Laboratory Procedure Manual

Analyte: ***Cryptosporidium parvum* Surface Antigens**

Matrix: **Serum**

Method: **Enzyme-Linked Immunosorbent Assays (ELISAs)**

Method No.:

Revised:

as performed by: *Parasitic Diseases Branch*
Division of Parasitic Diseases
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Important Information for Users

Centers for Disease Control periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 1999–2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
Lab17	LBDC1	Cryptosporidium (17 kDA)
	LBDC2	Cryptosporidium (27 kDA)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

IgG antibodies to two immunodominant *Cryptosporidium parvum* surface antigens are measured using experimental Enzyme-Linked Immunosorbent Assays (ELISAs) in an effort to gauge the level of exposure of the general population to this emerging parasitic disease. Purified recombinant antigens are immobilized on a plastic matrix in a 96-well plate format and the antibodies present in diluted human serum are bound to the antigen. Levels of IgG antibodies are then determined using a labeled anti-human IgG secondary antibody. Antibody levels are reported in an arbitrary unit value based upon a four parameter curve generated from a serial dilution of a strong positive control serum that is included on each ELISA plate. This assay measures only the level of antibodies present in a given serum sample and is not suitable for diagnosis of infection.

2. SAFETY PRECAUTIONS

This method is performed in a laminar-flow hood. Observe Universal Precautions. Wear gloves, lab coat, and safety glasses at all times. Treat all specimens as potentially positive for HIV and Hepatitis B. All leftover blood specimens and materials that have been in contact with blood must be autoclaved before disposal.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

A. Optical density (405 nm) readings are converted to arbitrary unit values using the Softmax PRO version 3.0 software (Molecular Devices Corp.). A four parameter curve is fitted to the two-fold serial dilution of the standard serum serial dilution (1:50 to 1:6400), and values are assigned based upon the curve from each individual plate run.

   After the data are calculated and the final values are approved for release by the reviewing supervisor, the results are transcribed by the data entry clerk into the NHANES IV database format which is located on the NCID/DPD PC network; data entry is proofed by the supervisor and clerk. After each NHANES stand is completed the supervisor will electronically transmit the anti-*C. parvum* IgG antibody values to Westat.

B. Files stored on the network are backed up nightly to tape by NCID/DPD LAN support staff and CDC.

C. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for archival of data.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

A. No special dietary instructions are given to donors.

B. Specimens for anti-*C. parvum* IgG antibody analysis should be fresh or frozen plasma or serum.

C. The optimal amount of sample is 250 µL; the minimum is about 20 µL.

D. Specimens may be stored in glass or in plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

E. IgG antibodies are stable for years at –20°C and below. Quality control pools for the HANES Lab are normally stored at –70°C for maximum stability. Several freeze-thaw cycles appear to have minimal effect on the specimen.

F. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was drawn.

G. Samples that are extensively hemolyzed may not give accurate test results.

H. In general, plasma or serum specimens should be transported and stored at no more than 4–8°C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at ≤ –20°C. Samples thawed and refrozen several times are not compromised. If the specimen needs to be divided because it contains more than one analyte of interest, transfer the appropriate amount of serum or plasma into a sterile Nalgene cryovial labelled with the participant's ID. There are no special timing requirements, handling requirements, or equipment requirements.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Instrumentation

(1) Molecular Devices VERSAmax microplate reader with Softmax PRO version 3.0 software (Sunnyvale, CA)

(2) Vortex mixer (Fisher Scientific Co., Fairlawn, NJ).

(3) Three Gilson Pipettemen, (Rainin Corp., Woburn, MA):

(a) A P-1000 200–1000µl pipetteman.

(b) A P-200 20–200 µl pipetteman.

(c) A P-20 2–20 µl pipetteman.

(4) One variable 5–50 µl 12-channel pipette (Labsystems Finnpipette, Fisher Scientific, Atlanta, GA.)

(5) Mettler model PM400 balance (Mettler Instruments Corp., Hightstown, NJ).

(6) Corning model 345 pH meter (Fisher Scientific).

B. Materials

(1) Deionized water, ≥ 1.0 megaOhm-cm at 25°C (Hydro Water Co., Atlanta, GA).

(2) Immulon 2 High Binding flat bottom microtiter plates, 96 well (Dynex technologies, Chantilly, VA).

(3) Biotinylated mouse anti-human IgG clone HP6017 (Zymed Laboratories, S. San Francisco, Cµ).

(4) Strepavidin-labeled alkaline phosphatase (Life Technologies, Gaitherburg, MD).

(5) *p*-Nitrophenylphosphate tablets, 5 mg (Sigma Chemical Co., St. Louis, MO).

(6) Diethanolamine ACS reagent (DEA) (99.9%) (Sigma Chemical Co.).

(7) Phosphate-buffered saline (PBS) (10 mM Na_2HPO_4 at pH 7.2 with 0.85% NaCl) (Life Technologies)

(8) Polyoxyethylenesorbitan Monolaurate (Tween-20) (Sigma Chemical Co.).

