Chapter 16: Molecular Methods for *B. pseudomallei* Bacterial Strain Typing

Chapter Overview and Introduction to *B. pseudomallei* Strain Typing

Bacterial strain typing, or the classification of different genetic variants of bacterial species, is important for disease diagnosis, treatment, and epidemiological surveillance. This is particularly relevant for bacteria exhibiting high levels of pathogenicity and virulence, including *B. pseudomallei*. *B*. *pseudomallei* is classified as a Tier 1 select agent by the US Centers for Disease Control and Prevention (https://www.selectagents.gov/). Potential outbreak scenarios thus warrant rapid public health response and high-resolution tools to quickly investigate source etiology.

In endemic regions, individual cases of melioidosis are typically the result of infection by a single environmental isolate of *B. pseudomallei*. Despite this, clonal or "point-source" outbreaks have been described on several occasions, including contamination of drinking water supplies in two remote Indigenous communities from Northern Australia (Currie et al. 2001; Sarovich et al. 2017), contaminated hand-washing detergent (Gal et al. 2004), as well as cases traced to domestic water wells/bores (McRobb et al. 2015). A handful of outbreaks have also been described in non-endemic settings. One of the most notorious of these outbreaks took place at a Paris zoo between 1975 and 1979. Aside from infecting numerous animals at the zoo, several horse and human melioidosis cases elsewhere in France also occurred as a result (Mollaret 1988). Outbreaks are also frequently reported in non-native animal species in endemic regions, such as in slender-tailed meerkats from Thailand and north Australian zoos (P. Kongmakee 2015; Rachlin et al. 2019), marine mammals from a Hong Kong Ocean Park (Hicks et al. 2000), exotic birds (Hampton et al., 2011), as well as numerous non-human primate species (Sim et al. 2018).

The following chapter discusses several of the more widely used methods for *B. pseudomallei* molecular strain typing, with specific protocols provided for multi-locus sequence typing (MLST), multilocus variable number of tandem repeats analysis (MLVA) and BOX-PCR. An overview of whole-genome sequencing (WGS) technology and its future implications for bacterial source-tracing are also presented at the end of the chapter.

General Approaches to Bacterial Strain Typing

A variety of molecular typing methods have been designed for epidemiological investigations of bacteria and for examining bacterial population genetics. Some methods are more useful for examining localized outbreaks, while others are more suitable for determining the global or long-term evolutionary relatedness of strains (Chan et al. 2001; Maiden et al. 1998). Several current approaches to *B. pseudomallei* strain typing include:

1. Multilocus sequence typing (MLST) – MLST involves sequencing 400-600 base pair fragments of DNA at seven different conserved genes. It enables small variations within a species to be detected. The method is highly discriminatory and can be used to compare the relatedness of isolates globally, but can be both time consuming and expensive, making it impractical for rapid outbreak scenarios (Maiden et al. 1998).
2. Multilocus variable number of tandem repeats analysis (MLVA) – MLVA is a molecular fingerprinting method based on PCR amplification and sequencing of repetitive DNA sequences termed “variable number tandem repeats” (VNTRs). It generates a specific MLVA pattern that is unique to the bacterial strain being investigated. MLVA is quicker to obtain results than MLST, however there are issues with reproducibility and inter-laboratory comparison (van Belkum et al. 1998).
3. Repetitive sequence-based PCR (rep-PCR) – Bacterial genomes contain multiple non-coding, repetitive regions of DNA. Rep-PCR uses these repetitive sequences to produce fragments of variable sizes. These can then be separated using gel electrophoresis, producing a

characteristic pattern of bands, sometimes called a genetic “fingerprint”. One type of rep-PCR frequently used for *B. pseudomallei* is the BOX-PCR (Versalovic et al. 1991).

For local outbreak investigations, molecular typing and fingerprinting methods that are highly resolving are necessary to distinguish small differences in genetically related strains. These types of assays are also useful for distinguishing small genetic changes during sustained outbreaks. The methods that have been used for this purpose include BOX-PCR and multiple-locus variable-number tandem repeat analysis (MLVA) (Maiden et al. 1998). During an outbreak or a cluster of cases, rapid high-resolution fingerprinting methods can be used alongside epidemiological data and more conserved typing techniques, such as MLST, to help discriminate and determine the relationship between isolates.

B. pseudomallei Multilocus Sequence Typing

Multilocus sequence typing (MLST) has become the most widely used method of *B. pseudomallei* strain typing globally, as it indexes variations within fragments of seven housekeeping genes that are selectively neutral. This allows for easy interlaboratory comparison and simple, unambiguous strain characterization (Maiden et al. 1998). Different allele numbers are assigned to each of the seven housekeeping alleles based upon point mutations that occur within each gene. Once assigned, the allele numbers of each of the housekeeping genes are arranged into a string of seven numbers to produce an allele profile. The allele profile corresponds directly to a specific sequence type (ST). An online MLST database has also allowed for standardization of strain types and has conferred the ability to analyze evolutionary relatedness between isolates (Maiden et al. 2013) (https://pubmlst.org/organisms/burkholderia-pseudomallei). Typing of *B. pseudomallei* using MLST scheme is useful to explore sequence type diversity in a particular geographical area (Cheng et al. 2008), track the source of melioidosis outbreaks (Inglis et al. 2000) and define whether recurrent

melioidosis is due to a relapse of the same bacterial ST or reinfection with a different ST (Maharjan et al. 2005; Rachlin et al. 2016).

The MLST scheme specific for *B. pseudomallei* was originally developed by Godoy *et al.* in 2003 and employs seven house-keeping alleles 400bp-600bp in length (Godoy et al. 2003; McCombie et al. 2006). The scheme has now been used to identify more than 1900 *B. pseudomallei* MLST sequence types (as of March 2021). In recent years, whole-genome sequencing (WGS) has provided a rapid alternative for extracting MLST profiles; however, the PCR-based Sanger sequencing method for MLST is still used by many researchers across the globe.

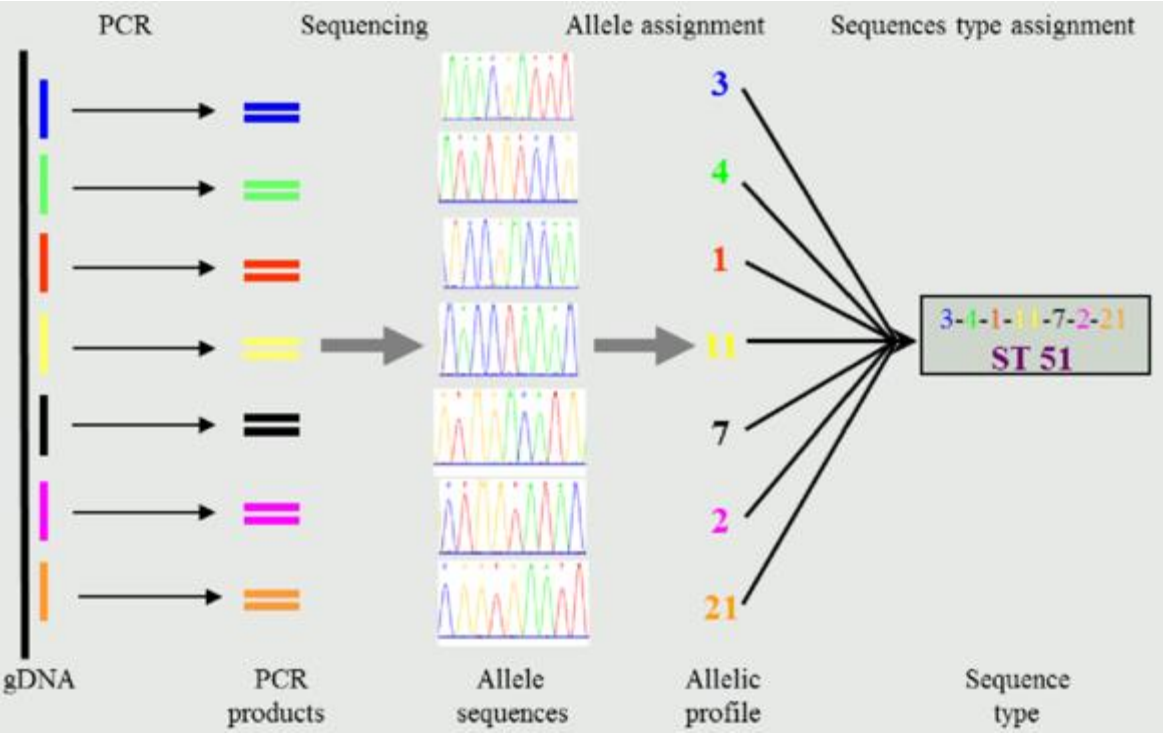


Figure 98- Schematic diagram of the typical bacterial MLST protocol. Allele numbers are assigned to each of the seven PCR amplified housekeeping gene fragments giving an allele profile for a bacterial isolate. Examination of the online MLST database using the allele profile results in ST assignment (Ruppitsch 2016).

B. pseudomallei MLST Protocol

General Comments Before Starting

PCR is very susceptible to contamination and additional precautions should be taken to minimize cross-contamination:

- Where possible, separate the areas where different PCR steps are performed, including PCR reagent assembly, addition of the DNA template, and detection of PCR products (see Chapter 12 for further detail).
- If individual rooms are not possible, separate laboratory bench spaces should be used for these steps. Separate pipettes, laboratory coats and gloves should be used in each of these areas.
- Working in a PCR laminar flow hood is also recommended to minimize cross-contamination.
- Decontamination of surfaces and equipment with a 10% bleach solution or DNAerase/commercial decontaminant solution followed by 70% ethanol should be performed after any handling of nucleic acids at the laboratory bench.
- Always use filter-barrier pipette tips and change and discard gloves frequently.
- Remove DNA templates, controls and other reagents from the freezer and allow them to thaw completely before use. Briefly vortex and centrifuge tubes for 30 seconds at 1000 rpm before using.
- It is good practice to keep reagents on ice while setting up the reaction plate and PCR master mix to prevent reagent degradation.
- Prior to beginning the PCR, plan the experiment by filling out and printing a plate template worksheet (see example below). Ensure sufficient quantities of primer working solutions and other PCR reagents are available and have been correctly calculated.
- Primer working stock solutions should be made up to 20 µM before starting. This will help to prevent primer contamination and limit repeat freeze-thaw cycles every time the reaction is performed.

- Care must be taken when interpretating results, particularly during inter-laboratory studies. Minor differences in electrophoresis conditions can change the distance bands migrate through the gel, confusing comparison between isolates.

Equipment and Reagents

- Primers (see Table 20 below)
- PCR positive and negative controls
- 10x PCR Buffer
- 10mM dNTP Mix
- Taq DNA Polymerase
- Q solution (QIAGEN) or Betaine solution (e.g. Sigma-Aldrich B0300)

NOTE- While not required, these additives are commonly used to enhance amplification of GC-rich sequences. 5% Dimethyl sulfoxide (DMSO) may also be used if these solutions are not available.

- $MgCl_2$
- PCR-grade water
- 10% bleach solution or commercial DNA decontaminant (e.g., DNAerase)
- 70% ethanol
- PPE (gloves, lab coats and safety glasses)
- Laboratory pens/markers
- Racks for 1.5mL microcentrifuge tubes
- Pipettes and filter/barrier pipette tips
- Sterile PCR microcentrifuge tubes
- Benchtop microcentrifuge
- 96-well polypropylene PCR plates (e.g. Thermo Scientific™ AB0600) or PCR strips (e.g. Axygen™ 0.2 mL Polypropylene PCR Tube Strips #16272591)
- PCR strip caps to cover wells

- Vortex
- Thermocycler (such as Eppendorf Mastercycler® or T100™ Bio-Rad)

Primers and PCR conditions for MLST of *B. pseudomallei*

The following primers should be used for the amplification of the seven house-keeping gene fragments:

Locus	Gene function	Product Size (bp)	Forward Primer (5'-3')	Reverse Primer (5'-3')
ace	Acetyl coenzyme A reductase	519	5'-CGGCGCTTCTCAAAACGATA-3'	5'-GAATCGCCTTCACCATGTC-3'
gltB	Glutamate synthase	522	5'-ACGCTCGCGATCGCGATGAA-3'	5'- TTCAGCACGAGCGTCTGCTG-3'
gmhD	ADP glycerol-mannoheptose epimerase	468	5'-GCAGTTCCTGTATGCGTC-3'	5'-GAAGCACTGGTACTTGCC-3'
lepA	GTP-binding elongation factor	486	5'-CATATTCGCAATTTCTCGATC-3'	5'-CACGAGCATCACGACGCCG-3'
lipA	Lipoic acid synthase	402	5'-GGCACCGCGACGTTCATG-3'	5'-GACCATCAGGCCCGATTTCG-3'
narK	Nitrate extrusion protein	561	5'-CTACTCGTGCGCTGGGAT-3'	5'-GACGATGAACGGCACCCAC-3'
ndh	NADH dehydrogenase	443	5'- AGTCGCGACGTTCTACAC-3'	5'- CGAGTTGCAGACGAGATA-3'

Table 20- House-keeping alleles used for *B. pseudomallei* MLST. Gene functions, primers and product sizes are also shown (https://pubmlst.org/organisms/burkholderia-pseudomallei) (Godoy et al. 2003).

NOTE- Alternative primers for the *B. pseudomallei* MLST assay are also described by Price et al. (2016) *J Med Microbiol* 65:992–7.

A. Preparation of DNA Template

For each of the PCR assays described in this chapter, DNA must be extracted from the specimen before it can be tested. **Molecular typing requires isolation of DNA from a pure bacterial culture**. This is done to ensure there is only one bacterial isolate extracted in the event there is a mixed or

"polyclonal" infection and ensures that there is sufficient quantity of bacterial DNA for typing. See Chapter 13 for specific protocols on DNA extraction from bacterial culture.

B. MLST PCR Set-Up

1. Create a plate map to keep track the samples being tested. An example of an appropriate plate map is shown below (Here, K96243 is used for the positive control).

	1	2	3	4	5	6	7	8	9	10	11	12	
A	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*ace* locus
B	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*gltB* locus
C	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*gmhD* locus
D	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*lepA* locus
E	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*lipA* locus
F	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*narK* locus
G	MSHR0338-5	K96243	MSHR7780	MSHR7781	MSMB0059	MSMB0263	MSMB0121	MSMB1588	MSMB0152	MSMB1011	NTC	Blank	<--*ndh* locus
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	

2. Prepare the PCR master mix reagents in the designated PCR clean room/area. The master mix should include all reagents **except the template DNA**. This should be done for all 7 MLST alleles/primer sets to be tested. Ensure two additional reactions are prepared when calculating master mix quantities to account for reagents lost during pipetting. Reactions should be performed in 50 µL volumes either in 8-well tube strips or in 96-well plates. When setting up the PCR reactions keep reagents on ice to prevent degradation.

Reagent	Volume (µL) per Reaction	Final Concentration
PCR grade water	27.75	
10X buffer	5.0	1X final concentration
25mM $MgCl_2$	3	1.5 mM final concentration
10 mM dNTPs	1	200 µM final concentration
20 mM forward primer	1	0.4 µM final concentration
20 mM reverse primer	1	0.4 µM final concentration
Q Solution/Betaine Solution	10	1X final concentration
Taq DNA polymerase	0.25	2.5U final concentration
Template DNA	1.0	
Final volume	50.0	

3. Gently mix the master mix then briefly spin in a microcentrifuge.
4. Pipette 49 µL of this master mix (for each MLST allele/primer set) into the appropriate well of a 96-well plate or PCR strips, according to the plate template worksheet.
5. Cover the wells using cap strip covers. Spray and wipe down the clean workspace with bleach or DNAerase and repeat this with 70% ethanol. Remove laboratory coat and gloves and put on a new pair of gloves. Carry the 96-well plate or PCR strips to the DNA template addition area.
6. Put on a new laboratory coat while keeping the same pair of gloves on. Carefully remove the cap strip covers from the 96-well plate or PCR strips. Open these slowly to prevent aerosolization and spraying of any liquid in the wells.
7. Add 1 µL of template DNA to each appropriate well of 96-well plate or PCR strip using the plate template worksheet for reference.
8. Add 1 µL water to the 'NTC' well.
9. Add 1 µL of the positive control DNA to the correct well. One positive control should be used for each master mix.
10. Cover the wells of each column of the PCR plate or each PCR strip using strip cap covers as you go and secure caps tightly.
11. Spray and wipe down the area with bleach or DNAerase and repeat with 70% ethanol. Remove laboratory coat and gloves. If possible, briefly spin the plate at 1000 rpm to bring down any liquid droplets. Transport the plate directly to the PCR thermocycler room/area and place it in the PCR thermocycler.

C. PCR Thermocycler Set-Up

1. Set the following PCR cycle on the thermocycler:

Stage	Time	Temperature	# Cycles
Initial denaturation	4min	95°C	1
Amplification	30sec	95°C	35
	30sec	62°C	

	30sec	72°C	
Final Extension	10min	72°C	1
Hold	--	4°C	--

2. Double check all temperature and times are correct and press start.
3. Once the reaction has finished, store the PCR products at 4°C until ready for subsequent product analysis or store at -20°C long-term.

D. Analysis of PCR Products by Gel Electrophoresis

It is useful to check for a successful PCR amplification using gel electrophoresis before beginning PCR product purification and sequencing.

1. While the MLST PCR is running, make up an agarose gel **(refer to Chapter 14- Conventional PCR for the full gel electrophoresis protocol).**
2. Once the PCR has finished running, turn off the PCR machine, briefly centrifuge the tubes and take the tubes into the post-PCR processing gel electrophoresis room.
3. Carefully load 5 μL of each DNA product (mixed with loading dye- 1-2 drops or approx. 2 uL) and the DNA ladder into the gel.
4. Run the gel for approximately 45-60 minutes at 100V (check frequently to ensure the products do not run off the gel).
5. Remove the gel from the tank and place on the gel doc to visualize the products.
6. A strong band should appear for each sample to be sequenced. Ensure these bands are the correct size. Only a single DNA fragment should have amplified. This may involve some optimization of the PCR annealing temperature. Since B*. pseudomallei* has a high G+C content, the use of additives such as DMSO with betaine "Q-Solution" can help to improve the amplification of PCR fragments (https://pubmlst.org/organisms/burkholderia-pseudomallei).

