotide sites. Isolates from animal/veterinary case (A), isolates from soil (S) and isolates from water (W).

## 4. Discussion

The isolates of this study showed low diversity, as only 12 STs were recovered from the 113 isolates suggesting a rather homogenous population structure. This finding was not surprising as Pearson et al. (2009) have also reported low diversity in *B. pseudomallei* possibly due to infrequent mutation and restricted recombination within the *B. pseudomallei* population. Moreover, Chapple et al. (2016) observed a restricted geographic distributions of *B. pseudomallei* STs recovered from the Northern Territory of Australia.

With the exception of ST46 and the novel STs, majority of the STs recovered in this study that included ST51, ST84, ST205 and ST289 have previously been recovered from humans and from the environments predominantly within the Asian region. However these STs have not been reported from animals such as seen in our study. ST46 have been isolated from humans, soil and one case of a monkey from Indonesia (Godoy et al., 2003). The ST46 appear to be more common in Malaysia as more than 50% of the deposited ST46 in the MLST database originated from Malaysia. ST164, which had previously been reported in clinical melioidosis, soil and water in Thailand (http://bpseudomallei.mlst.net)(McRobb et al., 2014), was recovered from soil sample in this study. All ST271 isolates deposited in the MLST database were from clinical cases from East Malaysia (Malaysian Borneo) and in this study, the ST271 was recovered from a case of melioidosis in sheep.

Our study found five (41.67%) novel *B. pseudomallei* STs, ST1130, ST1131, ST1338, ST1339 and ST1367. The ST1130 was predominantly (75%) found in veterinary cases, but was also recovered from soil and water (25%). A single isolate each of ST1338 and ST1367 obtained were from goat and horse respectively, while ST1131 and ST1339 were solely recovered from the soil.

This study suggests that the goat is the most affected species among the livestock and is infected by various STs of *B. pseudomallei* and this is consistent with the assertion of Choy et al. (2000) that goat is particularly susceptible to the melioidosis. None of the STs was found to be specific for water, suggestive that water possibly gets contaminated by soil carrying the organisms through rain water run offs, flooding, farming activities and construction activities. Vongphayloth et al. (2012) had previously incriminated soil containing *B. pseudomallei* to be the source of contamination of nearby waterways.

The eBURST comparison of all isolates showed that all STs from the present study have evolved from ST48 that belong to the Southeast Asia CC48. Two novel STs found in our study ST1131 and ST1339 linked large subgroups ST84 and ST289 to other members of CC48. At the time of preparing this manuscript, CC48 comprised of 2,078 isolates that resolved into 647 STs. All the ST48 deposited were from clinical and environmental sources from Thailand. In our study, ST271 found in sheep was closely related (SLV) to ST48. ST271 have previously only been recovered from human clinical cases (http://bpseudomallei.mlst.net).

Geo-clustering of *B. pseudomallei* genotypes have been suggested by some authors (Baker et al., 2011; Zulkefli et al., 2016). Vesaratchavest et al. (2006) reported that populations of *B. pseudomallei* have evolved independently in Australia and in Southeast Asia and hence the genotypes are not shared between the two regions. Currie et al. (2007), Pearson et al. (2009) and Dale et al. (2011) further demonstrated existence of the two distinct clusters that were formed by population of *B. pseudomallei* isolates recovered from Australia and Southeast Asia. The eBURST analysis showed that all STs from the present study have evolved from ST48 and belong to the Southeast Asia CC48. Likewise, Zulkefli et al. (2016) have observed a closer genetic relatedness of Malaysian isolates with all Southeast Asia strains in comparison to Australian strains. Nonetheless, eBURST diagram generated from the data in the MLST database for the present study revealed that six STs (ST46, ST89, ST105, ST301, ST594 and ST849) were reported in SEA, Australia and elsewhere. However, whether these STs are common to both regions is unclear as it is possible that those cases diagnosed in Australia (and elsewhere) could have originated from SEA. Similar incident has been demonstrated previously as a case of mistaken identity for ST60 that was reported to be shared between the two regions (Currie et al., 2007). In addition, a recent study of whole genome sequences of two *B. pseudomallei* STs shared by Australia and Cambodia De Smet et al. (2015) found substantial sequence diversity within the STs that correctly distinguished the Asian STs from the Australian STs which confirmed that the shared STs are due to homoplasy.

Neighbour joining (NJ) tree generated from the concatenated sequences of the MLST allelic profiles showed phylogenetic relationship among veterinary and environmental isolates, whereby isolates from both origins co-clustered in the same clade. Unlike a close relative of this organism, *B. mallei* (Nierman et al., 2004), and a few other organisms such as vancomycin-resistant enterococci (VRE) (Getachew, Hassan, Zakaria, & Aziz, 2013) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Lewis et al., 2008) that have been reported to be distinctive and clonal based on source of either environment, humans or animals, our study showed no association between *B. pseudomallei* and its origin. Majority of the *B. pseudomallei* STs obtained in this study from veterinary melioidosis cases are either similar or phylogenetically related to those found in the animal environments. Other previous studies (Finkelstein, Atthasampunna, & Chulasamaya, 2000; Godoy et al., 2003; McCombie, Finkelstein, & Woods, 2006; Podin et al., 2014) have also reported the recovery of similar STs from both clinical and environmental samples. Particularly, study by Mayo et al. (2011) lends support to this finding as they found matching *B. pseudomallei* ST from residential water and the resident suffering from melioidosis in Northern Australia. In addition, Zehnder et al. (2014) discovered identical *B. pseudomallei* ST linking the environment to animals and also to humans. Furthermore, McRobb et al. (2014) found no prominent ST bias among clinical or

environment isolates and suggested that all cultivable *B. pseudomallei* can potentially cause disease to both humans and animals.

## 5. Conclusion

In conclusion, this study provided information on the molecular characteristics and phylogeny of *B. pseudomallei* from animal melioidosis and the environment in Peninsular Malaysia. Two novel alleles *ace*97 and *lepA*69, and five novel STs, ST1130, ST1131, ST1338, ST1339 and ST1367 were recovered. The novel STs, ST1130 and ST1138 shared the novel allele *lepA*69. Isolates in the study were clonal and belong to the Southeast Asian CC48. Most STs previously reported in clinical melioidosis in humans and/or the environments were also present in animal melioidosis and the animal environment in this study. This suggests that any *B. pseudomallei* clones in the environment can potentially cause disease in animals and/or humans and no specific clones are more likely to cause infection in animals as compared to others. Therefore, even though direct zoonosis is uncommon, monitoring melioidosis occurrences in animals may provide insights into the bacterial strains infecting humans.

## 6. Conflict of Interest

All authors declare that no conflict of interest exists.

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