Biomarkers for Infectious Disease Diagnostics in the Developing World:

Diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhea* Infection in High-risk Women

John Todd, Paul Neuwald, Laura Penny and Mickey Urdea

August 2006

Halteres Associates, LLC

5858 Horton Street, Suite 550

Emeryville, CA 94608

510-420-6733

www.halteresassociates.com

Table of Contents

Halteres Associates, LLC

List of Tables and Figures

Diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhea* in High Risk Women

In this review of *Chlamydia trachomatis* (Ct) and *Neisseria gonorrhea* (Ng) diagnosis in high risk women, Section 1 discusses the current need for diagnostic products in resource-limited settings, given the clinical information and the user specifications required. In Section 2, we present the biomarkers, sample types, and technologies that would be most appropriate for diagnosis of Ct and Ng in women. Sections 4 through 7 outline the steps that need to be taken, if any, to develop these products. Finally, in Section 8, the recommended course of action, are presented.

1. Needs for Ct and Ng Diagnosis in Resource-Limited Settings

Although Ct and Ng are clearly distinct bacteria, they maintain many attributes in common, including their prevalence, location of infection, frequent co-infection, and resulting clinical complications in women. Therefore this manuscript will address their diagnosis in a parallel fashion. Both organisms infect the urogenital tract, throat, or rectum, and can be transmitted to a baby during delivery, leading to neonatal eye infection. Left untreated, infection in women often leads to pelvic sepsis, pelvic inflammatory disease, ectopic pregnancy, infertility, and chronic pelvic pain. It has been estimated that 70 to 75% of women remain asymptomatic when infected with Ct, and up to 80% of women remain asymptomatic when infected with Ng.[1,2] The lack of symptoms results in under–treatment, and because both organisms are readily transmitted sexually, this leads to transmission and increased disease incidence rates.

In the developing world, Ng and Ct infections remain significant health concerns. In Africa, the prevalence rates for Ng and Ct infection range from 2 to 66% in high risk populations (e.g., commercial sex workers) and 1 to 40 % in low risk populations. In Sub-Saharan African high risk populations, the median prevalence rates were estimated at 14% and 24% for Ct and Ng infection, respectively.[3] In 1999 the World Health Organization (WHO) estimated that there were 17 million new Ng cases and 16 million new Ct cases annually in Sub-Saharan Africa.[2] Therefore, pelvic inflammatory disease (PID), largely resulting from lower genital tract Ng or Ct infection, is a serious health concern in Sub-Saharan Africa.

The approach used by Ng and Ct eradication programs is to identify those potentially infected (the at risk population), to test for the presence of disease, and then effectively treat the individuals who are infected. In the case of Ng or Ct infection, the highest at risk population is female commercial sex workers (CSWs). Due to the sporadic availability of healthcare in resource-limited environments, it is of utmost importance to identify infected women and to provide treatment during a single healthcare encounter. In 1991 the WHO advocated the use of clinical syndromic algorithms (syndromic management) to identify women who potentially have Ng and/or Ct and to subsequently treat them. However, this syndromic management has proven ineffective in women. Several studies in developing nations have shown that the use of symptoms for diagnosis is neither sensitive nor specific, because the vast majority of infections are asymptomatic, and because there are other causes of vaginal discharge and pelvic pain.[2,4-6] In 2001, the Sexually Transmitted Diseases Diagnostics Initiative (SDI), WHO, and the Welcome Trust convened a panel of experts to discuss the need for diagnostic tests for sexually transmitted disease in developing

nations. They concluded that point-of-care rapid diagnostic tests (RDTs) for Ng and Ct remained the highest priority for screening asymptomatic patients in order to reduce the over treatment of women with vaginal discharge. An RDT would allow women to receive diagnosis and treatment during a single healthcare visit, which is important to the success of diseases management programs. The idea is to link diagnosis to treatment, and to prevent under-treatment of infections in women who do not return to the clinic to receive their diagnosis and treatment. Of importance, Gift et al. concluded that the performance of an RDT in such settings does not necessarily need to match the performance obtained in high- or moderate-complexity diagnostic laboratories in order to deliver a significant benefit in terms of reduction of morbidity and/or mortality. In "the rapid test paradox," they showed that even if the sensitivity of a test was less than its counterpart in a well-resourced diagnostic laboratory, if it can be used to diagnose patients that would otherwise not return to the clinic, it will lead to an increase in the number of infections treated.[7] These improved outcomes are most likely to be observed when the target population has a high prevalence of disease, such as with CSWs. In this scenario, a test with a high specificity and moderate sensitivity will result in a high positive predictive value (PPV) and provide value in disease management. Vickerman et al. recently noted that even with the low sensitivity of the currently available rapid tests (50 to 70%), modeling shows that they can outperform gold standard tests in populations with high sexual activity and/or low clinic return rates.[8]

The WHO SDI established criteria for an ideal test which would be required for broad acceptance in resource-limited diagnostic settings in developing countries. These criteria are symbolized by the acronym ASSURED: Affordable, Sensitive, Specific, User Friendly, Rapid and Robust, Equipment Free, and Deliverable to developing countries. In the broad context of ASSURED criteria, this manuscript reviews the currently available biomarkers and diagnostic approaches for Ct and Ng diagnosis, identifies their strengths and weaknesses for their use in resource-limited environments, and identifies newer approaches that have the potential to provide an impact in resource-limited settings.

To thoroughly understand the utility of the available biomarkers and the applicability of the different diagnostic approaches to resource-limited settings, it is first important to review the biology of the Ct and Ng organisms. The biology of *Chlamydia* species have been described in detail elsewhere.[9] Briefly, *Chlamydia* is a small obligate intracellular bacteria that preferentially infects columnar epithelial cells. They contain DNA, RNA, and ribosomes, and synthesize their own proteins and nucleic acids. They possess an inner and outer membrane, similar to gram negative bacteria. *Chlamydia* bacteria exist as two biological entities. The elementary body (EB) forms are individual infectious units that are 0.3 to 0.4 μM in diameter. EBs have a rigid outer membrane that is resistant to environmental factors and hence ensures their extracellular survival. EBs bind to receptors on host cells that initiate infection via cellular internalization. *Chlamydia* bacteria also exist as intracellular non-infectious reticulate bodies (RB) in a non-fusogenic vacuole, which is termed an inclusion. Once inside a cell, EBs reorganize to form RBs that may contain up to 500 progeny. At 18 to 48 hours post infection, some RBs convert back to EBs as the RBs continue to multiply. Eventually, the EBs are extruded as infectious units from the cell by reverse endocytosis and by cell lysis.

Historically, Ct species were divided into biological variants (or biovars) and serovars (defined by immune sera reactivity to species-specific outer membrane protein 1, or omp 1).[10 11,12,13] With the advent

of molecular biology technology, Ct species have been classified into 18 different variants based upon variation within the omp 1 gene.[13] The complete sequence of the Ct genome has been published by Stephens et al.[15]

Neisseria gonorrheae bacteria are gram-negative diplococci, ranging in size from 0.6 to 1.5 uM. The organism appears to have more than one complete genome copy per cell.[16] Its survival is aided by the presence of multiple antigenic variants in its surface proteins and lipooligosaccarides (LOS), allowing it to evade the host immune system. The genome has recently been sequenced.[17] The circular genome consists of 2.1 million base-pairs which encode 2,076 open reading frames. Thirty percent of the genome has been annotated based upon conserved protein structures in public databases. Ng primarily infects urogenital epithelia, with the uterine cervix serving as the initial site for infection in women. Gonococci bind to the CR3 receptor and are internalized into epithelial cells. After replication and sialation of LOS, Ng are released from the cervical cell where they invade adjoining epithelium. Ascending infection into the upper genital tract occurs in up to 45% of infected women and can result in PID, infertility, and ectopic pregnancy. Analysis of cervical secretions indicate that an antibody response is not generated in uncomplicated infections, and elevations in local levels of other host response factors such as IL-1, Il-6 and IL-8 have not been observed.[18] Pathogenic strains of Neisseria, including Ng and *N. meningitidis* produce extracellular IgA protease that uses IgA as its substrate.[19] Taken together, these findings further support the ability of Ng to evade the host immune system. Although Ng to do not appear to illicit a broad intense immune response, they due induce local production of TNF-alpha which damages ciliated cells in fallopian tubes and results in scarring.[20]

Ct and Ng infections are treated using inexpensive antibiotics in either a single dose or weekly regimens. Co-infection with Ct often occurs among patients that have Ng infection. Thus presumptive treatment of such patients has been suggested. As with all microbial infections, effective treatment is predicated upon susceptibility of the organism to the antibiotic chosen. The capability of Ng to develop antibiotic resistance has been observed and must be taken into consideration through either susceptibility testing, or by the empiric selection of antibiotics based on an understanding of the prevalence of specific antibiotic-resistant strains in specific geographic regions (discussed in later sections). The WHO has pointed out that in Africa, it appears that penicillin resistance is broadly disseminated among bacterial isolates, and Pham-Kanter has published that such resistance was found in up to 18% isolates in South Africa.[2, 21] Furthermore, tetracycline and fluoroquinolone resistance in Ng has been rapidly spreading in many parts of the world, including Africa.[22, 23] The issue of Ct resistance to antibiotics of choice (tetracycline, doxycycline, and azithromycin) has not been an issue until recently.[24, 25] The magnitude of such resistance is unclear but appears small.

2. Status of Current Diagnostic Approaches and Available Biomarkers

The currently used methods to diagnose Ct or Ng infection in women include microscopic observation of endocervical specimen smears for the presence of bacteria (e.g., gram stain), culture, antigen detection using immunodiagnostic methods, detection of host-derived antibodies using immunodiagnostic methods (serology), and the detection of nucleic acid sequences using a variety of approaches, including both direct probe hybridization and nucleic acid amplification. These methods and biomarkers are described below.