Refer to the troubleshooting guide in Chapter 14 if any of the reactions do not display bands on the gel, if there are multiple bands present in one lane, or if bands are very faint. An example of how PCR products should look is shown in the figure below (Figure 99) (Price et al. 2016).

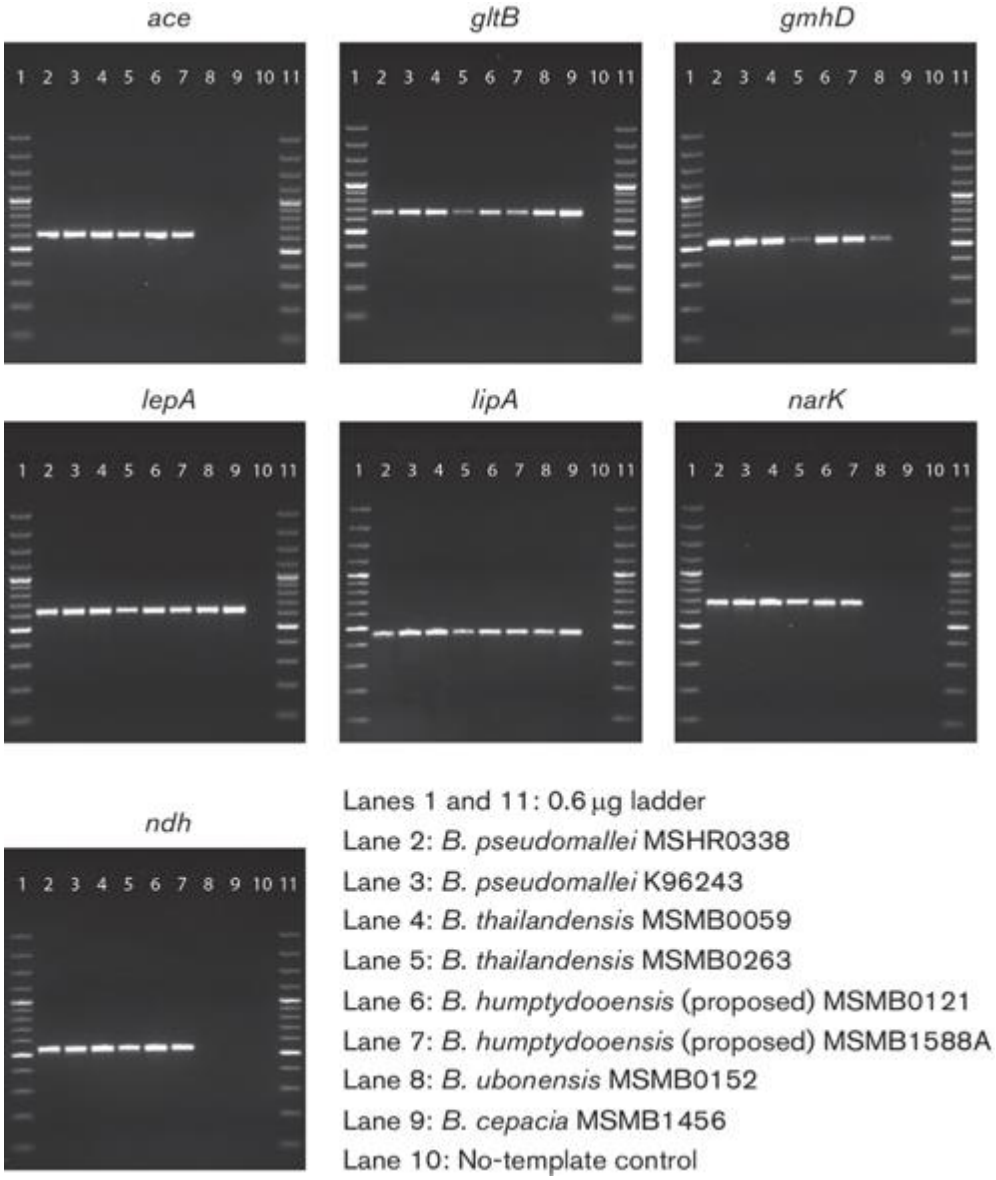


Figure 99- Example of successful amplification of the seven MLST gene alleles. Here, an alternative set of *B. pseudomallei* MLST primers (see the reference for the alternative primers above) are used to amplify members of the *Burkholderia pseudomallei* complex (*Burkholderia pseudomallei, Burkholderia humptydooensis, Burkholderia thailandensis*). Members of the *Burkholderia cepacia*

complex (*Burkholderia cepacia* and *Burkholderia ubonenesis)* do not amplify across all seven alleles (Price et. al 2016).

E. Purification of PCR Products

To perform MLST Sanger sequencing, the amplified products must be purified before sequencing reactions can be performed. This is done to remove any unincorporated PCR reagents and dyes. Purification can be carried out using one of several commercially available kits, such as QIAquick (QIAGEN), which utilize spin columns to purify the products. Likewise, the reaction products can be purified by ethanol precipitation methods or PEG_{8000}/2.5M NaCl precipitation. The inexpensive cost of reagents may make ethanol or PEG_{8000}/2.5M NaCl precipitation a more practical option in resource-poor labs. See below for a simple protocol for PEG_{8000}/2.5M NaCl purification.

NOTE- Sequencing generally requires about 10ng of purified PCR product per 100 bp product size (e.g., for a 500 bp PCR product, about 50 ng of template is required for sequencing) (QIAGEN 1998).

A protocol for QIAquick (QIAGEN) purification, can be accessed at:

https://www.qiagen.com/au/resources/resourcedetail?id=e0fab087-ea52-4c16-b79f-c224bf760c39&lang=en

For PEG_{8000}/2.5M NaCl Precipitation (Glenn 2021; Kusukawa et al. 1990):

Materials-

- 20% PEG (Polyethylene glycol), 2.5 M NaCl –
  - For 50mL, mix the following in a 50 mL conical flask:
    1. 10.0 g Polyethylene Glycol 8000
    2. 7.3 g NaCl
    3. Distilled H_2O up to 50 mL –
  - Place into an incubator set to 37°C for 20 minutes.

- 80% ethanol
- TLE = 10 mM Tris-HCl, 0.1 mM EDTA

Procedure

1. Add 50 µL of PEG to a sterile Eppendorf tube. Transfer the PCR product to the tube and mix thoroughly by pipetting up and down.
2. Incubate the mixture at 37°C for 15 min.
3. While the mixture is incubating, place the 80% ethanol on ice.
4. Once the mixture has been incubated, centrifuge tubes at high speed (~15,000 x g) for 15 minutes.
5. Use a sterile pipette to remove the supernatant and discard it.
6. Add 125 µL of cold 80% ethanol to the tube and centrifuge for two minutes. Carefully remove the supernatant with a pipette and discard.
7. Repeat step 6.
8. Evaporate residual ethanol using a speed-vac for 5-10 minutes or by opening the tube and leaving in a 37°C heat block. There should be no trace of the ethanol remaining.
9. Resuspend the pellet in 25 µL of distilled water or buffer of choice (e.g TEL buffer- Tris-HCl/EDTA) or make up to the appropriate concentration required for sequencing. Pipette up and down enough so that the DNA has fully gone into solution.
10. Laboratories should ensure they follow the protocols described by the sequencing platform being employed to ensure the correct concentration of product is sent.

F. Sanger Sequencing of Purified MLST PCR Products

The purified PCR gene fragments should be sequenced in both forward and reverse directions and then aligned using sequence alignment software. Sequencing each strand provides more confidence than if only a single strand is used to determine the base calls. The aligned sequences can then be trimmed to the correct length for comparison and examined for miscalled bases, insertions, gaps,

overlapping nucleotide signals and any sequence differences. High-quality sequencing is essential for MLST, as a single base pair change can result in a new allele number being designated (Maiden et al. 2013).

For *B. pseudomallei* MLST, the same 7 MLST primers are also used for sequencing that are used for PCR amplification. Primers should be diluted in PCR-grade water to a concentration of 10pmol/μL before sending for sequencing.

Preparing primer dilutions

- The easiest way to prepare primer dilutions is to make a 1 in 2 (1:2) dilution of a 20 μM primer stock.

  NOTE: 20 μM = (20mol/L) x (1L/1000,000 μL) x (1000,000 pmol/1μmol) = 20pmol/μL
- Send 20 μL of 10pmol/μL for every 5 samples to be sequenced.

Once the PCR products have been purified and primers have been appropriately diluted, they can be shipped to any commercial sequencing laboratory for standard Sanger sequencing (e.g. Macrogen, Seoul, Korea: https://dna.macrogen.com/pageLinkDnaSys.do?menuCd=SUP100200&layout=page_sub&link=%2Fsupport%2FretrieveGuideCes#none.

Ensure samples are appropriately sealed to prevent spillage or evaporation during transport and that sample information is filled out correctly based on the specific commercial sequencing laboratory being used. Sequencing can also be done in-house if available using commercially available kits (e.g. Applied Biosystems BigDye™ Terminator chemistry).

G. Analysis of Sequence Data and Allele Determination

- After the data has been obtained from the sequencer, the sequencing files (also termed trace files) must be examined for incorrect base calls. The complementary strands must also be aligned and trimmed so they are the appropriate length. These aligned and trimmed sequences are called “DNA consensus sequences”- or a sequence of DNA that represents the most commonly encountered nucleotides found at a specific location.
- Various computer programs can be used to assemble, align and edit the trace files from the DNA sequencer and can be used to create consensus sequences, such as CLC Genomics Workbench (QIAGEN) or ChromasPro (Technelysium- free online download).
- Follow the specific software protocols to assemble and align the sequences.
- Sequences that are inadequate in length (too short) or poor in quality (displaying two peaks at the same nucleotide position, or sequences exhibiting an inadequate concentration of DNA) should be discarded and the samples should be re-amplified and sequenced again.
- Forward and reverse sequences that are high in quality can be aligned to create a consensus sequence for the seven housekeeping genes for each *B. pseudomallei* isolate.

H. MLST Analysis

Once consensus sequences have been created, they can be copied into the MLST website for allelic analysis and sequence type (ST) determination (https://pubmlst.org/organisms/burkholderia-pseudomallei). The software examines if the sequences are the correct length and do not contain any unspecified characters. The user is then able to check the allele at a single locus, to enter an allele profile, to search for isolates in the database that match a specific allele profile, or simply browse the database. Sequences not already present in the database can be submitted to the database curator as a new allele. The database curator will then examine the sequence trace files and assign a number to the new allele profile to add to the database.

To check individual alleles:

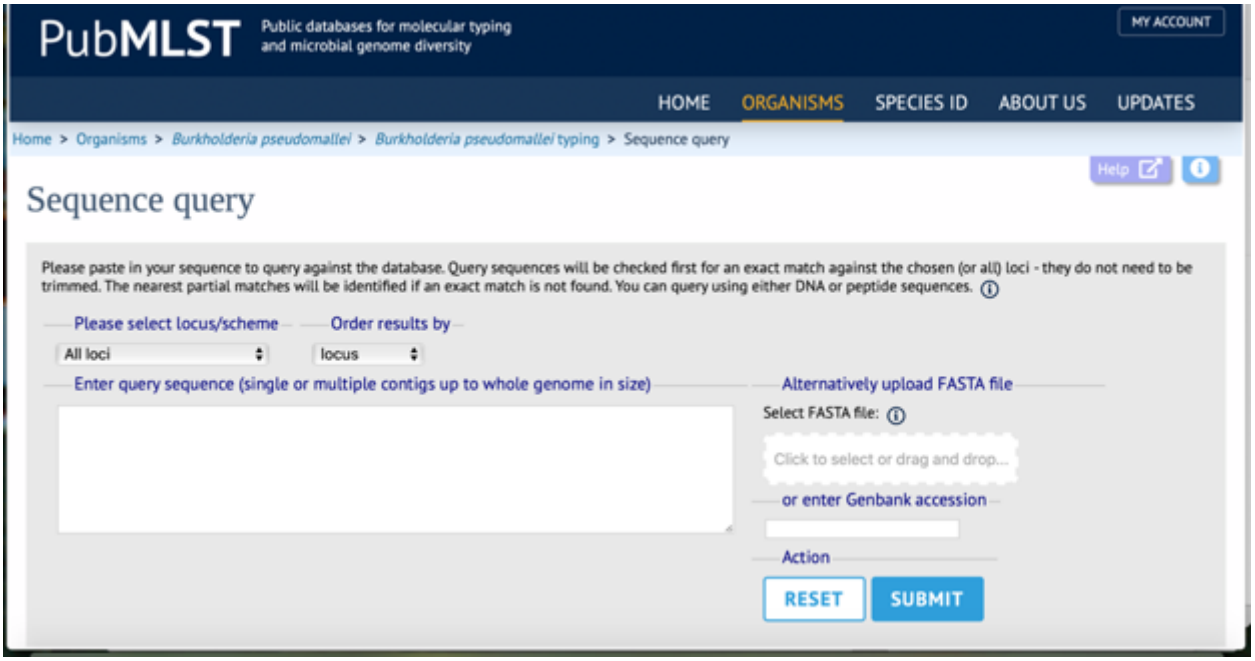
1. On the MLST website select “Single Sequence Query”

   (https://pubmlst.org/bigsdb?db=pubmlst_bpseudomallei_seqdef&page=sequenceQuery).

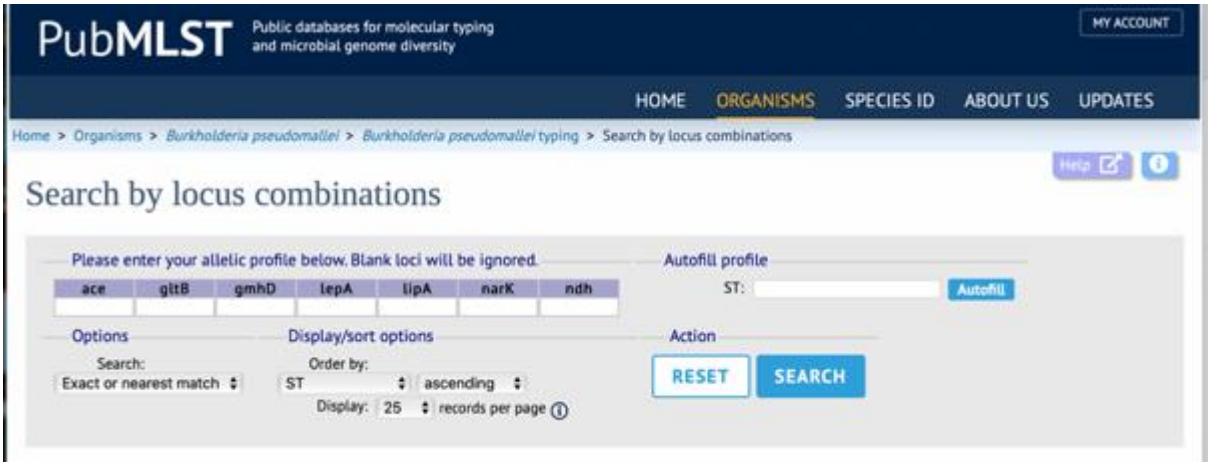
2. Choose the specific allele to be checked and paste your allele sequence into the box.

   Alternatively, you can upload individual sequence FASTA files.

3. Press submit. The number for your allele will be displayed at the top.

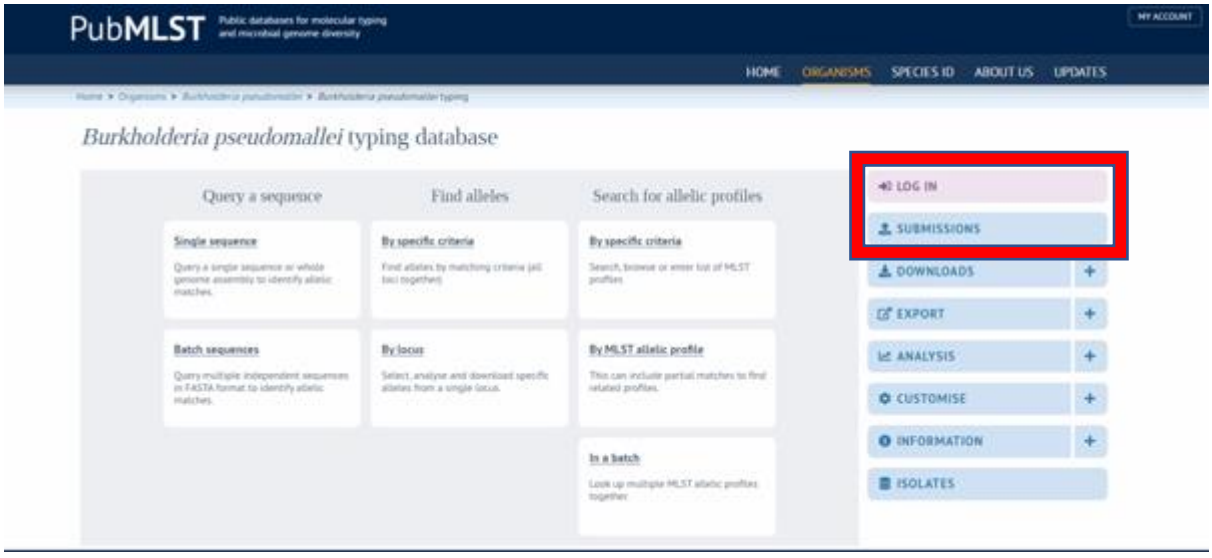


4. Write down the number that is assigned to each allele.
5. Go to “Search for Allelic Profiles by Specific Criteria”: https://pubmlst.org/bigsdb?db=pubmlst_bpseudomallei_seqdef&page=profiles&scheme_id=1.
6. Type in the number for each allele and press submit to view the designated ST type.

