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Guidelines for the Use of Molecular Biological Methods to Detect Sexually Transmitted Pathogens in Cases of Suspected Sexual Abuse in Children

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Abstract

Testing for sexually transmitted infections (STIs) in children presents a number of problems for the practitioner that are not usually faced when testing adults for the same infections. The identification of an STI in a child, in addition to medical implications, can have serious legal implications. The presence of an STI is often used to support the presence or allegations of sexual abuse and conversely, the identification of an STI in a child will prompt an investigation of possible abuse. The significance of the identification of a sexually transmitted agent in such children as evidence of possible child sexual abuse varies by pathogen.

While culture has historically been used for the detection of STIs in cases of suspected abuse in children, the increasing use of nucleic acid amplification tests (NAATs) in adults and the increasing proliferation of second-generation tests with better sensitivity and specificity has made inroads into the use of such tests in children, especially for diagnostic and treatment purposes. Acceptance by the medicolegal system for sexual abuse cases is still controversial and more test cases will be necessary before definitive use becomes standard practice. In addition, if these assays ever become legally admissible in court, there will be recommendations that more than one NAAT assay be used in order to assure confirmation of the diagnostic result.

Keywords

Chlamydia trachomatis; *Neisseria gonorrhoeae*; Nucleic acid amplification tests; Polymerase chain reaction; Sexual abuse; Strand displacement amplification; Transcription-mediated amplification; *Trichomonas vaginalis*

1. Introduction

The introduction of NAATs for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* has been a major advance in the diagnosis of STIs. Use of NAATs has supplanted standard culture methods for *N. gonorrhoeae* and *C. trachomatis* in many

[2]One method to ascertain laboratory and assay expertise is to participate in proficiency surveys. Several of these exist and most laboratories are required by their institutions to participate. The surveys provided by the Clinical Association of Pathologists (CAP) are the most commonly used.

[4]If cultures are being performed for any of the aforementioned organisms, proficiency can also be measured by participating in CAP surveys for the particular organism.

laboratories. Currently there are four commercially available NAATs for *N. gonorrhoeae* and *C. trachomatis*: polymerase chain reaction (PCR) (Amplicor, Roche Molecular Diagnostics, Indianapolis, IN), strand displacement amplification (SDA) (ProbeTec, Becton-Dickson, Inc., Sparks, MD), transcription-mediated amplification (TMA) (Aptima C2, GenProbe Inc., San Diego, CA), and real-time m2000 PCR (Abbott Molecular Diagnostics, Des Plains, IL) (1–4). NAATs are currently recommended by Centers for Disease Control and Prevention (CDC) as the diagnostic assays of choice for screening purposes (5). Use of such assays for sexual abuse is discussed in the new laboratory guidelines as useful in adults and potentially useful in children for diagnostic testing (5). Point-of-care tests (POC), which can be used by minimally trained healthcare workers, are not yet of sufficient sensitivity to be recommended for any screening or testing, but newer improved assays are under development. PCR, realtime PCR, and SDA are DNA amplification assays. TMA is an RNA amplification assay. All four assays have FDA approval for use in genital sites (cervix, vagina, self-collected vaginal swabs, and male urethra) and urine from adolescents and adults. None are currently approved for extragenital sites (pharynx or rectum) or have approval for any site in children. In the USA, since no company has sought FDA clearance for such sites, individual laboratories can perform independent verification for using amplification assays for testing such specimens for diagnostic purposes in order to remain Clinical Laboratories Improvement Act (CLIA) compliance (6). These diagnostic and controversial legal issues are discussed in this chapter (7).

1.1. NAATs for Gonococcus

Although NAATs offer several advantages over culture-based methods including higher sensitivity and enabling the use of noninvasive specimens (urine, vaginal swabs), these assays have some limitations, especially for detection of *N. gonorrhoeae*. The gonococcus (GC) has the capacity for genetic variation and recombination that can affect the genetic sequences that are targets for amplification, leading to potentially false-negative results. In addition *Neisseria* species also have the relatively rare characteristic of being fully competent for exogenous DNA uptake throughout their life cycle. This enables frequent horizontal interspecies exchange of genetic material between *Neisseria* species leading to false positive amplification results for gonorrhea, when nonpathogenic, commensal *Neisseria* species acquire gonococcal sequences and vice versa (8–10). All NAATs do not have the same performance parameters (11). PCR and SDA have both been demonstrated to have cross-reactivity with other *Neisseria* species including *N. cinerea, N. flavescens, N. lactamica, N. sicca*, and *N. subflava* (12). This has important diagnostic implications especially when testing extragenital sites including pharynx and rectum.

