DETECTION OF *BURKHOLDERIA MALLEI* AND *BURKHOLDERIA PSEUDOMALLEI* BY REAL-TIME PCR

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This protocol is an OIE-based method used at the EU-RL, all OIE-CFT based methods validated and used successfully in the proficiency tests can be used for this assay.

1. TOPIC AND SCOPE

This document describes the PCR methods for the detection of *Burkholderia* sp, *B. mallei* and *B. pseudomallei* according to the world organisation for animal health (OIE) international standard: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – Chapter 3.5.11, glanders and melioidosis (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.11_GLANDERS.pdf).

Several systems are described in this document:

- Detection of the *B. pseudomallei* complex (including *B. mallei*, *B. pseudomallei* and *B. thailandensis* species)
- Specific detection of *B. mallei* (fliP)
- Specific detection of *B. pseudomallei* (orf11)

For detection, the PCR systems implemented are based on the use of specific oligonucleotide primer pairs and probes labelled in the 5' with the FAM fluorochrome. The detection of the exogenous gene added at the sample extraction stage is carried out using the associated PCR kit (Diagenode, DICD-YD-L100) and includes primers and a probe labelled in 5' with the fluorochrome VIC. This internal control system makes it possible to check the correct operation of the DNA extraction and the possible presence of PCR inhibitors. It constitutes a control of the DNA extraction steps and of the PCR method.

2. MATERIAL TO BE EXAMINED

2.1. SERUM

The PCR diagnosis of glanders is performed on DNA extracted from tissues or swabs.
Upon reception, the samples should be kept refrigerated (5 ± 3°C) or frozen (≤ -16°C).

2.2. TRANSPORT OF SAMPLES

Samples must be stored at ≤ -16°C.

3. DILUENTS, REAGENTS AND OTHER PRODUCTS

3.1 REAGENTS

- TaqMan® Fast advanced Master (Applied Biosystems part number 4444557) or equivalent
- IPC primers and probes (Diagenode)
- Autoclaved ultrapure water
- TaqMan® oligonucleotide probes
- Oligonucleotide sense and antisense primers

Target	Name of primers and probe	Sequence (5' - 3')	Reference
B. mallei	fliP forw fliP rev fliP probe	CCC ATT GGC CCT ATC GAA G GCC CGA CGA GCA CCT GAT T FAM-CAG GTC AAC GAG CTT CAC GCG GAT C-TAMRA	Tomaso *et al.,* 2006
B. pseudomallei	orf11 forw orf11 rev orf11 probe	ATC GCC AAA TGC CGG GTT TC CAA ATG GCC ATC GTG ATG TTC FAM -TCG GCG AAC GCG ATT TGA TCG TTC-TAMRA	Thibault *et al.,* 2004
B. pseudomallei complex	aroA forw aroA rev aroA probe	CCG CTC GTG AAG GCG AAG CGC CAT CAG CTT GAT CGT GA FAM -CGA GCG TCG TCG AGA TCG-TAMRA	Laroucau *et al*., 2021

Tomaso *et al*. 2006. Development of a 5'-nuclease real-time PCR assay targeting fliP for the rapid identification of *Burkholderia mallei* in clinical samples. Clin Chem. 52(2):307-10.
Thibault *et al*. 2004. Identification and discrimination of Burkholderia *pseudomallei*, *B. mallei*, and *B. thailandensis* by real-time PCR targeting type III secretion system genes. J Clin Microbiol. Dec;42(12):5871-4.
Laroucau *et al*. 2021. A genetic variant of Burkholderia mallei detected in Kuwait: consequences for the PCR diagnosis of glanders. Transbound. Emerg. Dis. 2020;00:1-4.

4. EQUIPMENT AND PLASTIC/GLASS WARE

Conventional molecular biology laboratory equipment and in particular:

- Vortex
- Benchtop centrifuge
- Pipetting and micropipetting equipment
- 10, 20, 200 and 1000 µL filter tips
- 1.5 mL and 2 mL microtubes previously sterilised
- Plastic consumables (plates, microtubes, strips) compatible with the real-time thermocycler used
- Real-time thermocycler (e.g. AB 7500, ViiA7, QS7) and their *ad hoc* software.

