Sexually Transmitted Diseases

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Methods and Protocols

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Cover illustration: Chlamydial inclusion being exuded from host cell. Scaning electron micrograph supplied by R. W. Peeling.

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Preface

In recent years, molecular techniques have enhanced our ability to detect sexually transmitted infections and to conduct research to further our understanding of sexually transmitted diseases. Molecular methods to quantitate pathogen load have also been shown to be useful for the management of HIV and other viral STDs. Existing laboratory manuals for the clinical microbiology laboratory often do not include molecular methods for STDs. *Sexually Transmitted Diseases: Methods and Protocols* is intended to fill the need for a dedicated manual that covers all the fundamental aspects of molecular protocols for laboratory diagnosis, as well as research methodology for STDs, including HIV.

There are more than 12 types of molecular techniques described in this book covering nine major sexually transmitted pathogens. Although molecular methods for the detection of such pathogens as *Trichomonas vaginalis* are available in the published literature, they have not been included since they are not yet widely used for laboratory diagnosis or research.

Sexually Transmitted Diseases: Methods and Protocols is one of a series of books treating *Methods in Molecular Medicine*, published by Humana Press. It is intended as a stand-alone laboratory manual that will not require reference to any other sources. When a reagent or product for any protocol requires a unique source, the manufacturer is cited in the text. Unique features of books in this series are such regular elements as a "Notes" section at the end of each chapter that provides hands-on information on pitfalls to avoid, tips for problem solving, alternative strategies, along with other practical information that has accumulated during the authors' years of experience with molecular techniques. Such valuable information and insight are seldom found in journal articles or other publications.

Sexually Transmitted Diseases: Methods and Protocols should be of interest not only to clinical microbiologists who are new to molecular techniques, but also useful for laboratory scientists with an interest in STD/HIV research. Given the enormous psychological implications of a positive diagnosis for a sexually transmitted infection, laboratories currently employing commercial kits for routine detection of sexually transmitted pathogens can use methods described in this book to independently confirm positive results, and for qual-

ity assurance in general. Methods suitable for field studies described in this book may be of use for studies in developing countries. Duplex and multiplex PCR methods designed as panels for STD syndromes can be a cost-effective means of monitoring disease prevalence and validating algorithms for syndromic management, an important STD control strategy in developing countries.

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I

Overview

1

The Impact of Molecular Technology on STD Control

A Historical Perspective

P. Frederick Sparling

1. Introduction

1.1. The Way it Was

Sexually transmitted diseases have afflicted humankind for millennia, based on references to apparent gonorrhea or nongonococcal urethritis in the Old Testament (Leviticus). For most of history there has been no means of specific diagnosis, and clinical diagnosis of syndromes was fraught with error. Usually, this made no difference because there was no specific therapy and no means of prevention other than abstinence or monogamy, which was slightly effective at best (witness the very high prevalence of syphilis in much of Europe and the USA before advent of specific therapy, approaching 10% in many populations and 25% in some). Occasionally, syndromic diagnosis did cause serious consequences. If we could talk with John Hunter today, he certainly would bemoan the absence in his time of specific diagnostic tests for gonorrhea and syphilis. Had he had access to such tests, he certainly would not have inoculated himself with urethral exudate from a patient with gonorrhea and subclinical syphilis, resulting in the acquisition of both gonorrhea and syphilis *(1)*! Not only did he suffer from both diseases, but he also understandably but incorrectly concluded that both diseases had the same etiology, which held back the entire field until the discovery that *Neisseria gonorrhoeae* and *Treponema pallidum* were separate causes of the very distinctive diseases.

The understanding of specific etiologies of STDs and all infectious diseases required new technology. First was the visualization of bacteria by Gram's stain, and then culture of bacteria in vitro. Thus, in 1879 Neisser was able to visualize the organism that carries his name and correctly identified it as the

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cause of gonorrhea. Shortly thereafter, in 1882, it was cultured in vitro by Leistikow and Loeffler. Roughly concurrent were the discoveries of virulent *T pallidum* by inoculation of chimpanzees, visualization of *T. pallidum* in lesions by dark field microscopy, and development of useful if not specific serologic tests for syphilis (by Metchnikoff, Schaudinn, and Wassermann respectively). These late 19th century discoveries were revolutionary, and paved the way to our modern understanding of the epidemiology, transmission, and accurate diagnosis of these classic STDs. Once effective therapy became available about half a century later (discounting the arsenicals which were not effective enough to have a major impact on syphilis prevalence), reasonably accurate diagnostic tests helped immeasurably in finding and then treating asymptomatic cases of both gonorrhea and syphilis. This lead to dramatic declines in incidence and prevalence of both diseases after World War II in most of the world, and even more dramatic declines in the late complications of syphilis in particular.

Relative control of the two major STDs (gonorrhea and syphilis) had been achieved in the USA by the 1950s. Curiously absent, however, was discussion or concern over other STDs that we now recognize as of equal or greater significance. The problem was that the technological revolution had stalled, and had not gone far enough. There were bacteria that were very common indeed that did not grow on usual agar media, and did not stain by Gram's stain: the chlamydia. There were viruses to be discovered that were either clinically unknown or insufficiently appreciated on clinical grounds alone: human genital wart viruses, genital herpes viruses and many more. Discovery of their importance was only possible with development of cell culture technology for viruses and obligate intracellular bacteria such as the chlamydia. Development of electron microscopy, and especially in the 1980s of multiple molecular biology techniques as well as the expanded deployment of the still new technology of monoclonal antibody production, created a second and still ongoing revolution in our ability to detect, and therefore to understand, a variety of old and "new" STDs.

Understanding how far we have come in the past few decades may be illustrated by recollection of a formative experience in this author's training. During a three week course on venereology for new US Public Health Service officers assigned to the Venereal Disease Research Laboratories in 1964, there was little mention of Herpes simplex infections, and only the briefest discussion of "venereal" warts, which were discussed principally in terms of their differentiation from the secondary lesions of mucosal syphilis. There was actually more discussion about staphylococcal infections than there was about the complications of human papilloma virus infections, for the simple reason that there was yet no evidence that these common sexually transmitted agents are

the leading cause of cervical cancer. There was also scant, if any, discussion about asymptomatic carriers of gonococci, and no discussion at all about *Chlamydia trachomatis*. Rather, there was considerable discussion about the three principal "minor STDs": granuloma inguinale, chancroid, and lymphogranuloma venereum (LGV). All three were understood as clinical entities, but diagnostic tools were few and poor. It took the deployment of modern microbiology (culture in eggs, and later in cell culture) and good clinical inference before chlamydia were discovered to be common causes of cervicitis, urethritis, and salpingitis. It also took the development of molecular diagnostic tests for typing particular variants of human papilloma viruses (HPV) before it was realized that some but not all HPV were able to trigger the path to cervical cancer. Discovery of the rather astounding prevalence of HPV awaited discovery and deployment of the polymerase chain reaction (PCR).

In 1964, when I entered the field as a novice, testing for STDs was limited to the classic serologic tests for syphilis, updated by then to include the first generation of specific treponemal tests, as well as the still extant TPI immobilization test; dark field microscopy and rare animal inoculation to recover *T pallidum*; culture and microscopy for *N. gonorrhoeae*; group serology for LGV antigen; biopsy for granuloma inguinale; and Tzanck preparations and occasional culture for Herpes simplex virus. Nonspecific vaginitis (bacterial vaginosis) was diagnosed by examining for vaginal secretions for clue cells. There were no tests for either genital chlamydia or HPV, and of course none for HIV, which had not been recognized yet although the first human infections had already occurred. No one suspected sexual transmission of hepatitis viruses. Nongonococcal urethritis was simply defined as urethritis (in men, since the urethral syndrome in women had yet to be defined) in whom there was no evidence of gonococci. Trichomonas was recognized as a problem in women, but no one took seriously the possibility that men might harbor the organism, and some might have symptoms from it. Genital mycoplasmas were not yet on the scene. The vaginal microbial flora was virtually totally unknown; certainly, there was no thought that certain lactobacilli might be protective against infection by various pathogens.

1.2. The Way it Is

We live in a much more sophisticated world now, only a little over thirty years from that course on STDs at the Communicable Disease Center (CDC). We are aware of the dangers of silent infection by HPV in some persons, and the complications of asymptomatic or oligosymptomatic genital chlamydia infections. We actually understand a great deal about the molecular events that lead to cancer, in the case of HPV, or fallopian tube scarring and infertility, or Reiter's syndrome, in the case of genital chlamydia. Application of modern

microbiology techniques has clarified the roles of various organisms in bacterial vaginosis, and has helped to elucidate the role that bacterial vaginosis seems to play in more serious diseases such as salpingitis. We are on the verge of being able to rapidly and specifically diagnose the cause of syndromes such as genital ulcer syndrome that may have multiple etiologies. Now that we have effective therapy for genital herpes virus infections, it is helpful to have a multiplicity of diagnostic tests including culture so that we can know certainly who has herpetic infection. Much of the practice of genitourinary medicine, or STD control, as venereology has come to be known, now depends on increasingly effective and rapid diagnostic tests.