2A. Direct Microscopic Detection of Organisms Using Cytology Approaches

Microscopic observation of Ct and or Ng organisms on slides prepared from urogenital swab samples is considered a definitive diagnosis of active infection. In the case of Ng, a gram stain of the slide and 1,000X magnification can be used to observe diplococci within polymorphonuclear cells.[26] If urethral exudate is not present, as in the 70 to 80% of infections that are asymptomatic, then intra-urethral swab specimens are required, which are difficult to obtain in women compared to men. The sensitivity of Gram-stained specimens obtained from endocervical specimens, reported to be between 30 and 65%, is inadequate for clinical use. Adequate specificity requires a skilled microscopist.[1, 27] Therefore, even in well-resourced laboratories and primary care settings, Gram staining of urogenital specimens from females for the diagnosis of Ng is not recommended.[26]

Studies have evaluated the performance of cytologic techniques for the diagnosis of Ct infection in women.[28-30] Endocervical scrapings are placed on a microscope slide, dried, fixed and then tested for the presence of CT using fluorescently labeled antibodies. These studies have demonstrated a reasonably high concordance with culture-based detection methods (>90%). However, this method is difficult and tedious to perform. For instance, a technician must wait for at least 15 to 30 min in a very dark room to accommodate their eyes before beginning the microscopic examination. Consequently, the advent of higher-throughput technologies for the diagnosis of Ct infection have displaced this approach. Although the direct observation of an infectious organism in a clinical specimen is highly specific for the diagnosis of Ct and Ng; this approach would be difficult to perform in resource-limited settings, especially for specimens obtained from the endocervix. In the context of an ideal ASURRED test, the requirement for highly skilled technicians with expertise in microscopy, and the requirement for expensive fluorescence microscopes, rules out the wide-spread adoption of cytology in resource-limited field settings.

2B. Detection of Bacteria Using Culture-Based Methods

The traditional approach for the laboratory diagnosis of Ct and Ng in women has been culture of specimens obtained from urogenital regions. In the case of Ct, special care must be taken when collecting specimens for culture. Black pointed out that proficiency in specimen collection is paramount to accuracy in culture-based diagnostic testing for Ct.[31] Both sensitivity and specificity of tests results have been shown to be related to adequacy of specimen collection. Since Ct is an intracellular pathogen, specimen collection should include host cells that harbor the pathogen. Secretions that lack host cells are inadequate. Typically, a swab or cyto-brush is used to collect a specimen from within 1 to 2 cm of the endocervix. Alternatively, urethral swabs are used to collect specimens and pooled with endocervical specimens. Specimens are placed into a well-defined transport media, refrigerated, and transported to the laboratory within 24 hours of collection. At the laboratory, the specimen is inoculated into a confluent monolayer of susceptible cells. After 2 to 3 days of growth, the cells develop inclusions that contain both EBs and RBs. The inclusions are detected using a variety of immunological techniques, such as immunofluorescence microscopy using an anti-Ct antibody labeled with a fluorescent dye.[31,26] Culture-based diagnostic methods provide a high level of specificity and also provide a specimen for anti-microbial susceptibility testing. Regardless of these advantages, the sensitivity of this approach can be compromised by inadequacies in specimen collection and transport, as well as the technical complexity of the assay.

Specimen collection for Ng is similar to that for Ct; however, inclusion of epithelial cells is not required. Compared to Ct, the best culture results are obtained from inoculating the specimen immediately into nutritive medium and incubation in a CO_2-enriched atmosphere. Inoculated medium should be transported to the laboratory within 5 hours of specimen collection. In the laboratory, specimens are inoculated on selective or non-selective medium plates and incubated at 35°C in a CO_2-rich atmosphere. The plates are then monitored daily for the appearance Ng colonies. The identification of Ng is made with a Gram stain and an oxidase test on cultured isolates.[26, 32] Under quality-controlled conditions, the sensitivity for a single endocervical specimen culture has been estimated to be 80 to 95%.[33, 34] Sensitivity may be limited by poor specimen collection, or improper storage and transport.[1, 26]

Taken together, diagnosis of Ct and Ng by culture-based methods involves numerous steps, a highly proficient physician and laboratory, a long turnaround time of 3 to 5 days, and is relatively expensive. All of these attributes are inconsistent with those required in limited resource settings as described as an ASURRED test. Even in a well-resourced clinical setting and laboratory, the procedures required for the culture of these organisms can be challenging. In recent years other methods for testing have become increasingly popular and are discussed below.

2C. Bacterial Antigen Detection Using Immunodiagnostic Approaches

The first non-culture tests for the diagnosis of Ct and Ng included direct fluorescent antibody (DFA) tests and enzyme immunoassays (EIAs). More recently, antigen detection has been adapted to point-of-care test formats. These RDTs are based upon the detection of organism-specific antigen(s). The DFA test is performed on an endocervical or urethral swab specimen that has been smeared onto a microscope slide, dried, and fixed. After preparation, such slides do not require refrigeration but should be tested within 7 days of specimen collection. Monoclonal antibodies that detect the MOMP or LPS molecule of Ct, which have been conjugated to a fluorescent dye such as fluorescein, are then incubated on the slide, which is then observed using fluorescence microscopy for the presence of EBs. An advantage of this test is that the specimen can be examined for the required presence of columnar epithelial cells before the test is performed. There are numerous manufacturers of rapid DFA test kits. The assay can be performed in about 30 minutes. Although the test method is relatively easy and rapid, fluorescent microscopy is tedious, requires specialized equipment, a dark room, and extensive training.[1,26] Compared to traditional culture-based methods, the sensitivity is 80 to 90% and specificity is 98 to 99%.[31] DFA tests have not been developed as an initial test for the direct detection of Ng in clinical specimens.[26]

A wide variety of enzyme immunoassay (EIA) tests are commercially available for Ct, and are usually performed in a well-resourced laboratory setting. For testing females, an endocervical specimen is collected, transported, and stored without refrigeration. Upon arrival in the laboratory, the sample is then subjected to a procedure that releases EBs from the host cells. The released EBs are detected with enzyme linked polyclonal-anti LPS antibodies using a plate-based ELISA or tube assay format. Either colorimetric or fluorogenic enzyme substrates are used and measured in appropriate instrumentation. Specificity is obtained by repeat testing of reactive specimens in the presence of a Ct-specific anti-LPS monoclonal antibody that blocks and inhibits Ct-specific reactions. These tests are relatively rapid compared to culture, and require between 2 to 4 hours to complete. Sensitivity of EIAs with endocervical specimens ranges from 77 to 93% with specificities from 98 to 99%.[31] Most EIAS are less sensitive than

culture, and some EIAs are less sensitive than others. Recently, a new generation of the ELISA detection format has been introduced that employs a signal amplification strategy. Multiple copies of primary antibody and signal-generating secondary antibody are combined in a polymer.[35, 36] In a large evaluation study that included over 1,000 endocervical specimens, this signal amplification approach demonstrated a sensitivity of 92% and a specificity of 99.8 % compared to ligase chain reaction (LCR, discussed in Section 2E).[37] In another trial that examined 187 high-risk female sex workers, a sensitivity of 95% was obtained compared to methods that detect DNA sequences (discussed in Section 2E).[38, 39] These results are encouraging and demonstrate the potential sensitivity of EIA technology for the detection of Ct in female urogenital specimens in a well-resourced laboratory setting. EIAs for the detection of Ng antigens have been commercialized (e.g., Gonozyme, Abbott Labs). Although these tests have a high level of specificity (>97%), they display different sensitivities between male urethral and female endocervical specimens; with the latter being less sensitive.[32, 40]

Urine has been evaluated as an alternative specimen type for EIAs. In men, similar sensitivity can be obtained using urine or urethral swab specimens. Unfortunately, such studies have not been performed in women. Of note, though, urine specimens must be centrifuged to sediment cells that are subsequently used in the EIA analysis.[31]

Recently RDTs that use membrane lateral flow technology have been developed and commercialized for the detection of Ct and Ng antigens (see Table 1). All RDTs for Ct are similar in that they detect the LPS antigen produced by all Chlamydia species. Numerous versions of Ct RDT devices are commercially available, though some of the assay procedures are more complex than others. At least one assay requires heating (and hence has a power requirement), while other assays provide a device for filtration of endocervical swab specimens prior to testing. In general the RDTs are less sensitive and specific than the laboratory EIAs.[31] Compared to culture-based methods, sensitivities ranging from 52 to 85% have been reported for endocervical specimens.[41-44] These values are consistent with the manufacturers' claims. However, given that culture-based methods have sensitivities of only 50 to 85% compared to tests that detect nucleic acids (discussed in Section 2E), the true sensitivities of the RDT s may be in the 40 to 70% range.