5. SAMPLE PREPARATION

- Avoid inter-sample contamination by following the usual precautions (aliquot reagents; separate workstations; etc.).
- Work with sterile, single-use consumables.
- Include positive and negative extraction and PCR controls to ensure proper performance of the DNA extraction and PCR amplification steps and the absence of inter-sample contamination.

6. REAL-TIME PCR PROCEDURE

6.1 PREPARATION OF THE PCR REACTION MIX

- Determine the number of PCR reactions to be performed.
- Systematically include a positive control (T^{+}_{PCR}) and a negative control (T^{-}_{PCR}) of PCR.
- Thaw the primers/probe for each of the selected systems (or the corresponding mix) and the IPC mix (Diagenode probe/primers).
- Prepare the reaction mixt for a final volume of 20 µL per reaction and containing:

For the FAM/IPC system: (e.g. detection of the fliP target and IPC in a test sample)

Reagent	Volume needed per well		Final concentration
Master Mix Buffer (2X)	10 µL		1X
System 1 - forw (20 µM)	0.5 µL	or 1.4 µL of the corresponding Mix	0.5 µM
System 1 - rev (20 µM)	0.5 µL		0.5 µM
System 1 - probe (5 µM) **FAM**	0.4 µL		0.1 µM
IPC Diagenode (10X) **VIC**	2 µL		1X

H_2O	1.6 µL		/
Volume to be distributed per well	15 µL		/
DNA to be tested	5 µL		/
Final volume	20 µL		/

- Dispense 15 µL of reaction mix into each tube (or plate well)
- In the DNA deposit room, add 5 µL of DNA to be tested.
- For each set of analyses, it is essential to include:
  - a negative control (T^-_{PCR}) containing 15 µL of mix and 5 µL of water. This control is used to check that the reagents are not contaminated by the targets (negative PCR control).
  - a positive control (T^+_{PCR}) containing 15 µL of mix and 5 µL of the positive control obtained according to internal procedures. This positive target control is used to verify that the real-time PCR amplification reaction is running correctly under limit of detection conditions.
- Close the tubes with the special optical caps or seal the plate with optical film.
- Briefly centrifuge the tubes/plates using a suitable centrifuge.
- The tubes or plates are inserted into the real-time PCR thermocycler and the analytical follow-up sheet is completed.

6.2 PCR AMPLIFICATION

- Refer to the operating instructions of the instruments for programming depending on the thermocycler used. Wells are read in FAM/TAMRA(or BHQ) for *Burkholderia* targets and in VIC/None for the Diagenode IPC.

- The mix contains a passive ROX reference for Applied Biosystem devices. The fluorescence reading is taken at the end of the elongation step.

- Steps of the program used correspond to : 50°C for 2 min (UNG activation), 95°C for 20 s (Denaturation), followed by 45 cycles (95°C 3 s, 60°C 30 s) (Denaturation, hybridization, elongation, reading).

7. EXPRESSION AND INTERPRETATION OF RESULTS

7.1 DEFINITIONS

The term **"background noise"** refers to the uncharacteristic part of the curves observed during the first 10 to 15 cycles.

The term **"amplification"** refers to the presence of a curve with an increasing linear portion followed by a plateau. Any curve that does not have this aspect will be considered as an uncharacteristic curve (no amplification), such as a flat or sawtooth curve.

The threshold should be placed above the background noise, preferably in the middle of the linear part (in "logarithmic scale" display) common to all amplification curves.

Ct (for Cycle threshold) is the number of PCR cycles required to reach a threshold amount of fluorescence.

7.2 READING

The fluorescence reading is taken at the end of the elongation step in each cycle. The threshold cycle (Ct) is defined as the cycle number at which the fluorescence intensity crosses the threshold. This baseline is set manually or automatically (by the software associated with the thermal cycler used).

It is important to check the appearance of the amplification curve for each target, which should be in sigmoid form (logarithmic representation)

7.3 VALIDATION OF THE TEST

The test is validated if:

- The negative PCR (T^-_{PCR}) and extraction ($T^-_{Extraction}$) controls all have an undetermined target Ct value.
- The target Ct value of the positive controls (T^+_{PCR} and $T^+_{Extraction}$) meet the validation criteria as defined in the procedure « Control of test results ».

