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Isolation of Chlamydia from crocodile tissue samples

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ABSTRACT

This protocol is used for isolation of Chlamydia from crocodiles

1 Dissect tissue sample (approximate 50-100 mg) into small pieces.

- 2 Homogenize the small pieces of tissue sample with a pestle in 5 ml sucrose/phosphate/glutamate (SPG) buffer containing 500 ug/ml streptomycin, 500 ug/ml vancomycin, 50 ug/ml gentamycin, and 2.5 ug/ml fungizone and left at 4°C for 72 h.
- 3 Centrifuge the homogenized tissue sample at 250×g for 10 min and collect supernatant for cell culture inoculation.
- 4 Prepare monolayers of McCoy cells in 12-well plates by seeding 3.5×10^5 cell per well in cell culture medium (M199 medium (Gibco BRL Life Technologies Inc., NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies Inc., NY, USA), 0.2% NaHCO₃, 1× L-glutamine, 100 µg/ml streptomycin).
- 5 Inoculate 400 µl of supernatant into each well of monolayers cells.
- 6 Centrifuge 12-well plate containing infected cells at 1,000×g and 25°C for 1 h.
- 7 After centrifugation, incubate the infected cultures at 30°C in an atmosphere of 5% CO₂ for an additional 1 h.
- 8 Replace the inocula with fresh incubation medium (M199 medium (Gibco BRL Life Technologies Inc., NY, USA) containing 0.5 µg/ml cycloheximide and supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies Inc., NY, USA), 0.2% NaHCO₃, 10% glucose, 1× L-glutamine, 100 µg/ml streptomycin, 100 µg/ml vancomycin, 10 µg/ml gentamycin, and 1 µg/ml fungizone).
- 9 Further incubate the infected cultures at 30°C in an atmosphere of 5% CO₂ for 4–5 days.

- 10 Observe daily for the cytopathic effect (CPE) under an inverted microscope.

Chlamydia propagation

- 11
- 12 Prepare monolayers of McCoy cells in 12-well plates by seeding 3.5×10^5 cell per well in cell culture medium (M199 medium (Gibco BRL Life Technologies Inc., NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies Inc., NY, USA), 0.2% NaHCO_3 , 1× L-glutamine, 100 µg/ml streptomycin).
- 13 Thaw frozen stock of Chlamydia culture and place on ice.
- 14 Dilute culture stock to 1:10 in cell culture medium.
- 15 Inoculate 200 µl of diluted culture stock into each well of monolayers cells and add 200 µl of cell culture medium.
- 16 Centrifuge 12-well plate containing infected cells at 1,000×g and 25°C for 1 h.
- 17 After centrifugation, incubate the infected cultures at 30°C in an atmosphere of 5% CO_2 for an additional 1 h.

- 18 Replace the inocula with fresh incubation medium (M199 medium (Gibco BRL Life Technologies Inc., NY, USA) containing 0.5 µg/ml cycloheximide and supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies Inc., NY, USA), 0.2% NaHCO₃, 10% glucose, 1× L-glutamine, 100 µg/ml streptomycin, 100 µg/ml vancomycin, 10 µg/ml gentamycin, and 1 µg/ml fungizone).
- 19 Further incubate the infected cultures at 30°C in an atmosphere of 5% CO₂ for 4–5 days.
- 20 Observe daily for the CPE under an inverted microscope.

IFA

- 21
- 22 Prepare monolayer McCoy cells on glass coverslip (Ø 15 mm) in 12-well plates by seeding 3.5×10⁵ cell per well in cell culture medium (M199 medium (Gibco BRL Life Technologies Inc., NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies Inc., NY, USA), 0.2% NaHCO₃, 1× L-glutamine, 100 µg/ml streptomycin).
- 23 Thaw frozen stock of Chlamydia culture and place on ice.
- 24 Dilute culture stock to 1:20 in cell culture medium.
- 25 Inoculate 400 µl of diluted culture stock into each well of monolayers cells.

- 26 Centrifuge 12-well plate containing infected cells at 1,000×g and 25°C for 1 h.
- 27 After centrifugation, incubate the infected cultures at 30°C in an atmosphere of 5% CO₂ for an additional 1 h.
- 28 Replace the inocula with fresh incubation medium (M199 medium (Gibco BRL Life Technologies Inc., NY, USA) containing 0.5 µg/ml cycloheximide and supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL Life Technologies Inc., NY, USA), 0.2% NaHCO₃, 10% glucose, 1× L-glutamine, 100 µg/ml streptomycin, 100 µg/ml vancomycin, 10 µg/ml gentamycin, and 1 µg/ml fungizone).
- 29 Further incubate the infected cultures at 30°C in an atmosphere of 5% CO₂ for 3-4 days.
- 30 Observe daily for the CPE under an inverted microscope.
- 31 When CPE in cell culture occur approximately 10%, fix the infected cells with 1 ml of 4% paraformaldehyde for 24 h.
- 32 Remove 4% paraformaldehyde and wash fixed samples 3 times with 1 ml of 1× phosphate-buffered saline (PBS).
- 33 Add 1 ml of 0.2% Triton X-100 in PBS and incubate at 37°C for 30 min for permeabilization.

- 34** Wash fixed samples 3 times with 1 ml of 1×PBS.
- 35** Add 1 ml of 1% BSA and incubate at 37°C for 30 min for blocking of non-specific binding.
- 36** Transfer the coverslip to a humidity chamber and add 30 µl of 1:100 diluted primary antibody (Chlamydiaceae family-specific mouse monoclonal antibody directed against the chlamydial lipopolysaccharide (cat. no. sc-58106, Santa Cruz Biotechnology, Inc., TX, USA). Incubate the humidity chamber at 37°C for 1 h in the dark.
- 37** Transfer the coverslip to a 12-well plate and wash coverslip 3 times with 1 ml of 1×PBS.
- 38** Transfer the coverslip to the humidity chamber and add 30 µl of 1:200 diluted secondary antibody (Alexa Fluor 594-conjugated secondary goat anti-mouse antibody (cat. no. A11005, Life Technologies, CA, USA) and 2 µg/ml 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (Molecular Probes, OR, USA). Incubate the humidity chamber at 37°C for 1 h in the dark.
- 39** Transfer the coverslip to a 12-well plate and wash coverslip 3 times with 1 ml of 1×PBS.
- 40** Mount the coverslip onto microscope glass slides using 50% glycerol.
- 41** Seal the mounted coverslip with nail polish.

42 Visualize the image under an inverted fluorescence microscope.