May 28, 2021

Isolation of Chlamydia from crocodile tissue samples

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 dx.doi.org/10.17504/protocols.io.btcqnivw

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ABSTRACT

This protocol is used for isolation of Chlamydia from crocodiles

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0252081

DOI

dx.doi.org/10.17504/protocols.io.btcqnivw

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0252081

PROTOCOL CITATION

Somjit Chaiwattanarungruengpaisan, Metawee Thongdee, ladawan.sar 2021. Isolation of Chlamydia from crocodile tissue samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.btcqnivw

CREATED

Mar 16, 2021

LAST MODIFIED

May 28, 2021

PROTOCOL INTEGER ID

48240

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 3.5×105 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).

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

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12 Prepare monolayers of McCoy cells 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).

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.

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17 After centrifugation, incubate the infected cultures at 30°C in an atmosphere of 5% CO2 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 (∅ 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).

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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.

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42 Visualize the image under an inverted fluorescence microscope.

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