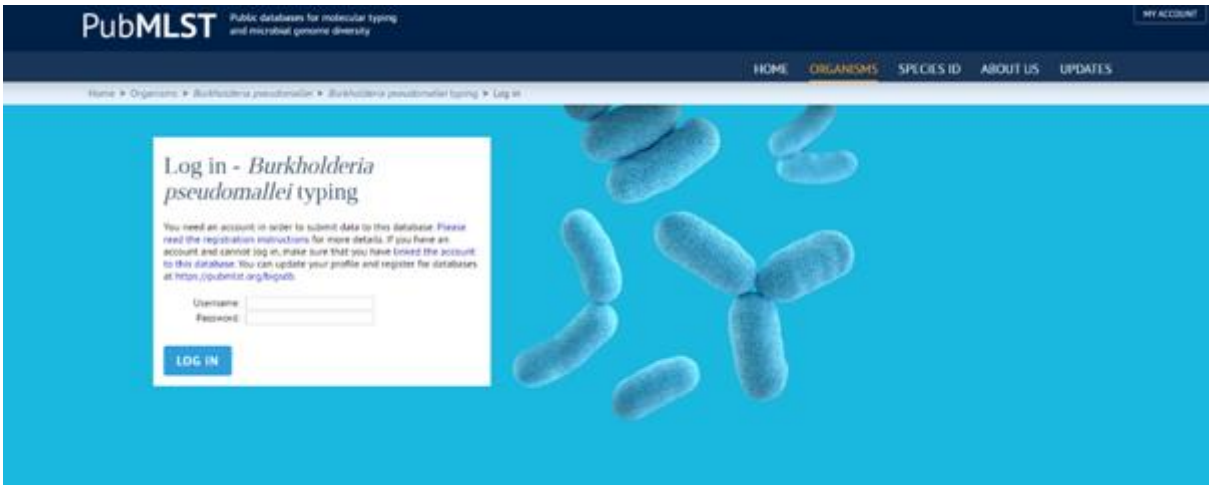


I. Submitting Data Using the Online Submission System

The automated submission system allows users to submit data (new alleles, ST profiles, or isolates) to the database. The MLST curator will then assign new alleles or STs and upload them to the database. New submissions can be made by clicking the ‘Typing” followed by “Submissions” tab on the database front page.



You must have an account in order to use the submission system. Once logged in, follow the instructions provided to submit MLST data.



NOTE- For any questions concerning how to use the *B. pseudomallei* MLST database or submitting isolate profiles, email the database curator (email found on the *B. pseudomallei* PubMLST homepage https://pubmlst.org/organisms/burkholderia-pseudomallei).

J. Phylogenetic Analysis Using MLST Data

The MLST online database has several links to various programs for further analyses. MLST data are frequently represented by a dendrogram (a tree diagram used to visualize evolutionary relationships and show the arrangement of clusters) using programs such as molecular evolutionary genetics analysis version-6 (MEGA 6) software (Tamura et al. 2013) (Figure 100) and eBURST analysis, which can be used to determine the evolutionary relatedness between isolates (Feil et al. 2004; Spratt et al. 2004) (Figure 101). A plugin for MLST eBURST analysis is available at https://pubmlst.org/bigsdb?db=pubmlst_bpseudomallei_seqdef&page=plugin&name=BURST&scheme_id=1&l=1). The global optimal based upon related sequence types (goeBURST) program, part of PHYLOViZ software, can also be used to examine *B. pseudomallei* isolate relatedness using MLST

data. geoBURST allows for higher discerning clustering based on double and triple locus variants (e.g. the number of alleles that differ from the ancestral genotype) and incorporates global MLST data (Francisco et al. 2009). See http://www.phyloviz.net/goeburst/ for more information and download options.

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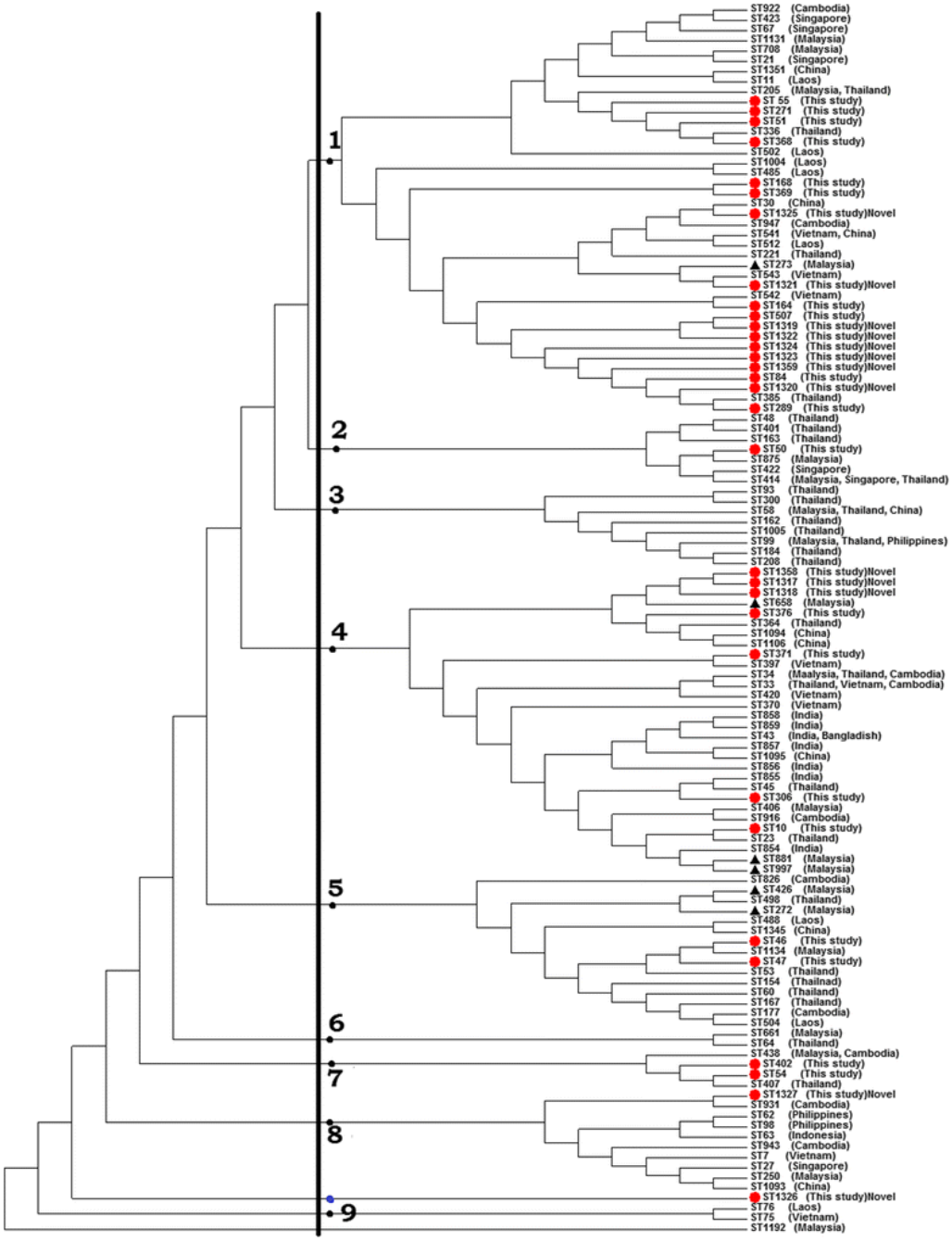


Figure 100- Here, the evolutionary relatedness of *B. pseudomallei* clinical isolates from Malaysia is compared to India, China and Southeast Asian isolates using a dendrogram based on an Unweighted

Pair Group Method with Arithmetic average (UPGMA) method in (MEGA 6) software (Zueter et al. 2018).

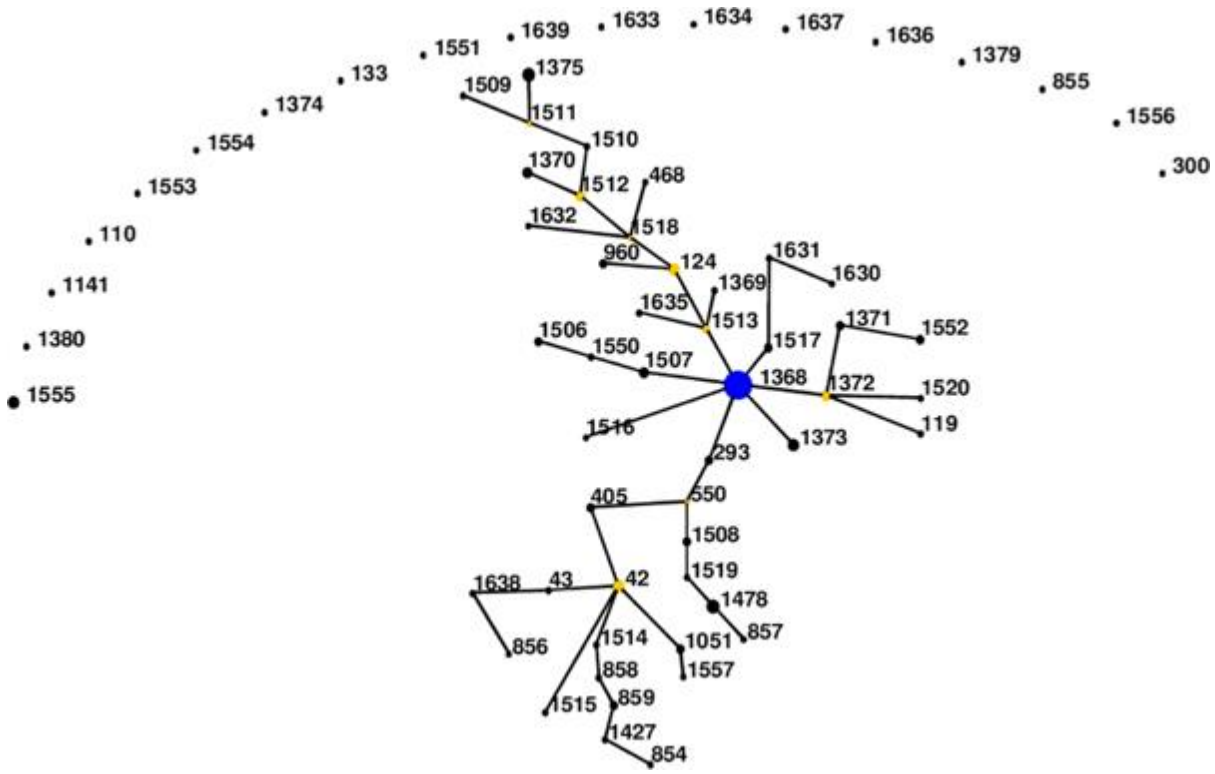


Figure 101- Genetic relationship of 130 Indian *B*. *pseudomallei* isolates using eBURST analysis. The blue dot demonstrates the founder (most ancestral genotype) and yellow dots refer to sub-group (secondary) founders. Each black dot represents single genotype (Kamthan et al. 2018).

Limitations of MLST

Although MLST is an effective and widely applicable scheme for examining *B. pseudomallei* strain relatedness and epidemiological surveillance, there are several limitations associated with its use. The scheme is incapable of determining minor variations amongst closely related isolates. MLST is not suitable to characterize small differences in strains from a potential point-source outbreak. Moreover, MLST can also take several days to weeks to obtain results, and is therefore unable to quickly distinguish the occurrence of an outbreak (Currie et al. 2007). In the event of a potential outbreak or case cluster of melioidosis, rapid and highly discriminatory fingerprinting methods, such as multiple-locus VNTR analysis (MLVA) or BOX-PCR, could be used to show how closely related isolates are. MLST may then be used for sequence typing and examining isolate divergence.

Methods for Rapid Strain Typing of *B. pseudomallei*

1. MLVA-4

   PCR-based variable-number tandem repeat (VNTR) loci is a widely utilized tool used for rapid strain typing of pathogens (Currie et al. 2009; Van Belkum 2007). Multiple-locus VNTR analysis (MLVA) is comprised of several tandemly repeated sequences of DNA that vary in copy number, producing different PCR-amplicons that can be visualised on a gel. MLVA allows for superior discrimination between closely related isolates (Liu et al. 2006; Van Belkum 2007). A 4-locus MLVA platform has more recently been developed for *B. pseudomallei.* This rapid and highly discriminatory typing tool can produce results within eight hours of receiving *B. pseudomallei* isolates. It has also been used on multiple occasions to identify point-source outbreaks of melioidosis (Currie et al. 2009).

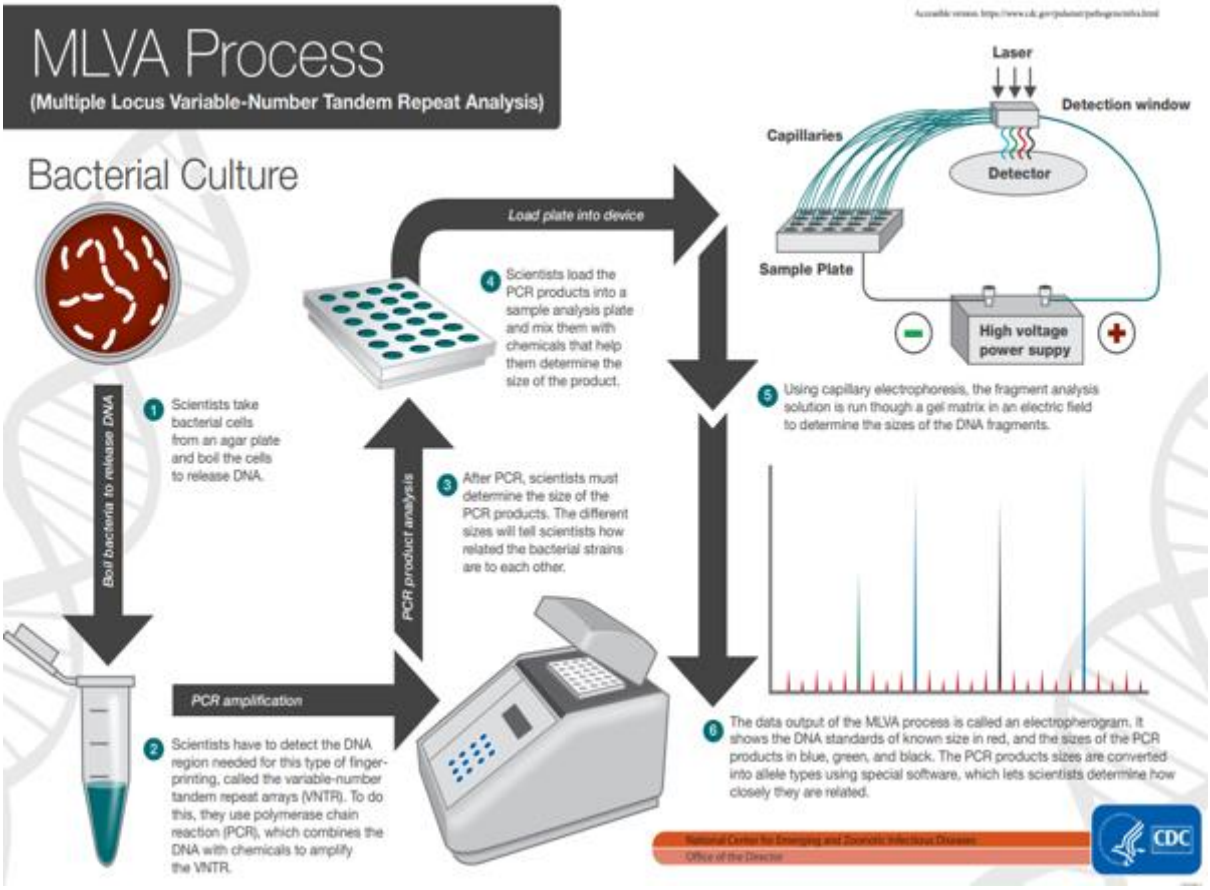


Figure 102- The MLVA process typically involves the detection of variable-number tandem repeats (VNTR) using PCR. Different sized products are used to determine how closely related bacterial strains are to one another (CDC 2016).

2. <u>BOX-PCR</u>

BOX-PCR was conceived as a simple and rapid tool to discriminate between *B. pseudomallei* isolates. It was initially adapted from methods used to differentiate *Burkholderia cenocepacia* isolates in cystic fibrosis patients but has since been demonstrated to be equally discriminatory in *B. pseudomallei* (Coenye et al. 2002; Currie et al. 2007). BOX-PCR can provide results within 10 hours of receipt of bacterial isolates, meaning it is a valuable tool for rapidly determining the relatedness of isolates during a possible outbreak scenario.

***B. pseudomallei* MLVA-4 Protocol**

The following protocol was provided by Menzies School of Health Research, Darwin, Australia. It has been adapted from Currie et al. *Emerg Infect Dis.* 2009; 15 (2):169-74. Contact mark.mayo@menzies.edu.au for further questions concerning the protocols described here.

NOTE- The protocols for MLVA-4 typing require specific data analysis software and thorough training of laboratory staff. This may make the technique impractical for many laboratories. In this instance, labs can also conduct BOX-PCR typing for rapid *B. pseudomallei* strain comparison (see the protocol below), as this method requires fewer technical equipment and training.

Technical Guides:

- GeneMapper Software- **http://tools.thermofisher.com/content/sfs/manuals/cms_042039.pdf**
- BioNumerics Software- **https://www.applied-maths.com/applications/mlva**

Primers:

LOCUS	2341	1788	933	389
Color-labeled forward primer sequence (5′ → 3′)	FAMGGCTTCGCACCC GCCCCATTTCAGC	PETGCGCGGCGAGAAC GGCAAGAACGAA	NEDATGGTGGCGGCC GTCGGCGAAAACC	VICGTTACAAGCGCGGG TCGGCAAGAGGCTGAA A
Reverse primer sequence (5′ → 3′)	GCACCGGGCGCGGC GCACTCG	GAGCATCGGGTGGGCG GCGCGTATTGAT	GCTCGAATGGGTGTA CGAAGGGCCACGCTG ATTC	GCCGGTGTTGAACGAG TGGGTGGCGTAAGC
Repeat sequence (5′ → 3′)	TTCGTGCGC	GTCGTGCGATCCTGCT	CGGCGAGGGAAA	GACGAACC
Minimum size (bp†)	111 (2)	235 (4)	171 (3)	221 (1)
Maximum size (bp)	243 (17)	382 (13)	337 (17)	292 (10)

†Error range in fragment sizing is ± 3 bp.