Several recent studies have examined the performance of NAATs for detection of *N. gonorrhoeae* from the pharynx and rectum, primarily in men who have sex with men (MSM). McNally et al. (13) reported that SDA had a low positive predictive value (PPV) for oral (30.4 %) and rectal specimens (73.7 %) in a population of MSM in Australia. Similar findings were reported by Schachter et al. (14) comparing PCR, SDA, and TMA in oral and pharyngeal specimens from MSM in California. Although the sensitivities of the NAATs were better than culture for detection of *N. gonorrhoeae* from both sites, PCR had a specificity of 78.9 % with oropharyngeal swabs. Specificities of SDA and TMA were ≥99.4

% for both anatomic sites. The overall prevalence of pharyngeal and rectal gonococcal infection in this population was 8.3 and 8.2 %, respectively. Bachmann et al. (15) examined the performance of PCR, SDA, and TMA compared to culture in pharyngeal specimens from males and females ≥15 years of age who acknowledged performing fellatio or cunnilingus. PCR was found to have a specificity of 73.0 % compared to 96.3 % for SDA and 98.6 % for TMA. The prevalence of oral infection in this population was 7.0 % in men and 9.1 % in women. Ota et al. also compared the detection of *N. gonorrhoeae* and *C. trachomatis* in pharyngeal and rectal specimens using the BD Probetec ET system, the Gen-Probe Aptima combo2 assay and culture, demonstrating the superiority of NAATs over culture (16). These prevalences in MSM and attendees at STD clinics are significantly higher than one would observe in children being evaluated for suspected sexual abuse. Therefore, the PPV of a positive result is highly relevant and significant in such a sexual abuse population, since low prevalence in a population directly lowers the probability (PPV) of a positive result being correct.

To date, there have been three published studies that compared NAATs to *N. gonorrhoeae* culture in children being evaluated for suspected sexual abused (17–19). All included urine specimens as well as vaginal swabs. Although the results of two of these studies suggested that the sensitivity of NAATs for *N. gonorrhoeae* was similar to culture, they both had several serious limitations (18, 19). The populations studied included adolescents up to18 and 20 years of age. In a study by Girardet et al. (20), only 48 of 203 (23.7 %) of the children enrolled were prepubertal and only 13 were male. Kellogg et al. (19) only evaluated girls; 58.5 % were ≥13 years of age, a large proportion of whom reported consensual sexual activity. Both studies utilized ligase chain reaction (LCR, LCx, Abbott Diagnostics), which was taken off the market in 2002 due to specificity concerns in the detection of *N. gonorrhoeae* (21). Kellogg et al. also evaluated PCR in addition to LCR (19). Other study limitations included the failure to use an independent reference standard in estimating test performance, failure to separately analyze test performance by age and gender (when applicable). The prevalence of *N. gonorrhoeae* infection in both studies was low (1.9 and 3.2 %) reducing the precision of sensitivity estimates. The number of extragenital specimens was also too low to assess test performance at those sites. Black et al. (17) recently evaluated the use of SDA and TMA using urine and genital swabs vs. culture for the diagnosis of *N. gonorrhoeae* and *C. trachomatis* in children, 0–13 years of age, evaluated for sexual abuse in four US cities. All children were tested at multiple sites for *N. gonorrhoeae* and *C. trachomatis* by culture and vaginal, urethral swabs and urine were also tested with SDA and TMA. Cultures of *N. gonorrhoeae* were performed at all sites using Thayer-Martin agar medium and positive results were confirmed at all sites by Gram stain, oxidase test, enzyme detection, and/or biochemical tests according to the site's standard protocol. Positive NAATs for *N. gonorrhoeae* were confirmed by an in-house PCR using an alternative target, the *Hinf* I fragment of the 4.2-kb cryptic plasmid (22). Of the 536 participants with complete data, none of the male children ($n = 51$) were positive for *N. gonorrhoeae* by any test at any site. Of the 485 female participants with complete data, 16 (3.3 %) had a positive result for *N. gonorrhoeae* by any test: 12 (2.5 %) by culture, 14 (2.9 %) by vaginal NAAT, and 14 (2.9 %) by urine NAAT. All participants who had a positive vaginal culture for *N. gonorrhoeae* had positive urine NAATs. There were discrepant results