RDTs for Ng antigen detection have been commercialized by numerous manufacturers, though they have not been rigorously tested in field trials using specimens from females and published in peer review articles. Therefore the available data on their performance is generally that provided by the manufacturers. Specimen collection procedures vary from kit to kit, but in general a swab specimen is placed into a tube containing a specimen diluent. Bacteria are released and disrupted in the diluent. A few drops of the diluent are placed into an immunochromatographic lateral flow device, and after 10 to 20 minutes, the test results can be observed visually by the appearance of specific lines on the test strip. Performance reported by manufacturers ranges from 91 to 98% and 96 to 98% for sensitivity and specificity, respectively, compared to culture-based methods. Although many of these tests are similar in format, some do have features or performance results that are of particular interest. PATH has developed an immunochromatographic test strip that is stable at ambient temperature. In two field trials, the first in South America, the test provided a sensitivity of 61% and a specificity of 96%, compared to LCR using vaginal swab specimens (see Section 2E). In the second study, which was a WHO-sponsored field trial in

Benin, Africa, PATH reported a sensitivity of 70% and a specificity of 97%. The reference method employed in this second study was not reported.[45] An intriguing report was made by Suzuki et al. using male urethral swabs. They reported a sensitivity of 94% and a specificity of 96% compared to culture. Importantly, they determined that specimens were stable for up to 45 hours at room temperature.[46] Biostar (Inverness Medical-Biostar, Louisville, USA) reported the results of their OIA GC test. This test detects the presence of L7/L12 ribosomal protein marker, which is claimed to be highly specific to Ng. This test is priced at $16 USD per test strip. Finally, First Diagnostic (Boca Raton, FL) announced the integration of a fluorescence-based technology into the one step lateral flow immunoassay format that will enhance sensitivity by 50 to 1,000 times, but in resource-limited environments this approach would will require a portable easy to use device for measuring fluorescence or other signaling molecules.

The performance of biomarkers used to detect Ct and Ng infection are presented in Table 1.

Table 1. Commercially Available Ct and Ng Rapid Diagnostic Tests Based on Lateral Flow Membrane Technology

Bacteria	Specimen	Details	Sensitivity	Specificity
C. trachomatis	Endocervical swab	Numerous manufacturers, some require sample prep, others heating. Testing in high-resource labs complete; field testing not complete.	50 to 80% vs. culture 40 to 70% vs. NAT	98 to 99.9%
N. gonorrhea	Endocervical swab	A few manufacturers; minimal testing reported	91 to 96% vs. culture	96 to 98% vs. culture
	Vaginal swab	PATH , 2 field tests reported	61 to 70% vs. LCR	96 to 98% vs. LCR
	Male uretheral swab	Tested in high-resource lab setting	94% vs. culture	96% vs. culture
	Endocervical swab or male urine	BioStar, novel ribosomal protein biomarker	ND	ND
	Endocervical swab	First Diagnostics, Fluorescence based highly sensitive	ND	ND

In summary, the detection of bacterial antigens in endocervical specimens using immunodiagnostic methods has several advantages compared to other biomarkers and diagnostic approaches. The specimen is relatively easy to obtain, the biomarkers appear to be stable over some length of time, and the assays can be completed fairly rapidly. The ELISA and tube EIA platforms have generally been found to be more sensitive than RDT immunochromatographic formats, but have the disadvantages of requiring a high level of resources, such as technical expertise, refrigerated reagent storage, specialized equipment and electricity. On the other hand, antigen detection using immunochromatographic RDTs does meet many of the ASURRED criteria. Though their specificity has been shown to be excellent (> 98%), their one deficiency at this time may be their sensitivity. It also remains to be determined how consistently

they will perform in resource-limited settings. The high specificity observed using RDTs would be likely to result in a reduction in inappropriate use of antibiotics in high prevalence disease populations, such as female CSWs in Africa.

2D. Detection of Host-Derived Anti-Bacterial Antibodies Using Immunodiagnostic Approaches

Johnson et al. from the CDC pointed out in their MMWR 2002 recommendation report that the detection of host-derived anti-bacterial antibodies (serology) has limited value in testing for uncomplicated Ct and should not be used for screening[26]. This is because previous chlamydial infection frequently elicits long-lasting antibodies that cannot be easily distinguished from the antibodies produced in a current infection. .While this may be true for developed nations where other biomarkers and approaches are available, one needs to understand the performance of such biomarkers and then place this performance in the context of the needs in resource-limited settings in developing nations. In the early 1980s, Ct antibody assays were adopted to the ELISA plate format and studies demonstrated a good correlation between ELISA results and the then-standard microfluorescence technique, wherein Ct are dried on a slide and detected using human sera for the presence of anti-Ct IgG.[10, 47] These findings were recently confirmed using numerous commercially available EIA kits.[48, 49] Serology assays were also used to identify active infection and it was shown that IgA antibody seropositivity (but not IgG) correlated with Ct culture from endocervical specimens, which is the gold standard method for detecting active infection.[50-53] This led to the hypothesis that anti-Ct IgA might be a dynamic marker for active CT infection. Subsequently, a number of studies have looked at the seroprevalence of IgG, IgM, and IgA anti-Ct antibodies using EIAs that contain a variety of binders (either specific antigen or whole organism-based).[54-56] The results indicated that IgA levels in serum correlate reasonably well with culture positivity using endocervical specimens, and that IgG levels are often found in high risk patients that are culture negative. In a large seroprevalence study, IgG, IgM, and IgA anti-Ct antibody was detected in 89%, 19%, 63%, respectively, of serum samples obtained from high risk female sex workers.[55] However, endocervical specimens from the same subjects demonstrated a true prevalence of infection of 9.6% by LCR. These results demonstrated that for every six to eight patients that test positive by IgA or IgG (respectively) only one is truly infected, demonstrating poor specificity. Although the sensitivity was high for these IgG and IgA tests (83 to 93%), it was very low for the IgM test (17%) compared to LCR. The low level of IgM sensitivity is not surprising in the context of reports that anti Ct IgM levels wane a few weeks after infection. Similar findings have been reported by others using a variety of EIAs that detect host-derived anti-bacterial antibodies.[49] Taken together, these results demonstrate that seroprevalence of different antibody classes does not correlate well with active infection. In the case of IgA, it is a poor indicator of active infection in high-risk women that have been previously treated. The exception is that a positive IgM reaction does correlate with active infection; however the sensitivity of IgM detection is low compared to other methods.

The detection of host-derived antibodies to Ng is hampered by the fact that the organism elicits a weak humoral antibody response. Hedges et al. used an ELISA with a laboratory strain of Ng, as well as the patient's homologous isolates, as antigens to study systemic and local antibody responses in males and females.[18] Even patients with previous Ng infections had only modest anti-Ng IgG and IgA antibody

titers. Their conclusion was that Ng does not elicit a strong humoral immune response during uncomplicated genital disease.

The available evidence indicates that the detection of host-derived anti-bacterial antibodies can be used to identify patients exposed to Ct (i.e. have a previous and/or current infection), but are not useful for distinguishing between an active and a previously treated infection. Existing serology biomarkers and testing approaches have limited utility testing populations that have been previously treated with antibiotics.

2E. Detection of Nucleic Acids Sequences

Nucleic acid testing has revolutionized testing for Ct and Ng in resource-rich settings. Because this approach uses a urine specimen, the sample is much easier to collect and the biomarkers are more stable during transport. In addition, this approach has significantly better sensitivity than culture-based methods, and allows testing for both Ct and Ng to be performed in the same assay using the same specimen.

The first approaches for detecting DNA sequences of Ct and Ng involved probe hybridization technology. In these approaches, a DNA probe with a reporter molecule (such as a chemiluminescent molecule) binds to specific nucleic acid sequences in the genome, and the binding event signals the presence of the target DNA sequences in the sample. Two commercially available assays are widely used, the Gen-Probe PACE test, which detects an rRNA target sequence, and the Digene Hybrid-Capture assay, which detects both a genomic DNA and a cryptic plasmid DNA target.[44,58] Technical expertise is similar to that required for EIAs, and specimens are stable for up to seven days at room temperature after they have been placed in the appropriate transport media. Both tests detect both organisms in a single specimen. Koumans et al. performed a meta-analysis of numerous studies that compared DNA-based Ng test performance to that of culture-based methods, and found a sensitivity of approximately 85%. They concluded that in instances where culture is problematic, this technology provides a reasonable alternative approach for the diagnosis of Ng.[59] The PACE assay detects rRNA sequences, present in very high copy numbers per organism (e.g. 10^4 rRNA copies per Ct EB). This approach of detecting nucleic acid sequences present in high abundance allows for the detection of as few as 10^3 EBs.[31] Compared to culture, sensitivities reported for these nucleic acid-based tests range from 70 to 95% and specificities range from 98 to 100%.[31] In high prevalence populations, sensitivities exceeding 90% have been reported.[60,59]

Soon after the introduction of hybridization-based methods, the next generation of tests emerged which utilize nucleic acid amplification (NAT) strategies. These tests are based upon amplification of the target nucleic acid sequences, using either polymerase chain reaction (PCR), transcription mediated amplification (TMA), ligase chain reaction (LCR), or strand displacement (SD) amplification. These methods can detect many target nucleic acid sequences, use a variety of instrumentation platforms, and have been reviewed previously. The components required for all four of these amplification strategies are commercially available and have been extensively evaluated for the diagnosis of Ng and Ct. Comparative studies and meta-analyses of published data have been performed on these various amplification test methods and it appears that they are even more sensitive than Ng and Ct culture.[61-62,59] Though

endocervical specimens can be used for testing, urine is works as well and is a much more convenient option.[62]

Whitley et al. have recently reported that the genetic diversity observed in Ng creates significant challenges in developing probes and primer pairs for use in both hybridization-based and amplification-based assays.[63] False positive results can occur from the frequent horizontal genetic exchange occurring within the *Neisseria* genus. False negative results can result from some Ng subtypes that either lack or contain modified nucleic acid sequences that are detected by the primers and probes. These concerns lead to the conclusion that a specific nucleic acid based test must be evaluated in any new patient population before it is employed in routine diagnosis.

