Trichomonas Vaginalis

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0. Public Release Data Set Information

This document details the Lab Protocol for NHANES 2001-2002 data.

A list of the released analytes follows:

Lab	Analyte	SAS Label	Description
l34_b	LBXTV	Trichomonas Vaginalis	Trichomonas vaginalis

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The PCR assay was described by Riley et al (1992). The primer pair used amplifies a 102 base pair region of the ferredoxin gene of *Trichomonas vaginalis.* This primer pair has been shown to readily detect a broad range of Trichomonas isolates from different geographical regions of the United States. These primers do not cross-react with human genomic DNA or with DNA from a broad range of other eukaryotic or prokaryotic microorganisms. In 1995 we developed a Trichomonas-specific DNA probe whose sequence is located internal to the primer pair for Southern blot hybridization to confirm the Trichomonas-specific nature of the 102 base pair product generated from the PCR assay.

2. SAFETY PRECAUTIONS

Standard, universal precautions will be strictly adhered to in handling the swab-based genital specimens to be analyzed for *Trichomonas vaginalis*. This will include the use of the following hospital-approved personal protective devices; laboratory coat, safety glasses and gloves for protection against agents such as HIV and Hepatitis B. This is in accordance with the CDC guidelines; MMWR 1988;37: (suppl. 4) 1-22. Guidelines for Prevention of Transmission of Human Immunodeficiency Virus and Hepatitis B Virus to Health-Care and Public-Safety Workers: A response to P.L. 100-607, the Health Omnibus Programs Extension Act of 1988. All biohazard waste generated from this testing will be properly discarded in double bagged, properly labeled biohazard waste containers that, when full, are placed in our on-site microwave for decontamination before disposal.

DNA visualization within the agarose gels requires the use of potentially hazardous ultraviolet light and ethidium bromide DNA intercollating dye. Personnel exposure to ultraviolet light will be minimized with the use of complete face shields designed to block UV ray transmission, as well as the use of long sleeved lab coats, gloves and the built in shield on the UV light box. Exposure to ethidium bromide, a chemical carcinogen will be minimized by incorporating it into the agarose gel. This allows for easier handling of a solid form of the waste rather than a liquid one which has the potential to be spilled.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES mobile unit contains a corresponding transmittal sheet and an ANSI data file (XXXXXXX.TXT) is emailed as an attachment. The data file, containing the specimen ID, collection date, and type of sample (i.e. swab) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. After the data is calculated and the final values are approved by the reviewing supervisor for release, all results are entered onto the NHANES ANSI data file by using Excel.

c. After the results are entered, back-up copies are made and stored in locked areas.

d. The results are emailed, as an attachment, to NCHS.

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

a. Vaginal fluid should be collected on a dry swab (dacron-tipped, plastic shaft) that is placed back into the plastic sheath after collection and maintained at 4° C from the time

of collection and throughout the shipping process when wet ice or cold packs can be placed in the container to maintain temperature.

b. Once the samples arrive at the contractor's institution, they will be stored at 4° C until they can be extracted and tested. This could be as much as 7 days from the date of collection. If analysis will not occur within this time frame, the specimens will be stored at -20° C for up to 2 weeks from the date of collection.

c. Sample testing, resulting and electronic data submission will occur within 3 weeks from the time the specimens were received by the contracting institution.

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

Perkin Elmer 9600 thermocycler

b. Standards Preparation

The positive control for Trichomonas PCR will consist of a low level positive control prepared from a frozen stock of a clinical isolate of Trichomonas vaginalis cultured in Diamond's broth at 37C for up to 3 days. The laboratory is routinely successful at culturing frozen stocks of Trichomonas vaginalis clinical isolates in Diamond's broth for a source of positive control DNA for our PCR assay. The low level positive control will consist of 10 microorganisms per PCR master mix reaction. This number will be calculated by performing a cell count from a freshly derived broth culture of Trichomonas. The 10 microorganisms per PCR master mix in the low level positive control was established because it is one dilution above the limit of detection for the assay (i.e. 5 microorganisms). The low level positive control will be useful in monitoring the constancy of the assay's level of sensitivity. A fall off in the assay's sensitivity will be picked up immediately by using a low level positive control, as compared to a high level positive control. The low level positive control will also assess the efficiency of the extraction and lysis step used in the sample preparation process.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A Perkin Elmer service representative performs semi-annual preventive maintenance checks on the 9600 thermocyclers in the clinical laboratory. This service includes well to-well temperature checks to ensure accurate temperatures in all wells of the instrument, along with replacement of the refrigeration fluid, cleaning, assessment of the computer software programs, and ramping parameters. The laboratory staff will perform daily, weekly, and monthly QC checks procedures as prescribed by the manufacturer. Each run will have a complete cycle printout attached to the PCR worksheet. This will ensure the accuracy of the cycling parameters.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF

RESULTS

a. Sample Preparation

For sample extraction, the swab will be taken out of the plastic transport sheath and the tip will be placed into a 1.5-ml Eppendorf tube containing 0.5 ml lysis buffer; Tris-HCI buffer, pH 8.3, containing 50 mM KCI, 2.5 MM MgC'2, and 1% Brij 35 detergent for 5 min at room temperature. The swab will be wrung out on the inside wall of the tube before being discarded. Approximately 200 µL of lysis buffer will be lost to the discarded swab, leaving approximately 300 µL of specimen remaining.

