Title: Obtaining Highly Purified *Toxoplasma gondii* Oocysts by a Discontinuous Cesium Chloride Gradient

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Key words: *Toxoplasma gondii*, cesium chloride, oocysts, discontinuous gradient, apicomplexan

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Abstract

Toxoplasma gondii is an obligate intracellular protozoan pathogen that commonly infects humans. It is a well characterized apicomplexan associated with causing food- and water-borne disease outbreaks. The definitive host is the feline species where sexual replication occurs resulting in the development of the highly infectious and environmentally resistant oocyst. Infection occurs via ingestion of tissue cysts from contaminated meat or oocysts from soil or water. Infection is typically asymptomatic in healthy individuals, but results in a life-long latent infection that can reactivate causing toxoplasmic encephalitis and death if the individual becomes immunocompromised. Meat contaminated with *T. gondii* cysts have been the primary source of infection in Europe and the United States, but recent changes in animal management and husbandry practices and improved food handling and processing procedures have significantly reduced the prevalence of *T. gondii* cysts in meat[1, 2]. Nonetheless, seroprevalence in humans remains relatively high suggesting that exposure from oocyst contaminated soil or water is likely. Indeed, waterborne outbreaks of toxoplasmosis have been reported worldwide supporting the theory exposure to the environmental oocyst form poses a significant health risk[3-5]. To date, research on understanding the prevalence of *T. gondii* oocysts in the water and environment are limited due to the lack of tools to detect oocysts in the environment [5, 6]. This is primarily due to the lack of efficient purification protocols for obtaining large numbers of highly purified *T gondii* oocysts from infected cats for research purposes. This study describes the development of a modified CsCl method that easily purifies *T. gondii* oocysts from feces of infected cats that are suitable for molecular biological and tissue culture manipulation[7].

Procedure:

1. General safety precautions when working with *T. gondii* oocysts

1.1 It is important to follow all safety precautions when working with *T. gondii* oocysts. In most healthy individuals, *T. gondii* infection is readily controlled by the immune system; however, a life-long infection results. Immunocompromised individuals are particularly susceptible to toxoplasmosis and should not handle *T. gondii* oocysts. Pregnant women should also not handle *T. gondii* oocysts, because infection can cause severe birth defects. For more details, see reference 8.

1.2 *T. gondii* oocysts should only be handled in a designated area and with trained personnel. Signs indicating *T. gondii* oocyst work is in progress must be posted to alert others entering the designated area.

1.3 Wear appropriate personal protective equipment (PPE) such as a lab coat, disposable gown, disposable gloves, and proper eye protection or a face shield when handling *T. gondii* oocysts.

1.4 Frequent glove changes are recommended. Do not handle any lab equipment with *T. gondii* oocyst contaminated gloves.

1.5 Always use metal autoclavable trays lined with a disposable absorbent liners when working with *T. gondii* oocysts. Ensure all racks, tubes, etc. used are either disposable or autoclavable.

1.6 All *T. gondii* waste must be autoclaved twice for at least one hour.

1.7 All non-disposable equipment (racks, trays, etc.) must also be autoclaved twice for at least one hour.

1.8 Vacuum lines used to aspirate liquids should be connected to a Vacushield™ filter to prevent contamination of the vacuum pump.

1.9 All affected laboratory bench-tops must be disinfected after completing work with *T. gondii* oocysts. Freshly made 10% hypochlorite should be liberally applied to the work area and allowed dry. The area must then be rinsed well with water.

2. Preparation of buffers and solutions

2.1 Prepare a 1 L 2.2 M solution of sucrose by dissolving 752.66 g of sucrose in 600 ml ddH_2O. Stir and heat gently using a heated stir plate to dissolve the sucrose. Once completely dissolved, bring volume to 1 L with ddH_2O. This should be followed by sterilization by autoclaving the solution for at least 20 minutes.

2.2 Prepare a 1L TE buffer (50 mM Tris-HCl, 10 mM EDTA), pH 7.2 by adding 6.05 g of Tris-HCl and 3.7 g of EDTA in 700 ml of ddH_2O, adjust pH to 7.2 then bring volume to 1 L with ddH_2O.

2.3 For the CsCl gradient, prepare a stock solution of CsCl with a specific gravity of 1.15 (1.15-CsCl) by adding 21.75 g of CsCl with 103.25 ml of TE Buffer. For Solution A, mix 30 ml of TE with 20 ml of 1.15-CsCl. For Solution B, mix 20 ml of TE with 30 ml of 1.15-CsCl and 12.5 µl of phenol red solution. For Solution C, mix 10 ml of TE with 40 ml of 1.15-CsCl (Table 1).

2.4 Prepare a 1L 1 N solution of sodium hydroxide (NaOH) by dissolving 40 g of NaOH, in 800 ml of ddH_2O. Once dissolved, bring volume to 1 L with ddH_2O.

2.5 Prepare a 1 L 2% (by volume) solution of H_2SO_4 mixing 20 ml of H_2SO_4 with 980 ml of ddH_2O.

3. Sucrose float

3.1 Add 10 ml of a fecal suspension of *T. gondii* oocysts, in 2% H_2SO_4, into a 50 ml conical centrifuge tube. It must be noted that fecal suspension refers to samples that have been pre-processed through a sucrose flotation procedure as previously described[8]. When the fecal samples are initially harvested from the infected cats they are processed through a sucrose float as described in reference 8. This additional sucrose float is necessary to further minimize fecal debris carried over to the CsCl purification process and obtain the purest *T. gondii* oocysts possible.