Nucleic acid biomarkers detected using either hybridization-based or amplification-based approaches have rapidly become the standard of care for diagnosing Ct and Ng in well-resourced laboratories. Both approaches have the advantage of using a specimen type (urine) that is significantly easier to collect properly. The amplification-based approaches have the advantage of being the most sensitive of all the approaches. However, these tests are currently expensive, require technical expertise, specialized equipment, a laboratory environment, and particular care to avoid false positives due to contamination, and are therefore unavailable in most healthcare settings in the developing world.[64, 65] The attributes of these testing approaches clearly do not meet the criteria for an ASURRED test.

A comparison of the currently available biomarkers, diagnostic approaches, and specimen types for diagnosing Ct and Ng infection is presented in Table 2. The ideal test incorporates most of the attributes delineated in the UNICEF/UNDP/World Bank/WHO ASSURED test criteria

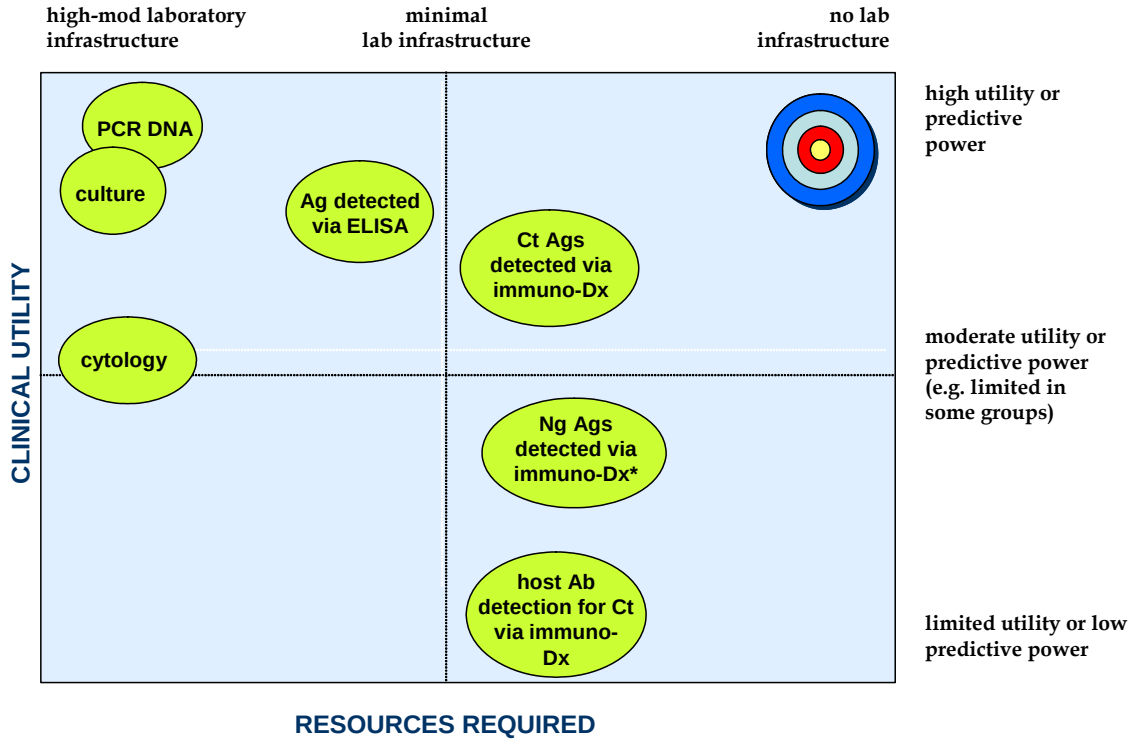
Table 2. Comparison of the Currently Available Diagnostic Approaches for Ct and Ng

Test Class	Test Name	Dx of active infection	Specimen	Resources Required	Time	Ease of use and interpretation	Performance (female urogenital specimens)
The Ideal Test (ASURRED Test Criteria)		Yes	Easily obtained, non-invasive	Disposable, no power required, Refrigerated or room temperature for stockpiling, 18 to 44°C for field use	10 to 15 minutes	Easy, visual, controls included	High sensitivity and specificity
Cytology	gram stain Ng DFA Ct	Yes	Endocervi-cal swab	Microscope, dark room, electricity; 2 to 8°C	30 minutes	Moderate to difficult; Specialized microscope	Ct: sens. 80-90% vs. DFA, spec. 99% Ng: sens. 30-65%, spec. >90%
Culture	Culture	Yes	Endocervic al swab	Highly specialized, transport issues; 2-8°C	Days	Moderate; skilled for interpretati on	Ct: sens. 50-85% vs NAT, spec. >99% Ng: sens. 80-90%, spec. >99%
Serology	Detect host antibo-dies to Ct (no test for Ng)	No	Serum or plasma finger prick	ELISA today; electricity, plate readers, skilled personnel; 2 to 8°C (better if in RDT format)	2 to 3 hours	Moderate: plate read-er, complex data inter-pretation	Ct: sens >98%, spec. varied* Ng: NA**
Immuno-diagnostic Tests	Antigen EIA	Yes	Endocervic al swab	Plate washer, reader, electricity, 2 to 8°C	2 to 4 hrs	Moderate	Ct: sens. 60-93% vs.cult, spec. 98-100% Ng: sens. <80%, spec. >95%
	Antigen-RDT	Yes	Endocervi-cal swab	Minimal supplies, some require heater & specimen prep device	15 to 20 minutes	Moderate	Ct: sens. 52-85% vs. cult, spec. 98-99% Ng: sens: 61-94%, spec. 96-97%***
Nucleic Acid Based Tests	Probe Hybridiza tion	Yes	Endocervic al swab - urine	Many lab supplies, electricity, high resource lab required	Hours	Moderate-Difficult	Ct/Ng combo: sens. 70-95%, spec. 98-100%
	Nucleic acid amplificat ion tests	Yes	Urine	High-resource lab required	Hours	Moderate-Difficult	Ct/Ng combo: sens. 70 to 100% vs. culture, spec. 98-100%

* Specificity varies depends upon population being tested. Since anti-Ct antibodies do not wane after treatment, the specificity of the test to diagnose an active infection in a population of previously treated individuals would be very low. In untreated populations, the specificity would be very high.
** Ng elicits a modest at best immunological response and hence serology is inappropriate.
*** Ct antigen RDTs have been extensively evaluated; Ng antigen RDTs have not been evaluated to the same degree as the Ct antigen RDTs.

The relative merits of the major diagnostic approaches discussed above are presented diagrammatically in Figure 1. In this figure, the ideal approach would be in the upper right quadrant, because of its ability to diagnose an active infection across all stages of disease (termed "broad Dx of active disease" in the figure) and the low level of resources required for successful implementation. In addition, approaches with potential for the broad diagnosis of active disease, but that currently require significant resources, are seen in the upper left quadrants.

Figure 1. Map of Currently Available Biomarkers and Diagnostic Approaches for Ct and Ng



* Clinical Utility might be higher. Sufficient peer-reviewed data is not available.

A significant point to take into consideration in the diagnosis and treatment of all infectious diseases is the identification of resistance to therapeutic options. In the case of Ct, the emergence of drug resistance appears to be relatively insignificant. This is part due to the fact that the chlamydial lifecycle occurs in relative isolation, within epithelial cells, which limits the opportunity for acquisition of antibiotic resistance genes from other organisms.[66] A relatively high frequency of Ng isolates, on the other hand, have been shown to be resistant to penicillin and/or tetracycline due to their ability to exchange plasmids that contain resistance elements.[21-23] The degree to which such isolates exist in developing nations is difficult to estimate, primarily due to the limited use of culture-based methods for diagnosis which are

essential for susceptibility testing (penicillin/tetracycline) or probe hybridization methods in the case of tetracycline.

A variety of test methods have been developed and are in use today to identify the susceptibility or resistance of Ng to specific therapeutic modalities. The first susceptibility method developed, and still in use, is the disk-diffusion susceptibility test, and variations thereof.[32] Disks containing known amounts of an antimicrobial agent are placed on the surface of an agar plate containing a nonselective medium that has been inoculated with a suspension of a strain of Ng to give a confluent lawn of growth. The antimicrobial agent diffuses into the medium, causing a zone of inhibition of growth of the strain around the disk corresponding to the susceptibility of the strain to the agent. Interpretative inhibition zone diameters have been established for susceptibility test results to permit classification of an isolate as being susceptible, intermediate (or exhibiting decreased susceptibility), or resistant to an antimicrobial agent. The problem with this approach is that a culture of the organism is required and hence this approach does not meet ASURRED test criteria. Nucleic acid hybridization and amplification technologies have been employed to identify gene sequences that are associated with antimicrobial resistance.[14, 67, 68] These tests, although promising, have yet to be validated in the clinic and furthermore based upon the testing platform do not meet ASURRED test criteria.

In the absence of the ability to test a specimen for drug resistance, and given that standard treatment regimens are expected to have a cure rate of 95% or more, an alternative approach is to understand the prevalence of regional Ng susceptibility and then use the appropriate therapy.[2,4,69] Such regional susceptibility analyses would need to be performed on a routine basis (e.g. annually) to monitor for rapid changes in susceptibility patterns. In resource-limited settings this is a difficult task. Although technical options are potentially available, , such as using the more "modern" transport systems where the specimen is collected, immediately inoculated, and then placed into a CO_2-rich container for transport, the challenge still remains toe to ensure that the inoculate is not exposed to temperatures greater than 37°C during transport. Furthermore surveillance of this type requires a well equipped microbiology laboratory, a data base, and finally sampling from the appropriate representative populations.

3. Current Deficiencies in the Diagnosis of Ng and Ct in Resource-Limited Settings

Although Ng and Ct can be easily diagnosed in a cost effective manner in clinical settings in high-resource settings, most of the biomarkers and tests used in the diagnostic process are generally inappropriate for resource-limited settings. Features that are desired for a test to diagnose Ng and Ct in resource-limited settings are described below.