1. Create a list of isolates to be tested and set up a plate template

For each sample, 4 individual PCR reactions must be done (hence MLVA-4).

A. Create a sample list with the sample names/IDs and a plate template to keep track of sample locations (see example below).

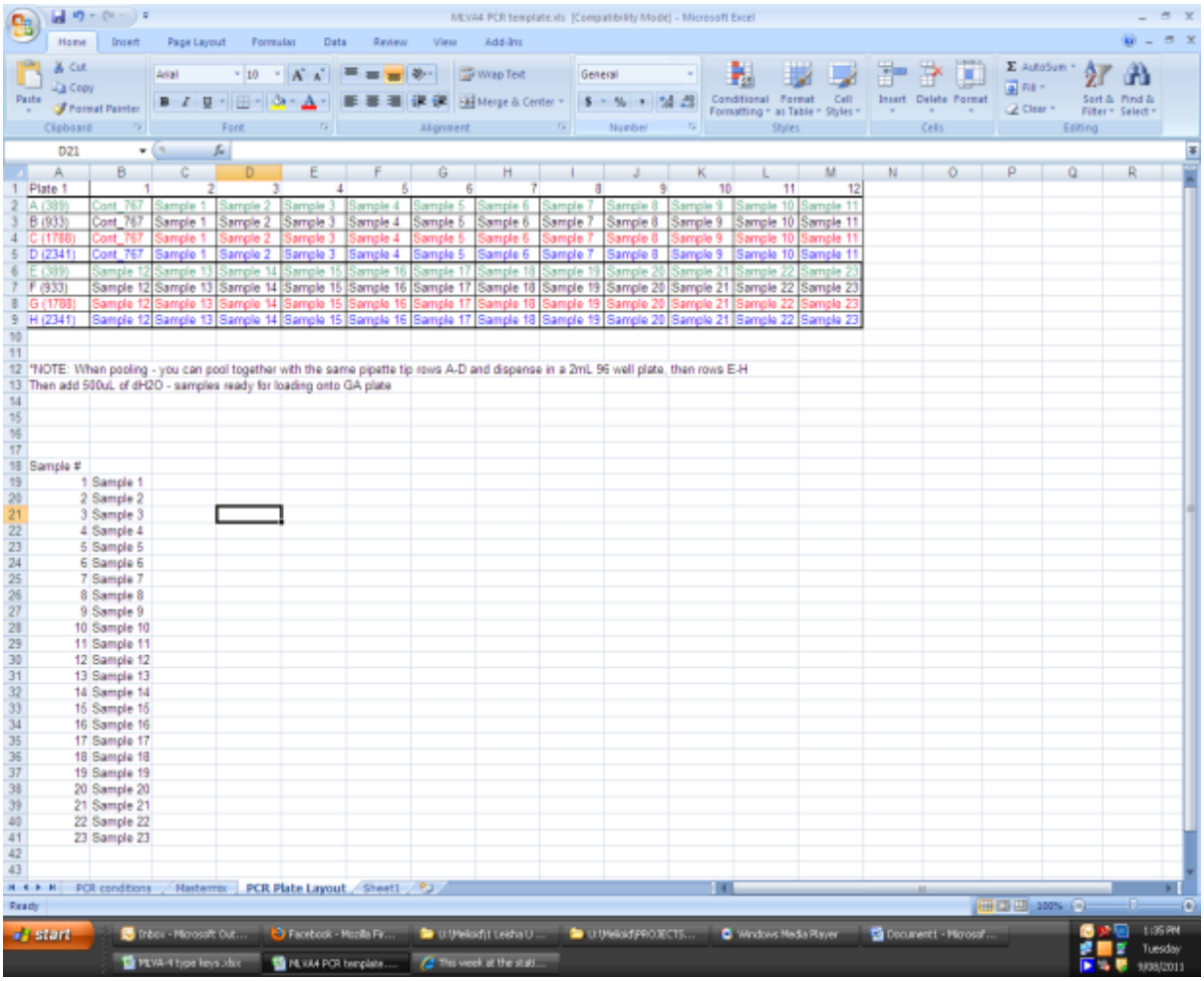


Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A (389)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
B (933)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
C (1788)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
D (2341)	Cont_767	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11
E (389)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
F (933)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
G (1788)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23
H (2341)	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23

*NOTE: When pooling - you can pool together with the same pipette tip rows A-D and dispense in a 2mL 96 well plate, then rows E-H
Then add 500uL of dH2O - samples ready for loading onto GA plate

Sample #	
1	Sample 1
2	Sample 2
3	Sample 3
4	Sample 4
5	Sample 5
6	Sample 6
7	Sample 7
8	Sample 8
9	Sample 9
10	Sample 10
11	Sample 11
12	Sample 12
13	Sample 13
14	Sample 14
15	Sample 15
16	Sample 16
17	Sample 17
18	Sample 18
19	Sample 19
20	Sample 20
21	Sample 21
22	Sample 22
23	Sample 23

B. Calculate the amount of master mix required based on the table below. Each reaction should contain 10µL of master mix and 1µL DNA template. Be sure to include a negative control, positive control and at least 2 additional samples to allow for reagents lost during pipetting.

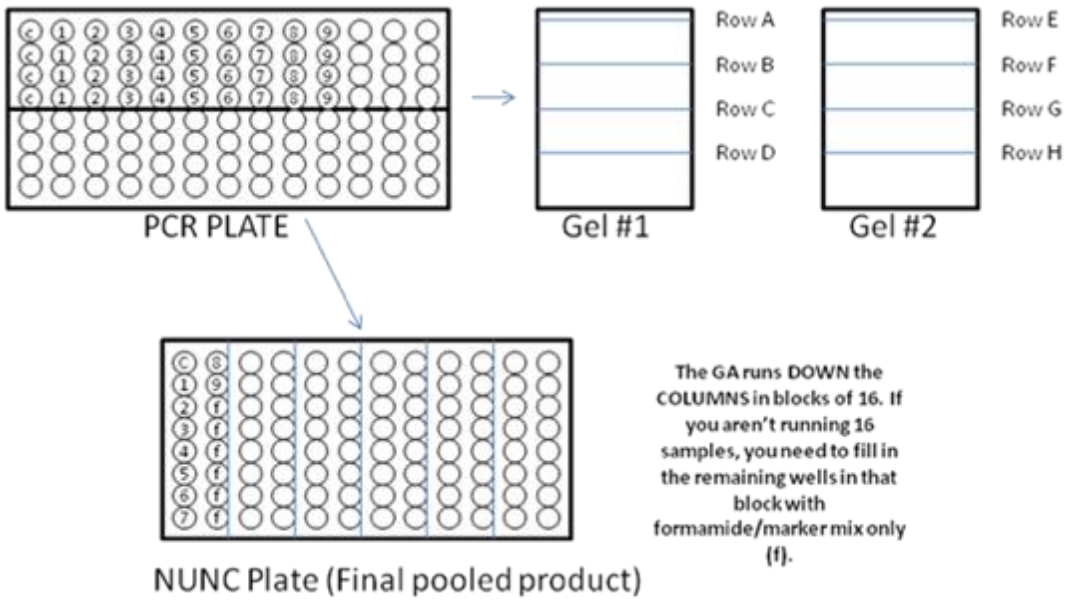
C. This will give the amount of master mix required for ONE SET OF PRIMERS. For MLVA 4, there are four primer sets: 389, 933, 1788 and 2341. Four master mix mixes should be created for each set of samples.

For Each MLVA Primer:					
	Start Concentration		Final Concentration.		Volume per reaction (µL)
PCR Buffer	10	X	1	X	1.1
$MgCl_2$	25	mM	3	mM	1.32
dNTPs	2	mM	0.2	mM	1.1
Betaine (Q solution)	5	M	1.2	M	2.64

Hot Star Taq	5	u/ul	0.08	u/ul	0.18
Forward Primer	20	uM	0.2	uM	0.11
Reverse Primer	20	uM	0.2	uM	0.11
PCR Water					3.4
DNA template					1
Total					11

2. Setting up the PCR

A. Prepare the four master mixes in the PCR clean room.
B. Ensure all surfaces and pipettes are cleaned before and after preparing the master mix with 10% bleach or DNA decontaminant, followed by 70% ethanol.
C. Take your master mixes to the DNA template addition area.
D. Draw a line across the 96-well PCR plate between rows D and E. Aliquot 10µL of master mix across the PCR plate in rows A-D, and then again in rows E-H (as shown below).



E. Add 1µL of the DNA control in column 1, only in rows A-D.
F. Continue adding samples across the plate (1-12) in rows A-D. Sample 13 -24 will be in rows E-H.
G. Seal PCR plate with strip cap covers or with PCR film (e.g. BIO-RAD Microseal 'B' film). If using film be sure to use a plate sealer/roller to completely seal and prevent evaporation during thermal cycling.
H. Briefly centrifuge the plate to remove bubbles.

3. Running the PCR

A. The PCR thermal cycling conditions are as follows:

Step	Temp. (°C)	Time
1	94	5 minutes
2	94	30 seconds

	68	30 seconds
	72	30 seconds
	Cycle 34 times	
3	72	5 minutes
	15	hold

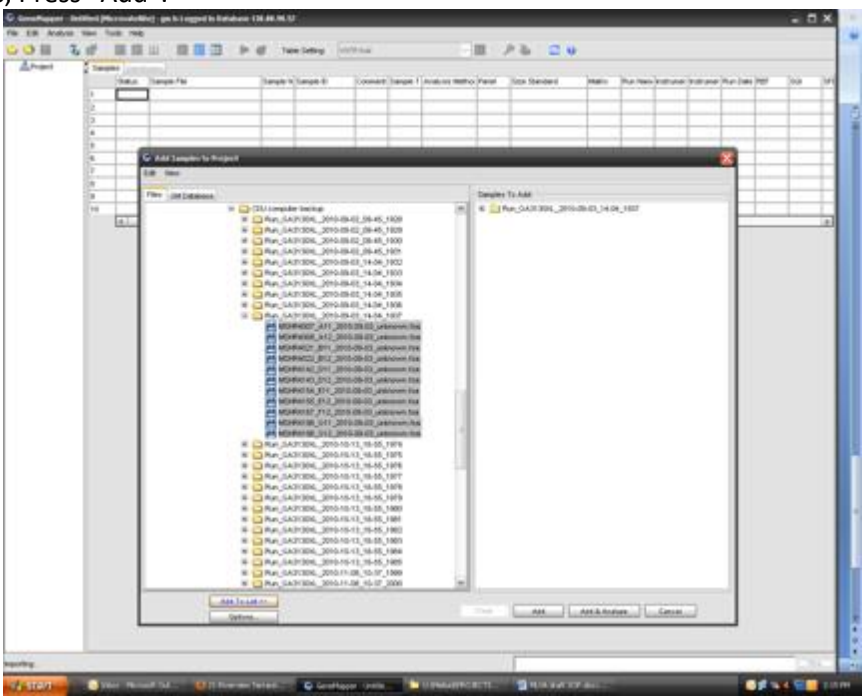
B. Briefly centrifuge the plate after the PCR has finished running.

4. **Running a gel to confirm PCR products**
   A. Make up a 1% 200 mL agarose gel for every 12 samples tested (e.g., if you have 23+control (24 total) samples in a 96 well plate, you will need 2 x 200mL gels).
   B. Follow the instructions found in "Chapter 14- Convention PCR" covering how to prepare an agarose gel for electrophoresis.
   C. In the first well of each row in the gel, add 6 µL of 100bp marker.
   D. Mix 2 µL loading dye with 5 µL of PCR products from row A and add the gel. Repeat this for the first 12 samples in rows A-D of the PCR plate (As shown in the image above).
   E. Be sure to mark the running tank with "samples A-D".
   F. With the second 200mL gel, repeat steps C-E using the DNA from rows E-H of the PCR plate.
   G. Run the gels at 100mV for ~30 minutes. It is important to keep checking the gel and where the dye front is. These are small PCR products; it is important make sure the products don't run off the gel.
   H. Visualize the gel using a gel documentation system.
   I. Check that all 4 products have amplified for each sample.
      a. If there are no bands at all on the gel (i.e. only a marker), the PCR has failed – repeat the PCR reaction again.
      b. If you have bands in 3 of the regions for a sample but one hasn't worked, repeat the PCR on the single region that has not work.
      c. If all 4 regions have failed (i.e. no bands) for a sample (but other samples are ok), the DNA extraction may not have worked for that sample and needs re-extraction.

5. Pooling the PCR Products for each sample
   A. PCR products must be pooled together before being sent for DNA sequencing.
   B. Pool products in a 96-well round-bottom plate (such as a 2mL NUNC plate) or into sterile closable microcentrifuge tubes (e.g. 1.7 mL Eppendorf tubes) if there are only a few samples.
   C. Take 3.5uL from row A of the PCR products. Transfer to column A of the round-bottom plate where the products will be pooled (as shown in the image above).
   D. Take 3.5uL of row B, C and D of the PCR products. Transfer to column A of the round-bottom plate. All 4 amplified regions for each sample will now be pooled.
   E. Take 3.5uL each from Row E, F, G and H of the PCR products and transfer to column B of the round-bottom plate.
   F. Carefully document and label which samples are in which wells of the round-bottom plate or in which tubes they have been placed.

6. Dilution of the pooled product
   A. To each of the pooled products add 500 µL of distilled H_2O. Pipette up and down to ensure it is well mixed.
   B. The samples can now be sent to any commercial sequencing laboratory (e.g. Macrogen, Seoul, South Korea).

7. Analyzing the sequencing results

A. Sequenced product sizes must now be analyzed. The following describes a protocol using "GeneMapper", which is a genotyping software package that provides DNA sizing for all Applied Biosystems Genetic Analyzers.
B. Go to File→ New Project. Select "Microsatellite".
C. Go to File→ Add samples to project.
D. Browse to find where the sample list created at the beginning of the procedure is stored.
E. Select/highlight the sample files (.fsa files) and Press "Add to list". Once you have all of your samples, Press "Add".
    a. 
F. You now need to tell the program which analysis methods you wish to use. In the first cell of each column that needs changing, select the required method. Then click at the top of the column (to highlight the whole column). Then press CONTROL D to auto fill for all samples. Select the appropriate analysis method, panel and size standard (these will need to be manually set when running the protocol for the first time).
G. The fragments are now ready to analyze: Click the Green Play button at the top of the screen.
H. Review the panel of indicators. The panels should appear green if the protocol has worked. If there are any yellow triangles or stop signs, this needs to be checked.
    a. Usually a yellow triangle or stop sign indicates there is a problem with the size standard and the program has been unable to call the size properly. See below on how to fix a size standard.

Fixing a size standard:

a. Select the sample that is red or yellow.
b. Click on the "Size Match Editor" icon.
c. Look at the plot – are there smooth, well defined peaks there? Are all the numbers on the peaks correct?
    a. If there are no numbers, you will need to add in the numbers by clicking on the peak and then right clicking – Add – then select the number (use the chart to guide you on which peak represents the right number). Click "Apply" and "Override SQ".
    b. If there are numbers, double check that they are in the right space.
    c. If there are no numbers and there are no nice peaks, the reaction has failed and will need to be repeated.

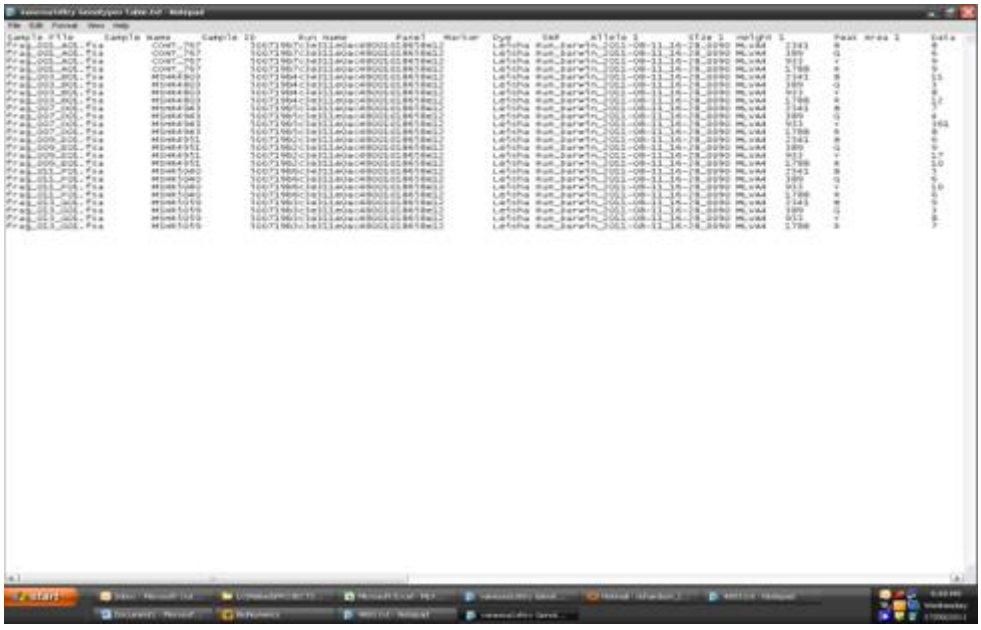
d. Once everything has been checked and all the samples display a green sign, proceed with the panel binning:

8. Panel Binning

A. Select the "Genotypes" tab. Check that all samples display the appropriately matching alleles.
B. Edit→ Select All.
C. Tools→ Panel Manager. Press the "+" next to MLVA 4. Select the MLVA 4 tab that appears.
D. Select→ "Apply" then "OK".
E. Go to Tools→ Table Editor settings. Click on the "Genetypes" tab then select "OK".
F. Click the "Display Plots" Icon – (multi coloured peaks).
G. You will now check all 4 markers for each isolate.
    a. If all 4 have a peak that falls in the grey areas (assigned "Bins"), they should have a box under the peak with the peak details.
    b. If the peak trace is messy, the reaction (PCR) will need to be repeated.
H. Click File→ "Save Panel" then "Exit".
I. Click File→ "Export table". The table should be saved as a .txt file for upload into BioNumerics Software.
J. Ensure that all the raw data files and the GeneMapper files/exported table are saved in an appropriate folder.