And then of course, there is HIV. This is such a big development that it threatens to dwarf other STDs in the public mind, and it has become a specialty within a specialty as new treatments and tests rapidly evolve. Here we are almost completely dependent on serologic tests, sophisticated molecular analyses of the state of the immune system, and the extent of the "viral load" to guide our diagnosis, prognosis, and treatment. It is safe to say that had this infection evolved in another era, like the one described in 1964 at the CDC course mentioned above, it would have taken much longer at best to discover the cause, and we might still be groping for useful tests and therapies. Because HIV occurred in the era of "molecular medicine," we have collectively made amazing strides in understanding the disease and in beginning to control it. The point is, we depend on really good tests, and are fortunate to have more and increasingly better tests at our disposal for all the STDs, including HIV. This book is an attempt to capture this rapidly moving field, in a form that will be useful to practitioners in the laboratory, and to clinicians who desire deeper understanding of the basis of the new tests that are rapidly entering practice.

2. What Will Be the Impact of the New Tests?

There are so many new tests that it is difficult to try to predict their impact Because many are relatively expensive, their deployment will be somewhat limited, certainly excluding much of the developing world where the STD problems are worst. New inexpensive tests are needed that can be used in the field. Development of reliable, stable simple sensitive and specific antigen detection tests for virtually all of the major STDs, would have a huge impact, because much of the world still relies on syndromic diagnosis through algorithms. This need is exemplified by the announcement by the Rockefeller Foundation of a prize of one million US dollars for development of a simple nonculture test for gonorrhea and chlamydia, suitable for use in the developing world. At the time of this writing there are attempts to create such a test, but certainly there has been no announcement of a winner. Thus, despite our excitement about the plethora of useful new molecular tests for a variety of STDs, there is still much work to be done.

Putting these cautionary comments aside, there is room for real optimism about what has been accomplished in recent years. The new tools at our disposal are already making a difference in many ways. I will illustrate the power of the new tests, and also what we still need, by focusing on a few of the areas that are covered in detail in later chapters of this book (*see* Chapters 2, 3, 5, 8, 11, 12, 13).

2.1. Gonococcal Infection

The advent of PCR and the related ligase chain reaction (LCR) tests has made it possible to detect current or very recent infection by amplifying gonococcal DNA in patient secretions, including urine and vaginal secretions *(2,3)*. The revolutionary impact of these tests is based on their ability to detect infection in women without doing an invasive pelvic exam, which is slow and something most women would rather avoid. This is possible because screening urine or vaginal fluids is at least as sensitive as screening cervical secretions. Some argue that DNA-based tests might be a problem because they do not allow testing of isolates for antimicrobial sensitivity, and there is no means for strain typing as can be done by several techniques with live isolates. However, there are new technologies on the horizon including chip-based DNA sequencing *(4)* that will allow detection of genes such as beta lactamase, directly from patient secretions, and similar methods almost certainly can be used to perform molecular strain typing based on the DNA sequence of porin or other genes. The DNA-based diagnostic tests are a real advance in our ability to diagnose gonorrhea, particularly in screening high prevalence populations for infection, especially in women. Curiously, we still do not have an effective serological test for gonorrhea, and no one is working on this problem to the best of my knowledge.

2.2. Genital Chlamydia Infection

Virtually the same comments apply as were made about gonococcal infections, but the impact here is even greater because culture tests for chlamydia are so much more difficult and expensive than they are for gonorrhea. Antigen detection tests for chlamydia *(5)* were developed that were quite sensitive and specific, but the new nucleic acid based tests are clearly more sensitive than any previous tests, and there is good evidence that they are specific as well *(6)*. DNA remains detectable for many days in patient secretions after effective treatment, so DNA-based amplification tests cannot be used as a test of cure *(7)*. This undoubtedly applies to all infectious diseases. Screening for genital chlamydia infections appears to have an impact on community prevalence *(8)*, and wider use of the DNA-based tests will hasten the decline of chlamydia in societies that can afford to use these tests. Of course, cost is an issue, even in rich countries such as the USA. Cost effectiveness analyses will be needed

before managed care companies or health departments can fully commit to using these excellent tests *(9)*. As with gonococcal infection, we lack a useful serological test for genital chlamydia. There is exciting evidence that correlates serum antibody responses to certain antigens (especially the heat shock protein of approx 60 kDa) with increased likelihood of late complications of disease, particularly salpingitis, ectopic pregnancy, and tubal infertility *(10,11)*, but the tests are not sufficient at present for individual diagnostic use. Interesting as the serological results are, they remain in the province of research laboratories.

2.3. Syphilis

Development of DNA-based technologies for syphilis diagnosis will help in a couple of ways: detection of the etiologic agent in genital ulcers, through use of multiplex panels, and detection of *T pallidum* in tissues especially cerebrospinal fluid (CSF), in order to help make more accurate diagnosis of neurosyphilis. A charitable assessment of the present state of the art of diagnosis of neurosyphilis is that it is problematic *(12)*. PCR tests of CSF will hopefully allow differentiation between the many causes of CNS pleocytosis in AIDS patients, and will help determine whether patients with serological evidence of syphilis have active infection of the central nervous system. Clinicians are in great need of help in both of these arenas. Unlike the cases with gonorrhea and chlamydia, we lack good data at present to determine the role of these tests in management of possible neurosyphilis; hope is high but data are needed.

The DNA sequence of *T pallidum* was completed very recently. One hopes that analysis of the genomic sequence will lead to insights about the physiology of the organism, and therefore to solutions of the very old problem of in vitro cultivation outside of animals. Availability of the DNA sequence will predictably enable the development of molecular strain typing tools, which has been entirely lacking in syphilis research until now. One envisions the PCR-based sequencing from patient materials of genes that are known to be variable in different isolates, as a means of better understanding the evolution of the organism within an individual, and as it moves between individuals. There is no reason that this cannot be done as effectively for *T pallidum* as it can now be done for *C trachomatis* and *N. gonorrhoeae*, and HPV and HIV. Development of better serological tests based on knowledge of the DNA sequence of pathogenic and nonpathogenic treponemes is another development that can be anticipated with reasonable confidence.

2.4. HPV Infection

Nearly all of our current understanding about the epidemiology and pathogenesis of HPV disease is owing to the development of molecular methods for typing isolates, and detecting the virus in patient materials in the absence of

culture *(13,14)*. A most interesting recent development is the creation of serological tests based on artificial pseudovirus particles, the result of expressing particular genes as recombinant proteins in vitro. These tests, which are discussed in Chapter 11 of this book, are effective tools for epidemiologic studies of disease prevalence *(15)* in the same manner that specific serological tests for herpes virus infection have allowed estimations of the true prevalence of genital herpes infections.

2.5. HIV Infection

We are dependent to a large measure on molecular methods to assess who has very early HIV disease, the extent of disease in virtually everyone who is being considered for treatment, and for following the response to treatment *(16)*. Availability of chip-based sequencing technology is beginning to be deployed already as a tool to determine the sensitivity of HIV to particular antiviral agents, and we can look forward to much more widespread use of these techniques. Indeed, the state of the art is so dependent on viral load testing by molecular methods that the absence of these tests in developing countries is a real dilemma for clinicians trying to deploy the new but expensive antiviral therapies for HIV wisely. Assays for infectious amounts of virus in secretions, based on quantitative assays for HIV RNA in patient samples, has shown that other "minor" STDs, such as gonorrhea, have important effects on increasing the shedding of HIV in secretions, and therefore presumably increasing the risk of sexual transmission of HIV *(17)*.

3. Conclusion

I have made no attempt to be encyclopedic about the history of testing for STDs. However, this brief appraisal makes it clear that there has been a revolution in our ability to apply sensitive and specific tests for diagnosis, and as an aid to therapy in a variety of STDs. Indeed, the development of such tests has been directly linked to improved understanding of the epidemiology and natural history of many STDs, as for instance HPV and HIV. We have come a very long way from the initial (successful) effort to make a serological test for syphilis based on crude extracts of syphilitic liver tissue. In retrospect, it is not surprising that Wassermann's original serological test for syphilis actually discovered increased serum antibody responses to what we now understand is a normal tissue antigen:diphosphatidyl glycerol. If the original tests for STDs sometimes depended as much on serendipity as the prepared mind and good scientific reasoning, we certainly have now moved to a time and place where hard science forms the basis for most of the new tests that are being developed at a nearly breathtaking pace. New tests as well as a more open acceptance of the importance of STDs have transformed the entire field. What we knew just 34 years ago pales in comparison to what we now know.