1. Easy to perform test with two to three manipulations that can be completed in approximately 15 minutes (ASSURED test criteria). This is a critical issue and must be strongly weighted because technical expertise is often limited in clinical field settings and furthermore, diagnosis must be made during the clinical encounter so that the woman can be treated and enabled with partner notification. Without such notification, a woman could be diagnosed and successfully treated and then re-infected at a later time. In addition, partner notification and subsequent treatment will minimize further transmissions by the partner. The only tests that meet this feature are the RDTs for Ct and Ng antigens that use immunochromatographic technology.

2. An easy to interpret test under field conditions that does not require specialized equipment. The RDTs for Ct and Ng antigens are currently the easiest to interpret, and the only test format that requires minimal training.

3. Insensitive to environmental challenges, including temperature extremes both during reagent storage and testing. All of the currently used tests must be stored in controlled conditions that range from refrigeration to a maximum of 30°C. None of these tests have been validated for use in extreme environmental conditions, including high humidity, elevated temperatures, and dusty atmospheres.

4. Works on a readily available specimen in the priority of urine > finger prick whole blood > serum>vaginal swab > endocervical swab > urethral swab; with the latter two being difficult to obtain in a resource limited setting. Urine has been shown to be an excellent specimen for both Ng and Ct diagnosis using nucleic acid sequence biomarkers. All the other test formats require a specimen that is more difficult to adequately obtain, and require a properly trained health care worker to obtain the specimen.

5. Has reasonable sensitivities and specificities compared to standardized laboratory testing methods. In resource-limited environments, one has to be practical and assume that test performances will not exactly match those obtained with the same specimens tested in an established laboratory setting. Nevertheless, one also needs to consider sensitivity, specificity, positive and negative predictive values in the context of the population being tested. The concept of Gifts "rapid test paradox" must be taken into consideration here.[7] Gift pointed out that field testing with a rapid test that has a relatively lower sensitivity compared to an established test can still result in significantly more patients being treated. This is because with a rapid test the patient can be treated in a single diagnosis-treatment encounter. In separate diagnostic-treatment encounters, a specimen would be obtained from a patient, sent to the lab for testing; only to have the patient never return to receive treatment. It is fair to assume that a test that could be readily deployable and adopted in areas of high disease prevalence, such as Africa, could significantly improve health outcomes; even with a less than ideal sensitivity. In this context, it is important to note that data suggests that the specificity of the existing RDTs is high and this minimizes the potential of over-diagnosis and over-treatment.

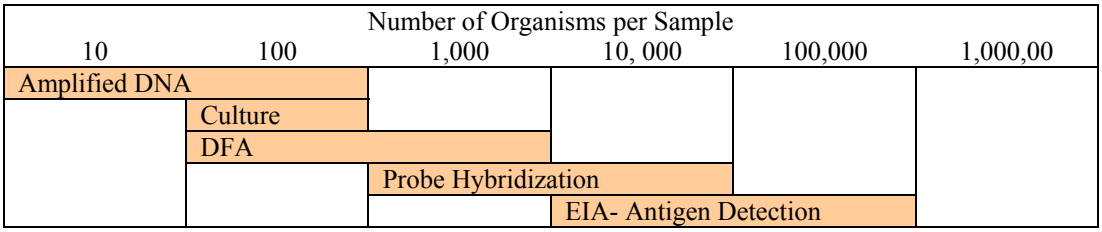
6. Can differentiate between active infection and previously treated infection. The RDTs that detect Ng and Ct antigens are the only test format that can currently provide this information in a resource-limited setting.

7. Can identify active infection early in disease as well as at later stages. The nature of Ng and Ct infection is that the organisms replicate at the site of infection and spread to other urogenital regions during the course of disease progression. Thus all the approaches except the detection of host-derived anti-bacterial antibodies can achieve this goal.

4. Opportunities to Improve the Clinical Performance of Existing Biomarkers

The key to develop a test that meets the ASSURED criteria is to use a diagnostic approach that is easy to implement on a single, easily obtainable specimen type, which detects biomarkers that are present at concentrations that are within the sensitivity range of the analysis system. To put this into perspective, Figure 2 depicts the minimal number of Ct organisms that need to be in a sample to be detected using currently available biomarkers and testing platforms. This minimal number is not the number of organisms in the entire specimen that is collected; but rather it is the number of organisms that must be in the fraction of the sample that is actually tested after it has been prepared. In this figure, the data are in numbers of Ct EBs detected. This table was adapted from Black.[31]

Figure 2. Relative Limits of Detection of Different Technologies Used to Diagnose Ct.



Clearly higher sensitivity is desirable, but practical limitations may limit the ability of a high-sensitivity test to be implemented. In this context, there are two opportunities to improve the clinical utility of existing biomarkers.

4A. Opportunity to Improve the Detection of Bacterial Antigen Biomarkers Detected Using RDT Immunochromatographic Format

The currently available Ct and Ng RDTs need to be evaluated to assess:

1. The minimal number of organisms required to obtain a detectable readout.

2. The impact of using a vaginal swab or urine specimen on test performance. For instance, do substances in the native microbial community (e.g., mucous, epithelial cells, blood cells, etc.) impact test performance?

3. The quality and type of vaginal swab or urine specimen that must be obtained. For example, for detection of a Ct infection, should the specimen be obtained with the idea of maximizing the number of columnar epithelial cells, because the organism resides inside these cells? It will be important to determine the number of organisms that can be reasonably collected in a "typical" specimen.

4. The sensitivity and specificity when a vaginal swab or urine is employed as the specimen type. The idea would be to determine if urine or even a urine concentrate/filtrate contains sufficient organisms for use in RDTs that detect bacterial antigens.

5. The ease by which samples are prepared for analysis

6. The robustness of the RDT products under a variety of conditions, such as high temperatures that are likely to be encountered. It will be important to understand when stability is affected to the point that the product performance falls. The product performance should also be studied to understand the impact of humidity, atmospheric dust, bright sunlight and low light.

Obtaining answers to these questions in a controlled laboratory environment will set up the next series of events. For instance, if the above studies indicate that the test has sufficient sensitivity to detect organisms in a typical specimen, such specimens can be easily obtained, and the RDT products possess reasonable robustness, the conclusion might be to advance the RDTs into field trials. Another scenario might be that the number of organisms is sufficient but that the RDT device is not stable under conditions typically found in a resource-limited environment. This could invoke a re-formatting of the device using stabilizers, new chemistries, or even new antibodies as binders to detect the antigens. On the other hand, if the number of organisms in a typical specimen is too low for the sensitivity of the testing system, either the sensitivity of the system or, new specimen processing devices could be considered. To improve sensitivity, some of the signal amplification approaches that have already been applied to plate-based EIAs might be considered.[36] In the case of a new specimen processing device, the concept would be to develop a device that is used to concentrate the collected specimen into a testing sample that is used in conjunction with the RDT device. This idea could involve: Placing the vaginal swab or urine specimen into a tube of a few mL of saline, pouring the saline solution into a conical device that contains a filter on one end, allowing the solution to flow through the device (fed by gravity or a small battery operated vacuum pump), and collecting the organisms on the filter either by size or immunotrapping on microparticles. Once collected, a few drops of a lysing solution that would disrupt cells and bacteria could be applied to the device, and the resultant sample could be collected for immediate application to the RDT device.

Although it is tempting to advance these RDT devices into field trials, significant information needs to be obtained first in a controlled laboratory setting. In such a setting, variables that are encountered in resource-limited field testing can be mimicked and tested. This type of evaluation will allow for an assessment of the potential success of field trials and provide guidance in the strategic decision-making processes.

On final point to consider is that studies have demonstrated that Ct and Ng are present as co-infections in a high percentage of high risk women.[3] Thus one alternative would be to focus on the easiest test and introduce it into resource-limited field testing. If this test was for Ct, for example, a significant number of Ng infections would be detected using Ct as a surrogate. In this case the use of broad spectrum antibiotics would be required in treatment regimens. Although this approach would not be considered acceptable for screening and treatment in high-resource settings, it is an approach that could potentially have a significant impact on decreasing both Ng and Ct infections. Of note, co-infection of HIV, Ng, and Ct is common in high risk females in Africa, and it is believe that Ng and or Ct infection may enhance susceptibility to infection with HIV.[3] Thus, in high risk women recently diagnosed with HIV, asymptomatic co-infection with Ng and/or Ct should be evaluated.

4B. Opportunity to Implement a Field-Ready, Easy-to-Use Nucleic Acid-Based Test Platform

As described earlier, tests that detect nucleic-acid biomarkers are not ready for deployment in resource-limited settings. One approach is to make these testing platforms more suitable to resource-limited settings. Others have attempted this and the best system today for such applications is marketed by Cepheid. Even this single-use, disposable cartridge PCR-based platform is not close to meeting ASURRED criteria.[70] Regardless, if an appropriate platform was available, it will be required to stabilize reagents for use in resource limited settings and to adapt existing DNA testing methods to the platform for diagnosis of Ng and Ct in a combination test using urine as a specimen.

5. Known Biomarkers That Have Not Yet Been Clinically Validated

Opportunities exist to validate other known molecules for Ct and Ng that have not yet been evaluated for their utility as biomarkers of infection. One example of this would be a combination RDT device for Ct and Ng antigen detection. The goal would be develop a system that could detect the presence of Ng and/or Ct in the same device. This would minimize cost as well as testing time and resources. Another approach would be to test an alternative specimen for the presence of Ng or Ct antigens. As suggested in Section 4, it would be important to understand the number of Ct and Ng organisms that are found in urine, possibly first void urine. Urine is a specimen of choice for Ng and Ct testing using NAT-based methods, and male urine has already been shown to be a suitable specimen for antigen testing. Therefore it will be important to know if there are detectable levels of biomarkers for these bacteria in female urine. Are the levels a function of disease spread (e.g. from endocervix to urethra)? If it turns out that the levels are relatively low, it could be possible to design and develop a simple gravity fed device that collects and concentrates epithelial cells and organisms from urine. The use of urine as a specimen would make specimen collection easier, potentially avoid social and cultural issues, as well as provide a very "clean" sample for use in the RDT antigen device.