Proteinase K at a concentration of 200 ug/mL will be added to the lysis buffer containing the specimen. The samples will be incubated for 60 min at 56° C before inactivating the proteinase K at 95° C for 10 min. After enzyme inactivation, the samples will be sonicated for 1 min. Samples are now ready to be analyzed for the presence of Trichomonas DNA and Beta globin DNA by PCR in separate reactions. Screening each specimen for Beta globin is included as a control measure to interpret Trichomonas negative PCR results. Samples lacking amplifiable Beta globin DNA may contain inadequate cellular material, or inhibitors of the PCR assay.

Trichomonas PCR primer sequences: (Riley et al., 1992)
TVA5: 5'-GAT CAT GTT CTA TCT TTT CA-3' (Sense)
TVA6: 5'-GAT CAC CAC CTT AGT TTA CA-3' (Antisense)

Beta globin primer sequences: (Bauer et al., 1991)
BGL-1: 5'- CTT CAT CCA CGT TCA CC-3'
BGL-2:5'-GAA GAG CCA AGG ACA GGT AC-3'

Master Mix formulation for Trichomonas or Beta globin PCR:

Vol.	Reagent	Final Concentration
10 uL	1OX Tris-HCI buffer, pH 8.3	10 mM Tris-HCI, 50 mM KCI
10 uL	25 mM $MgCl_2$	2.5 mM
16 uL	dATP, dCTP, dGTP, dUTP	200 uM each dNTP
0.5 uL	Taq DNA polymerase	1.5 Units
1 uL	Uracil-N-Glycosylase	1 Unit
1.25 uL	TV or BGL primers	0.25 uM/0.5 uM
55.0 uL	sterile distilled water	
95 uL		

b. PCR Amplification of Trichomonas or Beta Globin DNA:

Five microliter of the extracted specimen will be added to 95 µL of PCR master mix and analyzed for Trichomonas and Beta globin DNAs in individual reactions. 40 cycles of amplification, including 1 min at 95° C (denaturation), 1 min at 57° C (annealing), and 1 min at 72° C (elongation) will be carried out in a Perkin Elmer 9600 thermocycler. The sizes of the Trichomonas and Beta globin amplified products are 102, and 268 base pairs, respectively. A positive Trichomonas control will be run with each batch of up to 26 specimens to be analyzed for Trichomonas DNA. The positive control will be a low level positive containing 10 microorganisms of Trichomonas vaginalis. The positive control for the Beta globin PCR assay will consist of WBC's isolated from a buffy coat preparation of whole blood. A negative control, no DNA reaction will be included within each batch of samples for both the Trichomonas and Beta globin PCR assays.

c. Agarose Gel Electrophoresis For Detection Of PCR Products:

All PCR amplified samples will be analyzed for the amplified DNA product by agarose gel electrophoresis using a 3% Nusieve GTG agarose in 1X TBE buffer. Our largest agarose gel box can accommodate up to 30 samples at one time, 15 wells in the upper row, and 15 wells in the lower row. For either the Trichomonas or Beta globin PCR runs, each agarose gel electrophoresis run will include up to 26 specimens, a low level positive control for Trichomonas, or a positive control for Beta globin, as well as a negative control and 2 sets of molecular weight markers; one each for the upper and lower 15 wells. Wells 1-13 will contain samples, Well 14 will contain the low level positive control, or Beta globin positive control, Well 15 will contain a molecular weight marker, Wells 16-28 will contain samples, Well 29 will contain the negative control, and Well 30 will contain a duplicate molecular weight marker. The predicted size of the PCR product is 102 base pairs. The much fainter band running below the Trichomonas 102 base pair band is the excess Trichomonas specific primers. The dark band running below the primers is the brome phenol blue dye front.