in two cases (both SDA-positive and TMA-negative). One of these girls was positive in urine and negative by vaginal swab, the other was positive both by urine and swab. All SDA-positive results for *N. gonorrhoeae* were confirmed to be true positives by a species-specific *N. gonorrhoeae* PCR. Three girls had discrepant results by site: two were vaginal swab positive and urine negative; one was vaginal swab negative and urine positive.

The 2010 CDC STD Treatment Guidelines now recommend that NAATs can be used for detection of *N. gonorrhoeae* in vaginal swabs and urine from girls being evaluated for suspected sexual abuse. However, NAATs were not recommended for use in boys or extragenital specimens, as there are no supporting data. As some NAATs cross-react with other *Neisseria* species, it was also recommended that clinicians consult with an expert before selecting an assay for use in this population. Although confirmatory testing was not specifically mentioned, it was suggested that specimens be retained for further testing if necessary (23).

1.2. NAATs for C. trachomatis

As described previously in the section on gonorrhea, there are several FDA approved NAATs for the simultaneous detection of *N. gonorrhoeae* and *C. trachomatis*: PCR, SDA, and TMA. NAATs are currently approved by the FDA for detection of *C. trachomatis* from genital sites (cervix, vagina, urethra) and urine from adolescents and adults. None are approved for extragenital sites (pharynx or rectum) or have approval for any site in children. These methods have been found to have excellent sensitivity for detection of *C. trachomatis*, usually well above 90 %, in genital specimens and urine from adult men and women, while maintaining high specificity (14). A new genetic variant of *C. trachomatis* was discovered in Sweden in 2006, which was found to have a mutation in the sequence of the cryptic plasmid at the target site for Roche PCR rendering the organism undetectable by this assay (24). Recent data from Sweden reports that this variant is now responsible for 20–65 % of all detected chlamydial infection in counties where PCR was used. So far, this variant appears to be limited primarily to Sweden with a few isolates being identified in Norway and Denmark. Spread of the variant in Sweden was associated with use of PCR as the NAAT for diagnosis of *C. trachomatis* infection. It has not yet been detected in the United States (25).

The recent multicenter study by Black et al. (17) mentioned above, also evaluated the use of SDA and TMA using urine and genital swabs (vagina and urethra) compared to culture for diagnosis of *C. trachomatis* in children, 0–13 years of age. Cultures for *C. trachomatis* were performed at the clinical or hospital laboratories of each center, according to their own standard protocols. All sites transported swab specimens at 4 °C for *C. trachomatis* culture in either commercial Chlamydial or viral transport medium. Culture protocols at all sites included the isolation of *C. trachomatis* in cycloheximide-pretreated McCoy cells, either in shell vials, 24-well, or 96-well tissue culture plates. The inoculated cell monolayers were incubated at 35–37 °C for 48–72 h followed byfixation with ethanol, methanol, or acetone. Thefixed monolayers were stained to detect chlamydial inclusions withfluorescein-conjugated Chlamydia genus-specific or *C. trachomatis* species-specific monoclonal antibodies. One laboratory also performed a single passage of the inoculated cell monolayers onto a fresh monolayer after 48 h of incubation. The commercial NAAT tests were