There is a fundamental need to conduct clinical studies to validate these combinations of specimen types and biomarkers. There is no need to conduct these studies at the remote sites of eventual test use; because the development and evaluation studies can take place in any standard clinical laboratory with sophisticated capabilities. This would allow for an immediate and thorough validation of the approach, including identification of strengths, weaknesses, and determination of key performance criteria such as sensitivity and specificity. This study is especially important to perform because broad and deep analysis of Ng and Ct antigen variability has not been performed on isolates obtained from developing nations. Whiley et al. recently pointed out that Ng has significant genetic variability across subtypes and this variability can impact NAT performance.[63] It is thus conceivable that some subtypes may show diminished binding to antibodies, especially monoclonal antibodies that were developed for antigen detection and validated using bacterial strains that are found in resource-rich settings.

6. Approaches for the Discovery of Novel Biomarkers for Active Ct and Ng Diagnosis

6A. Opportunity for Advances in Ct Serology

Due to its ease of use and broad adoption on RDT platforms for a variety of infectious agents (including HIV and syphilis) serology remains an intriguing approach for the diagnosis of Ct (immune response is

nominal for Ng). There is precedence for its utility in RDTs. The problem is that the available evidence indicates that IgG and IgA antibody levels do not sufficiently differentiate active from previously treated infection. These findings may reflect more the way the studies were performed than the biology. Most studies have used either whole organisms or antigens shown to be the most immunogenic. The humoral immune response is complex and produces a plethora of IgG, IgA, IgE, IgM and other classes of immunoglobulins that are directed against a variety of the pathogen's proteins, lipopolysaccharides, and other structures at different times after infection. It is conceivable that some of these structures elicit relatively weak humoral immune responses and that these responses wane once the pathogen is eradicated from the host. Straightforward experiments could be performed using gel electrophoresis followed by Western immunoblotting using whole Ct as an antigen and pre/post treatment human sera. Since the Ct genome has been sequenced and open reading frames identified, individual antigen preparations could be made as well and tested in a dot blot, micro bead, or ELISA type of format. Alternatively, different classes of lipopolysaccharides could be isolated and used as test antigens. The idea would be to identify antigens that react with pre-therapy sera but are non-reactive post therapy (3 to 9 months post therapy). If found, such antigens could then be formatted into ELISA tests and large panels of sera could be tested for the antigens' ability to differentiate active from previous infections. Another similar approach could be to use vaginal swab material as the specimen, instead of serum, and test for the presence of antigen-specific immunoglobulins, including secretory IgA and inflammatory IgE classes. This approach would be multi-pronged, starting at discovery, leading to clinical validation and finally adoption of the antigens to a rapid test RDT format.

6B. Opportunity for Markers of Systemic or Localized Immune Activation

Another approach to differentiate active from previous infection might be the use of a test for immune modulators or inflammatory agents as a combination test for serology. The idea would be to test a patient that has a positive Ng/Ct serology result using another companion test that measures the presence of elevated cytokines, for example. Reports in the literature are conflicting on the levels of cytokines in urogenital secretions obtained from females infected with Ct and/or Ng.[18, 71] It is conceivable that a local inflammatory response might induce hyper production of cytokines in urogenital regions. Immunoassays are commercially available from many vendors in an ELISA format for the quantification of cytokines in numerous types of specimens. These assays could be used in discovery programs using well-annotated specimens, which are readily available in highly endemic areas. The process used for such discovery might be as follows:

1. Literature search to identify candidate cytokines.

2. Assembly of specimens representing all stages of infection, including those successfully cured.

3. Perform complete Ct and Ng serology work-up and correlate with clinical information available for the specimens.

4. Using the urogenital specimens and candidate cytokines, perform the appropriate assays.

5. Perform bioinformatic analysis of the resultant data. It will be important to note the quantitative levels of the individual cytokines in the context of the sensitivity expected field testing device (i.e. make sure that quantitative cytokine levels can be readily measured in the test device).

In the data analysis it will be important to identify potential cytokine biomarkers that are not only significantly different (statistically) in the study populations but also provide useful information for the management of individual patients in the target population. Any biomarkers identified will require additional validation in prospective studies using the intent-to-treat population.

7. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

As in any study, it is of utmost importance to collect specimens from the correct study populations and ensure that integrity is maintained. In Africa, for instance, the prevalence of Ct and Ng ranges up to 20 to 60% in female CSWs, thus there appears to be an ample number of subjects for specimen collection in an environment where they could be categorized and frozen. To perform the cytokine based discovery described above, the numbers of subjects in each category does not need to be large (approximately 100 to 200 for each disease) because the analysis will look for strong trends, not small trends that are slightly statistically significant. Serology would need to be performed on all specimens as well as a NAT test to rule in active infection. If a study was to evaluate 15 different candidate cytokine markers, and specimens were to be tested in duplicate, and assuming that 96-well ELISA plates were used according to SOPs, approximately 200 ELISA plates would be required.

8. Discussion and Recommendations for the Improvement of Diagnostics for *Chlamydia trachomatis* and *Neisseria gonorrhea* Infection in High-Risk Women

The following recommendations are presented for consideration, based upon the deficiencies of current diagnostic test methods for deployment in resource-limited settings and the opportunities for improving the deployment of existing biomarkers.

8A. What clinical information and user specifications are required for the design and development of the diagnostic products needed for Ng and Ct?

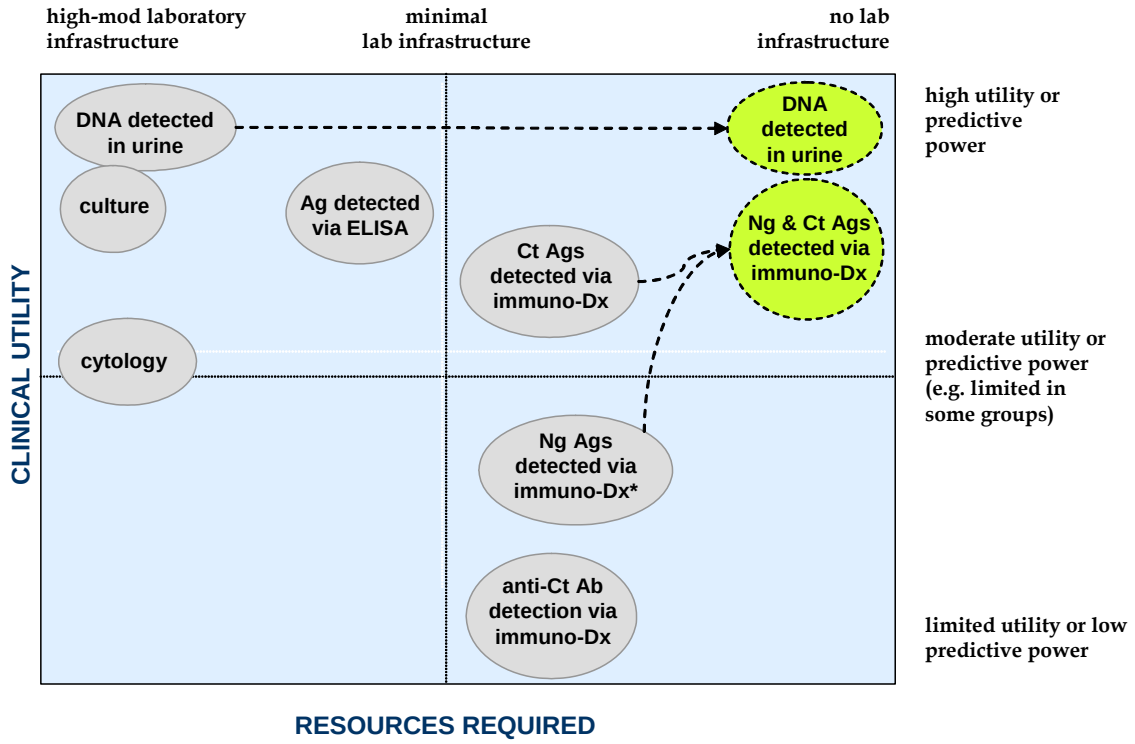
The assay should be able to identify both Ng and Ct with a reasonable level of sensitivity (approximately 85%, or greater) and specificity (approximately 90%, or greater) from a single, easily obtained specimen. The test needs to use a format that is easy to perform in resource limited settings.

8B. What biomarkers, sample types, and technologies are most appropriate for Ng and Ct?

The most likely candidates for practical specimen types are vaginal swab or urine. These specimens have been shown to be appropriate for nucleic acid (pathogen DNA) based tests but have not been validated for use in immunoassay based antigen detection technologies. Antigen based technologies have been developed for point of care settings as RSTs and are most appropriate for Ng/Ct diagnosis; however, reagent stability is probably an issue. Nucleic acid test platforms are more challenging. Endocervical specimens are inappropriate due to their potential difficulty of collection in a resource limited setting.

The future diagnostic tests for Ng/Ct in resource-limited settings are depicted in Figure 3. In this Figure, the current approaches are shown as light gray spheres and the future approaches are shown in color. The position of each sphere on the graph illustrates the resource requirements (x-axis) of the test method and the clinical utility of the test in a resource-limited setting (y-axis). In this Figure, therefore, an ideal biomarker and test method will be in the upper right quadrant, with a high predictive power and low resource requirements.