9. Preparing the GeneMapper export file for import into BioNumerics

A. Open the GeneMapper export .txt file in the BioNumerics software.



B. Select all (CTRL – A).
C. Open an excel worksheet and paste (CTRL –V) the GeneMapper export table.
D. Remove all columns except for:

Sample Name	Marker	Dye	Allele 1	Size 1	Height 1	Peak Area 1	Data Point 1

E. Remove the letters from the Sample Name for all isolates. BioNumerics can only import sample names based off the "Key" identifier. This means you will need to change the Sample name of all samples to include only the # (e.g. MSHR1234 should become 1234).
F. Assign the "MLVA number" (this number won't be uploaded into BioNumerics but will be manually added later).

a. Open your saved document that contains information about the key and the "Bin" ranges.
b. To determine the MLVA number, click on the "MLVA 4 type key" tab. Using the filter function in the spreadsheet, enter in the allele combinations given for each sample. A MLVA4 number for the sample will be given based on the combination.
c. If the combination does not exist, add it to the end of the list and give it the next "MLVA number" in sequence.
d. Write down this number separately as you will be manually adding this info in later.

G. Now delete the allele columns from the spreadsheet. The file cannot be uploaded into BioNumerics with the allele column still present.
H. The file should look like this:

Sample File Name	Marker	Dye	Size	Height	Peak Area	Data Point
4803	2341	B	188.61	375	4883	2780
4803	389	G	235.6	548	6599	3576
4803	933	Y	230.45	508	5660	3487
4803	1788	R	364.47	200	5035	5853
4943	2341	B	153.14	498	5883	2093
4943	389	G	243.29	653	7794	3549
4943	933	Y	361.04	292	3974	5542

I. Open up a new Excel document. Copy the results for one sample into the document:

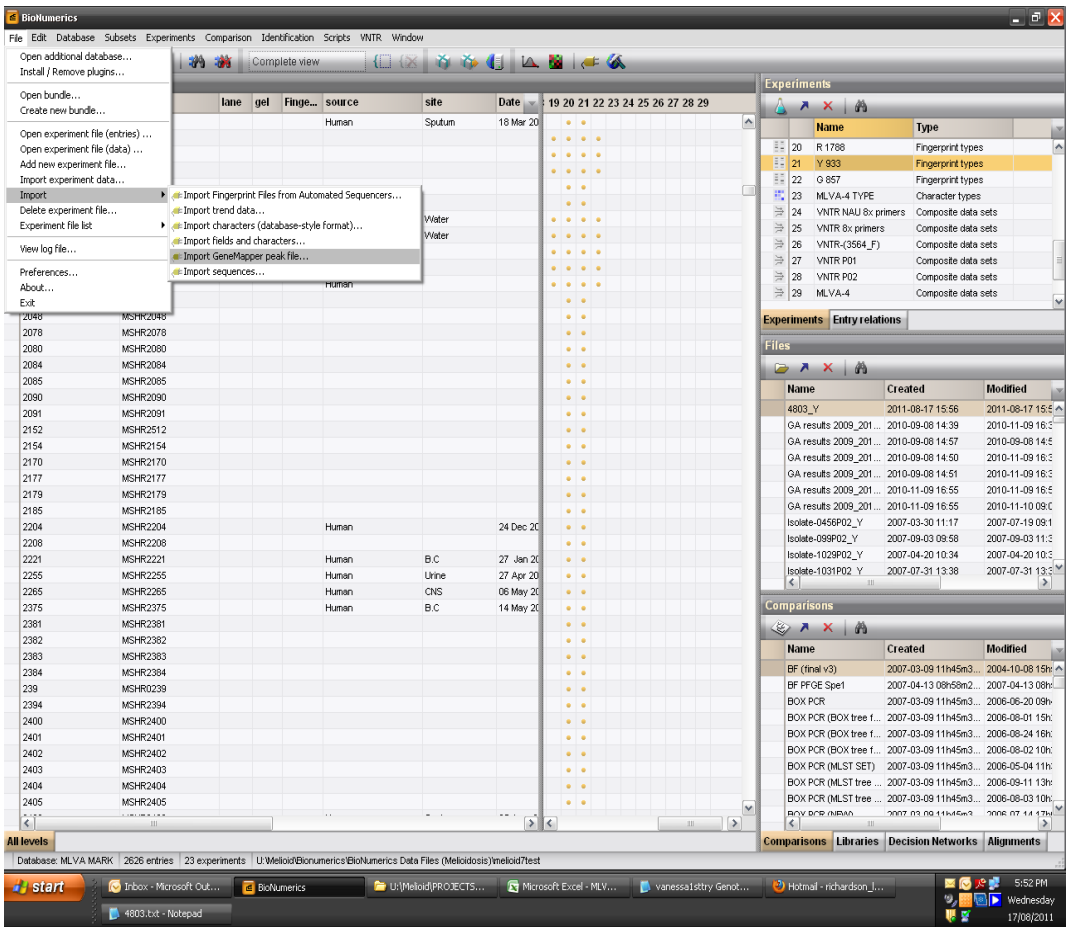
Sample File Name	Marker	Dye	Size	Height	Peak Area	Data Point
4803	2341	B	188.61	375	4883	2780
4803	389	G	235.6	548	6599	3576
4803	933	Y	230.45	508	5660	3487
4803	1788	R	364.47	200	5035	5853

J. Save the document as a tab delimited file with the sample name as the file name (i.e. MSHR4803.txt)
K. Open up another new Excel document. Paste in the next sample (with the headings). Save the document as a tab delimited file with the sample name.

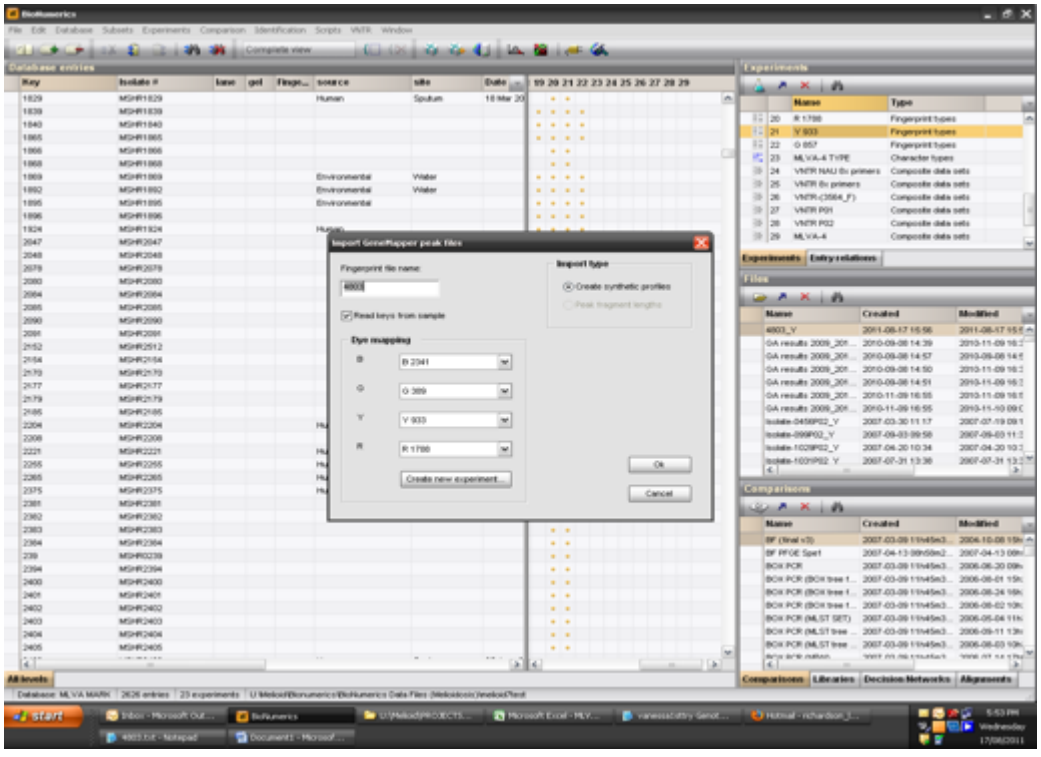
Sample Name	Marker	Dye	Size	Height	Peak Area	Data Point
4943	2341	B	153.14	498	5883	2093
4943	389	G	243.29	653	7794	3549
4943	933	Y	361.04	292	3974	5542
4943	1788	R	298.83	295	6824	4482

L. Do this for every isolate (saving each isolate/sample as its own file. Be sure to include the heading for each file). This may seem unnecessary, but it is difficult to remove a result from BioNumerics if a mistake is made unless each sample has its own file.
M. Open up the BioNumerics software.
N. Import the GeneMapper trace/peak file: File→ Import→ Import GeneMapper Peak File.

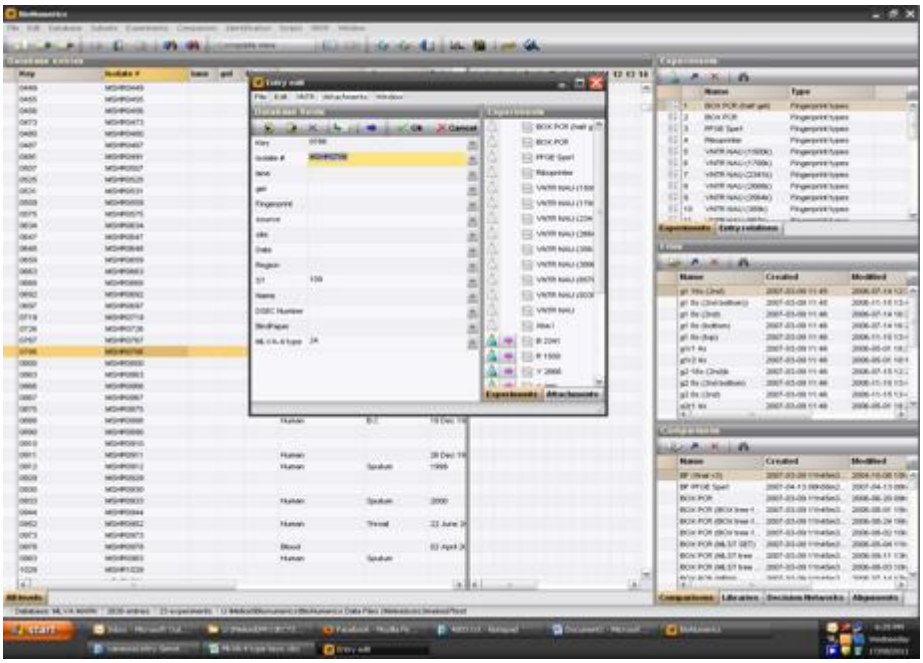
O. Select the tab delimited .txt file just created and open the file.



P. The next screen should appear automatically.



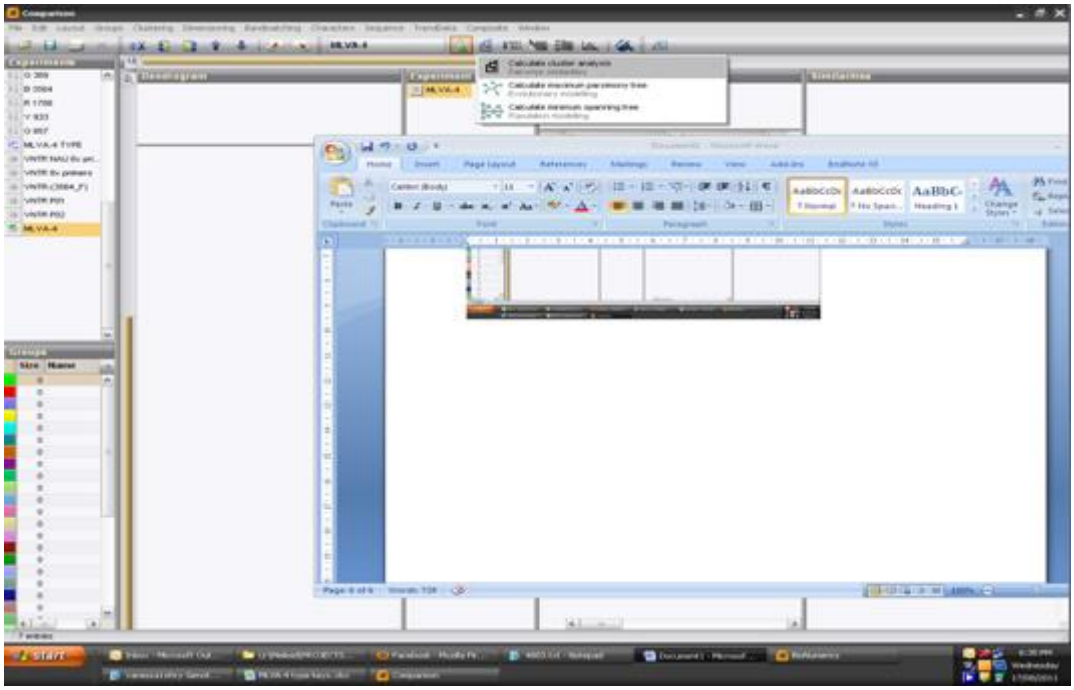
Q. Leave the boxes as they are. Click OK. The file will now download.
R. Repeat this for every sample you are uploading.
S. Find the uploaded isolates by locating their “Key” numbers on the left-hand side of the page.
T. Double click on the isolate. Enter the information you have about the isolate including the newly assigned MLVA4 number.



U. Click “OK” when finished.

To create a dendrogram:

a. Hold down the “CTRL” key and select (using the mouse) the isolates you wish to use in the dendrogram. A small orange arrow will appear next to the selected isolates. The tree will need to be rooted using an appropriate *B. pseudomallei* isolate.
b. Once all the isolates have been selected, click the “Comparisons” tab in the bottom right hand corner.
c. Select the “Create new comparison” icon.
d. The isolates will appear in a new window.
e. On the left side of the screen, in the Experiments box, scroll down to the bottom to MLVA4. Click this experiment (a green tick should appear over the MLVA4 icon).
f. Select the “Calculate cluster analysis” icon.
g. Choose “Calculate cluster analysis – Pairwise similarities”.



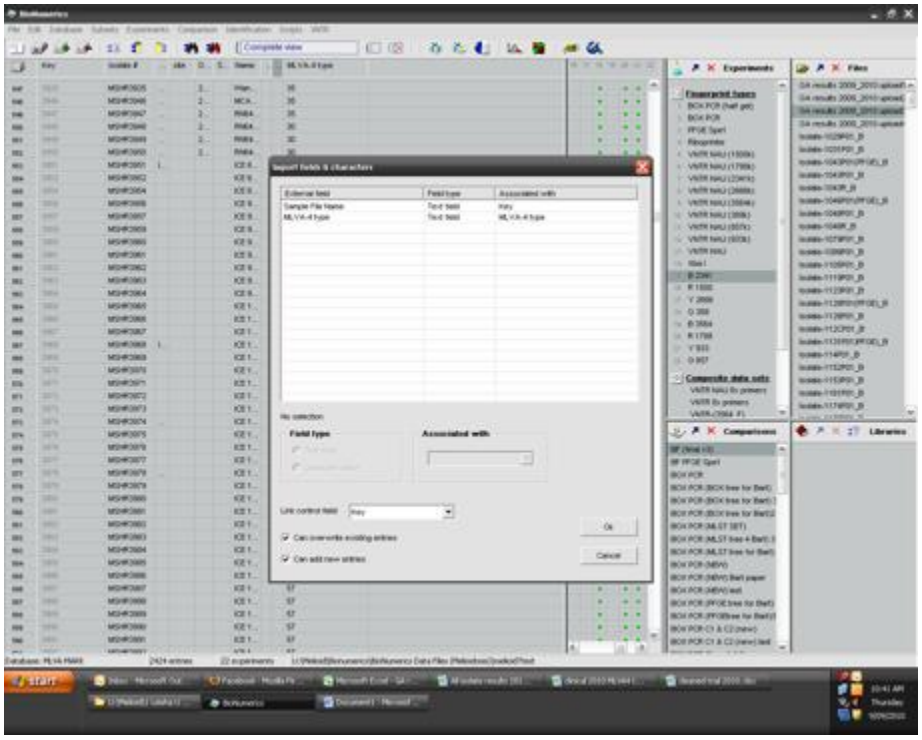
h. Use the default settings: Average from experiments and UPGMA for the "dendrogram type". Click OK.
i. To print: Click print preview.
j. Move the fields to suit what you wish to print.

Uploading sample information into BioNumerics:

A. Create a .txt file (start in excel and then save as .txt tab delimited file) that has the Sample names as the Key (e.g.MSHR4145 has a key of 4145) with the information in the following columns that you wish to import.

Sample File Name	MLVA-4 type
4018	15
4021	110
4022	44
4023	16
4024	16
4025	16
4026	16
4027	16
4028	16

B. In BioNumerics, open File→ Import→ Import fields and characters.
C. Locate the .txt file to import via the browse function. Click OK.
D. The wizard identifies the columns present in the file. You now need to link them to a field in BioNumerics.



E. Click on the first row. Make sure the “Field Type” function has “text” selected. You now link it with “Key” by selecting it from the drop-down box list.
F. Do the same for all other external fields until all are completed
G. “Link control field” should be set to “Key”
H. Click OK to upload.