performed at the CDC (SDA and TMA). All samples were processed and tested according to manufacturer's protocols except for the TMA tests which were performed on previously frozen urine or swabs collected in the BD ProbeTec sample collection medium. Test results that were positive by SDA for *C. trachomatis* were confirmed using an in-house PCR targeting the *ompA* gene, performed at the CDC (26). Fifteen (3.1 %) of 485 female participants had a positive result for *C. trachomatis* by any test (7 [1.4 %] by culture; 11 [2.3 %] by vaginal NAAT; 13 [2.7 %] by urine NAAT). None of the male participants had any positive cultures or NAATs for *C. trachomatis*. All participants who had a positive vaginal culture for *C. trachomatis* also had positive urine NAAT. Two prepubertal female children had positive *C. trachomatis* cultures from rectal swab specimens, but negative vaginal swab specimens by both culture and NAATs, and negative urine NAATs. No other participants had positive rectal cultures. There were no discrepant results in any of the participants tested by two commercial NAATs for *C. trachomatis* (ProbeTec and Aptima Combo 2). All *C. trachomatis*-positive results were confirmed to be true positives by DNA sequence genotyping. When NAAT results were compared by the type of specimen, only one girl had a discrepant result for *C. trachomatis* (vaginal swab negative, urine positive). The sensitivity of vaginal culture for *C. trachomatis* was 39 % in all girls studied (n = 485). In contrast, the sensitivities of urine and vaginal swab NAATs were 100 and 85 % in all female children, respectively, for detection of *C. trachomatis*.

The results of Black et al. (17) suggest that NAATs, specifically SDA and TMA, can be used for detection of *C. trachomatis* in girls being evaluated for suspected sexual abuse. However, the same limitations apply as for use of these assays for detection of *N. gonorrhoeae*: (1) as the prevalence of *C. trachomatis* in this population is low, confirmatory testing is necessary. (2) One cannot extrapolate from these results to other NAATs, specifically PCR and use in specimens other than vagina and urine in girls. (3) One cannot make any recommendations on the use of these assays in prepubertal boys. Performing a confirmatory NAAT may be problematic as most hospital laboratories only use one assay. Some of the more recently available commercial NAATs, such as TMA (Aptima Combo 2), offer an alternate target confirmation method that can be used on the same testing platform; however, there are no data on the use of this confirmatory test in this setting. Additional options include sending blinded specimens to an independent or reference laboratory for confirmation testing, confirming a NAAT-positive result by culture test (requires a separate, invasive specimen), or use of a second, alternate technology commercial NAAT (probably the preferred option). Confirmatory testing originally recommended by CDC is no longer recommended by CDC for routine genital samples from adults and sexually active adolescents being testing for routine screening and diagnostic testing (27). However, for cases of suspected sexual abuse, confirmatory testing by a second NAAT should be performed and the laboratory should always use a newer "second generation" NAAT with the highest sensitivity possible, preferably with a different target (11). The 2010 CDC STD Treatment Guidelines recommended that NAATs can be used to detect *C. trachomatis* in vaginal swabs and urine from girls being evaluated for suspected sexual abuse (23). However, NAATs were not recommended for use in boys or extragenital specimens, as there are no data. Specimens collected from children for forensic applications should be retained

in the laboratory for purposes of additional testing, in accordance with local policies and procedures (see Notes 1–[5]).

1.3. NAATs for T. vaginalis

Background—Trichomonas infections, caused by the parasite *Trichomonas vaginalis*, are highly prevalent sexually transmitted infections (STIs) worldwide, with estimates of 7–8 million infections annually in the United States (28). As such, they represent the most common curable STI in young, sexually active women (29, 30). Trichomonas infections have been associated with poor reproductive outcomes such as low birth weight (LBW) and premature birth (31, 32). However data on trichomonas infections in the setting of child sexual abuse are limited. Most published studies of STIs in sexually abused children have testing for *T. vaginalis* has been limited to girls presenting with vaginal discharge (20, 33–36).

Rarely *T. vaginalis* can be transmitted vertically from mother to infant (vaginal, urine) during parturition (37, 38). These infections may persist for several months after birth. Care should be taken in interpretation when trichomonads are reported present in urine specimens from children collected for another purpose. As the morphology of *Pentatrichomonas (Trichomonas) hominis*, a nonpathogenic intestinalflagellate, is very similar to that of *T. vaginalis*, care must be taken to make sure that specimens are not contaminated with fecal material which can occur with bagged urine specimens.

Trichomoniasis has primarily been screened for at gynaecologic visits in the antenatal or family planning setting by visualization of the trichomonads on wet mount microscopy, but this methods in relatively insensitive; culture is more sensitive, but not as sensitive as NAAT assays, which are not yet FDA cleared.