(9) Magnesium chloride, hexahydrate (Sigma Chemical Co.).

(10) Hydrochloric acid (HCl), concentrated, "Baker Analyzed" (J.T. Baker Co.). (36.5 to 38.0%).

(11) Sodium bicarbonate anhydrous ACS reagent (Sigma Chemical Co.).

(12) Recombinant antigens are produced by the methods of Priest et al. 1999 and 2000.

(a) The following two deoxyoligonucleotides were designed for the directional cloning of the C. parvum 27-kDa antigen (GenBank accession number U34390) into the BamHI and EcoRI restriction enzyme sites of the pGEX 4T-2 expression vector (Pharmacia Biotech, Uppsala, Sweden): Cp23 5'-primer (5'-CGC GGA TCC ATG GGT TGT TCA TCA TCA AAG-3') and Cp23 3'-primer (5'-GCG GAA TTC ATT AGG CAT CAG CTG GCT TG-3'). The 27-kDa antigen-coding sequence was amplified from 260 ng of genomic DNA by using 100 µM

concentrations of Cp23-5' and Cp23-3' and AmpliTaq DNA polymerase as directed by the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.). The following amplification protocol was used: 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min followed by 1 cycle of 72°C for 15 min.

(b) A PCR product was generated for the 17-kDa antigen using an identical protocol and the following two deoxyoligonucletides: Cp17 5'-primer (5'-CGC GGA TCC GAA ACC AGT GAA GCT GCT GC-3') and Cp17 3'-primer (5'-GCG GAA TTC TTA ATC CTT CAA AAG AAC TGT G-3')

(c) Plasmid-containing insert was transformed into *Escherichia coli* strain HB101 cells (Life Technologies, Frederick, Md.). The sequence of the resulting clone was confirmed by automated DNA sequencing.

(d) Recombinant *C. parvum* antigens/*Schistosoma japonicum* glutathione-S-transferase (GST) fusion proteins were purified from isopropyl-*b*-D-thiogalactopyranoside (IPTG) induced cell cultures using glutathione Sepharose 4B as directed by the manufacturer (GST Bulk Purification Module, Pharmacia Biotech).

(e) The *C. parvum* proteins with an additional GlySer dipeptide at the amino terminus were released by overnight cleavage with thrombin at room temperature and then separated from uncleaved fusion protein and the GST cleavage product by passage over glutathione Sepharose 4B resin.

(f) Protein purity was monitored by both SDS polyacrylamide gel electrophoresis and Western blotting with a monoclonal antibody against the native 27-kDa antigen (C6B6), native 17-kDa antigen (C6C1), and with serum samples from infected humans.

C. Reagent Preparation

(1) 0.05% Tween-20 PBS: dissolve 0.5 mL of Tween-20 in 1.0 L of PBS.

(2) 0.3% Tween-20 PBS: dissolve 3.0 mL of Tween-20 in 1.0 L of PBS.

(3) M $MgCl_2$: dissolve 203.3 g of $MgCl_2 \bullet 6H_2O$ in 1.0 L of deionized water.

(4) 10% DEA/3 mM $MgCl_2$ solution. Dissolve 50.0 mL of DEA in 448.5 mL of deionized water. pH solution to 9.8 concentrated HCl. Filter sterilize (0.2 µm). Add 1.5 mL of 1.0 M $MgCl_2$. Store at room temperature.

(5) M Sodium bicarbonate. Dissolve 10.6 g of sodium bicarbonate in 1.0 L of deionized water. pH to 9.6 with concentrated HCl and filter sterilize (0.2 µm). Store at 4°C.

D. Antigen solutions:

(1) Cp23: Dilute 900 ng of recombinant protein in 5.0 mL of 0.1 M sodium bicarbonate solution at pH 9.6 and vortex.

(2) Cp17: Dilute 1500 ng of recombinant protein in 5.0 mL of 0.1 M sodium bicarbonate solution at pH 9.6 and vortex.

(3) Secondary antibody: Dilute 5µL of biotinylated mouse anti-human IgG antibody into 50. mL of 0.05% Tween-20 PBS and vortex briefly.

(4) Alkaline phosphatase: Dilute 10 µL of streptavidin-labeled alkaline phosphatase into 5.0 mL of 0.05% Tween-20 PBS and vortex briefly.

(5) Substrate: Dissolve 1 tablet of *p*-nitrophenylphosphate in 5.0 mL of 10% DEA with 3.0 mM $MgCl_2$ at pH 9.8 and vortex until completely dissolved.

E. Standards Preparation

(1) Control sera are diluted 1:50 using 0.05% Tween-20 PBS. Store at 4°C for up to 1 month.