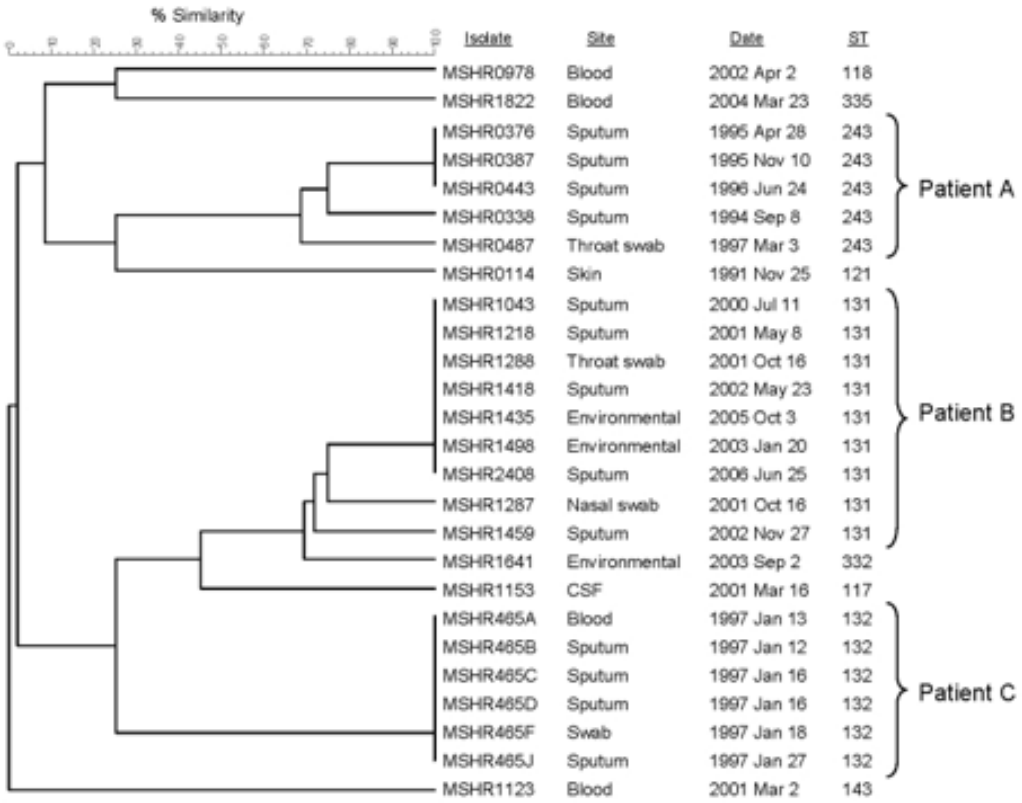


Figure 102- Dendrogram showing MLVA-4 profiles for isolates from three patients with melioidosis, along with corresponding isolate numbers and multilocus sequence types (Currie et al. 2009).

***B. pseudomallei* BOX-PCR Procedure** (adapted from Currie et al. 2007)

Primer:

BOX-A1R: (5'-CTACGGCAAGGCGACGCTGACG-3')

A. PCR Set-Up

1. Create a PCR plate template to keep track of where samples will be placed.
2. Calculate the master mix volumes needed for the number of samples to be tested based on the table below. Remember to include 2 additional samples to account for reagent lost during pipetting.

Reagent	Concentration	Volume per reaction (µL)
10X PCR Buffer	1X	2.5
$MgCl_2$ (25mM)	4.5 mM	4.5
dNTPs (2mM)	200µM	2.5
Q or Betaine Solution	1X	5
Hot Star Taq (QIAGEN)	5U	0.25
BOX-A1R Primer (30µM)	1.05µM	0.875
PCR Water		7.375
DNA template		2
Total		25

3. Prepare the master mix in the PCR clean room/area.
4. Gently mix the master mix then briefly spin in a microcentrifuge.
5. Pipette 23 µL of the master mix into the appropriate well of a 96-well plate or PCR strips according to the plate template worksheet.
6. Cover the wells using cap strip covers. Spray down the clean workspace with bleach or DNAerase and follow with 70% ethanol. Remove laboratory coat and gloves and put on a clean pair of gloves. Take the 96-well plate or PCR strips to the DNA template addition area.
7. Put on a new laboratory coat, keeping the same pair of gloves on. Very carefully, take off the cap strip covers from the 96-well plate or PCR strips. Open these slowly to prevent aerosolization and spraying of any liquid in the wells.
8. Add 2 µL of template DNA to each appropriate well of 96-well plate or PCR strip according to the plate template worksheet.
9. Add 1 µL water to the 'NTC' well.
10. Add 1 µL of the positive control DNA to the correct well.
11. Cover the wells of each column of the PCR plate or each PCR strip using strip caps as you go and secure caps tightly.
12. Wipe down the workbench area with bleach or DNAerase, followed by 70% ethanol. Remove laboratory coat and gloves. If possible, briefly spin the plate at 1000 rpm to remove any

droplets. Transport plate to the PCR thermocycler room/area and place it in the PCR thermocycler.

13. Place tubes/plates in the conventional PCR machine.
14. Set the following thermal cycling conditions and press start:

Stage	Time	Temperature	# Cycles
Initial denaturation	5min	95°C	1
Amplification	1min	95°C	35
	1min	40°C	
	2min	72°C	
Final Extension	10min	72°C	1
Hold	--	4°C	--

B. Running the Gel

1. Make a 2% agarose gels for the required number of samples to be run (follow the procedures outlined in Chapter 14- Conventional PCR).
2. Load the gel using a 100bp ladder and 2 µL DNA loading dye mixed with 10µL PCR product.
3. Place the lid on the gel tank, plug electrodes into a power pack setting it to run at 100mV.
4. Once ready lift gel from the tank and visualize results on Gel Doc machine.

C. Results Interpretation

BOX-PCR gel results should always be interpreted in the proper epidemiological context and the gels alone should not be used to confirm an epidemiological connection or outbreak. It is always important to confirm the link with another more robust typing method, such as MLST or whole-genome sequencing (see below). The quality of the gel electrophoresis results and the variability and the prevalence of the pattern in question should always be considered.

Only isolates displaying identical banding patterns should be considered as potentially being the same isolate/strain and the possible cause of an infection cluster, patient relapse, or point-source

outbreak. If the samples in question display different BOX-PCR banding patterns they should be considered unique, unrelated isolates (e.g., not part of the same cluster or a patient reinfection rather than relapse). An example is shown in Figure 103 below. Here, BOX-PCR has been used to demonstrate that a melioidosis patient has been reinfected with a new strain of *B. pseudomallei* and is unlikely to have had a relapse in their primary infection, as two distinct banding patterns are present.

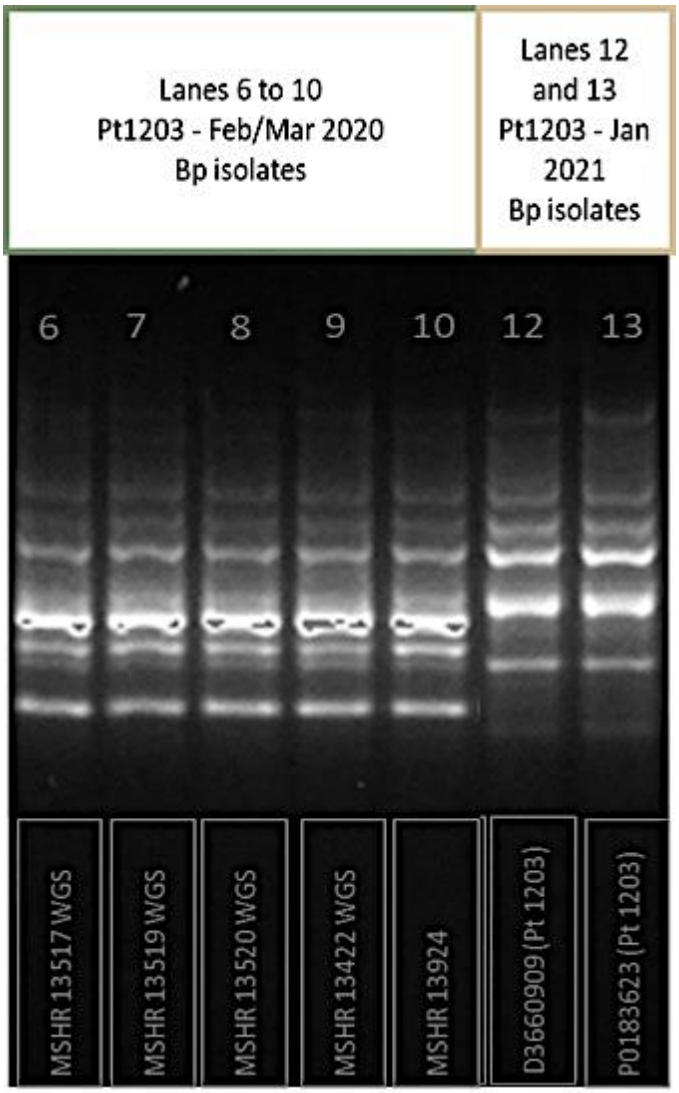


Figure 103- The BOX-PCR banding patterns in lanes 6-10 of the gel, which are isolates cultured from the patient's initial infection, have a unique banding pattern and are different than isolates cultured from the same patient's subsequent infection nearly one year later. This likely indicates that the patient was infected by a different strain of the bacteria and has not been reinfected by the same strain (e.g. from a single point-source) or have had a relapse in infection due to inadequate treatment (image courtesy of Menzies School of Health Research, Darwin Australia).

MLVA-4 and BOX-PCR Limitations

While both BOX-PCR and MLVA-4 have proved useful for smaller regional surveys of *B. pseudomallei*, a serious hindrance to their widespread practical application thus far has been the difficulty involved in data sharing, limiting inter-laboratory isolate evaluation (Cheng et al. 2005). These rapid strain typing methods can lack reproducibility and robustness and variations in results have been shown to occur between different labs or PCR machines (Currie et al. 2007). MLVA also requires trained and skilled laboratory technicians. It is recommended that these schemes be used in conjunction with a more robust and reproducible typing method, such as MLST, to confirm the event of an outbreak or case cluster and to verify the relatedness or divergence of strains.

Next Generation Sequencing for Bacterial Strain Typing

The arrival of the first high-throughput, rapid next-generation sequencing (NGS) technology has signalled an important shift in microbial genetics research. Since then, sequencing platforms have become more advanced in their data quality, cost, and accessibility, with whole-genome sequencing (WGS) platforms providing a comprehensive method for analyzing entire bacterial genomes (Edwards & Holt 2013; Köser et al. 2012). In addition to providing a much wealthier source of genetic information, WGS now also supersedes many genotyping methods in pricing. For example, WGS now costs less to carry out than conventional Sanger sequencing-based MLST.

Current WGS platforms are comparatively fast and can be generated in-house in some laboratories. Much of the data generated is also available through online public databases, including the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/genbank/), allowing for extensive comparative analysis (Edwards & Holt 2013). The high-resolution data generated from WGS can be utilized to investigate various aspects of *B. pseudomallei* genetics, including the spread of drug resistance (Webb et al. 2017), within-host evolution (Price et al. 2010; Köser et al. 2012), as well as outbreak source attribution through the examination of isolate genome-content variability (McRobb et al. 2015; Sarovich, D. S. et al. 2017). Moreover, WGS has the

ability to extract MLST sequence types directly from reads, permitting a highly precise method of MLST typing of *B. pseudomallei* (Edwards & Holt 2013; Lam et al. 2012). The Bacterial Isolate Genome Sequence database (Jolley & Maiden 2010) now allows for the PubMLST website to use all levels of sequence data, including complete, finished genomes. Where resources permit, WGS and *in silico* MLST could be used after rapid fingerprinting of isolates to confirm an outbreak or case cluster and to further examine the relatedness of strains.

***B. pseudomallei* WGS Data Analysis**

When applying WGS to study the molecular epidemiology of bacterial pathogens such as *B. pseudomallei*, it is useful to examine variations in a genetic sequence, or set of sequences (Schürch et al. 2018). Genome variants can take several forms, including nucleotide insertions, when additional nucleotides are found in a sequence, nucleotide deletions within a sequence, or a single base change, referred to as a single nucleotide polymorphism (SNP) (Schürch et al. 2018). These variations can arise through point mutations, homologous recombination or discrepancies in genome content (Bryant et al. 2012). Most studies rely on SNP data for population and epidemiological analysis since variability between bacterial strains can be translated into measures of distance in core genome alignments, or can be indexed to assign unique allelic profiles (Schürch et al. 2018). Additionally, SNPs can be found even in regions of high homology, or regions of shared ancestry not recently affected by recombination, and thus can more accurately reflect evolutionary time (Pearson et al. 2009). SNPs are also more informative than other currently used molecular markers due to intrinsically slow mutation rates and extensive distribution across the entire genome. Large numbers of shared SNP loci can thus facilitate robust characterization amongst closely related isolates (Pearson et al. 2009).

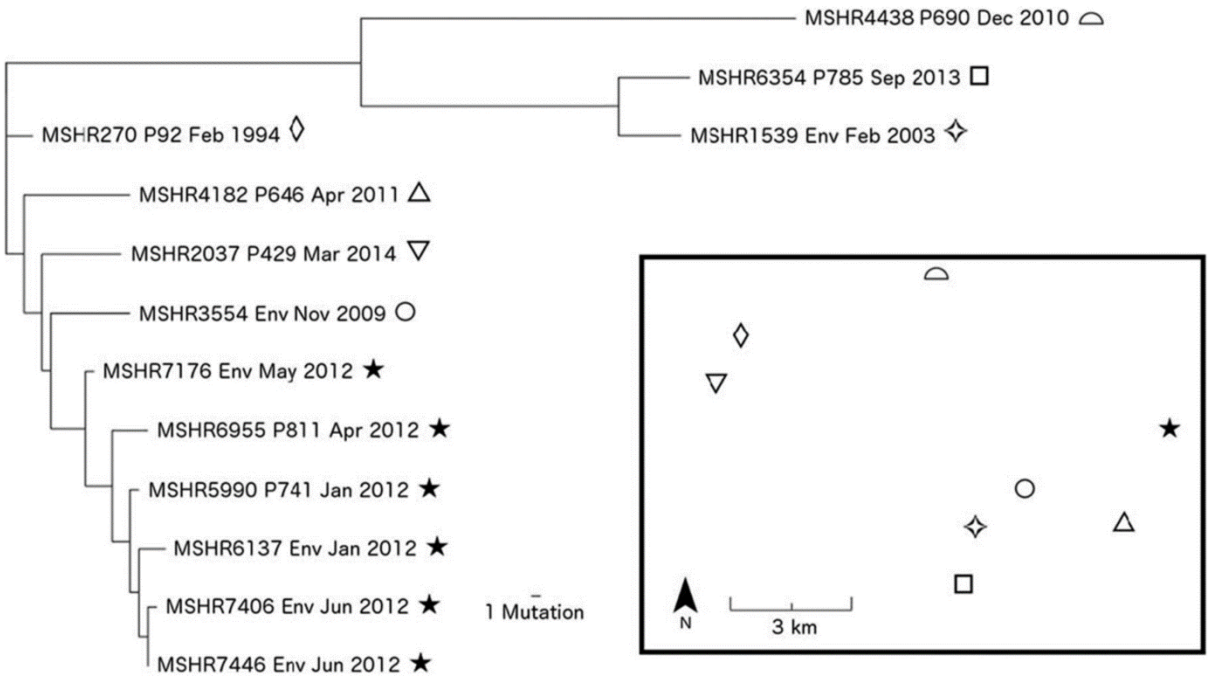


Figure 104- Whole-genome sequencing data from a melioidosis outbreak in the Darwin region of northern Australia. Here, a phylogenetic tree was constructed using combined SNP and indel data from *B. pseudomallei* environmental and clinical isolates to demonstrate that the source of a melioidosis outbreak was from a contaminated domestic water supply (McRobb et al. 2015).

Identification of insertions, deletions, or SNPs begins with mapping and aligning sequence reads to a closely related reference genome (Li & Homer 2010). Variant determining algorithms have quality filters that then attempt to define variants, versus sequencing errors. Alignments are normally performed using a microbial SNP pipeline, such as Snippy, NASP, or SPANDx (Sahl et al. 2016; Sarovich & Price 2014; Schürch et al. 2018). The resulting alignment can then be used for phylogenetic analysis. To increase resolution, genomes chosen for the reconstructed phylogeny are normally closely related to one another. This allows for discrimination between isolates that differ by only a few SNPs, which is especially important in facilitating investigations into outbreak source attribution (Pearson et al. 2009).