7.4 VALIDATION OF THE SAMPLE RESULT

The result of each sample is validated if :

- The Ct obtained for the sample with the exogenous IPC PCR is ≤ 37.
    - If the exogenous non-target IPC Ct is > 37, or indeterminate (-), dilute the total DNAs 1:10 in ultrapure water and repeat the target and exogenous IPC PCRs from this dilution.
    - If the Ct of the IPC and target PCR are again > 37 or undetermined, the sample will be considered unusable (presence of PCR inhibitors; lysed or rotted sample...).
- The Ct obtained with the target PCR is positive (with a characteristic curve), even if the IPC PCR is indeterminate or > 37.

7.5 EXPRESSION OF RESULTS

Depending on the target (*B. mallei* or *B. pseudomallei* or *B. pseudomallei complex*):

- The result is reported as "***B. mallei*** (or *B. pseudomallei* or *B. pseudomallei complex*) **genome not detected**" when the Ct obtained with the target PCR is indeterminate (-).

- The result is reported as "***B. mallei*** (or *B. pseudomallei* or *B. pseudomallei complex*) **genome detected**" when the sample has a target Ct < 40 with a characteristic amplification curve (sigmoidal appearance in logarithmic display).

- Special case: if the sample is unusable (presence of PCR inhibitors; lysed or putrefied sample...), the result is returned as "**uninterpretable**".

Sample	Result of the IPC PCR	Result of the *B. mallei* PCR	Conclusion	Result of the 2nd IPC PCR	Result of the 2nd *B. mallei* PCR	Conclusion
Lyzed or putrified sample	*Sample rejected*		*Request for a new sample*			
Compliant sample	IPC positive	*B. mallei* negative	Negative for *B. mallei*			
	IPC positive	*B. mallei* positive	Positive for *B. mallei*			
	IPC negative	*B. mallei* negative	Inhibited sample - Carry out a dilution (see 7.4)	IPC negative	*B. mallei* negative	uninterpretable
				IPC negative	*B. mallei* positive	Positive for *B. mallei*
				IPC positive	*B. mallei* negative	uninterpretable
				IPC positive	*B. mallei* positive	Positive for *B. mallei*
	IPC negative	*B. mallei* positive	Positive for *B. mallei*			

Sample	Result of the IPC PCR	Result of the *B. pseudomallei* PCR	Conclusion	Result of the 2nd IPC PCR	Result of the 2nd *B. pseudomallei* PCR	Conclusion
Lyzed or putrified sample	*Sample rejected*		*Request for a new sample*			
Compliant sample	IPC positive	*B. pseudomallei* negative	Negative for *B. pseudomallei*			
	IPC positive	*B. pseudomallei* positive	Positive for *B. pseudomallei*			
	IPC negative	*B. pseudomallei* negative	Inhibited sample - Carry out a dilution (see 7.4)	IPC negative	*B. pseudomallei* negative	uninterpretable
				IPC negative	*B. pseudomallei* positive	Positive for *B. pseudomallei*
				IPC positive	*B. pseudomallei* negative	uninterpretable

				IPC positive	*B. pseudomallei* positive	Positive for *B. pseudomallei*
	IPC negative	*B. pseudomallei* positive	Positive for *B. pseudomallei*			

Sample	**Result of the IPC PCR**	**Result of the *B. pseudomallei complex* PCR**	**Conclusion**	**Result of the 2nd IPC PCR**	**Result of the 2nd *B. pseudomallei complex* PCR**	**Conclusion**
Lyzed or putrified sample	*Sample rejected*		*Request for a new sample*			
Compliant sample	IPC positive	*B. pseudomallei complex* negative	Negative for *B. pseudomallei complex*			
	IPC positive	*B. pseudomallei complex* positive	Positive for *B. pseudomallei complex*			
	IPC negative	*B. pseudomallei complex* negative	Inhibited sample - Carry out a dilution (see 7.4)	IPC negative	*B. pseudomallei complex* negative	uninterpretable
				IPC negative	*B. pseudomallei complex* positive	Positive for *B. pseudomallei complex*
				IPC positive	*B. pseudomallei complex* negative	uninterpretable
				IPC positive	*B. pseudomallei complex* positive	Positive for *B. pseudomallei complex*
	IPC negative	*B. pseudomallei complex* positive	Positive for *B. pseudomallei complex*			