The challenge is to deploy these tests and those that will follow in the most cost effective manner, and to try to use them as adjuncts not only to treat, but also to help in prevention. Perhaps the next generation of molecular tests will include very inexpensive tests that are suitable for use in the whole world.

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II

Molecular Techniques for the Detection of Sexually Transmitted Pathogens

2

Neisseria gonorrhoeae

Detection and Typing by Probe Hybridization, LCR, and PCR

Charlotte A. Gaydos and Thomas C. Quinn

1. Introduction

1.1. Taxonomy

Neisseria gonorrhoeae, first described by Neisser in 1879, is a Gram-negative, nonmotile, nonspore-forming diplococcus, belonging to the family *Neisseriaceae*. It is the etiologic agent of gonorrhea. The other pathogenic species is *Neisseria meningitidis*, to which *N. gonorrhoeae* is genetically closely related. Although *N. meningitidis* is not usually considered to be a sexually transmitted disease, it may infect the mucous membranes of the anogenital area of homosexual men *(1)*. The other members of the genus, which include *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria cinerea*, and *Neisseria flavescens*, which are related to *Neisseria gonorrhoeae*, and saccharolytic strains, such as *Neisseria subflava*, *Neisseria sicca*, and *Neisseria mucosa*, which are less genetically related to the aforementioned, are considered to be nonpathogenic, being normal flora of the nasopharyngeal mucous membranes *(2)*.

1.2. Clinical Significance

Gonococcal infection may be either symptomatic or asymptomatic, and can cause urethritis, cervicitis, proctitis, Bartholinitis, or conjunctivitis. Gonorrhea is the most frequently reported bacterial infection in the US. In males, complications may include: epididymitis, prostatitis, and seminal vesiculitis. In homosexuals, rectal infection and pharyngitis can occur. In females, most cases are asymptomatic, and infections of the urethra and rectum often coexist with cervical infection. Complications can include pelvic inflammatory disease, pelvic pain, ectopic pregnancy, infertility, Fitz-Hugh Curtis syndrome,

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chorioamnionitis, spontaneous abortion, premature labor, and infections of the neonate, such as conjunctivitis. Other serious sequelae, such as disseminated gonococcal infection (DGI), occur rarely and can result in septicemia, septic arthritis, endocarditis, meningitis, and hemorrhagic skin lesions *(2)*.

1.3. Standard Diagnostic Methods

1.3.1. Direct Smear Examination

A direct Gram-stain may be performed as soon as the specimen is collected on site, or a smear may be prepared and transported to the laboratory. Urethral smears from males with symptomatic gonorrhea usually contain intracellular Gram-negative diplococci in polymorphonuclear leukocytes (PMNs). Extracellular organisms may be seen also, but a presumptive diagnosis of gonorrhea requires the presence of intracellular diplococci. The sensitivity of such smears in males is 90–95.0% *(3)*. However, endocervical smears from females and rectal specimens require diligent interpretation because of colonization of these mucous membranes with other Gram-negative coccobacillary organisms. In females, the sensitivity of an endocervical Gram-stain is estimated to be 50–70% *(3)*.

1.3.2. Antigen Detection

Gonococcal antigen may be detected by an enzyme immunoassay (EIA) (Gonozyme, Abbott Laboratories, Abbott Park, IL) for a presumptive diagnosis. This EIA is about as sensitive and specific as a Gram stain in males, but is less sensitive for use with endocervical specimens *(4,5)*.

1.3.3. Culture

The isolation and identification of *N. gonorrhoeae* are the currently accepted gold standard for the diagnosis of gonococcal infections *(2)*. Specimens should be inoculated onto nonselective media (chocolate agar), on which all *Neisseria* spp. will grow, or selective media, such as modified Thayer-Martin (MTM), Martin-Lewis (ML), or New York City (NYC). Selective media contain antimicrobial agents to inhibit commensal bacteria, nonpathogenic *Neisseria* sp., and fungi. MTM contains vancomycin, colistin, trimethroprim lactate, and nystatin. ML contains the same antibiotics, except nystatin is replaced by anisomycin. NYC, a clear medium containing hemolyzed horse blood and plasma with yeast dialysate, contains vancomycin, colistin, trimethroprim lactate, and amphotericin B. The selective media allow the growth of *N gonorrhoeae* and *N. meningitidis*, while inhibiting, for the most part, commensal neisseria. Rarely, some strains of gonococci are susceptible to vancomycin and trimethoprim *(2)*. Thus, specimens from normally sterile sites, such as blood, cerebrospinal fluid, and joint fluid, should be plated onto both nonselective and selective media. Because pathogenic neisseria are nutritionally and

environmentally fastidious, the ideal method for transporting organisms is to plate the specimens directly onto the selective or nonselective medium, and immediately incubate the plates in an increased humidity atmosphere of 3–5% CO_2, at 35–37°C. The CO_2-enriched atmosphere is important, and with the advent of commercial zip-locked bags with CO_2 generating tablets, specimens should not be transported in Stuart's or Amies medium.

Two levels of identification of isolated organisms may be used: presumptive and confirmatory. An isolate may be presumptively identified as *N. gonorrhoeae* when a Gram-negative, oxidase-positive diplococcus has been isolated. Confirmatory identification requires that biochemical, fluorescent antibody, chromogenic enzyme substrate, serological, or coagglutination tests be performed to distinguish the isolate from *N. meningitidis*, and *Branhamella catarrhalis*, *Kingella denitrificans*, as well as nonpathogenic *Neisseria* spp. Many of these methods are commercially available, none are perfect, and most require an isolated subculture or colony.

1.4. The Molecular Expansion

Methods for the identification of difficult to isolate bacteria and viruses by molecular probing and DNA amplification have forever changed the science of microbiology and infectious diseases.

1.4.1. Nonamplified Probe Assay

A DNA probe hybridization assay has been developed based on the fact that complementary nucleic acid strands will bind together in a stable double strand. No amplification of the nucleic acid occurs. The target sequence of the ribosomal RNA of *N. gonorrhoeae* is hybridized by a chemiluminescent labeled single-stranded DNA probe, which is complementary to it. After the removal of the nonbound probe, the resulting stable DNA–RNA hybrid is measured in a luminometer (the GenProbe Leader®). The results of the assay are calculated by determining the difference between the chemiluminescence of the specimen and the mean of the results from the negative reference. An advantage of this method is that there are no stringent transport conditions required, which makes it attractive for use with specimens that must be transported to an off-site laboratory.

Molecular techniques for the identification, sequencing, and amplification of genes from *N. gonorrhoeae* have obviated the requirement for viable organisms for the diagnosis of infections and for epidemiological typing studies. In particular, the technology to amplify DNA from clinical specimens is so powerful that theoretically a single-gene copy in a sample can be detected. The power of the amplification methods has also led to the use of nonconventional specimens types that are unsuitable for culture, such as urine, vaginal swabs, and tissue from the upper reproductive tract *(6,7)*.

The ability to amplify DNA has not been without problems associated with its use, however. Because even a single DNA sequence can be amplified, this power has led to problems of contamination in the laboratory frustrating scientists and questioning the interpretations of positive tests. Technology was soon developed to prevent crosscontamination of specimens with laboratory amplicons *(8)*.

1.4.2. Ligase-Chain Reaction (LCR) for Genital Specimens and Urines

Birkenmeyer and Armstrong first reported the use of LCR for the detection of *N. gonorrhoeae* by testing two probe sets against the *opa* genes and one against the *pilin* gene *(9)*. The use of four hapten conjugated probes allowed the amplification of DNA from 136 isolates of *N gonorrhoeae* and none of 124 nongonococcal strains, including *N. meningitidis*. The probes sets were designed for regions of the gonococcal genes, so that gap-filling and ligation happened at sites of mismatch for other neisseria. The short gap formed after hybridization with the adjacent oligonucleotides was filled by DNA polymerase in the presence of dGTP, with the DNA ligase from *Thermus thermophilus* sealing the nick *(9)*.

Thermocycling of the reaction was performed in an automated temperature cycler and consisted of 27–33 cycles of two temperature steps: denaturation to separate DNA strands, a lower temperature to allow annealing, gap-filling, and ligation of the oligonucleotide probes. After cycling, a 40-µL portion was used to detect amplified products (amplicons) by using an automated Microparticle Capture Enzyme Immunoassay (Abbott Laboratories). Antifluorescein-coated microparticles were used to capture the ligated amplicons, which contained the hapten-labeled probes: the capture hapten, fluorescein, and the detection hapten, biotin. The captured products were detected by an antibiotin alkaline phosphate conjugate, which used methylumbelliferyl phosphate substrate, to produce a fluorescent product, methylumbelliferone, at a rate proportional to the amount of ligated amplicon *(9)*.