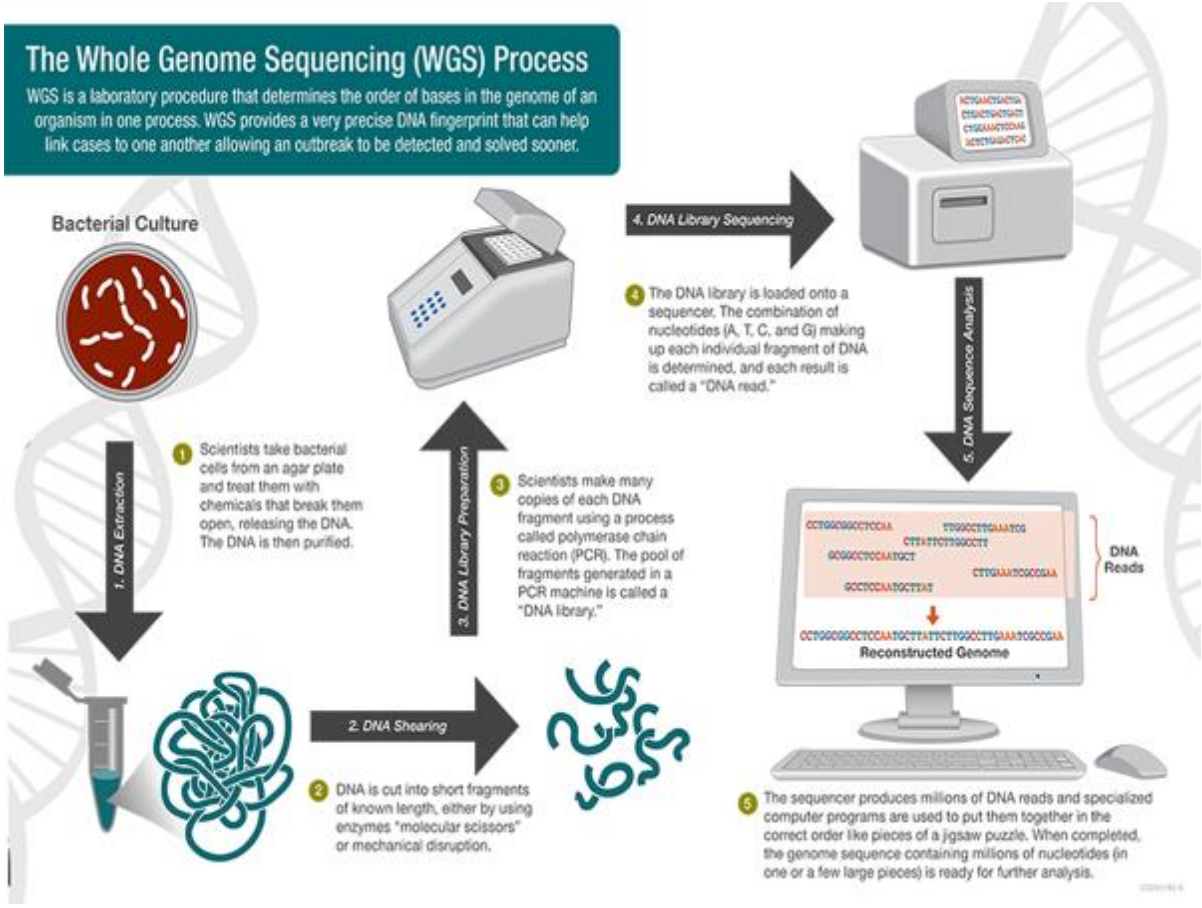


Figure 105- Whole-genome sequencing normally consists of DNA extraction, shearing the extracted DNA into small fragments, creating a “DNA library” that includes many copies of each DNA fragment. Each fragment is then sequenced to create a read, and finally, analysis of the sequenced products occurs (CDC 2013).

Most commercial sequencing laboratories now offer fast, well-priced WGS sequencing facilities. **However, due to the complexity of WGS and the extensive bioinformatics training required to properly analyze and interpret WGS data, it is best to contact a diagnostic reference laboratory or research laboratory with WGS capabilities and expertise if considering implementing the technique for the first time.**

Chapter Summary

The preceding chapter presents an overview of bacterial molecular characterization and several common methods used for *B. pseudomallei* strain typing. Bacterial strain typing is important for disease diagnosis, treatment, and epidemiological surveillance, particularly for highly pathogenic species such as *B. pseudomallei*. Potential outbreaks of melioidosis warrant rapid public health response and high-resolution molecular typing tools, such as those described above, are needed to quickly investigate the infection etiology.

Chapter 17: *B. pseudomallei* Suspected Isolate Reporting, Storage and Data Management

Chapter Overview

While melioidosis is not a notifiable disease in all countries, it is recommended that suspected cases be reported to local or national sentinel laboratories that can assist with diagnostic confirmation, epidemiological investigation, and provide important biosafety guidance. The U.S. Centers for Disease Control (CDC) and U.S. Department of Agriculture (USDA) have identified biological agents and toxins that they consider to be a threat to public health because of their potential use as bioterrorism agents. These are referred to as "select agents". Their transfer, possession, use, and disposal are strictly regulated in the United States. *B. pseudomallei* is classified as Tier 1 select agent (https://www.selectagents.gov) and its identification should be reported to a Laboratory Response Network (LRN) laboratory or sentinel laboratory as soon as possible upon identification. The following chapter describes the appropriate protocols for reporting and transporting *B. pseudomallei* to reference laboratories.

Reporting a Suspected or Confirmed *B. pseudomallei* Isolate

Any oxidase-positive, indole-negative, gram-negative rod that is resistant to gentamicin and colistin but sensitive to amoxicillin/clavulanic acid should be suspected as *B. pseudomallei* until this identification is ruled out (Trinh et al. 2018). For suspected or confirmed cases, clinical laboratories should refer to local and national disease authorities and comply with the specific reporting requirements. This typically involves reporting cases and laboratory results and may also mean that the isolate concerned is subject to specific biosafety and biosecurity measures. Laboratories that typically perform bacterial work on an open bench should transfer all suspected cultures to a class II BSC or higher where possible, especially when performing potentially aerosol-generating

procedures. See Chapter 4 for further detail regarding *B. pseudomallei* biosafety (ASM 2016; BMBL 6th ed. 2020; Peacock et al. 2008).

Although the risk of laboratory acquired infection with *B. pseudomallei* is relatively low compared with some other bacterial select agents, clinical laboratories should perform a risk assessment and develop protocols to prevent accidental exposure. This includes strictly enforcing standard laboratory procedures and GLP, conducting culture work for suspected isolates inside of a BSC, and wearing appropriate personal protective equipment (PPE), as described in Chapter 4 (BMBL 6th ed. 2020). If an accidental exposure occurs, the risk of exposure should be determined based on the guidelines described in Chapter 4 (APHL 2017; Peacock et al. 2008).

Data Management and Record Keeping

A data management system should be used to keep track of specimens and isolates processed by the clinical laboratory and those that are being stored long-term. A computerized record keeping system is recommended, but handwritten laboratory logbooks may be used where these are unavailable. Small or resource-poor laboratories may find that paper records are sufficient. However, computerized methods are recommended for laboratories that handle larger quantities of clinical specimens and isolates. Commercial laboratory information management systems (LIMS), such as Freezerworks, may also be used where resources permit.

Regardless of whether the data management and recordkeeping system is computerized or paper-based, all data must be accurately and reliably recorded and easily accessed. Human errors in record keeping are very common and great care should be taken to avoid errors. It is best to use a single unique specimen or isolate reference along with at least two other data items such as patient name, date of birth or hospital number. Bar code printers and scanners reduce the risk of human

transcription errors. It is also critical that laboratory data is linked with epidemiological data to ensure the accuracy.

It is recommended that individual laboratories maintain several records, including:

- Detailed records of every isolate.
- Secured access to isolates.
- Safety, security, and emergency response plans.
- Training records.
- Reference lab transfer forms (if used): The designated reference laboratory should advise on any required transfer forms. Always ensure the latest versions of these forms are being used.
- Safety and security incident reports.

Storage of Isolates

If isolates need to be sent to a reference laboratory for confirmation or further testing, they should be stored appropriately to ensure viability. Isolates should always be confirmed as pure cultures before they are stored. Fresh cultures (e.g., ~48 hours growth) should always be used when storing isolates. Aseptic techniques should be used when preparing isolates for storage to prevent contamination.

Short-term storage of *B. pseudomallei* can be carried out on agar slants/slopes (e.g. on tryptone soya agar slants) or in other liquid broth medium containing glycerol for 1-2 weeks. Instructions for preparation of agar slants and glycerol broth are described below. Slants can be produced as a 4 mL slant in a 7 mL screw-cap tube or 1 mL slant in a 2 mL screw-cap tube. They should be stored at 4°C until transporting. Broth should be frozen at -20°C (or -70°C for longer-term storage).

Tryptone Soya Agar Slope Protocol

1. Weigh out 15 grams of TSB powder (Tryptone Soya Broth – Oxoid CM0131 or locally available equivalent).
2. Put the TSB powder in a 1 L autoclavable glass beaker or flask containing a magnetic stirrer on top of a magnetic hot plate.
3. Add 500 mL of distilled water to the bottle and turn the stirrer on.
4. Allow the solution to mix thoroughly until all the powder is dissolved.
5. Add 5 grams of Bacteriological Agarose and stir for 5 minutes.
6. Autoclave for 30 minutes at 121°C or sterilize in a pressure cooker if an autoclave is not available.
7. Take the bottles out of the autoclave/pressure cooker once finished and allow to cool slightly (until the agar is at ~60°C).
8. Make 1 mL aliquots of the agar into 2 mL screw-cap tubes, or 4 mL slants in 7 mL screw-cap tubes. If the agar starts to solidify before finishing, sit the bottle in the water bath set at 60°C for 5 minutes and continue once it is liquid again.

Tryptone Soya Broth Containing 15% Glycerol

1. Weigh out 30 grams of TSB powder.
2. Add the TSB powder to a large 2 L glass beaker or flask containing a magnetic stirrer on top of a magnetic hot plate.
3. Add 850 mL of distilled water to the beaker and turn the stir plate on.
4. Allow to mix until all the powder is dissolved.
5. Add 150 mL of glycerol and stir for a further 5 minutes (or until all glycerol is mixed).
6. Divide the broth into 2 x 500mL glass bottles.
7. Autoclave for 30 minutes at 121°C or sterilize in a pressure cooker if an autoclave is not available.

8. Take the bottles out of the autoclave/pressure cooker once finished and allow to cool.
9. Once cool, aliquot 1 mL in to 2 mL screw cap tubes. Store at 4°C until needed.

Isolate Shipping and Transfer

Upon notification to the local health department of suspected *B. pseudomallei*, laboratories should be prepared to appropriately package and ship specimens or isolates. See the packaging and shipping section in Chapter 6 for additional information about appropriate packaging and shipping procedures.

Transport of diagnostic specimens and infectious substances should be done so that it minimizes any potential risks and also protects the viability of the isolate or specimen. The shipment of infectious substances or diagnostic specimens by air should comply with local, national, and international guidelines. International air transport regulations may be found in the IATA publication *Dangerous Goods Regulations*. https://www.iata.org/en/publications/dgr/. See Chapter 6 for more detail.

Agar slopes/slants in screw-cap tubes can be shipped at room temperature (25°C) (IATA 2019; WHO 2020).

***B. pseudomallei* Isolate Security, Destruction and Decontamination**

If suspected specimens and/or isolate cultures are to be transferred to a reference laboratory, they should be transported within 7 days of confirmed identification where possible. During this time, specimens and isolates should be secured against theft, loss, or release until they have been transferred. If a laboratory is unable to transfer isolates or store them appropriately long-term at -70°C, isolates should be inactivated or destroyed. Suspected or known loss of a specimen or associated materials must be reported immediately. Individuals not associated with the diagnostic laboratory should not have access to specimens or isolates and should be secured in locked cabinets,

rooms, or other containers. Rooms should also be secure against entry by unauthorized personnel (ASM 2016).

If a lab decides to transfer any specimens or cultures, staff should contact the local reference laboratory and work to ensure that the correct paperwork and protocols are followed. Copies of sample destruction logs, and documented reports should be maintained.

If a laboratory chooses to destroy any specimens or isolates, it is also critical that these are properly inactivated prior to disposal. Isolates should not be directly discarded into the biohazardous waste stream like other infectious medical waste materials. Inactivation using an autoclave/pressure-cooker or chemical decontamination should be done before disposal.

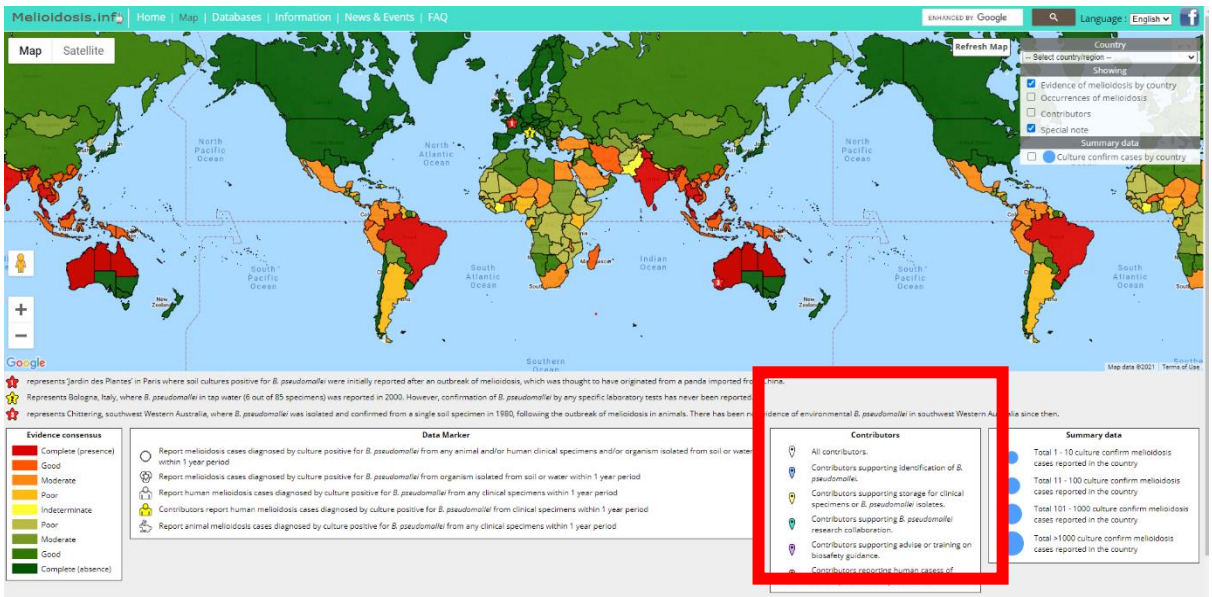
Chemical Decontamination Process (APHL 2016)

1. Prepare a fresh 1:10 bleach solution in any receptacle large enough to submerge all containers and plates suspected to contain *B. pseudomallei*.
2. Working inside of a BSC, place culture containers in the bleach solution so they are fully submerged and leave in the bleach solution. This should be done overnight.
3. Once overnight inactivation has occurred, discard the bleach solution down the drain with tap water running.
4. Package all inactivated culture plates and containers with other biohazardous waste for final treatment and disposal.

Checklist of appropriate steps to take if *B. pseudomallei* is to be transferred to a local or national reference laboratory for confirmation or storage:

____ 1. Contact the laboratory where the specimen/isolate will be transferred.
____ 2. Package the suspected isolate(s) and other clinical specimens (aspirates, biopsies, sputum specimens) according to local and national regulations and send to laboratory.
____ 3. Note who performed worked on cultures and if work was done inside of a BSC. Write down if any additional clinicians were involved in specimen collection (Peacock et al. 2018).
____4. Notify public health authorities such as the local health department epidemiologist or health officer as required by the local or national disease reporting regulations.
____5. Notify the infection control/disease team so the patient can be given appropriate treatment and an epidemiological investigation can be done.
____6. Discuss with the laboratory about whether any further specimens should be submitted for testing.
_____7. If *Burkholderia pseudomallei* is ruled out, continue with identification using local protocols.

In countries where cases of melioidosis have not previously been reported or where there are no regulatory authorities in place to report cases, the website www.melioidosis.info also offers methods to report culture-confirmed cases of melioidosis, provides support for *Burkholderia species*



identification, submitting unusual isolates, and advice or training on biosafety techniques. It can also help a laboratory to locate nearby Biosafety level 3 facilities to store *B. pseudomallei* clinical isolates. Contributors can be found on the main webpage map (https://www.melioidosis.info/map.aspx- red box).

Laboratories are also able to report cases by clicking on "Report cases" on the primary tab on the website (shown below- red box). If the email application does not open, please copy the form, fill out the requested information and email it to melioidosis.info@tropmedres.ac.

Chapter Summary

B. pseudomallei is classified as a Tier 1 select agent by the U.S. CDC because of its high virulence and potential use as a bioterrorist weapon. Accordingly, many countries strictly regulate the transfer, possession, use, and disposal of *B. pseudomallei* isolates and contaminated specimens. The preceding chapter describes the appropriate protocols for reporting, transporting and documenting suspected *B. pseudomallei* isolates or specimens. While melioidosis is not a notifiable disease in all countries, it is recommended that suspected cases be reported to local or national sentinel laboratories or public health authorities who can assist with diagnostic confirmation and provide

biosafety guidance. The website www.melioidosis.info also offers methods to report culture-confirmed cases of melioidosis, provides support for *Burkholderia species* identification and advice or training on biosafety techniques.

Chapter 18: Melioidosis Surveillance and Filing a Clinical Case Report Form

Chapter Overview and Introduction to Disease Surveillance

Surveillance encompasses the collection, classification, analysis, interpretation, and reporting of health-associated data that enables public health decision making and action. Disease surveillance is accomplished through the ongiong collection, evaluation and dissemination of morbidity, mortality, and health data. Effective surveillance is essential to any prevention and control program and for effective public health decision making (CDC 2012).

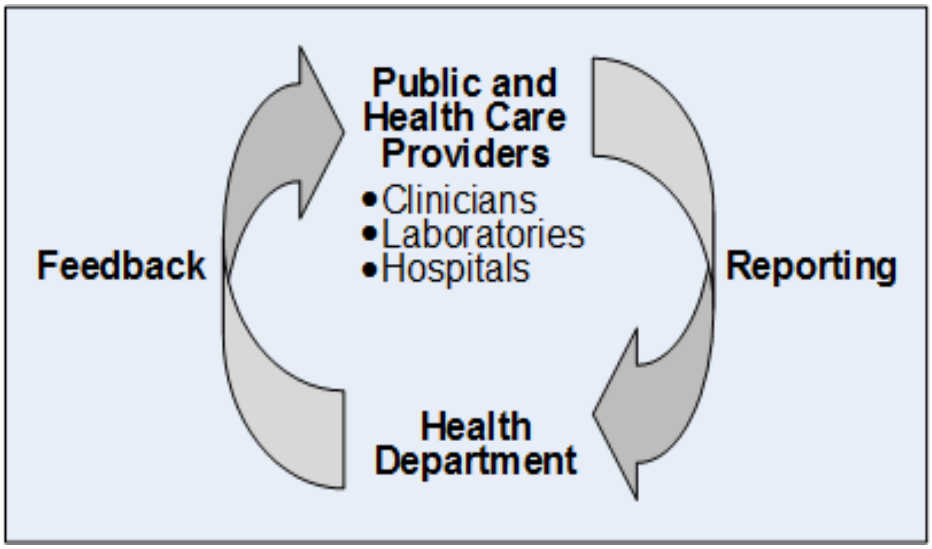


Figure 106- Surveillance starts with local healthcare providers like clinicians, laboratories, and hospitals and health departments. Feedback is provided by health departments back to local public and healthcare providers (CDC 2012).