Trichomonas vaginalis PCR assay: Estimating assay sensitivity. Known numbers of cultured Trichomonas vaginalis microorganisms were added to 250 uL of lysis buffer and processed as described above. Lanes 1-8 represent 200, 100, 50, 25, 20, 15, 10, and 5 microorganisms, respectively added to individual PCR reactions and amplified for the presence of Trichomonas DNA. Lane 9 is a positive control. Lanes 10 and 11 represent negative controls; no DNA control and lysis buffer blank, respectively. Lane 12 contains a 100 base pair molecular weight ladder. The 102 base pair band is the predicted size of the Trichomonas-specific product.

d. Southern blot confirmation for Trichomonas PCR positive samples:

All Trichomonas PCR positive samples will be confirmed using Southern blot hybridization. The agarose gel containing the Trichomonas PCR positive samples will be denatured for 30 min at room temperature in a solution containing 0.5 M NAOH and 1.5 M NaCl, and then neutralized for 30 min at room temperature in a solution containing 1.0 M Tris-HCI, pH 7.0 buffer and 1.5M NaCl. The DNA within this gel is then transferred via capillary action onto a positively charged nylon membrane (Immobilon-Ny+, Millipore Corporation) using 2X SSC made from a 2OX SSC stock which consists of 175.3 g NaCl, 88.2 g sodium citrate in 800 mL distilled water. Adjust pH to 7.0, q.s. to 1 liter volume. The DNA on the membrane will be permanently affixed to the membrane using ultraviolet light. The membrane will be pre-hybridized in a solution containing 1% SDS, 1M NaCl, 10% dextran sulfate. The same formulation used to make the prehybridization solution will be used to prepare the hybridization solution, with the addition of 2.5 pmoL of the Digoxigenin tail-labeled Trichomonas TV JJP DNA probe (TVJJP DNA probe sequence: 5'-CTC TGA GTC TTC TTC TAG AGG TC-3'). After probe hybridization at 60° C for 1 hour, the 102 bp band will be visualized using the Genius detection system according to the manufacturer's directions (Boehringer Mannheim). If the 102 bp product is specific to Trichomonas, it will hybridize to the probe and be seen as a dark band on the developed X-ray film. If the PCR product is not specific for Trichomonas, no signal will be visualized when the exposed X-ray film is developed.

Southern blot analysis using the Digoxigenin tail-labeled TVJJP DNA probe. Lane 1; Trichomonas negative genital specimen, Lane 2; Trichomonas positive genital specimen, Lane 3; Positive control, Lane 4; Negative control.

e. Sample rejection criteria:

1) Unlabeled, inadequately labeled or illegibly labeled specimens.

2) Specimens collected on improper swabs; e.g. calcium alginate swabs, or swabs

whose shafts are made of wood or metal, as both situations are known to be inhibitory to the PCR assay.

3) Specimens arriving more than 7 days after the date of collection.

4) Specimens shipped at ambient temperature, without wet ice or cold packs.

f. Limits of detection (LOD) for the Trichomonas PCR assay:

The LOD for our Trichomonas PCR assay is less than or equal to 5 Trichomonas vaginalis microorganisms in the PCR master mix reaction. This level of sensitivity was duplicated in multiple experiments.

9. REPORTABLE RANGE OF RESULTS

A zero would not be a reportable value in the Trichomonas PCR assay. A normal value for Trichomonas PCR would be a negative result, interpreted as an absence of, or an undetectable level of Trichomonas vaginalis in the sample. The results of the Trichomonas PCR test would be finalized as follows:

Positive for Trichomonas: A sample will be considered positive if it yields a 102 base pair fragment after PCR amplification that is recognized by the Trichomonas specific DNA probe upon Southern blot hybridization.

Negative for Trichomonas: A sample will be considered negative if it does not yield the 102 base pair fragment after PCR amplification, or when the PCR product's identity cannot be confirmed by Southern blot hybridization.

Uninterpretable; A sample will be considered Uninterpretable if the specimen was found to be Trichomonas and Beta Globin negative by PCR. This could be due to either the presence of inhibitors or the lack of DNA or both.

10. QUALITY CONTROL (QC) PROCEDURES

The positive control for Trichomonas PCR will consist of a low level positive control prepared from a frozen stock of a clinical isolate of Trichomonas vaginalis cultured in Diamond's broth at 37° C for up to 3 days. The laboratory is routinely successful at culturing frozen stocks of Trichomonas vaginalis clinical isolates in Diamond's broth for a source of positive control DNA for our PCR assay. The low level positive control will consist of 10 microorganisms per PCR master mix reaction. This number will be calculated by performing a cell count from a freshly derived broth culture of Trichomonas. The 10 microorganisms per PCR master mix in the low level positive control was established because it is one dilution above the limit of detection for the assay (i.e. 5 microorganisms). The low level positive control will be useful in monitoring the constancy of the assay's level of sensitivity. A fall off in the assay's sensitivity will be picked up immediately by using a low level positive control, as compared to a high level positive control. The low level positive control will also assess the efficiency of the extraction and lysis step used in the sample preparation process.

Semi-annual repeat testing in a blinded fashion is performed. Six specimens, some positive and some negative, will be analyzed by PCR. The result of the repeat test will be compared to that of the original test. The two results should demonstrate an overall agreement of at least 90% over the year to be satisfactory. If possible, specimens will be shared from other

laboratories that also performed PCR for Trichomonas vaginalis. Results of all proficiency testing will be recorded.