When the assay was tested for sensitivity, signals 2.2.–3.3 times background were generated for as low as 1.1 gonococcal cell equivalents/LCR reaction. At 2.7×10^2 cell equivalents/LCR, signals 21–162 times background were generated, depending on which probe set was used. For specificity assays, none of 124 nongonococcal strains produced signals above background, when tested at 1.3×10^6 cells/assay *(9)*.

Preliminary testing of 100 genital specimens demonstrated a sensitivity of 100% and a specificity of 97.8%. Bloody and heavily exudative negative specimens did not show loss of positive signal when spiked with gonococcal DNA *(9)*.

A multicenter trial demonstrated that the overall sensitivity and specificity of LCR (Abbott) for *N gonorrhoeae* were 97.3 and 99.8%, respectively, from 1539 female endocervical specimens *(10)*. When culture was compared to

resolved true-positive specimens, the sensitivity and specificity for culture were 83.9 and 100%, respectively. There were three culture-positive specimens that LCR did not detect, which may have been caused by the presence of inhibitors. However, culture missed 18 specimens that were positive by LCR and that were confirmed to be true positives by using one of the alternative probe sets developed by Birkenmeyer *(9)*. The specimens used were from both high-prevalence (15.9%) and low-prevalence (2.7%) populations. The additional detection of positives of LCR over that of culture ranged from 13.5% for STD clinics to 25.9% for obstetrical/gynecology clinics (Lee et al., personal communication). Thus, the advantage of the LCR assay for screening in low-prevalence populations is of great value.

In the multicenter trial, which included both male urethral swabs and urine from 1639 males, LCR had a sensitivity and specificity of 98.5 and 99.8% for 808 urethral swabs and 99.1 and 99.7% for urine, respectively *(10)* (Lee et al., personal communication). LCR demonstrated a 5% extra pickup of positive specimens compared to culture.

Smith et al. used LCR to screen urine for *N. gonorrhoeae* from 283 women attending an STD clinic and compared the results to culture of the endocervix and urethra *(11)*. Positive LCR results were obtained for 51 of 54 women with culture positive cervical or urethral specimens. Two of 229 women with both cervical- and urethral-negative cultures had a positive LCR result. Discrepant testing with alternative LCR probe sets revealed that the three urine LCR-negative/endocervix and urethral culture-positive specimens were from truly infected patients, whereas the analysis indicated that the two urine LCR-positive/endocervix and urethral culture-negative specimens were truly positive also. Thus, the resolved sensitivity, specificity, positive predictive, and negative predictive values for LCR of urine were 94.6, 100, 100, and 98.7%, respectively *(11)*.

1.4.3. PCR for Genital and Nongenital Specimens

Although there are currently no FDA-approved commercial PCR assays for the detection of gonococcal organisms, Ho et al. demonstrated that PCR is highly sensitive and specific for use in clinical specimens to detect *N. gonorrhoeae (12)*. Clinical trials by Roche Molecular Systems (Branchburg, NJ) for the coamplification of *N. gonorrhoeae* and *Chlamydia trachomatis* in genital swabs and urine specimens are currently in process. (Commercial PCR assays for *N. gonorrhoeae* have been available in many countries, including Canada, for many years.) Preliminary results from preclinical trials indicated that the assay is highly sensitive and specific for gonococci *(13,14)*. For males, the resolved sensitivity and specificity for urethral swabs were 97.3 and 98.9%, respectively, and for urine, 94.4 and 98.2%, respectively. In contrast, the sensitivity of urethral culture was only 76.6%. For females, the resolved sensitivity

and specificity of endocervical swabs were 95.2 and 97.7%, respectively, and for urines, 88.9 and 94.3%, respectively, whereas the sensitivity of endocervical culture for gonorrhoea was 71.4% ***(13)***.

The method of Ho et al. used primers from the *cppB* gene, which is carried on both the chromosome and the 4.2-kb cryptic plasmid of *N. gonorrhoeae* to amplify DNA successfully from 33 gonococcal strains. None of the 12 other *Neisseria* spp. or 13 genital commensal bacteria produced the expected 390-bp product by gel electrophoresis. *Neisseria denitrificans* gave an amplified product, but of 190 bp. The specificity of the gonococcal-amplified product was confirmed by the use of the restriction enzyme *MspI*, which cleaved the amplicon product into 2 fragments of 250 and 140 bp. When the procedure was used for 52 clinical specimens, 34 of 34 culture positives were successfully detected by PCR. In addition, PCR identified two culture-negative cases of gonorrhoea, which were confirmed positive by testing with an ELISA method (Gonozyme, Abbott).

In addition to the use of PCR in genital specimens, there have been several other applications of the PCR technology to detect *N. gonorrhoeae* in nongenital specimens. Primers for the structural gene of the outer membrane protein III, which is universally present in all gonococcal strains, were used in conjunction with a set of nested primers to amplify DNA in 11 of 14 synovial fluids from arthritis patients from whom cultures were negative for *N. gonorrhoeae* ***(15)***. The sensitivity was 78.6%, and the specificity was 96.4%. Samples from Reiter's syndrome patients were negative by contrast. Muralidhar et al. detected gonococcal DNA in synovial fluid from five of eight arthritis patients with systemic infection of *N. gonorrhoeae* ***(16)***. Two culture-negative patients were positive by PCR, and all patients with positive synovial fluid cultures were PCR-positive.

1.4.4. Multiplex PCR for Detection of C. trachomatis *and* N. gonorrhoeae *in Genitourinary Specimens*

Mahony *et al.* published a multiplex PCR (M-PCR) for both of these organisms ***(6)***. Standard PCR techniques were used, and the primers for *N. gonorrhoeae* were HO1 and HO3, which amplify a 390-bp fragment of the cppB gene on the cryptic plasmid ***(12)***. Ho's method is described above. The primers for *C. trachomatis* were KL1-KL2, which amplifies a 241-bp fragment of the genetically conserved plasmid ***(17)*** (*see* Chapter 3). First-void urine and urethral swabs from males and female endocervical swabs were tested. The sensitivity of M-PCR for detecting *N. gonorrhoeae* in urethral specimens was 92.3% (12 of 13 positives), compared to culture and for *C. trachomatis* 100% (22 of 22 positives). The specificities were 100% for both *N. gonorrhoeae* (178 of 178) and *C. trachomatis* (187 of 187).

1.4.5. Epidemiological Typing of N. gonorrhoeae Isolates

O'Rourke et al. used PCR followed by restriction-fragment-length polymorphism to perform *opa* gene typing successfully *(18)*. The method appeared to be highly discriminatory and could differentiate between isolates of the same auxotype/serotype class. Identical opatypes were obtained from known sexual contacts. From one STD clinic, there were 41 opatypes from 43 consecutive isolates.

Eleven distinct and highly variable opa genes from *N. gonorrhoeae* were amplified using primers that only amplified the region encoding the mature Opa proteins.

This chapter will address the use of two FDA-approved DNA tests for *N. gonorrhoeae*: the unamplified probe test (Pace 2, GeneProbe, San Diego CA) and the amplified DNA test, LCR (Abbott Laboratories). Also discussed will be the PCR test for the coamplification of both *N. gonorrhoeae* and *C trachomatis* (Roche Molecular Systems). Several other noncommercialized PCR applications are available to identify both of these organisms (multiplex PCR) *(6)* for use with body fluids, such as synovial fluid *(15,16)* and for epidemiological typing systems *(18)*, and these will be reviewed (*see also* Chapter 9 for more details on typing).

2. Amplified DNA and Nonamplified Probe Assays

2.1. Probe Hybridization (Pace 2, GenProbe) for Clinical Swabs

2.1.1. Specimens

The test is FDA-approved for the detection of *N. gonorrhoeae* in male urethral and female endocervical swab specimens, as well as for the identification of *N gonorrhoeae* from culture isolates (*see* **Notes 1–3**). Only swabs available from the Pace collection kit should be used, and only the GenProbe transport media can be used to transport specimens to the testing site (*see* **Note 4**).

2.1.2. Materials

Almost all materials are provided in the commercial kit and include: Probe Reagent, Hybridization Buffer, Selection Reagent, STD Separation Reagent, STD Wash Solution, Positive Control, STD Negative Reference, Polystyrene Tubes, and Sealing Cards. Available separately from the manufacturer are the Detection Kit (Reagents I and II) and the Magnetic Separation Unit.

2.1.3. Equipment

Luminometer (Leader®) is available only from GenProbe (San Diego, CA)
Covered water bath (60°C)
Vortex mixer.