Figure 3. Future Approaches for Diagnosing Ng and Ct, and Their Utility in Resource-Limited Settings.



8C. Recommended Course of Action and Resources Required

There are several potential sets of activities that would be required to develop the future diagnostic tests for Ng and Ct.

One of the first steps in the development of a test for Ng/Ct will be to fill in the gaps in knowledge about the levels of Ng and Ct antigens in vaginal swab and urine specimens. Once the levels of such antigens are established, a variety of RDTs can be tested for requisite sensitivity. It may turn out that a specific level of antigen concentration is required (especially in the case of urine) to match RDT performance or clinical performance parameters. If these studies demonstrate that antigen detection will not meet the minimum performance needs then a pathogen DNA based test will be required. This will necessitate the development of a nucleic acid based detection systems that is appropriate for resource limited settings.

Potential Path Forward 1: Detection of Ng and Ct antigen using RDTs

Before moving RSTs forward into clinical evaluation it will be imperative to understand the level of Ng and Ct antigens in urine and/or vaginal swab specimens, to understand the robustness of RDTs themselves, and to determine the clinical utility of the presence or quantification of the antigens. Understanding the level of antigen in specimens can be performed in a straightforward manner using prospectively collected matched specimens. Such specimens can be collected in an urban setting where disease prevalence is high (such as an STD clinic in Africa) and evaluated either in an appropriately equipped laboratory near the collection site or frozen or otherwise stored for subsequent shipping and testing. This information will be used to elucidate the sensitivity and specificity required and to establish assay performance specifications. In the case of urine or even vaginal swab specimens, a concentration step may be required. This can be tested via centrifugation or other methods of specimen preparation in the laboratory. There are four potential outcomes from this analysis: 1. There is(are) sufficient quantity of a key antigen(s) in the specimen; 2. There is(are) sufficient antigen(s), but concentration is required; 3. There is marginally insufficient antigen present; and 4.The antigen(s) are not sufficiently informative.

In parallel with understanding the level of antigen in specimens, the RDTs must be evaluated for robustness and stability under conditions that mimic those encountered in resource limited settings. This can be done in an established laboratory using repository specimens. It is essential that user specifications for testing and test kit storage be rigorously established and accurately reflect those that will be encountered in the field. If they (separate kits for Ng and Ct) pass this evaluation then the existing kits are considered ready for field trials. If they fail, then the root cause for failure needs to be understood and the RDTs need to be re-developed. If an RDT for Ng or Ct passes and the other fails, then both should be re-engineered into a single combination Ng/Ct RDT. It may be that the RDT passes robustness testing, but the level of antigen in the specimen is marginal to achieve desired sensitivity (#3 in the preceding paragraph). In this case the RTD(s) should be re-engineered to achieve requisite sensitivity.

If a sufficient level of antigen can be demonstrated in a vaginal swab or urine specimen and a robust RDT is documented then they should be advanced to field trials. Such trials will involve low risk because potential causes of failure have been identified and mitigated. If there is a need to antigen concentration from urine or vaginal swab specimens, then such a concentration device will need to be developed and validated under appropriate user specifications. Finally if the results demonstrate that there is insufficient antigen in a specimen for use with a RDT, even with concentration, then potential path forward #2 will be required.

Potential Path Forward 2: Adoption of DNA Testing to a Rapid, Robust Platform

A global impact would be made with a system that could easily detect genomic Ng and Ct DNA in urine using a simple inexpensive system. Significant effort will be required to develop a DNA amplification and/or detection device that parallels the convenience, robustness, and costs of lateral flow RDT testing devices. However, if developed, such a device would prove invaluable, not only to addressing limitations of Ct and Ng diagnosis, but on a grander scale by providing a means for the diagnosis of many other

pathogens using urine or oral fluid specimens. Designing and developing such a DNA analysis device may be an expensive and multi-year, project, involving multi-disciplinary teams with broad skill sets.

If such a system were available, it would be straightforward to adapt the nucleic acid amplification tests used today with urine specimens (standard of care in developed nations). This would involve validating pathogen genome sequences being amplified and detected, demonstrating stability of testing reagents under conditions that are appropriate for resource limited settings. In one sense it might be easier to develop a new test system from scratch against well defined user specifications vs. modifying existing reagent kits to meet such specifications.

Summary of Recommendations

In summary, we recommend that the gaps in knowledge about the stability of existing RDT systems be fulfilled. We also recommend a detailed study be conducted to understand the relationship between the levels of Ng/Ct in urine and vaginal swab specimens. Improvements in RDT kit robustness as well as development of a specimen concentration procedure may be required. These activities should be performed in a parallel manner to understand the probability of existing RDTs meeting the performance criteria required in resource-limited environments. If these kits have a low probability of success in field trials, even after modifications, then efforts should be focused on developing a robust nucleic acid diagnostic platform and reagent systems for use in resource limited settings.

References

1. Centers for Disease Control. Sexually Transmitted Diseases. Clinical practice guidelines. Atlanta.
2. www.who.int.
3. Mati, J. STDs/AIDS and Reprodructive Health in Sub-Saharan Africa. www.reproline.jhu.edu/english/4morerh/4std/stdproc-c.htm
4. Tapsall, J. Antimicrobial resistance in neisseria gonorrhoeae. http://www.who.int/csr/resources/publications/drugresist/WHO_CDS_CSR_DRS_2001_3/en/ (2001).
5. Dallabetta, G., Gerbase AC, and Holmes, KK. . Problems, solutions and challenges in syndromic management of sexually transmitted diseases. Sex Transm Infect 74, S29-33 (1998).
6. Mabey, D. a. P., R. Rapid diagnostic tests for sexually transmitted infections. Internation Planned Parenthood Federation www.ippf.org (2002).
7. Gift, T. L., Pate, M. S., Hook, E. W., 3rd & Kassler, W. J. The rapid test paradox: when fewer cases detected lead to more cases treated: a decision analysis of tests for Chlamydia trachomatis. Sex Transm Dis 26, 232-40 (1999).
8. Vickerman, P., Peeling, R. W., Watts, C. & Mabey, D. Detection of gonococcal infection : pros and cons of a rapid test. Mol Diagn 9, 175-9 (2005).
9. Schachter, J. a. S., W. Chlamydia (ed. Murray, P.) (ASM Press, Wash DC, 1995).
10. Finn, M. P., Ohlin, A. & Schachter, J. Enzyme-linked immunosorbent assay for immunoglobulin G and M antibodies to Chlamydia trachomatis in human sera. J Clin Microbiol 17, 848-52 (1983).
11. Grayston, J. T. & Wang, S. New knowledge of chlamydiae and the diseases they cause. J Infect Dis 132, 87-105 (1975).

12. Grosskurth, H. et al. Impact of improved treatment of sexually transmitted diseases on HIV infection in rural Tanzania: randomised controlled trial. Lancet 346, 530-6 (1995).
13. Sturm-Ramirez, K. et al. Molecular epidemiology of genital Chlamydia trachomatis infection in high-risk women in Senegal, West Africa. J Clin Microbiol 38, 138-45 (2000).
14. Sanchez-Pescador, R., Stempien, M. S. & Urdea, M. S. Rapid chemiluminescent nucleic acid assays for detection of TEM-1 beta-lactamase-mediated penicillin resistance in Neisseria gonorrhoeae and other bacteria. J Clin Microbiol 26, 1934-8 (1988).
15. Stephens, R. S. et al. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 282, 754-9 (1998).
16. Tobiason, D. a. S., H.S. The obligate human pathogen, Neisseria gonorrjoeae is polyploid. PLOS Biology 4 (2006).
17. http://microgen.oufse.edu.
18. Hedges, S. R., Mayo, M. S., Mestecky, J., Hook, E. W., 3rd & Russell, M. W. Limited local and systemic antibody responses to Neisseria gonorrhoeae during uncomplicated genital infections. Infect Immun 67, 3937-46 (1999).
19. Mulks, M. a. P., A. . IgA protease production as a characteristic distibguishing pathogenic from harmless neisseriacae. N.E J.Med 299, 973-76 (1978).
20. McGee, Z. A. et al. Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. Microb Pathog 12, 333-41 (1992).
21. Pham-Kanter GB, S. M. a. B. R. Sexually transmitted diseases in South Africa. Genitourin Med 73, 76
(1997).
22. Knapp, J. S. Neisseria gonorrhoeae resistant to ciprofloxacin and ofloxacin. Sex Transm Dis 25, 425-6 (1998).
23. Lind, I. Antimicrobial resistance in Neisseria gonorrhoeae. Clin Infect Dis 24 Suppl 1, S93-7 (1997).
24. Robinson, A. J. & Ridgway, G. L. Concurrent gonococcal and chlamydial infection: how best to treat. Drugs 59, 801-13 (2000).
25. Somani, J., Bhullar, V. B., Workowski, K. A., Farshy, C. E. & Black, C. M. Multiple drug-resistant Chlamydia trachomatis associated with clinical treatment failure. J Infect Dis 181, 1421-7 (2000).
26. Johnson, R., Newhall, WJ, Papp, JR et al Screening tests to detect chlamydia trachomatis and neisseria gonorrhoeae infections. MMWR Recommendations and reports 51, 1-27 (2002).
27. Hook, E. W. a. H., H.H. Gonoccoccal infections in the adult (ed. Holmes, K. K., Sparling, P.F, Mardh, P-A, et al.) (McGraw-Hill, New York, NY, 1999).
28. Inhorn, S. L. et al. Chlamydia trachomatis and Pap testing from a single, fluid-based sample. A multicenter study. J Reprod Med 46, 237-42 (2001).
29. Lentrichia, B. B., Hecht, S. S., Lapen, D. & Corkill, M. K. Potential for routine concurrent determination of chlamydia and cervical abnormalities by single fluid-based sampling. Prim. Care Update Ob Gyns 5, 149-150 (1998).
30. Szarewski, A. et al. Use of the Cytobrush for concurrent cytology and chlamydia sampling: a comparison of immunofluorescence and culture for detection of chlamydia. Int J STD AIDS 2, 367-8 (1991).
31. Black, C. M. Current methods of laboratory diagnosis of Chlamydia trachomatis infections. Clin Microbiol Rev 10, 160-84 (1997).
32. Knapp, J. a. R., R. Neisseria and Branhamella (ed. Murray, P.) (ASM Press, Wash DC, 1995).
33. Lewis, D. A., Forster, G. E. & Goh, B. T. The falling accuracy of microscopy in the diagnosis of gonorrhea--a cause for concern? Genitourin Med 71, 136 (1995).
34. Myziuk, L., Romanowski, B. & Brown, M. Endocervical Gram stain smears and their usefulness in the diagnosis of Chlamydia trachomatis. Sex Transm Infect 77, 103-6 (2001).
35. Notomi, T., Okadome, A., Ariyoshi, A. & Nagayama, A. [Basic evaluation of Chlamydia antigen detection by EIA using a dual amplification enhanced immunoassay method]. Kansenshogaku Zasshi 72, 45-53 (1998).