3.2 Neutralize the 2% H_2SO_4 by adding 6 ml (3/5 volumes) of 1 N NaOH to the fecal suspension. Mix well by vortexing.

3.3 Add an equal volume (16 ml) of 2.2 M sucrose creating a final concentration of 1.1 M to the fecal suspension and mix well by vortexing.

3.4 Carefully overlay the sucrose/fecal suspension with 10 ml ddH_2O using a 10 ml pipette. Centrifuge the suspension at 1,200 x g for 20 min at room temperature with no brake.

3.5 Carefully collect the top water and interphase layers and transfer to a new 50 ml conical centrifuge tube by pipetting from the air-water interface without swirling the pipette. It is important to minimize sucrose carryover while collecting the interphase layer.

3.6 Mix the remaining sucrose/fecal pellet solution by vortexing the tube.

3.7 Repeat steps 3.4 and 3.5.

3.8 Bring the volume of the two oocyst interphase solutions to 50 ml with ddH_20 and centrifuge the tubes at 2,000 x g for 10 minutes at room temperature.

3.9 Aspirate the supernatant from each tube and resuspend pellets with 5 ml TE buffer and pool the oocyst suspension together. The total pooled volume should be 10 ml.

4. CsCl gradient

4.1 Prepare a discontinuous CsCl gradient in a 50 ml polycarbonate Oak Ridge tube by carefully underlaying each layer using a 50 ml syringe with an 18 gauge blunt-ended, autoclavable, steel needle, and a 2 way stop cock. Note: Phenol red is added to Solution B to easily distinguish between the gradient layers (Table 1).

4.2 Slowly add the following solutions to the tube in the order listed below. It should be noted that the flow rate should not exceed more than 0.5 ml/sec.

1. 10 ml of the TE/oocyst suspension sample
2. 8 ml Solution A
3. 8 ml Solution B
4. 8 ml Solution C

4.3 Centrifuge the Oak Ridge tube at 12,000 x g for 60 min at 4 ^{o}C with no brake. Use of a fixed angle rotor is acceptable, but a high speed swinging bucket results in better separation of the oocysts from the suspension sample with minimal fecal debris smears along the side or the tube. The extent of the smears depends on the

composition of the fecal suspension and thus it may be necessary to perform two CsCl gradients if the fecal suspension is extremely “dirty.”

4.4 Following centrifugation, collect the opaque/white oocyst containing band between solutions A and B. To minimize contamination with fecal debris, go directly to the oocyst band without disturbing the gradient and collect the oocyst interphase using a 10 ml pipette. Transfer the oocyst interphase to a new 50 ml conical centrifuge tube. Try to minimize the amount of CsCl solution aspirated while collecting the oocyst interphase as it may pose a problem in pelleting the oocysts during the wash step.

4.5 Wash oocysts with 30-40 ml of ddH_20. Centrifuge the tube at 2,000 x g at room temperature for 10 min with no brake. Carefully aspirate the supernatant without disturbing the oocyst pellet. Repeat the wash 2 additional times.

4.6 At the end of the final wash, carefully aspirate the supernatant and resuspend the pellet in 10 ml 2% sulfuric acid and store at 4 °C until use. Oocysts are now ready for further manipulation. Oocyst purity can be checked microscopically by the absence of fecal debris in the sample.

Acknowledgments:

We would like to thank Nichole Brinkman and Michael Ware for technical review of the manuscript and Dr. Abu Sayed and Linda Ransick for technical and administrative support. The United States Environmental Protection Agency through its Office of Research and Development partially funded and collaborated in the research described here under contract number EP-D-06-096 to Dynamac, Inc. and an interagency agreement number DW-12-92289801-0 to USDA. It has been subjected to Agency review and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. MJS current address: Dynamac, Inc. Cincinnati, OH.

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185 Table 1: Cesium chloride solution

Solution	Specific gravity	TE (ml)	1.15-CsCl (ml)
A	1.05	30	20
B*	1.11	20	30
C	1.125	10	40

186 *12.5 µl of phenol red is added to solution B

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Reagent	Vendor	Catalog #
1 way Luer Lok stopcock	Promega	A7261
10 ml pipettes	Fisher	13-618-11-E
18 gauge blunt-ended needle	Fisher	14-825-16H
50 ml conical centrifuge tubes	Fisher	05-539-13
50ml conical tube adaptors	Fisher	05-375-51
50 ml Luer Lok Syringe	BD	309663
Centrifuge	IEC	IEC6466K
Cesium Chloride	Sigma	C4036
EDTA	Sigma	E5134
Fixed angle high speed rotor	IEC	IEC7685C
Oak Ridge tube adaptors	Fisher	04-974-011A
Oak Ridge tubes, 50 ml	Nalgene	05-529C
Phenol Red 2000x solution	Sigma	P8421
Protective disposable gown	Fisher	18-567
Sodium Hydroxide	Sigma	S8045
Sucrose	Sigma	S8501
Sulfuric Acid	Sigma	339741
Swinging bucket rotor	IEC	IEC6555C
Tris-HCl	Sigma	T6066
VacuShield Filter	Gelman	629-4402
Versidry Bench Protector Pads	Fisher	14-206-37

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