2.2. LCR for Genital Specimens (Abbott)

2.2.1. Specimens

Only swab specimens collected with the Abbott Lcx Swab Collection and Transport Kit may be used. Storage conditions are as follows:

1. Prior to sample preparation: at 2–30°C, 4 d, and at –20°C, 60 d (*see* **Note 5**).
2. After sample preparation: at –20°C, 90 d.
3. After amplification: at 15–30°C, 72 h

Sample processing steps include:

1. Allow samples that have been frozen after collection to thaw at room temperature
2. Heat in a dry heating block at 97 ± 2°C for 10 min
3. Allow to cool to room temperature for 15 min

2.2.2. Materials

Almost all materials and reagents are provided in the commercial kit and include: unit dose tubes, positive calibrator and negative controls, and the unit pack of LCx enzyme immunoassay detection reagents. Additional materials required include aerosol barrier pipet tips and pipetters.

2.2.3. Equipment

Perkin-Elmer Cetus Thermocycler (Emeryville, CA)
Dry heat block.
Microcentrifuge
LCx automated enzyme immunoassay machine (Abbott)

2.3. LCR for Urine Specimens (Abbott)

2.3.1. Specimens

Specimen collection (males and females) (*see* **Note 6**):

1. Instruct the patient not to urinate for 1 h before collection of the urine
2. Instruct the patient to collect the first 15–20 mL of voided urine (the beginning part of the urine stream) into an empty sterile collection cup
3. Refrigerate the specimen immediately at 2–8°C or freeze at –20°C or lower.

2.3.2. Specimen Transport and Storage

1. From the collection site, urine specimens can be shipped to the laboratory at 2–8°C or frozen, and must arrive within 24 h of shipment On arrival, the urine may be stored at 2–8°C or frozen until processed.
2. In the laboratory, prior to sample processing, urine specimens stored at 2–8°C must be processed within 4 d of specimen collection Urine specimens stored at –20°C or below must be processed within 60 d of specimen collection. Once frozen, urine should not be thawed until ready for processing and testing

3 After sample preparation, the processed urine may be frozen at –20°C for 60 d After amplification, the samples may be stored up to 72 h at 15–30°C

2.3.3. Materials

The materials are as outlined above, except that sterile microcentrifuge tubes and plastic, single-use, 1-mL transfer pipets for processing the urine samples are required.

2.3.4. Equipment

The equipment is the same as outlined above, except that the microcentrifuge must be capable of speeds of 9000*g*.

2.4. PCR for Genital Specimens

2.4.1. Specimens

Urethral or cervical swabs may be collected in saline and transported at room temperature. For urine collection, transport, and storage, please refer to **Subheadings 2.3.1.** and **2.3.2.**

2.4.2. Materials

1 Two 20-mer oligonucleotide primers. HO1: (5'GCTACGCATACCCGCGTTGC3') and HO3: (5'CGAAGACCTTCGAGCAGACA3').
2 *Msp*I restriction enzyme (Gibco/Bethesda Research Labs, Gaithersburg, MD).
3 General PCR reagents may be purchased separately from many suppliers or may be purchased in kit form from Perkin-Elmer (Cetus, Emeryville, CA) These include 200 µ*M* each deoxyribonucleotides (dATP, dCTP, dTTP, dGTP), 1X PCR reaction buffer (50 m*M* KCl, 10 m*M* Tris, pH 8.3, 100 µg/mL bovine serum albumin, 1 5 m*M* $MgCl_2$), and *Taq* polymerase
4 General-laboratory supplies, such as aerosol barrier pipet tips, PCR tubes, mineral oil, electrophoresis running buffer, mol-wt markers, agarose, and ethidium bromide (EtBr) stain are also required ***(19)***.

2.4.3. Equipment

Perkin-Elmer Cetus Thermocycler
Dry heat block
Microcentrifuge.
Electrophoresis apparatus and power supply.

2.5. Epidemiological Typing

2.5.1. Specimens

Specimens are obtained from gonorrhoea patients or stock strains. *N gonorrhoeae* isolates are streaked across GC agar (Difco) plus IsoVitaleX (Becton Dickinson).

2.5.2 Materials

Tris-HCl, EDTA, lysozyme, phenol/chloroform/isoamyl alcohol, ethanol, protease K, Triton-X, TE buffer, standard PCR reagents, agarose, GeneClean (Bio101, Inc), [α-P32]-dCTP, nondenaturing polyacrylamide gel, and X-ray film.

Oligonucleotide primers: The upstream primer corresponds to nucleotides 663–684, and the downstream primer corresponds to nucleotides 1227–1208 in the numbering scheme of Bhat et al. *(20)*. Opa-up: (5'GCGATTATTTCA GAAACATCCG-3') and Opa down: (5'-GCTTCGTGGGTTTTGAAGCG-3').

Restriction enzymes: *Taq*I, *Hin*PI, and *Hpa*II.

2.5.3. Equipment

Thermocycler (Perkin-Elmer Cetus)
Water bath
Electrophoresis apparatus

3. Methods

3.1. Probe Hybridization (Pace 2, GenProbe)

The detailed procedure may be found in the package insert. A brief description is included herein.

3.1.1. Sample Preparation

Vortex swab, express liquid from swab, and discard the swab.

3.1.2. Reagent Preparation

Probe reagent: Vortex the probe reagent, and warm the hybridization buffer at 60°C for 30–40 s. Place 6.0 mL of hybridization buffer into the lyophilized probe reagent. Allow to stand for 2 min and vortex.

Separation suspension: Calculate the volumes of selection reagent and separation reagent needed for the number of tests to be performed. Mix two reagents in a ratio of 10 mL of selection reagent and 0.5 mL of separation reagent. (Stable for 6 h at room temperature.)

3.1.3. Hybridization

1. Label tubes. Include three tubes for the negative reference, and one for the positive control. Insert tubes into rack of the magnetic separation unit
2. Vortex controls and specimens for 5 s Pipet 100 µL of the controls and specimens into the bottom of the respective tubes
3. Pipet 100 µL of the probe reagent into the bottom of each tube Cover the tubes with sealing cards, and shake the rack 3–5 times.
4. Incubate the tubes in the 60°C water bath for 1 h Ensure that the leader is prepared for use and that sufficient volumes of detection reagents I and II exist

3.1.4. Separation

1. Remove rack from water bath and remove sealing cards. Pipet 1 mL of mixed separation suspension into each tube.
2. Cover the tubes with sealing cards, and shake vigorously three to five times. Incubate the rack in the 60°C water bath for 10 min.
3. Remove rack from water bath, remove sealing cards, and place the rack on the magnetic separation unit for 5 min at room temperature
4. Holding the rack and unit together, decant the supernatant, shake unit, and blot tubes three times for 5 s each. Do not remove rack from unit. Fill up each tube with wash solution, and allow the tubes to remain on the unit for 20 min at room temperature
5. Decant supernatants, and shake unit two to three times before righting Do not blot. Separate rack from the unit, and shake the rack to resuspend the pellets

3.1.5. Detection

Set up the leader software. Wipe each tube to remove residue on outside tube. Ensure pellets are resuspended, and insert into the leader, following prompts. Read three negative reference tubes, positive control, and then specimen tubes.

3.1.6. Results and Interpretation

The results are calculated based on the difference between the response in relative light units (RLU) of the specimen and the mean of the negative reference. The leader prints the specimen response and a negative or positive interpretation, as compared to the cutoff value.

A specimen is considered positive if the difference is greater than or equal to 300 RLU, and negative if the difference is <300 RLU (*see* **Notes 1–4**).

3.1.7. Pace 2 (GenProbe) for Identification of Culture Isolates

When testing organisms isolated from chocolate or modified Thayer-Martin agar plates, prepare a bacterial suspension in saline having the same turbidity as #1 barium sulfate MacFarland standard, mix well, add 100 µL to a GenProbe transport tube, and vortex. All further directions are as stated above. A specimen is considered positive if the cutoff value is 10,000 RLU above the mean of the negative reference.

3.2. LCR for Genital Specimens

The detailed procedure may be found in the package insert. A brief description follows.

1. Prepare a positive calibrator and negative control vial by adding 100 µL of activation reagent to each vial After the heated specimens are cooled to room temperature, add 100 µL of the specimen from the transport tube to unit dose tubes,

which have been pulse-centrifuged to remove condensation from the top of the unit dose tube. After all specimens have been added to the unit dose, add 100 µL of each of the positive calibrator and negative controls to each of the unit dose tubes Set up 2 positive calibrator and 2 negative controls for each 20 specimens.

