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Evaluation of *Burkholderia cepacia* Complex Bacteria Pathogenicity Using *Caenorhabditis elegans*

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[Abstract] This protocol describes two biological assays to evaluate pathogenicity of *Burkholderia cepacia* complex (Bcc) strains against the nematode *Caenorhabditis elegans.* Specifically, these two assays allow one to identify if the under-investigated Bcc strains are able to kill the nematodes by intestinal colonization (slow killing assay, SKA) or by toxins production (fast killing assay, FKA). The principal differences between the two assays rely on the different killing kinetics for worms.

[Background] The *Burkholderia cepacia* complex (Bcc) occupies a critical position among Gram-negative multi-drug resistant bacteria. It consists of at least 20 closely related species. Many Bcc strains are multi drug and pandrug-resistant opportunistic human pathogens caused problematic lung infections in immune-compromised individuals, including cystic fibrosis (CF) patients. The use of non-vertebrate host model can be useful for dissecting virulence and pathogenicity determinants as well as identifying novel therapeutic targets (Kothe *et al.,* 2003).

There are a good number of assays for detecting Bcc virulence against a large panel of host models, in liquid or in solid surface. However, some of those are mostly focused on phenotypic observations, which are difficult to detect and have a low reproducibility (Cardona *et al.*, 2005). Herein, we developed two assays based on the analysis of surviving worms, which is a more reproducible and allows easy and fast comparison among the Bcc strains tested. In addition, these assays permit the detection of death mechanisms of Bcc towards nematode.

These killing assays allow us to identify bacterial strains that are able to colonize the nematode intestine and produce diffusible toxins capable of killing the host.

Materials and Reagents

1. 15 ml Falcon tubes (Corning, Falcon®, catalog number: 352095)
2. 3.5 cm diameter agar plates (Corning, catalog number: 430165)
3. *Caenorhabditis elegans*
4. *Burkholderia cepacia* complex (Bcc) strains
5. *E. coli* OP50
6. NaOH
7. Bleach (Aurora)

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8. NGM agar
9. PGS agar
10. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
11. Tryptone (Conda, catalog number: 1612)
12. Yeast extract (Conda, catalog number: 1702)
13. Peptone (Conda, catalog number: 1602)
14. European agar (Conda, catalog number: 1800)
15. Magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, catalog number: M7506)
16. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C1016)
17. Cholesterol (Sigma-Aldrich, catalog number: C3045)
18. Glucose (Conda, catalog number: 1900)
19. Sorbitol (Sigma-Aldrich, catalog number: S1876)
20. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
21. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S5136)
22. LB broth (see Recipes)
23. NGM agar medium (see Recipes)
24. PGS agar medium (see Recipes)
25. M9 buffer (see Recipes)

Equipment

1. Centrifuge
2. 20 °C chamber
3. 37 °C shaking incubator
4. Dissecting microscope
5. Platinum loop

Software

1. Graph-pad Prism 5 software

Procedure

A. Day 1
   1. Synchronize worms with bleaching protocols: use *C. elegans* plates with many gravid hermaphrodites (Stiernagle, 2006). Wash the plates with sterile H_2O.
   2. Collect the liquid in a sterile 15 ml Falcon tube. Add H_2O to a total volume of 3.5 ml.
   3. Mix 0.5 ml 5 N NaOH with 1 ml bleach. Make this solution fresh just before use! Add to the centrifuge tube with the worms.

4. Vortex the tube for a few seconds. Repeat vortexing every 2 min for a total of 10 min.
5. Spin the tube in a centrifuge for 30 sec at 1,300 *x g* to pellet the released eggs.
6. Aspirate to 0.1 ml.
7. Add sterile H_2O to 5 ml. Vortex for a few seconds.
8. Repeat steps A6 and A7.
9. Transfer the eggs in the remaining 0.1 ml of liquid to the edge of a clean NGM plate seeded with an *E. coli* OP50 lawn and incubate at 20 °C.
   Note: If you use C. elegans mutants that do not change their intrinsic nature under high temperature, you can incubate eggs at 25 °C as some mutants grow slower than WT.
10. Inoculate Bcc strains in 15 ml Falcon tubes containing 3 ml of LB and incubate the tubes at 37 °C for 24 h in shaking condition (220 rpm).
    Note: As controls, use E. coli OP50 in the place of Bcc strains.

B. Day 2
1. Normalize Bcc cultures at 1.7 OD/ml and seed 50 µl of the culture on 3.5 cm diameter plates containing 3 ml of NGM agar (slow killing assay, SKA) or PGS agar (fast killing assay, FKA). Incubate the plates O/N at 37 °C.
   Note: Incubation of Bcc strains at 37 °C should never exceed 16 h. Do not store Bcc seeded plates at 4 °C, as many of these pathogens are very sensible to temperature and this can cause variation.
2. Check the developmental stage of worms. After 24 h worms should be at larval stage L2 or L3 when grown at 20 °C.