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

Trichomonas and Beta globin PCR assays will be run in batches. A total of 28 samples will be run at one time, including up to 26 specimens, along with one negative control (no DNA control) and one low level positive control for Trichomonas, or positive control for Beta globin. These 2 batches of samples can be amplified concurrently in a Perkin Elmer 9600 thermocycler. The cycling temperatures during each run will be monitored and reviewed after each run. Any malfunctions in the cycling parameters will be noticed so that failed runs can be repeated immediately.

Failed runs can occur in which the positive control is not positive, or where the negative control is not negative. For failed runs in which the positive control is not positive, the prepared samples will be reanalyzed by PCR, using a new tube of the same lot of positive control. Positive controls are prepared in sufficiently large batch sizes to maintain stability for up to 6 months at -80° C. When ready to use, an individual aliquot of the control is thawed and used only once, never refrozen or reused. If the repeat run using a fresh tube of the positive control is also negative one must consider the PCR master mix to be problematic. Fresh Trichomonas master mix would be made up and tested with the positive control. If the new master mix is OK, then the run could be repeated using the new master mix.

Failed runs containing a contaminant 102 base pair band in the negative control well would be considered a false positive result. In this case, all samples within that run would have to be reamplified but not before an extensive decontamination protocol is carried out. These steps would include cleaning all pipettes, bench top surfaces, laminar flow hoods and thermocycler surfaces with an enzymatic DNase; DNA Away (Molecular BioProducts, Fisher Scientific).

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. The presence of PCR inhibitors in a specimen may cause false negative test results.

a.

b. The presence of spermicides in excess of 1%, surgical lubricants in excess of 10%, or feminine powder sprays may have an inhibitory effect on the P.CR assay. These agents should not be used immediately prior to specimen collection.

b. Specimens that are grossly mucoid may cause inhibition of PCR assays.

c. Swabs collected on a woman who is actively menstruating may contain sufficient levels of blood to interfere with the PCR assay.

d. Mucoid or bloody sample characteristics will be noted on a sample log sheet.

e. The use of the Beta globin PCR assay as an internal control will be helpful in interpreting the Trichomonas PCR-negative specimens.

f. Ideally, a dry swab with a dacron tip and plastic shaft (Medical Packaging SP-2D, distributed by Fisher under the name of Swab-Pak) should be used to collect the specimen. The swab should be placed within the vagina to collect sufficient fluid from an adequate sampling area. At a minimum, the swab must be moistened during this

procedure, and at best, should be saturated with fluid. The specimen containing swab should be placed back into the accompanying pre-labeled plastic sleeve and maintained at 4° C after collection and during transport (wet ice) to the contractor laboratory.

13. REFERENCE RANGES (NORMAL VALUES)

A normal value for Trichomonas PCR would be a negative result, interpreted as an absence of, or an undetectable level of Trichomonas vaginalis in the sample.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable to this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Once received by the contractor, the specimens can be stored at 4° C for up to 7 days from date of collection before sample processing and testing takes place. If this time will be greater than 7 days, the samples will be frozen at -20° C for up to two weeks from date of collection before performing sample processing and testing. Before freezing, the swab would be placed into 500 µL volume of lysis buffer (lacking proteinase K enzyme), wrung out and the swab discarded. The resulting fluid, approx. 300 µL within the Eppendorf tube will then be stored at -20° C for up to one additional week before PCR testing would take place following sample extraction and sonication to meet the 3 week turn around time from date of receipt at our institution.

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

The laboratory has three 9600 thermocyclers, along with three gel boxes, and two electrophoresis power supply units. This level of instrumentation backup is more than adequate if an instrumentation failure was to occur. The laboratory will be able to easily perform the PCR amplification and detection assays on the specimens sent to us from the stands within the necessary 3 week turnaround time frame.

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

Not applicable for this procedure.

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

For the NHANES 2001-2002 residual urine samples are stored at $\leq$-20^{o}C for 6 years after analysis and then discarded.

19. Summary Statistics and QC graphs

These assays are PCR assays with a positive, negative or uninterpretable result.

References:

Jordan JA. PCR Detection of Trichomonas vaginalis from Vaginal Secretions Using a 5' Exonuclease Assay and Fluorogenic Probe. International Congress of Sexually Transmitted Diseases, Seville, Spain, October 19-22, 1997.

RP Nugent, MA Krohn and SL Ellier. Reliability of Diagnosing Bacterial Vaginosis Is Improved by a Standardized Method of Gram Stain Interpretation. J Clin Microbiol 1991; 29:297-301.

Bauer BA4, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, Reingold A, Manos MM. Genital human papillomavirus infection in female university students as determined by a PCR-based method. JAMA 1991; 265:472-477