ANNEX 2

Laboratory Operating Procedure (LOP)
Protocol for Cultivation of *Clostridioides difficile* from faecal and environmental samples

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A. Description

The purpose of this procedure is to provide a harmonizing framework in the cultivation and identification of *Clostridioides difficile* for further toxin gene detection and typing, antimicrobial susceptibility testing and WGS. This procedure applies to *C. difficile* from faecal (human, pigs, wild animals) samples, as well as pig manure and environmental (soil and water) samples.

B. Equipment and material

See procedures.

C. Procedures

1. Protocol for faecal samples (human, pigs, wild animals) and pig manure

1. *Clostridium difficile* culture (plating and enrichment)

   a) Inoculate faecal sample (approx. 0.5g) in 10 ml *C. difficile* broth supplemented with CDMN selective supplement and 0.1% sodium-taurocholate;

   b) Plate 100 µl of this mixture onto CDMN agar base with CDMN selective supplement (CDMN agar) and/or on ChromID® *C. difficile* agar; in alternative perform ethanol shock using the same amount of faeces and 1 mL of ethanol (96-100%). Incubate 30 min to 1h at room temperature to select for spores, and then inoculate 100 µl of this suspension onto CDMN agar and/or on ChromID agar;

   c) Incubate plates for 2-3 days, and enrichments for 7 days, at 37°C, under anaerobic conditions; for the enrichments, change the atmosphere every 2-3 days of incubation;

2. After the 7 days of enrichment, mix 1 ml of the mixture with 1 ml of ethanol (96-100%) and incubate 30 min to 1h at room temperature to select for spores;

3. Centrifuge mixture at 5,000 x g for 10 min, remove supernatant and resuspend the pellet in 200 µl NaCl (0.85%);

4. Plate 100 µl of the pellet onto CDMN agar and/or on ChromID agar and incubate anaerobically at 37°C for 2-3 days;

5. For each of the plates, pick up to 20 presumptive *C. difficile* colonies and subculture onto blood agar plates;

6. Confirm species by MALDI-TOF or molecular methods (PCR, Real-Time PCR);

7. Perform toxin gene detection and typing of the isolates by PCR-ribotyping and/or MLST;

8. For selected isolates (different ribotypes or STs), perform antimicrobial susceptibility testing by disk diffusion to the following antibiotics: clindamycin, erythromycin, imipenem, metronidazole, moxifloxacin, rifampicin, tetracycline and vancomycin (protocol from One Health IMPART project);

9. Perform cryopreservation of the isolates, using TSB with 10% of glycerol;

10. Perform WGS of all unique isolates (different ribotypes/STs, or same ribotype/STs with different AST).

2. Protocol for soil samples

(adapted from Janezic S et al, 2016 doi:10.1371/journal.pone.0167101)

1. Resuspend one sample of 25 g soil in 90 ml of sterile water and the other sample of 25 g soil in 90 ml of *C. difficile* broth for enrichment;

2. Incubate the enrichment at 37°C for 7 days under anaerobic conditions, changing the atmosphere every 2-3 days of incubation; After this period of incubation proceed as the described below for the sample in water (steps 3-11);

3. Process immediately the sample in water, by centrifugation of 50 ml of soil suspension at 50 x g for 2 min;

4. Transfer 40 ml of the supernatant to a new sterile tube and centrifuge again at 50 x g for 2 min;

5. Subject the supernatant (~30 ml) to heat shock, at 70ºC for 20 min;

6. Filter the entire volume through 0.2 µm cellulose nitrate sterile membrane filter (Whatman) using Milipore filtering system;

7. After filtration, place filters on ChromID® *C. difficile* agar and incubate anaerobically at 37ºC for 3-5days;

8. After incubation, pick up to 20 presumptive *C. difficile* colonies from each filter and subculture onto blood agar plates;

9. Swab the remaining bacterial growth from the filter, resuspend in 700 µl of ethanol (96-100%) and incubate at room temperature for 30 min to 1h;

10. Centrifuge 5,000 x g for 10 min and after centrifugation inoculate the pellet onto ChromID® *C. difficile* agar plates and incubated anaerobically for 2 days;