2. Place the tubes into the thermocycler in order according to prepared template containing a map of the specimen numbers. Start the thermocycler by selecting the preprogrammed gonococcal thermocycling file, and push start The thermocycling file consists of 40 cycles of the following 3 cycles. 97°C for 1 s, 55°C for 1 s, and 62°C for 50 s.
3 At the end of the thermocycling step, remove the unit dose tubes, and briefly pulse-centrifuge the tubes to remove condensation from the tops of the tubes. Insert the unit dose tubes into the wheel of the LCx EIA machine containing the carriers for the unit doses. Insert a pack of the EIA reagents and push start The machine should have previously passed the necessary daily and weekly quality-control checks for temperature, reagents (specimen diluent and inactivation reagent volumes), and optical density, specified by the manufacturer.
4 At the end of the cycle, the machine will issue a tape with the calibration and negative control results, a calculated cutoff value, and will indicate the result for each specimen from 1–24. Each thermocycler run requires that 2 LCx EIA cycles be run

3.2.1. LCR for Urine Specimens

1 Allow the urine to thaw if frozen. Mix the urine by swirling. Using an aerosol barrier pipet, transfer 1 mL of urine into a urine microfuge tube from the urine specimen preparation kit.
2 Centrifuge the urine at ≥9000*g* for 15 min (± 2 min) in a microcentrifuge.
3 Using a fine-tipped, disposable, plastic transfer pipet or a pipetter with a 1-mL aerosol pipet tip, aspirate and discard the urine supernatant, being careful not to dislodge the pellet, which may be translucent. The removal of the supernatant must be performed within 15 min of centrifugation.
4. Using aerosol barrier pipet tips, add 1.0 mL of Lcx urine specimen resuspension buffer. Close the lid, and vortex until the pellet is resuspended Secure the top with a cap lock
5 Preheat the dry bath to 97°C (±2°C), which will require 40 min. Insert the specimens into the wells of the dry bath, and allow the heat block to stabilize back to temperature. Heat the specimen for 15 min.
6. Allow the specimens to cool to room temperature for 15 min (±5 min). Pulse-centrifuge the cooled specimen for 10–15 s Test the processed specimen immediately or store for up to 60 d at –20°C or below prior to testing.
7 Using aerosol barrier pipet tips, add 100 µL of each processed urine specimen to the labeled unit dose amplification vial. Prepare and test controls as indicated for swab testing. Place the controls and specimens into the thermocycler, and initiate the cycling as described above.
8. The detection assay is performed as previously described for swab specimens.

3.3. PCR for Genital Specimens

3.3.1. Specimens

Obtain clinical specimens by using phosphate-buffered saline prewet swabs and transport in 2 mL of phosphate-buffered saline. They may also be frozen at –20°C. For processing, vortex the specimen and remove the swab. Centrifuge the specimen for 5 min at ≥9000*g* to pellet the cells. Remove the supernatant by aspiration and resuspend the cells in 100 µL of 1X PCR buffer containing Tween-20 (45%) and proteinase K (200 µg/mL). Incubate the suspension at 50–60° C for 1 h. and heat to 95°C for 10 min to destroy the proteinase K. Fifty microliters are used for testing.

3.3.2. Amplification

1. The total reaction volume for each PCR will be 100 µL. A master mix may be made and dispensed (50 µL) to each tube, and the specimen (50 µL) can be added last. For each PCR reaction, the following are needed: 10 µL primer HO1, 10 µL HO 3, 10 µL dNTP mixture, 10 µL 10X PCR buffer, 0.5 µL *Taq* polymerase, 9.5 µL molecular-grade water. Multiply each reagent volume by the desired number of PCR tests plus negative and positive controls plus approximately three extra for pipeting bubbles, and make a master mix (*see* **Note 7**).
2. Dispense 50 µL to each tube.
3. Add 50 µL of prepared specimen or control specimen, add one drop of sterile mineral oil, and place all tubes into the thermocycler.
4. Program the thermocycler to perform 40 cycles of the following: denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 74°C for 30 s.

3.3.3. Detection

Analyze 10 µL of amplified product by agarose or acrylamide gel electrophoresis. Use 1–2 µL of tracking dye according to standard electrophoresis methods ***(19)***. Stain the gel with ethidium bromide, and examine it with UV light for the presence of the expected 390-bp fragment.

3.3.4. Restriction Digestion

For those specimens and controls producing the expected 390-bp band, digest 5 µL of the amplified PCR product with 5 U of *Msp*I according to manufacturer's directions at 37°C for 2 h in a final volume of 20 µL, using the buffer supplied by the manufacturer. Examine the digested products for the specimens and controls again by electrophoresis for the two expected fragments of 250 and 140 bp, respectively. This demonstrates the specificity of the original amplified product for sequences specific for the *cppB* gene, since the restriction site sequence is contained in the PCR product.

3.4. Epidemiological Typing of PCR

3.4.1. Preparation of Chromosomal DNA

After overnight incubation, resuspend the confluent growth from a plate in 1 mL of 50 m*M* Tris-HCl and 20 m*M* EDTA, pH 7.4. Add lysozyme (20 µL of 10 mg/mL). Incubate for 10 min at room temperature. Add an equal volume of 2% Triton-X and 50 m*M* Tris-HCl, pH 7.4. Freeze on dry ice and rethaw to ensure full cell lysis

3.4.2. DNA Extraction

Transfer the lysate (500 µL) to a microfuge tube, extract twice with phenol/chloroform/isoamyl alcohol (25.24:1), once with chloroform, and precipitate the DNA with ethanol. Dry centrifuged pellet, and resuspend in 100 µL Tris-HCl and 1 m*M* EDTA, pH 7.4 (TE buffer).

3.4.3. Opa Typing

Perform PCR amplification for 25 cycles in a 100-µL volume containing 0.5 µg of each primer, 200 ng of chromosomal DNA, 10 m*M* Tris-HCl, pH 8.5, 0.2 m*M* of each dNTP, 2.5 m*M* $MgCl_2$, 50 m*M* KCl, and 0.2 mg/mL gelatin. Heat samples to 95°C, cool over a 20 min period, and add 2.5 U of *Taq* DNA polymerase. The cycling program: 72°C for 2 min, 95°C for 1 min, 68°C for 2 min. Extend the final extension reaction at 72°C for 5 min. Cool the samples over a 30-min period to 4°C.

3.4.4. Electrophoresis

Apply the entire PCR reaction to 1% agarose gel and electrophorese. Extract the 550-bp opa gene fragments from the gel, and purify using GeneClean Resuspend at 25 µg/µL in TE buffer.

3.4.5. Restriction Digestion

Digest the fragments at 65°C for 2 h with 2 U of *Taq*I (or *Hpa*II or *Hin*PI) in a volume of 20 µL using the buffer recommended by the supplier. End-label the resulting digests with [a-32P]-dCTP, fractionate on a 6% nondenaturing polyacrylamide gel, and expose to X-ray film as described by Zang et al. *(21)*.

3.4.6. Interpretation of Results

Compare the overall patterns of DNA fragments from the opa genes of the different gonococci with each other and with those in existing databases. The images on the X-ray films can be captured as tagged format files (TIFF) using a Hewlett-Packard ScanJet IIcx high-resolution scanner. Cluster analysis can be performed by GELCOMPAR software (Applied Maths, Kortrijk, Belgium)

Isolates whose *Taq*I opa types are identical or are similar can be investigated further using *Hpa*II and *Hin*PI enzymes.

3.5. Conclusion

Because commercial applications and kits for the molecular amplication of DNA from genitourinary and other specimens are becoming available, routine clinical laboratories must address whether they can successfully adapt to the molecular identification procedures of *N. gonorrhoeae* and other organisms that cause STDs. Manufacturers must also address such issues as specimen preparation, the presence of inhibitors, and the possibility of contamination. As these and other issues are resolved, the amplified technology will move from the research arena to the routine clinical laboratory. Innovative tests, which allow for the testing of several organisms in a single assay, will simplify testing modalities and will help drive costs down. Future cost-effective analyses, which take into consideration the costs of the disease sequelae that are prevented from happening, will also justify the higher costs of amplified DNA testing modalities.

4. Notes

1. The Pace 2 assay has been approved only for use with genital swab specimens and culture suspensions, and must not be used for other specimen sources
2. Grossly bloody specimens (>80 µL in 1 mL transport) may interfere with performance of all the molecular assays
3. Molecular methods should not be used for suspected cases of child abuse, rape, or in other instances where adverse psychosocial outcomes may occur. Culture should be used for all such cases and in any medicolegal situation
4. Clinical evaluations of the assay probe hybridization have supported the reported high sensitivity and specificity of the assay reported in the package insert Of published reports, the sensitivity ranged from 96.3 to 100% and specificity from 98.8 to 99 6% *(22–26)* Additionally, Limberger reported specimens were stable for up to 1 mo storage at room temperature *(25)* Another advantage of this assay is that the same specimen can be used for the detection of *C trachomatis* using the same test technology The DNA probe assay has also been reported to be reliable for a test of cure assay as early as 6–11 d after treatment *(27)*
5. A great advantage of both LCR and PCR assays for genital swabs is that the specimen in the transport tube is stable at room temperature (or at 4°C) for up to 4 d, thus removing the need for stringent transport conditions required for cultures In addition, the same swab can be used for the detection of *C trachomatis*. Both of these facts make LCR and PCR tests desirable assays for use in large screening programs.
6. Because urines are easily obtained, noninvasive specimens, the ability to use them for screening purposes for gonorrhea offers a great advantage in terms of large public health screening programs, when there is no opportunity to obtain a cervi-

cal or urethral specimen, and for young sexually active patients, such as high school students, who are not in contact with a health clinic. Additionally, because urines can be refrigerated or frozen, there are no stringent transport conditions, as required for culture

7. In order to prevent contamination of PCR assays with DNA from prior PCR amplicons, specimen preparation should be performed in a room separated from where the PCR products are detected and from where the PCR is set up In addition, it is becoming increasingly important to adapt a chemical method to assure decontamination of reagents used in PCR assays. Commercial companies have these methods built into their assays Several methods exist, such as the use of the enzyme uracil *N*-glycosylase and isopsoralen, and have been compared and explained in detail *(8)* It is strongly recommended that one of these method be used in all laboratories using any noncommercial PCR assay, routinely.