POC assays—There are several FDA-cleared POC tests available including the Affirm VP III Microbial Identification System (Becton Dickinson) test, which is a direct nucleic acid probe hybridization test for detection of *T. vaginalis*, *Gardnerella vaginalis*, and *Candida* spp. (39). It has been reported to have sensitivities of 80–90 % in adult women with vaginitis compared to culture as the reference standard. Culture, however, has now been demonstrated to be of lower sensitivity than NAAT assays (40, 41). The Affirm VP III has not been validated or approved for use in genital specimens from prepubertal girls or urethral specimens from men.

A relatively new POC test (Genzyme, Inc.), test shows promise with better sensitivity than wet preparation and culture, but it has not been studied in prepubertal girls (42).

NAAT assays—As previously mentioned, at present there is no FDA-cleared commercial NAAT assay for trichomonas. Several research PCR assays have been reported and appear

[1]Troubleshooting may be necessary when assays are not performing correctly as when positive and negative controls for a particular kit are performing incorrectly. In cases such as these, companies have technical representatives, who will work with laboratory personnel to correct the problem.

[5]As always, for diagnostic laboratories, quality assurance measures should be in continual use for each diagnostic assay, continued laboratory staff proficiency should be maintained, and adequate training of new laboratory professionals is required.

to perform with sensitivities >90 %; greater sensitivity than wet preparation or culture (42–49). There is one commercially available analyte-specific reagent (ASR) based on TMA (GenProbe, Inc., San Diego, CA) that is FDA cleared for purchase of reagents, but not in a "kit" format (49). Clinical trials for FDA clearance of this NAAT assays have been performed and have been submitted to the FDA. More research is needed to ascertain how this test performs with samples from prepubertal girls and in men.

Sensitivity comparisons using NAATs—When comparisons have been performed with NAATs, the sensitivity and specificity of wet-preparation microscopy for trichomonas have been estimated to be between 50–60 % and >90 %, respectively, whereas sensitivity and specificity of PCR for trichomonas have been shown to be both >90%, respectively (46, 47, 50). A study of multiple etiologies of cervicitis in STD clinics, using both a TMA-based research NAAT (49) and another research PCR-based NAAT (45) for trichomonas, demonstrated an overall prevalence of 15.3 % for trichomonas compared with 11.9 % using wet-preparation microscopy (51). Because of the lower sensitivity of the wet-preparation method for diagnosis of TV, a significant percentage of infections may be routinely missed, which is of concern in cases of suspected sexual abuse. The increased sensitivity of NAAT assays in women over traditional methods of diagnosis such as wet preparation is evidence for the need for more future research determining use of NAAT assays for the diagnosis of trichomonas.

2. Materials

The materials for commercially available and FDA-cleared diagnostic tests are provided in the kits provided by the manufacturer. For NAAT assays, these include positive and negative controls, primers, polymerase enzyme, buffers, and reagents. For culture, additional supplies such as Thayer Martin plates for gonorrhea culture, as well as generic biochemical tests for gonorrhea and Diamonds media for trichomonas culture are required. Additional materials needed are consistent with standard laboratory supplies and include items such as the equipment platform for the particular commercial assay, in addition to standard laboratory supplies such as pipettes, tips, plastic ware and tubes, gloves, biohazard bags, and miscellaneous equipment such as plate washers, incubators, refrigerators, freezers, etc.

3. Methods

Methods differ for each diagnostic test being processed (gonorrhea, chlamydia, or trichomonas) and are rigorously standardized for commercially available and FDA-cleared assays. Manufacturer's package inserts must be followed exactly according to the provided protocol. If manufacturer's directions are altered as in the case of using a chlamydia of gonorrhea for rectal samples for example, each laboratory must perform their own verification study and document it carefully in order to remain CLIA compliant for laboratory inspectors (see Notes 1–[3]) (6).

[3]Another method to maintain laboratory and test excellence for amplified tests is to routinely perform "swipe" tests by collecting moistened swab samples from the environmental surfaces in the laboratory and equipment on a monthly basis and to run them in an amplified test that is in use to monitor for amplicon contamination in the environment. If any such samples test positive, rigorous cleaning is required and a repeat monitoring "swipe" test should be performed.

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