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Fluorescent in vitro model to assess invasion and intracellular maturation of Bd in A6 cells (Plos One)

PLOS One

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ABSTRACT

The largest current disease-induced loss of vertebrate biodiversity is due to chytridiomycosis and despite the increasing understanding of the pathogenesis, knowledge unravelling the early host-pathogen interactions remains limited. *Batrachochytrium dendrobatidis* (Bd) zoospores attach to and invade the amphibian epidermis, with subsequent invasive growth in the host skin. Availability of an *in vitro* assay would facilitate in depth study of this interaction while reducing the number of experimental animals needed. We describe a fluorescent cell-based *in vitro* infection model that reproduces host-Bd interactions. Using primary keratinocytes from *Litoria caerulea* and the epithelial cell line A6 from *Xenopus laevis*, we reproduced different stages of host cell infection and intracellular growth of Bd, resulting in host cell death, a key event in chytridiomycosis. The presented *in vitro* models may facilitate future mechanistic studies of host susceptibility and pathogen virulence.

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MATERIALS

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MATERIALS

Distilled Water Contributed by users

Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 Thermo Fisher Scientific Catalog #A11011

HBSS with calcium and magnesium Contributed by users

HBSS without calcium and magnesium Contributed by users

Triton X-100 Sigma Catalog #93426

Fetal bovine serum Contributed by users

Paraformaldehyde Sigma Aldrich Catalog #P6148

Leibovitz's L-15 Medium Thermo Fisher Catalog #11415049

CellTracker™ Green CMFDA Dye Thermo Fisher Catalog #C7025

ProLong™ Glass Antifade Mountant Thermo Fisher Catalog #P36980

Rat Tail Collagen Coating Solution Sigma Aldrich Catalog #122-20

Calcofluor White stain Sigma Aldrich Catalog #18909-100ML-F

polyclonal antibody against Bd raised in rabbit Contributed by users

24-well tissue culture plates

glass coverslips

1 Prepare Cell Medium A:

L15 medium: 70%

Distilled water: 20%

Fetal bovine serum 10%

2 Prepare Cell Medium B:

L15 medium: 40%

Distilled water: 55%

Fetal bovine serum: 5%

3 Coat coverslips with Rat tail collagen:

Add glass coverslips in a 24-well tissue culture plate. Coat the glass coverslips at 37°C for 2 hours. Therefore, carefully aspirate the Collagen Coating Solution and add 200 µl per well (so per coverslip). After 2 hours, rinse the coated surface twice with HBSS-. Coated tissue culture ware may be used immediately or air-dried and stored at 4 °C for up to one week.

4 Seeding A6 cells:

- Detach A6 cells from a cell culture flask using trypsin
- Centrifuge for 5 min at 1500 rpm
- Wash the cells with 70% HBSS- and in meantime count the cells
- Centrifuge for 5 min at 1500 rpm
- Resuspend the cells in cell medium A to reach a concentration of 10e5 cells per mL
- Seed the cells at a concentration of 10e5 cells per well (so add 1 mL) which contains a collagen-coated glass coverslip
- Let the A6 cells attach for 2 hours at 26°C and 5% CO₂
- After 2 hours, wash the cells with 70% HBSS+
- The cells are now ready to be exposed to *Bd* spores

IMPORTANT NOTE: Include a control well that can be used to check the cells throughout the entire protocol via light microscopy.

5 *Bd* infection of A6 cells:

- Isolate *Bd* zoospores and spin them down for 5 min at 3000 rpm (20°C)
- Remove the supernatant, resuspend them in cell medium B and count the spores
- Dilute the spores to a concentration of 10e6 spores/mL in cell medium B
- Add 1 mL of the spores suspension (= 10e6 spores) to the wells containing A6 cells (on a coverslip). As such the spores are seeded at a MOI of 10:1.
- Incubate for 2 hours at 20°C 5%CO₂.
- After 2 hours gently wash the infected cells three times with 70% HBSS+ to remove non-adherent spores
- Replace the cell medium B with cell medium A for X days (depending on the experimental setup) at 20°C, 5% CO₂

IMPORTANT NOTE: *Bd* zoospores lose their motility when exposed to cell medium A.

IMPORTANT NOTE: Include a control well that can be used to check the motility of the spores throughout the entire protocol via light microscopy.

6 Staining of *Bd*-infected A6 cells: (Work in the dark)

- Wash the infected cells 3 times with 70% HBSS+
- Stain the infected cells with 3 µM celltracker green CMFDA in cell medium A for 45 min at 20°C, 5% CO₂

7 Staining of extracellular *Bd*: (Work in the dark)

- Remove the green celltracker and wash with 70% HBSS+
- Add 200 µL CalcoFluor White (10 µg/mL in cell medium A) for 10 min

- Wash 3 times with HBSS+

8 Fixation and staining of *Bd*: (Work in the dark)

- Fix the infected cells with 0.5 mL of 3% paraformaldehyde for 10 min
- Wash 2 times with 70% HBSS+
- Permeabilise with 200 µL of 0.1% triton in 70% HBSS+ on room temperature during 2 min.
- Wash 2 times with 70% HBSS+
- Incubate 1 hour (up to 1.30 hour) with a primary antibody (1/1000):
 - * Anti chytrid antibody produced in rabbit -> Thomas et al. 2018

Thomas V, Blooi M, Van Rooij P, Van Praet S, Verbrugghe E, Grasselli E, et al. Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. Transbound. Emerg. Dis. 2018; 65: e478-e488.

- Wash 3 times with 70% HBSS+
- Incubate for 1 hour with a secondary antibody (1/500):
 - * Alexa Fluor 568 goat anti-rabbit IgG
- Wash 3 times with 70% HBSS+
- Mount the coverslips using ProLongGold antifade mountant
- Use fluorescence microscopy to analyse *Bd*-A6 cell interactions