NOV 15, 2019

Fluorescent in vitro model to assess adhesion and invasion of Bd in PAK

PLOS One

Elin Verbrugghe¹

¹Ghent University, Faculty of Veterinary Medicine, Wildlife Health Ghent

Elin Verbrugghe

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.

OPEN ACCESS

DOI:
[dx.doi.org/10.17504/protocols.io.8ihhub6](https://doi.org/10.17504/protocols.io.8ihhub6)

External link:
<https://doi.org/10.1371/journal.pone.0225224>

Protocol Citation: Elin Verbrugghe 2019. Fluorescent in vitro model to assess adhesion and invasion of Bd in PAK . **protocols.io** <https://dx.doi.org/10.17504/protocols.io.8ihhub6>

MANUSCRIPT CITATION:
 Verbrugghe E, Rooij PV, Favoreel H, Martel A, Pasmans F (2019) *In vitro* modeling of *Batrachochytrium dendrobatidis* infection of the amphibian skin. PLoS ONE 14(11): e0225224. doi: [10.1371/journal.pone.0225224](https://doi.org/10.1371/journal.pone.0225224)

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

Created: Oct 21, 2019

MATERIALS

Last Modified: Nov 15, 2019

MATERIALS

PROTOCOL integer ID:
28969

Keywords: Primary
amphibian keratinocytes,
Batrachochytrium
dendrobatidis, chytrid, in vitro

☒ Distilled Water **Contributed by users**

☒ HBSS with calcium and magnesium **Contributed by users**

☒ HBSS without calcium and magnesium **Contributed by users**

☒ Fetal bovine serum **Contributed by users**

☒ Paraformaldehyde **Sigma Aldrich Catalog #P6148**

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

☒ CellTracker™ Red CMTPIX Dye **Thermo Fisher Catalog #C34552**

☒ CellTracker™ Green CMFDA Dye **Thermo Fisher Catalog #C7025**

☒ Hoechst 33342, Trihydrochloride, Trihydrate, 100 mg **Thermo Fisher Catalog #H1399**

☒ ProLong™ Glass Antifade Mountant **Thermo Fisher Catalog #P36980**

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

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 **Staining of Primary Keratinocytes (PAK): (Work in the dark)**

- Add 1 ml of 3 µM Celltracker green CMFDA in cell medium A to the PAK

- Resuspend carefully

- Incubate for 30 min at 20°C 5% CO₂

- Centrifuge for 5 min at 1500 rpm and 20°C

- Resuspend the pellet in 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 PAK attach for 1 hour at 20°C and 5% CO₂
- After 1 hour, 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 **Staining of *Bd* zoospores: (Work in the dark)**

- Isolate *Bd* zoospores and spin them down for 5 min at 3000 rpm (20°C)
- Remove the supernatant and resuspend them in 3 µM celltracker red CMPTX solution for 45 min at 20°C. Important here is that the 3 µM celltracker solution should be diluted in CELL MEDIUM B in order to ensure the motility of the zoospores.
- After 45 min, spin the spores down for 3 min at 3000 rpm
- Resuspend them in cell medium B and count the spores
- Dilute the spores to a concentration of 10e6 spores/mL in cell medium B

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 ***Bd* infection of PAK cells: (Work in the dark)**

- Add 1 mL of the spore suspension (= 10e6 spores) to the wells containing celltracker-labelled PAK 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 cell medium B with cell medium A for another 2 hours to assess ADHESION or 22 hours to assess invasion

IMPORTANT: Use the control well to check the adhesion/invasion of the spores to/in the cells via light microscopy.

7 **Visualisation of early *Bd*-PAK interactions: (Work in the dark)**

ADHESION: 4 hours post infection (2 hours with cell medium B and 2 hours with cell medium A)

INVASION: 24 hours post infection (2 hours with cell medium B and 22 hours with cell medium A)

- Wash the infected cells 3 times with 70% HBSS+
- Fix the infected cells with 0.5 mL of 3% paraformaldehyde for 10 min
- Stain the nuclear content with Hoechst
- Wash 2 times with 70% HBSS+
- Mount the coverslips using ProLong Gold antifade mountant
- Use fluorescence microscopy and confocal laser scanning microscopy to analyse *Bd*-PAK

interactions