C. Day 3
1. Wash synchronized worms at larval stage 4 (L4) off plates with M9 buffer and collect them in 15 ml Corning tubes.
2. Wash worms 2-3 times with M9 buffer to remove residual bacterial cells.
3. Spot 30-40 L4 worms on the plates seeded with Bcc strains (5 replicas).
   Note: Before adding the worms, plates should be kept at room temperature to cool them down after the incubation at 37 °C.
4. Count worms at time 0. Incubate the plates at 20 °C and perform daily count of surviving worms up to day 5 (for FKA) and day 6 (for SKA).
   Note: A worm is considered dead when it no longer responds to gentle touch with a platinum wire.
5. At the end of the experiment, calculate the average percentage of surviving worms.
6. Evaluate Bcc pathogenicity. The virulence ranking (VR) ranges from 0 to 3 and it is based on the percentage of surviving worms after the period of observation. A Bcc strain is considered to be non-pathogenic (VR = 0) when the percentage of worms alive at the conclusion of the period

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of observation ranges from 100% to 80%; VR = 1 corresponds to a percentage of worms alive between 79% to 50%; VR = 2 corresponds to a percentage of worms alive between 49% to 6%; finally, the VR is considered 3 when the percentage of surviving worms was ≤ 5% (Figure 1).

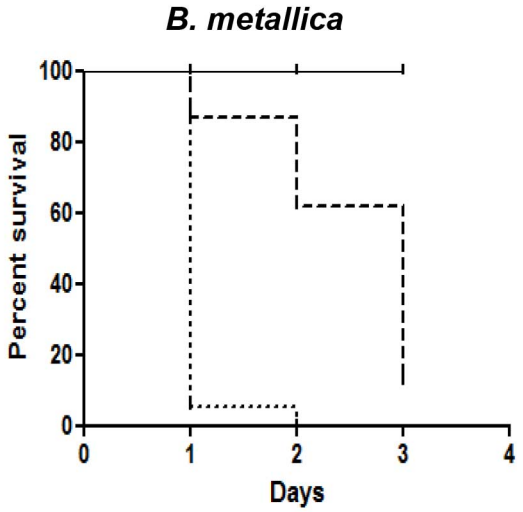

Figure 1. Kaplan-Meier survival plots for L4 stage WT worms fed with *E. coli* OP50 (solid lines), *Burkholderia metallica* on NGM (dashed lines), *Burkholderia metallica* on PGS (dotted lines). n: Number of worms at day 0. The pathogenicity of Bcc strain *B. metallica* on SKA (n = 80) was compared with the ability on FKA (n = 113).

Data analysis

Kaplan-Meier survival curves can be generated and analyzed using Graph-pad Prism 5 software. Comparisons vs. control for both the *C. elegans* are performed using Fisher's exact test to account for possible non-Normality in the data. Bonferroni-Holm correction of *P*-values is used to account for the multiple comparisons performed. More details can be found in Tedesco *et al.* (2015).

Notes

1. These protocols can be very useful for the identification of virulent Bcc strains and have a high reproducibility. However, slight variation in percentage of mortality can be observed, as the nematode is an *in vivo* model. To minimize those variations, worms and Bcc strains should be maintained always in the same conditions, using the same incubation times and temperatures.
2. The principal concern relies on nematode's larval stage. Only worms at larval stage 4 should be used. This is because worms at larval stage L3 or L2 can be more susceptible to pathogens, while older worms carry eggs, which can hamper the counting process.
3. Timing is central. Please plan your experiment so that the experiment could be done using plates and animals that were prepared at the same time.

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4. Bcc strains can display different VR in the two assays. In our study we found a high variation in pathogenicity: strain *B. metallica* LMG 24068 and *B. stabilis* LMG 14294 had maximum score in both assays, while *B. cepacia* LMG 1222 had VR = 3 in FKA and VR = 1 in SKA. *B. multivorans* LMG 13010 instead showed no pathogenicity at all VR = 0 in both assays.

Recipes

1. LB broth (1 L)
   10 g NaCl
   10 g tryptone
   5 g yeast extract
   Add H_2O to 1 L
   Sterilize by autoclaving.
2. NGM agar medium (1L)
   2.5 g peptone
   17 g European agar
   2.9 g NaCl
   Add H_2O to 1 L
   Sterilize by autoclaving.
   After solution cools down, add 1 ml autoclaved/sterile 1 M $MgSO_4$, 1 ml autoclaved/sterile, 1 M $CaCl_2$, 25 ml autoclaved/sterile 1 M KPO_4, 1 ml cholesterol 5 mg/ml dissolved in 100% ethanol.
3. PGS agar medium (1 L)
   12 g peptone
   12 g glucose
   27.25 g sorbitol
   17 g European agar
   2.9 g NaCl
   Add H_2O to 1 L
   Sterilize by autoclaving.
   After solution cools down, add 1 ml autoclaved/sterile 1 M $MgSO_4$, 1 ml autoclaved/sterile, 1 M $CaCl_2$, 25 ml autoclaved/sterile 1 M KPO_4, 1 ml cholesterol 5 mg/ml dissolved in 100% ethanol.
4. M9 buffer (1 L)
   3 g KH_2PO_4
   6 g Na_2HPO_4
   5 g NaCl
   Add H_2O to 1 L
   Sterilize by autoclaving.
   After solution cools down, add 1 ml autoclaved/sterile 1 M $MgSO_4$.

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