Regulations often exist for reporting certain diseases. These diseases are usually of high epidemic potential or with high morbidity or mortality rates and are known as “notifiable” diseases. Due to the high morbidity and associated case fatality rates, melioidosis is included on several national

notifiable diseases lists, particularly in endemic regions of the world including Thailand and Australia (Hinjoy et al. 2018).

A case of melioidosis in a human should result in a report and investigation where possible. As the disease is rare or not well known in many countries, continuing education programs for healthcare workers and laboratory staff should include information on recognition and appropriate diagnostic tools and techniques. Certain at-risk populations, such as diabetics and those with underlying immunosuppressive conditions, may warrant more proactive surveillance, especially during periods of heavy rainfall or other adverse weather events (Suputtamongkol et al. 1999).

Surveillance Levels and Feedback

Infectious disease surveillance is conducted across multiple levels of government, enabling integrated and enhanced monitoring and feedback. Within a country, these levels can be broken down into: 1) local (e.g. clinician, hospital, laboratory), 2) intermediate (e.g. city, district, county, state), and 3) central/national surveillance (WHO 1999; WHO 2008).

1. Local Level Surveillance

The first effort to identify cases is normally focused at the local level (e.g., healthcare practitioners, clinics, hospitals, and laboratories) where an initial diagnosis is likely to be made. The primary responsibilities at this level include diagnosis and case management, reporting of cases and case and outbreak investigation (where resources permit). This is also the point at which surveillance data should first be collected and simple tabulation and graphing of data is performed (WHO 2008).

Local healthcare staff should complete a case-report form on every suspected or confirmed case of melioidosis (see an example case report in Figure 107 below). The form may be completed by a local healthcare professional or by health authorities at the local level. This should be done to establish

any possible sources of infection, including recreational or occupational hazards that may have led to exposure to contaminated soil or water.

Healthcare staff may develop a data collection form tailored to the specific details of that disease or a specific outbreak cluster. They may also use a generic case report form. Regardless of the case form that is used, it should include the following information (CDC 2012):

a. Identifying information- The patient's name, address, and telephone number are important in case physicians or investigators need to contact them for additional questions and to notify them of laboratory results. This information also helps to verigy if there are any duplicate records, while addresses allow the dispersal of infections to be mapped and visualized.
b. Demographic information- Information such as age, sex, race, occupation, etc. will help to characterize the populations at risk in that area (e.g., rice farmer, construction worker).
c. Clinical information- Clinical data will allow health investigators to establish whether the case definition for melioidosis has been met. Date of symptom onset is needed to chart the time course of the disease and possible time of exposure. Additional information, including length of illness, hospitalization or death, will help aid in the characterization of the infection.
d. Risk factor information- This information should be specifically tailored to melioidosis. For example, determining whether the patient is diabetic or has an immunosuppressive condition, or has had any recent notable exposure to soil or water.
e. Reporter information- The case report should include the name or information about reporter (typically a physician, clinic, hospital, or laboratory). Investigators will sometimes need to contact this individual for additional clinical information or to feedback any results.

Melioidosis Clinical Case Report Form

Patient ID: _____

Date of Birth (DoB): ___ / ___ / _____
(mm / dd / yyyy)

Name: _____
(given) (surname)

Residential Address: _____

Contact Phone #: _____

Gender: M / F

Race: 1 = White 2 = Asian 3 = Black or African American
4 = American Indian or Alaska Native
5 = Native Hawaiian or Other Pacific Islander 6 = Other

Occupation: _____

Recent Travel History: _____

Risk Factors

Diabetic:	Y / N	Smoking:	Y / N	Excessive Alcohol Use:	Y / N	Renal Disease:	Y / N
Chronic Lung Dis:	Y / N	Occ. Exp:	Y / N	Recreational Exposure:	Y / N	Inoculation Event:	Y / N
Malignancy:	Y / N	Immunosuppression:	Y / N	Cystic Fibrosis:	Y / N	Oral Steroid	Y / N
		Other:	_____	_____			

Admission No: __ Date: ___ / ___ / _____	Diagnosis Date ___ / ___ / _____	Discharge Date ___ / ___ / _____

Blood culture:
0 = -ve
1 = +ve
2 = not done

Died: 0 = no
1 = yes- initial illness
2 = yes- relapse
3 = yes- unrelated

Date: ___ / ___ / _____

	Treatment

Septic shock: Y / N	**ICU admission:**	**Y / N**	**Inotropes:**	**Y / N**
	Ventilated:	**Y / N**	**Renal Replacement Therapy:**	**Y / N**

Diagnoses: 1=Primary, 2=Secondary		
__ pneumonia	__ genito/urinary	__ bacteremic no focus
__ skin abscess(es)	__ soft tissue abscess(es)	__ CNS
__ osteomyelitis	__ septic arthritis	__ other clinical focus

Abscesses	
Prostate:Y / N	Spleen:Y / N
Liver:Y / N	Kidney:Y / N
Other: _____ _____ _____	

Chest XRay		
Left	**Right**	**Coding**
Upper: __	Upper: __	1 = consolidation lobar
Lingula: __	Middle: __	2 = other discrete cons.
Lower: __	Lower: __	3 = extensive patchy
		4 = patchy localised

Treatment	**Antibiotic**	**Dose**	**Duration**	**Compliance**
Intensive 1				
Intensive 2				
Eradication 1				
Eradication 2				

Blood			
WCC: _ _ _ _	Neutrophil: _ _ _	Lymphocytes: _ _ _ _	Sodium: _ _ _ _
Potassium: _ _ _ _	CO2: _ _ _	Urea: _ _ _ _	Creatinine: _ _ _ _
ALT: _ _ _ _	Bilirubin: _ _ _	GGT: _ _ _ _	Protein: _ _ _ _
	Albumin: _ _ _	Platelets: _ _ _ _	CRP: _ _ _ _
HbA1c (closest to admission) _ _ _ Date __/__/__			

Serology: Indirect Hemagglutination Assay (IHA)						
Date	**Titer**	**Date**	**Titer**	**Date**	**Titer**	**Coding**
__ / __ / __		__ / __ / __		__ / __ / __		1 = <1/20, 2 = 1/20,
__ / __ / __		__ / __ / __		__ / __ / __		3 = 1/40, 4 = 1/80,
__ / __ / __		__ / __ / __		__ / __ / __		5 = 1/160, 6 = 1/320,
__ / __ / __		__ / __ / __		__ / __ / __		7 = 1/640, 8 = 1/1280,
__ / __ / __		__ / __ / __		__ / __ / __		9 = >1/1280, 10 = not done

Figure 107- Example of a patient case report form that may be used if a case of melioidosis infection is suspected or confirmed in an individual. The form is adapted from Menzies School of Health Research and Royal Darwin Hospital, Darwin, Australia and can be tailored based on individual data and epidemiological reporting requirements.

If there is any apparent temporal and/or geographical clustering of melioidosis cases based on the data collected, additional investigations may need to be occur. These can include:

a. Collecting related soil and/or water samples for the detection of *B. pseudomallei* in the environment.
b. Investigating the genetic relatedness of isolates using molecular methods where resources allow.
c. Notifying local clinicians and residents about the cluster and about preventative measures that can be taken (e.g., chlorination or UV sterilization of domestic water supplies).

Local health authorities are normally expected to gather primary data and organize initial epidemiological investigations targeted at identifying and controlling the source of infection (including identification and investigation of additional cases and tracing possible sources of infection) as well as initiating control measures. They are also responsible for reporting to the intermediate level (e.g. state) or to the central/national level (e.g. federal), depending on the government structure (WHO 1999).

2. <u>Intermediate Level Surveillance</u>

Depending on the political structure of a country, data from local surveillance and local case reports may be managed at an intermediate level, such as a state health department. The intermediate level often collates and analyzes data from local levels to examine epidemiological links and trends. The intermediate level may also help with epidemiological investigations and monitoring of control measures and typically reports data to the central/national level.

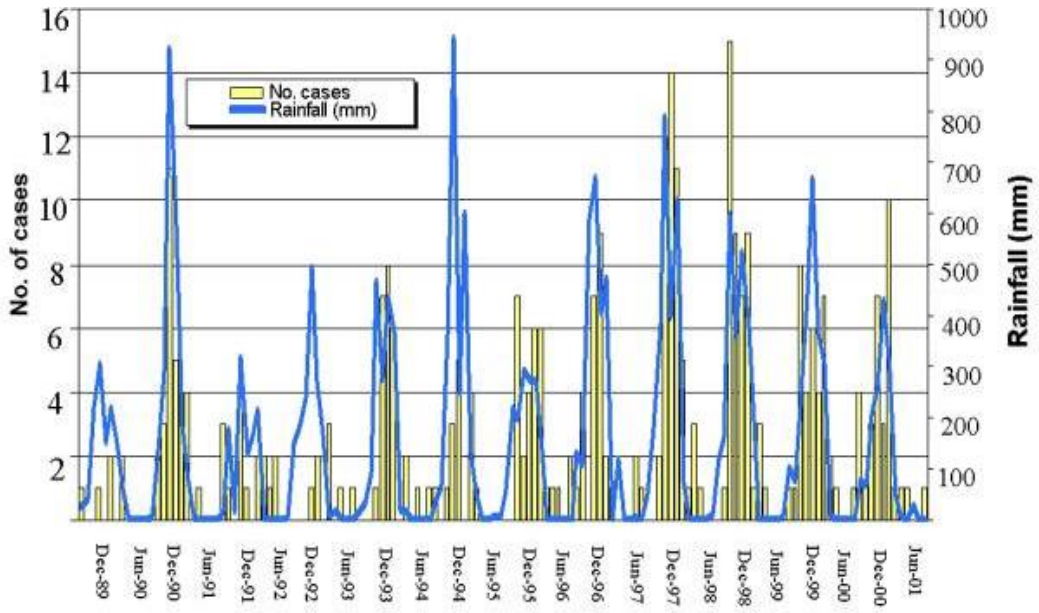


Figure 108- Surveillance of melioidosis cases; monthly rainfall vs. the number of melioidosis cases during a 12-year period in the Northern Territory, Australia. Here, the data is used to show that rainfall is associated with an increase of melioidosis cases (Currie & Jacups 2003).

3. Central/National Level Surveillance

Cases of melioidosis cases should be reported to the central/national level. This level will typically analyze and report data back to intermediate and local levels, devise national policies and allot resources based on the reported data. The central level may also offer practical and technical support to intermediate and/or local level laboratory or epidemiological staff and report summary surveillance data to international health authorities (WHO 2008).

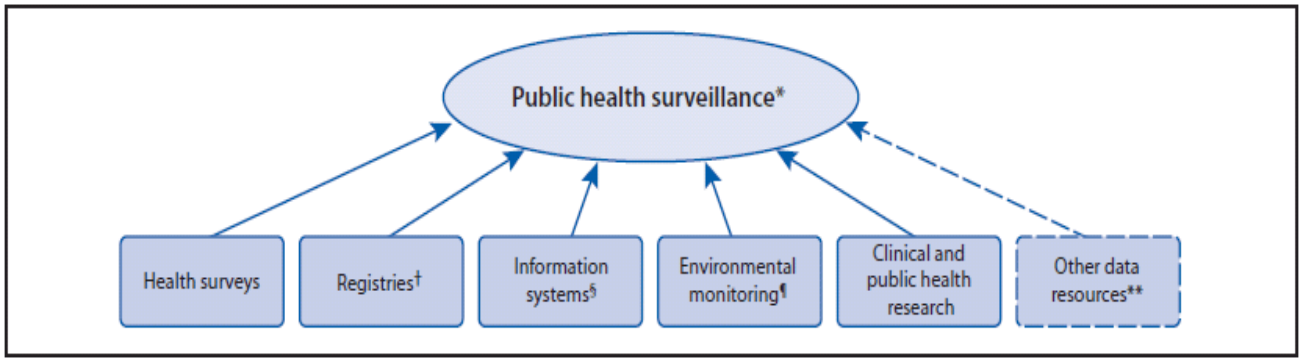


Figure 109- Conceptual framework for public health surveillance. Effective surveillance requires continuous collection, analysis, interpretation, and distribution of results so that effective action can be taken (Porta 2008).

Chapter Summary

Effective surveillance is essential to any disease prevention and control program. It is accomplished through the collection, analysis, and interpretation of case reports and associated health data, and the dissemination of data to those involved in disease control and public health decision making. A single case of melioidosis in a human should always result in a patient case report and investigation where possible. This information should be communicated to intermediate and central levels of surveillance, enabling integrated and enhanced monitoring and feedback of melioidosis infection.

Glossary

Aerobic bacteria- Bacteria that utilize oxygen for growth and oxygen-based metabolism.

Agglutinate- the formation of clumps of cells or particles by specific antibodies to surface antigenic components.

Algorithm- A procedure or formula for solving a problem, based on conducting a sequence of specified actions.

Aliquot- To divide a portion of the whole, especially one of two or more samples of something that have the same volume or weight**.**

Ashdown media- A selective culture medium for the isolation and characterization of *Burkholderia pseudomallei.*

Augur- A drilling device used for making holes in wood or in the ground that typically has a rotating blade. The rotation of the blade causes the material to move out of the hole being drilled.

Autoclave- A machine that uses steam under pressure to kill harmful bacteria, viruses, fungi, and spores.

Broth microdilution- A method used to test the susceptibility of microorganisms to antibiotics where wells are filled with broth containing different concentrations of the antibiotic.

Chaotropic- a substance that disrupts the structure and denatures molecules such as proteins and nucleic acids (e.g. DNA and RNA).

Chelating- A chemical compound that binds tightly to metal ions.

Electrophoresis- A laboratory method used to separate DNA, RNA, or protein based on size and electrical charge. An electric current is used to separate molecules through a gel.

Eluate- A solution that is available for analysis after an elution is performed.

Elution- A process in which particles are separated and extracted based upon size, shape and density by washing in a solvent (liquid substance capable of dissolving other substances).

Endemic- Natural to, native, confined to, or widespread within a place or population of people.

Enrichment broth- A liquid medium that contains different nutrients and is used to culture bacteria and other microorganisms.

Exonuclease- Enzymes that work to remove nucleotides from linear single-stranded DNA in the 3' to 5' direction.

Fluorophore- A fluorescent chemical compound that is able to re-emit light when energy is applied.

Geographic Information System (GIS)- A system designed to capture, store, analyze, and display geographical data that can be referenced to locations on the earth.

Gold standard- Something that serves as a reference that others can be measured or judged against.

Gram-positive- Bacteria that have a thick peptidoglycan layer, no outer lipid membrane and retain the color of the crystal violet stain in the Gram stain.

Gram-negative- Bacteria that have a cell wall composed of a thin layer of peptidoglycan and have an outer lipid membrane. The structure of their cell wall is unable to retain the crystal violet stain so they are colored by the counterstain.

Hemolysis- The rupturing or breakdown of red blood cells that leads to the release of their contents into the surrounding fluid.

Heterogeneous- Populations, samples or results that are different or mixed.

Homogenize- To blend different elements into a mixture that is the same throughout.

Inoculate- A process where a small streaking loop is dipped into a solution containing bacterial cells and is used to streak an agar plate with the bacteria.

Inoculum- A small amount of material containing bacteria, viruses, or other microorganisms that is used to start a culture.

Intercalating- A substance that inserts itself into the DNA structure of a cell and binds to the DNA.

Latent infection- An infection that typically does not cause noticeable symptoms and can last long periods of time without becoming active and causing symptoms.

Latex agglutination- A laboratory test used to check for specific antibodies or antigens in different body fluids such as saliva, urine, cerebrospinal fluid, or blood.

Lysis- Breaking down the membrane of a cell by viral, chemical, or physical means.

MacConkey agar- A selective culture medium used for the isolation and differentiation of Gram-negative enteric bacilli.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)- A rapid and high-throughput method for microbial identification using specific protein signatures.

McFarland Standard- References used to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. This is done to standardize microbial testing.

Minimum Inhibitory Concentration Testing (MIC)- A test used to determine the lowest concentration of an antimicrobial agent that will prevent visible growth of a microorganism.

Oligonucleotide- Short single strands of synthetic DNA or RNA that serve as the starting point for many molecular biology applications.

Oxidase test- A diagnostic test used to identify bacteria that produce cytochrome c oxidase, an enzyme that is part of the bacterial electron transport chain.

Pathogenic- Capable of causing disease.

Personal Protective Equipment (PPE)- PPE is clothing or equipment designed to be worn to protect a person from the risk of injury or illness.

Polymerase Chain Reaction (PCR)- A technique used to amplify small segments of DNA into many copies.

Post-Exposure Prophylaxis (PEP)- Treatment used to reduce the likelihood of acquiring an infection after potential exposure.

Quantify- To count or express something using numbers.

Quencher- Substances capable of absorbing energy from a fluorophore (such as a fluorescent dye) and re-emitting that energy as either heat or visible light.

Sapronotic- An infection that is a sapronosis, or a disease transmissible from the environment (e.g. soil, water, decaying plants). The organism must be able to grow and replicate in the environment.

Seropositive- Showing a significant level of serum antibodies, or other immunologic marker in the serum, indicating previous exposure to the infectious agent of interest.

Spatial autocorrelation- The degree to which one object is similar to other nearby objects. It is the tendency for samples or sites that are close together to have similar values.

Supernatant- The fluid lying above a precipitate/pellet following the centrifugation of a suspension.

TBSS-C50- An enrichment broth often used for soil culture that contains a threonine-basal salt solution plus colistin at 50 mg/liter.

Titer- A measurement of the amount or concentration of a substance in a solution. It usually refers to the amount of antibodies found in blood.

Vortex- To mix (as the contents of a test tube) by rapid spinning or circular motion.

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