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3

Molecular Diagnosis of *Chlamydia trachomatis* Infections by Probe Hybridization, PCR, LCR, TMA, and Q-β Replicase

Max A. Chernesky and James B. Mahony

1. Introduction

The use of cell cultures for the laboratory diagnosis of *Chlamydia trachomatis* infections was popularized during the 1970s and 80s *(1–4)*. The techniques required live organisms and were restricted to specialized laboratories. During the 1980s the detection of chlamydia-specific antigens was extensively used and compared to cell culture, provided a less stringent transportation of clinical specimens. Both direct fluorescent antibodies (DFA) and enzyme immunoassay (EIA) systems were commercialized *(5–7)* and algorithms for confirming false positives were popularized *(8,9)*. The first nucleic acid detection assay for *C. trachomatis* was evaluated during this time frame with comparisons to culture and antigen detection *(10–14)*. In the early 1990s, the following amplified nucleic acid assays detecting *C. trachomatis* gene fragments were developed: polymerase chain reaction (PCR), ligase-chain reaction (LCR), Q-β replicase-amplified hybridization (QBRAH), transcription-mediated amplification (TMA), and nucleic acid sequence-based amplification (NASBA). Extensive evaluations of PCR and LCR have shown, through discordant analysis and expansion of the reference standard for positives, that these amplified assays are 20–30% more sensitive than culture, antigen, or nonamplified nucleic-acid detection methods *(15–29)*. This range in sensitivity (for PCR and/or LCR) is influenced by the rate of inhibitors of amplification found in clinical specimens. The multispecimen evaluations with the use of several different assays have shown both PCR and LCR, for example, to be highly specific if appropriate anticontamination precautions are exercised. Thus, the proper use of these assays should provide added sensitivity without a need to

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confirm positives *(28,29)*. Recent data have shown the successful use of amplified probes on noninvasive specimens, such as first-void urine (FVU) in both men *(22,23)* and women *(22,24,25)* and introitus specimens in women *(31)*, which should enable the implementation of chlamydia screening programs in asymptomatic men and women.

2. Materials

2.1. Solutions

1. Lysis buffer. 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 2 5 m*M* $MgCl_2$, 0.01% gelatin, 1% Tween 20, and 200 µg/mL proteinase K
2. TE buffer: 10 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA.
3. Chaos buffer for RNA extraction: For 100 mL combine the following. 50 g GuSCN (4 2 *M*), 2.5 mL 20% Sarkosyl, 1 25 mL 2 *M* Tris HCl, pH 8.0, dH_20 to 99 3 mL, and filter these through a 0 45-µm Millipore filter Then add 0.7 mL β-mercaptoethanol/100 mL just prior to use.
4. PCR reaction buffer (10X): 500 m*M* KC1, 100 m*M* Tris-HC1 pH 8.3, 25 m*M* $MgC1_2$, 0.1% (w/v) gelatin
5. PCR master mix for 30 reactions· Glass-distilled sterile H_2O (2325 µL), 10X PCR reaction buffer (300 µL), dNTP mix, 12.5 m*M* each (48 µL), primer #1 200 m*M* (7 5 µL), primer #2 200 m*M* (7 5 µL), Cetus Ampli *Taq* (5 U/mL) (12 µL)
6. LCR reaction mix: 50 m*M* HEPES, pH 7 8, 10 m*M* $MgCl_2$, 10 m*M* NH_4Cl, 100 m*M* KCl, 1 m*M* DTT, 10 µg/mL BSA, 0 1 m*M* NAD, 50 µ*M* dCTP, or appropriate dNTP for gap-LCR, thermostable DNA ligase from *Thermus thermophilus* (2 U/50 µL) from Molecular Biological Resources (Milwaukee), 10^{11} mol of each of four primers.
7. NASBA 4X primer mix: primer 1 (0.8 µ*M*), primer 2 (0.8 µ*M*), DMSO (120 µL), sterile water (bring up to 200 mL).
8. 2.5X buffer mix (5 mL)· 1 *M* Tris (500 µL), 1 *M* $MgCl_2$ (150 µL), 2 *M* KCl (262 5 µL), 100 m*M* dNTPs (125 µL each), 100 m*M* ATP (250 µL), 100 m*M* CTP (250 µL), 100 m*M* UTP (250 µL), 100 m*M* GTP (187.5 µL), 100 m*M* ITP (62 5 µL), sterile water (2.962 m)
9. NASBA buffer-primer mix (1X)· 2 5X buffer (10 µL), sterile water (1 75 µL), 4X primer mix (6.25 µL).
10. NASBA enzyme mix (1X): BSA (0 13 µL), RNase H 1 2 U/µL (0 12 µL), T7 RNA polymerase 40 U/µL (0.66 µL), AMV RT 10 U/µL (0 32 µL), 4 *M* sorbitol (0.685 µL), 1 *M* DTT (0 125 µL).
11. Q-β replicase sample processing buffer: 8 *M* GuHCl, 0.6% Sarkosyl (or CTAB), 100 m*M* Tris-HCl, pH 7.8, 20 m*M* EDTA.
12. Q-β replicase probe dilution buffer: 100 m*M* Tris-HCl, pH 7.8, 20 m*M* EDTA.
13. Q-β replicase low-salt buffer: 100 m*M* Tris-HCl, pH 8 1, 20 m*M* EDTA, 25 m*M* NaCl, 0 2% Sarkosyl, 0.05% BSA, 0.05% Bronopol.
14. Q-β replicase GuSCN release buffer 240 m*M* Tris-HCl, pH 7 8, 60 m*M* EDTA, 3 *M* GuSCN, 0.6% Sarkosyl

15 Q-β replicase GuHCl detection buffer: 100 m*M* Tris-HCl, pH 7.5, 20 m*M* EDTA, 8 *M* GuHCl
16 Q-β replicase buffer: 220 m*M* Tris-HCl, pH 7 8, 40 m*M* $MgCl_2$, 1 2 mL each GTP, ATP, CTP, UTP, 2 μg/mL propidium iodide, 25% glycerol, 100 μg/mL Q-β replicase.
17 Q-β replicase high-salt buffer 100 m*M* Tris-HCl, pH 8.1, 20 m*M* EDTA, 300 m*M* NaCl, 0.2% Sarkosyl, 0 05% BSA, 0 05% Bronopol.

2.2. Equipment

All methods require a microfuge, vortex mixer, heat block and micropipeters with adjustable volumes. PCR and LCR both require a thermal cycler. There are several companies that sell thermal cyclers, most of which use heat blocks that accept thin-walled 0.5-mL PCR tubes. Perkin Elmer (Branchburg, NJ), the leader in this field, sells both a 24- and 96-well model, which has a heated lid for oil-free amplification. Roche Molecular Systems (Branchburg, NJ) has recently developed an automated system called Cobas Amplicor™ that has two or four 12-well heating blocks and a built-in EIA detection station. LCR from Abbott Diagnostics (Chicago, IL) requires an LCx analyzer for detection of specific LCR products. Gen Probe's PACE®2 and TMA assay (San Diego, CA) require a leader 550 luminometer, a water bath, and a dry heat block. Q-β replicase *(32,33)* uses a magnetic block separator, an eight-channel aspirator, and a 96-well kinetic fluorometer containing a 37°C heat block (Gene-Trak Systems, Framingham, MA) for measuring binding of propidium iodide to amplified RNA. In-house DNA or RNA amplification assays require agarose-gel electrophoresis, Southern blottings, or hybridization equipment.

3. Methods

3.1. Specimens

C. trachomatis nucleic acid (DNA or RNA) can be detected in a wide range of clinical specimens, including urethral and cervical swabs, FVU, vaginal introitus swabs, and rectal swabs using a variety of molecular techniques. Commercial tests employ different unique extraction protocols that may use detergents, heat, or both to release DNA and RNA from infected cells. Extraction of nucleic acids for in-house detection methods can be performed using a variety of methods, including a combination of the following techniques: phenol-chloroform, guanidium isothiocynate spin columns, silica particle adsorption. Some examples of nucleic acid extraction kits are GENECLEAN II (BIO IOI Inc., Vista, CA) and X-TRAX™ (Gull Laboratories, Salt Lake City, UT).