(2) Standard curve is generated by 1:2 serial dilutions of a strong positive control serum in 0.05% Tween-20 PBS. The following dilutions are used: 1:50; 1:100; 1:200; 1:400; 1:800; 1:1600;

1:3200; and 1: 6400. Store at 4°C for up to 1 month.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Working Standards

Not applicable for this procedure.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

Samples and control sera are allowed to thaw overnight at 4°C. 96-Well plates are coated with antigen in bicarbonate buffer (50 µL per well) overnight at 4°C.

B. Sample Preparation

Aliquots of each sample (10 µL) are diluted to 1:50 with the addition of 500 µL of 0.05% Tween-20 PBS, vortexed briefly, and stored at 4°C until assayed.

C. Operation

(1) The plates were washed in 0.05% Tween-20 PBS and blocked with 100 µL per well of 0.3% Tween-20 PBS for 1 h at 4°C.

(2) After a series of three washes (subsequent washes were all with 0.05% Tween-20 PBS), 50 µL aliquots of serum diluted 1:50 with wash buffer were added to each well. All serum samples were tested in duplicate. A two-fold serial dilution (1:50 to 1:6400) of a strong positive control was used to generate a standard curve on each individual plate. Two buffer blanks, three confirmed positive sera, and a battery of four serum samples known by Western blot assay to be negative for C. parvum antibodies were also included on each plate.

(3) Plates were incubated 2 h at room temperature, and then washed four times with wash buffer.

(4) A biotinylated mouse monoclonal antibody against human IgG (clone HP6017, Zymed Laboratories, 50 µl of a 1:1000 dilution in wash buffer) was added to each well and incubated for 1 h at room temperature.

(5) Following four washes, the wells were filled with alkaline phosphatase-labeled streptavidin (Life Technologies, 50 µl of a 1:500 dilution in wash buffer) and incubated an additional hour at room temperature.

(6) After four washes (the final wash for 10 min at room temperature), p-nitrophenylphosphate substrate was added in 3 mM $MgCl_2$ and 10% diethanolamine at pH 10, and the color was allowed to develop until the 1:50 positive control wells had reached an absorbance of about 1.5 at 405 nm.

(7) Absorbances were measured at 405 nm using a Molecular Devices VERSAmax microplate reader. Antibody levels of the unknown samples were assigned a unit value based on the 8-point positive control standard curve with a four-parameter curve fit. The 1:50 dilution of the positive control was arbitrarily assigned a value of 6400 units.

(8) Unknown samples with absorbance values above the standard curve were diluted further and reassayed. Arbitrary unit values were expressed per microliter of serum.

D. Recording of Data

Data were stored using the Softmax PRO software.

E. Replacement and Periodic Maintenance of Key Components

Not applicable for this procedure.

F. Calculations

Optical densities at 405 nm were converted to arbitrary unit values per µL of serum using the Softmax PRO software.

G. Special Method Notes

None.

9. REPORTABLE RANGE OF RESULTS

0–6400 arbitrary units per µL of serum or plasma.

10. QUALITY CONTROL (QC) PROCEDURES

Three known positive sera and four negative sera were included on each ELISA plate along with an eight-point standard curve. The four-parameter curve that is fitted to the standard curve values must have a correlation coefficient of >0.98 or the run is rejected. As with the unknowns, the values for the positive control standards must have a C.V. of less than 15% for the duplicate wells. Because this assay is experimental, a definitive value for the known positive samples has yet to be established.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If a curve with a high correlation coefficient cannot be generated from the standard curve values, the assay is repeated. If, upon repetition, the curve is not improved, a new lot of ELISA plates or a new dilution of standards are used.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The assays are experimental and we have no indications of the limits of the tests. The assays do appear to be inhibited to some as yet undetermined level by the presence of large amounts of hemolysis products in the sample.

13. REFERENCE RANGES (NORMAL VALUES)

Because this assay is experimental there are no reference ranges for this type of testing.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Because this assay is experimental there are no critical call limits for this type of testing.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at 4°C prior to loading of the ELISA plate. After the samples have been added to the wells at room temperature, they are again returned to 4°C until the assays are completed and then autoclaved for disposal.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If the test system fails the specimens are stored frozen until the system is operational.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

    There are no critical call limits for this type of testing.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

    Local PCs, the DPD network, hard copies, and 3 inch computer disks are used to keep records and track specimens. All records, including related QA/QC data, are required to be maintained for 10 years. Only numerical identifiers (e.g., case ID numbers) should be used.

REFERENCES

1. Priest JW, Kwon JK, Arrowood MJ, and Lammie PJ. Cloning of the immunodominant 17-kDa antigen from *Cryptosporidium parvum*. Mol. Biochem. Parasitol. 2000;106:261–271.
2. Priest JW, Kwon JP, Moss DM, Roberts JM, Arrowood MJ, Dworkin MS, Juranek DD, and Lammie PJ. Detection by enzyme immunoassay of serum immunoglobulin G antibodies that recognize specific *Cryptosporidium parvum* antigens. J. Clin. Microbiol. 1999;37:1385–1392.