11. After incubation, pick up to 10 presumptive *C. difficile* colonies and subculture onto blood agar plates;

12. Confirm species by MALDI-TOF or molecular methods (PCR, Real-Time PCR);

13. Perform toxin gene detection and typing of the isolates by PCR-ribotyping and/or MLST;

14. For selected isolates (different ribotypes or STs), perform antimicrobial susceptibility testing by disk diffusion to the following antibiotics: clindamycin, erythromycin, imipenem, metronidazole, moxifloxacin, rifampicin, tetracycline and vancomycin (protocol from One Health IMPART project);

15. Perform cryopreservation of the isolates using TSB with 10% of glycerol;

16. Perform WGS of all unique isolates (different ribotypes/STs, or same ribotype/STs with different AST).

3. Protocol for water samples

(adapted from Janezic S et al, 2016 doi:10.1371/journal.pone.0167101)

1. Subject the two water samples (2*50 ml) to heat shock by incubation at 70ºC for 20 min;

2. Filter each one (50 ml) through 0.2 µm cellulose nitrate sterile membrane filter (Whatman) using Milipore filtering system;

3. After filtration, place one of the filters onto selective agar ChromID® *C. difficile* plates and incubate anaerobically at 37ºC for 3-5 days;

4. With the other filter perform the enrichment. Place the filter onto a sterile tube with 10 ml of *C. difficile* broth and incubate anaerobically at 37ºC for 7 days, changing the atmosphere every 2-3 days of incubation;

5. After enrichment, vortex the mixture, remove the filter and centrifuge 5 mL of the mixture at 5,000 x g for 10 min, remove supernatant and resuspend the pellet in 1 ml of ethanol (96-100%) and incubate 30 min to 1h at room temperature to select for spores;

6. Plate 2-3 drops on ChromID® *C. difficile* agar and incubate anaerobically at 37°C for 2-

3 days;

7. After plates incubation, pick up to 20 presumptive *C. difficile* colonies from each filter and subculture onto blood agar plates;

8. Swab the remaining bacterial growth from each filter, resuspend in 700 µl of ethanol (96-100%) and incubate at room temperature for 30 min to 1h;

9. Centrifuge 5,000 x g for 10 min and after centrifugation inoculate the pellet onto ChromID® *C. difficile* agar and incubate anaerobically for 2-3 days;

10. After incubation, pick up to 20 presumptive *C. difficile* colonies subculture onto blood agar plates;

11. Confirm species by MALDI-TOF or molecular methods (PCR, Real-Time PCR);

12. Perform toxin gene detection and typing of the isolates by PCR-ribotyping and/or MLST;

13. For selected isolates (different ribotypes or STs), perform antimicrobial susceptibility testing by disk diffusion to the following antibiotics: clindamycin, erythromycin, imipenem, metronidazole, moxifloxacin, rifampicin, tetracycline and vancomycin (protocol from One Health IMPART project);

14. Perform cryopreservation of the isolates using TSB with 10% of glycerol;

15. Perform WGS of all unique isolates (different ribotypes/STs, or same ribotype/STs with different AST.

Appendix 1

Media and supplements:

- CDMN, *Clostridium difficile* moxalactam norfloxacin selective supplement (Ref. SR0173, Oxoid)
- CDMN agar, *Clostridium Difficile* Agar-Basis (Ref. CM0601, Oxoid)
- ChromID® *C. difficile* agar, ready to use plates (Ref. 43871, bioMérieux)
- Sodium-taurocholate (Ref. 86339, Sigma-Aldrich)
- COH, Blood agar plates (Ref. 43041, bioMérieux)
- TSB, Tryptone soya broth (Ref. CM0129, Oxoid)

***C. difficile* broth composition:**

Recipe:	**gm/litre**
Proteose peptone	40.0
Disodium hydrogen phosphate	5.0
Potassium dihydrogen phosphate	1.0
Magnesium sulphate	0.1
Sodium chloride	2.0
Fructose	6.0
pH 7.4 ± 0.2 @ 25°C	