36. Okadome, A., Notomi, T., Nomura, S. & Nagayama, A. Reactivity of a dual amplified chlamydia immunoassay with different serovars of Chlamydia trachomatis. Int J STD AIDS 10, 460-3 (1999).
37. Chernesky, M. et al. Comparison of a polymer conjugate-enhanced enzyme immunoassay to ligase chain reaction for diagnosis of Chlamydia trachomatis in endocervical swabs. J Clin Microbiol 39, 2306-7 (2001).
38. Tanaka, M. et al. Evaluation of a new amplified enzyme immunoassay (EIA) for the detection of Chlamydia trachomatis in male urine, female endocervical swab, and patient obtained vaginal swab specimens. J Clin Pathol 53, 350-4 (2000).
39. Tanaka, M. et al. Detection of Chlamydia trachomatis in vaginal specimens from female commercial sex workers using a new improved enzyme immunoassay. Sex Transm Infect 74, 435-8 (1998).
40. Schachter, J. et al. Enzyme immunoassay for diagnosis of gonorrhea. J Clin Microbiol 19, 57-9 (1984).
41. Blanding, J. et al. Comparison of the Clearview Chlamydia, the PACE 2 assay, and culture for detection of Chlamydia trachomatis from cervical specimens in a low-prevalence population. J Clin Microbiol 31, 1622-5 (1993).
42. Ferris, D. G. & Martin, W. H. A comparison of three rapid chlamydial tests in pregnant and nonpregnant women. J Fam Pract 34, 593-7 (1992).
43. Grossman, J. H., 3rd, Rivlin, M. E. & Morrison, J. C. Diagnosis of chlamydial infection in pregnant women using the Testpack Chlamydia diagnostic kit. Obstet Gynecol 77, 801-3 (1991).
44. Kluytmans, J. A. et al. Performance of a nonisotopic DNA probe for detection of Chlamydia trachomatis in urogenital specimens. J Clin Microbiol 29, 2685-9 (1991).
45. www.path.org.
46. Suzuki, K. et al. Evaluation of a rapid antigen detection test for Neisseria gonorrhoeae in urine sediment for diagnosis of gonococcal urethritis in males. J Infect Chemother 10, 208-11 (2004).
47. Evans, R. T. & Taylor-Robinson, D. Development and evaluation of an enzyme-linked immunosorbent assay (ELISA), using chlamydial group antigen, to detect antibodies, to Chlamydia trachomatis. J Clin Pathol 35, 1122-8 (1982).
48. Bax, C. J. et al. Comparison of serological assays for detection of Chlamydia trachomatis antibodies in different groups of obstetrical and gynecological patients. Clin Diagn Lab Immunol 10, 174-6 (2003).
49. Jones, C. S., Maple, P. A., Andrews, N. J., Paul, I. D. & Caul, E. O. Measurement of IgG antibodies to Chlamydia trachomatis by commercial enzyme immunoassays and immunofluorescence in sera from pregnant women and patients with infertility, pelvic inflammatory disease, ectopic pregnancy, and laboratory diagnosed Chlamydia psittaci/Chlamydia pneumoniae infection. J Clin Pathol 56, 225-9 (2003).
50. Samra, Z. & Soffer, Y. IgA antichlamydia antibodies as a diagnostic tool for monitoring of active chlamydial infection. Eur J Epidemiol 8, 882-4 (1992).
51. Cevenini, R. et al. Serum specific IgA antibody to Chlamydia trachomatis in patients with chlamydial infections detected by ELISA and an immunofluorescence test. J Clin Pathol 37, 686-91 (1984).
52. Clad, A., Freidank, H., Plunnecke, J., Jung, B. & Petersen, E. E. Chlamydia trachomatis species specific serology: ImmunoComb Chlamydia bivalent versus microimmunofluorescence (MIF). Infection 22, 165-73 (1994).
53. Hanuka, N., Glasner, M. & Sarov, I. Detection of IgG and IgA antibodies to Chlamydia trachomatis in sera of patients with chlamydial infections: use of immunoblotting and immunoperoxidase assays. Sex Transm Dis 15, 93-9 (1988).
54. Morre, S. A. et al. Comparison of three commercially available peptide-based immunoglobulin G (IgG) and IgA assays to microimmunofluorescence assay for detection of Chlamydia trachomatis antibodies. J Clin Microbiol 40, 584-7 (2002).
55. Rabenau, H. F., Kohler, E., Peters, M., Doerr, H. W. & Weber, B. Low correlation of serology with detection of Chlamydia trachomatis by ligase chain reaction and antigen EIA. Infection 28, 97-102 (2000).

56. Theunissen, J. J. et al. Detection of IgG, IgM and IgA antibodies in patients with uncomplicated Chlamydia trachomatis infection: a comparison between enzyme linked immunofluorescent assay and isolation in cell culture. Int J STD AIDS 4, 43-8 (1993).
57. Mahony, J. B., Chernesky, M. A., Bromberg, K. & Schachter, J. Accuracy of immunoglobulin M immunoassay for diagnosis of chlamydial infections in infants and adults. J Clin Microbiol 24, 731-5 (1986).
58. Modarress, K. J. et al. Detection of Chlamydia trachomatis and Neisseria gonorrhoeae in swab specimens by the Hybrid Capture II and PACE 2 nucleic acid probe tests. Sex Transm Dis 26, 303-8 (1999).
59. Koumans, E. H., Johnson, R. E., Knapp, J. S. & St Louis, M. E. Laboratory testing for Neisseria gonorrhoeae by recently introduced nonculture tests: a performance review with clinical and public health considerations. Clin Infect Dis 27, 1171-80 (1998).
60. Melton m, H. m., Pawlowicz D. in 95 general meeting of the americal society for microbiology 138 (american society for microbiology, 1995).
61. Cook, R. L., Hutchison, S. L., Ostergaard, L., Braithwaite, R. S. & Ness, R. B. Systematic review: noninvasive testing for Chlamydia trachomatis and Neisseria gonorrhoeae. Ann Intern Med 142, 914-25 (2005).
62. Johnson, S. J., Green, D. H., Reed, D. A. & Wood, L. S. Semiautomation of nucleic acid-based assays for Chlamydia trachomatis and Neisseria gonorrhoeae. Clin Chem 47, 760-3 (2001).
63. Whiley, D. M., Tapsall, J. W. & Sloots, T. P. Nucleic acid amplification testing for Neisseria gonorrhoeae: an ongoing challenge. J Mol Diagn 8, 3-15 (2006).
64. Archibald, L. K. & Reller, L. B. Clinical microbiology in developing countries. Emerg Infect Dis 7, 302-5 (2001).
65. Mabey, D., Peeling, R. W., Ustianowski, A. & Perkins, M. D. Diagnostics for the developing world. Nat Rev Microbiol 2, 231-40 (2004).
66. Binet, R. & Maurelli, A. T. Frequency of spontaneous mutations that confer antibiotic resistance in Chlamydia spp. Antimicrob Agents Chemother 49, 2865-73 (2005).
67. Giles, J. et al. Use of applied biosystems 7900HT sequence detection system and Taqman assay for detection of quinolone-resistant Neisseria gonorrhoeae. J Clin Microbiol 42, 3281-3 (2004).
68. Vernel-Pauillac, F., Falcot, V., Whiley, D. & Merien, F. Rapid detection of a chromosomally mediated penicillin resistance-associated ponA mutation in Neisseria gonorrhoeae using a real-time PCR assay. FEMS Microbiol Lett 255, 66-74 (2006).
69. Roy, K., Wang, S. A. & Meltzer, M. I. Optimizing treatment of antimicrobial-resistant Neisseria gonorrhoeae. Emerg Infect Dis 11, 1265-73 (2005).
70. Kolberg J., Penny L., Todd J. & M. Urdea. An Assessment of the Technological Issues and Options for Point-Of-Care Diagnostic Tests in Resource-Limited Settings; Report presented by Halteres Associates to the Bill and Melinda Gates Foundation. (2005).
71. Cohen, C. R. et al. Increased interleukin-10 in the endocervical secretions of women with non-ulcerative sexually transmitted diseases: a mechanism for enhanced HIV-1 transmission? Aids 13, 327-32 (1999).