Specimens to be tested by Amplicor™ PCR or LCx should be processed according to the manufacturer's instructions. The following protocol can be used for extraction of DNA and RNA for in-house assays.

3.2. DNA Extraction

1. For isolation of DNA for tissue samples or swabs, transfer cells or tissue to a 1 5-mL microfuge tube and pellet. For urine samples or specimens collected in transport media, spin approx 0 1 mL of sample in a microfuge tube 20–30 min at 14,000 rpm.
2. Discard the supernatant and process the pellets by adding a 100 µL vol of freshly prepared lysis buffer.
3. Incubate the specimens at 55°C for 1 h or overnight at room temperature.
4. Heat-inactivate proteinase K by heating samples at 94°C for 10 min if the crude specimen is to be amplified directly.
5. Briefly spin the tubes in a microfuge to pellet the condensation.
6. Add an equal volume (200 µL) of phenol/$CHCl_3$/isoamyl alcohol (50.48.2) to the lysate, and mix by inversion.
7. Centrifuge for 5 min at 14,000 rpm to separate the phases
8. Transfer the top aqueous phase to a fresh microfuge tube, and add an equal volume (approx 200 µL) of $CHCl_3$/isoamyl alcohol (98·2 v/v)
9. Mix well, and centrifuge as above.
10. Transfer the aqueous phase to a fresh microfuge tube, and add 3 *M* sodium acetate, pH 5.2, to a final concentration of 0 25 *M*.
11. Add 2 vol of 95% ETOH (400 µL), and precipitate the DNA at –70°C for 30 min or at –20°C overnight.
12. Centrifuge the samples at 14,000 rpm at 4°C for 30 min.
13. Wash the pellet once with 70% ETOH, and centrifuge at 14,000 rpm for 10 min at 4°C.
14. Air-dry the pellet and resuspend in H_2O or TE buffer

3.3. RNA Extraction

All solutions must be RNase-free and should be prepared with double-filtered deionized water using disposable plastic pipets and diethylpyrocarbonate-(DEPC) treated glassware. DEPC-treated water should be used throughout to prevent degration of RNA. Negative and positive patient specimens should be added as controls for the extraction procedure.

1. Transfer 100 µL of serum, 100 mg of homogenized tissue, or 10^4–10^6 mammalian cells to a 1 5-mL microfuge tube Resuspend tissue and cells in 100 µL of dH_2O or TE buffer
2. Add 600 µL of chaos buffer (with mercaptoethanol added!).
3. Incubate for 30 min at room temperature
4. Add 700 µL of phenol/CHCl3/isoamyl alcohol, and mix well by inversion
5. Centrifuge for 10 min at 14,000 rpm.
6. Transfer aqueous phase to a fresh 1.5-mL microfuge tube, and add 700 µL of $CHCl_3$/isoamyl alcohol
7. Mix well by inversion, and centrifuge as above
8. Transfer upper aqueous phase to a fresh 1.5-mL microfuge tube.

9. Add 3 *M* NaOAc, pH 5.2, to a final concentration of 0.25 *M*, and add 1 vol of isopropanol
10. Mix well by inversion. Store at –70°C for at least 30 min or –20°C overnight.
11. Centrifuge for 30 min at 4°C at 14,000 rpm.
12. Wash pellet once with 70% EtOH
13. Dry pellet, and dissolve in 10–30 µL of dH_2O

3.4. Test Protocols

3.4.1. Nucleic Acid Hybridization (NAH)

GEN-PROBE's PACE®2C assay detects both *C. trachomatis* and *Neisseria gonorrhoeae* using a single swab. The specimen is treated to release 16S rRNA target, which is then reacted with an acridinium ester-labeled DNA probe. The probe target hybrid is captured onto magnetic beads and detected by chemiluminescence using a luminometer. The PACE®2C assay reagents are prepared by GEN-PROBE and are proprietary; however, the approach is straightforward and could be adapted in the research laboratory for use in detecting specific sequences. The following is the PACE®2C procedure:

1. Express the fluid from the swab, vortex, and transfer 100 µL to a labeled tube
2. Add 100 µL of probe reagent.
3. Incubate at 60°C for 1 h
4. Add 1 mL separation solution containing negative beads
5. Incubate at 60°C for 10 min.
6. Decant solution using magnetic block separator
7. Fill each tube with wash solution, and let stand 20 min.
8. Decant wash solution, and add substrate.
9. Read tubes in leader luminometer.

3.4.2. Polymerase Chain Reaction (PCR)

Several in-house PCR assays and one commercial assay have been described for the detection of *C. trachomatis* in clinical specimens. These employ primers for the amplification of the cryptic plasmid, the major outer membrane protein, cysteine-rich protein, or 16S RNA genes. The following method uses KL1-KL2 plasmid primers, and has proven to be both sensitive and specific on cervical and urethral swabs:

1. Prepare PCR master mix containing KL1-KL2 primers for 30 × 100 µL reactions:
   KL1 5'-TCC GGA GCG AGT TAC GAA GA.
   KL2 5'-AAT CAA TGC CCG GGA TTG GT.
2. Aliquot 90 µL of the master mix into a 0.5-mL microfuge tubes, and cover with 75 µL of light mineral oil
3. Add 10 µL of DNA or processed specimen. Include positive and negative controls. It is useful to include a sensitivity panel consisting of dilutions of total

cellular control DNA ranging from 1 fg to 1 pg. Interspersing negative controls in a large run is a useful way of detecting carryover contamination if uracil-*N*-glycosylase is not used (*see* **Notes 1** and **2**)

4. Amplify for 40 cycles as follows using a Perkin Elmer thermocycler model 9600: 30 s at 94°C (denaturation), 30 s at 55°C (annealing), 60 s at 72°C (extension) The last cycle should be followed by an extension at 72°C for 8 min.
5. Analyze amplification products by electrophoresizing 10 µL on a 2% agarose gel for 40 min at 140 V (*see* **Note 3**)
6. Confirm the specificity by Southern hybridization using FITC-labeled KL3 oligonucleotide probe (5'-TGA CTA ATC TCC AAG CTT AA-3') and horseradish-peroxidase-labeled antifluorescein antibody.

Chlamydia Amplicor™ from Roche Molecular Systems is a plasmid-based PCR that uses CP24-CP27 primers to amplify a 207-bp fragment that is captured onto a 96-well microtiter plate with immobilized oligonucleotide probe. Specific amplicon is detected using HRP–avidin conjugate, which binds to the biotinylated amplicon. Carryover contamination is prevented by incorporation of dUTP and uracil-*N*-glycosylase with cleaves previously amplified product at each uracil base rendering the DNA nonamplifiable. Specimens with an absorbance below 0.2 are interpreted as negative, whereas specimens with an absorbance above 0.5 are considered positive. Specimens with absorbances between 0.2 and 0.5 are considered equivocal and must be repeated in duplicate to determine if they are true positives (specimens with at least two of three results having an absorbance >0.25). The presence of spermicides in excess of 1% or surgical lubricants in excess of 10% or excess mucus or blood in cervical samples may have an inhibitory effect on amplification.

The Cobas Amplicor™ is a newly developed semiautomated assay that has two 12-specimen rings and uses magnetic particles for the detection of amplified DNA by chemiluminescence. An optional internal control reagent is available to verify negative results by ruling out the presence of inhibitors in clinical specimens.

3.4.3. Ligase-Chain Reaction (LCR)

LCR utilizes four oligonucleotide primers (instead of two used in PCR), a thermophilic DNA ligase to ligate the contiguous primers, and a thermal cycler to cycle the reaction between the denaturation temperature (94°C) and the ligation (72°C) temperature A variation of LCR called gapped LCR employs *Taq* polymerase to fill in one nucleotide prior to the ligation of adjacent primers. Abbott Diagnostics has commercialized LCR (LCx™ Chlamydia for the direct detection of *C. trachomatis* plasmid DNA in endocervical and urethral swabs and first-void urine specimens). The assay employs a microparticle EIA to detect specific LCR product with the automated IMx instrument. Four oligo-

nucleotide probes are designed to be complementary to the target sequence, so that in the presence of target, the probes bind adjacent to one another. They are then enzymatically ligated to form an amplification product that serves as a target for further rounds of amplification. Ligated product is captured by antibody immobilized onto the surface of microparticles via a ligand attached to the end of one primer and then detected by an enzyme-conjugated antibody directed at a second reporter molecule at the distal end of the other primer. The amplification product accumulates exponentially and is detected on the LCx analyzer. The following is a generic LCR protocol for urine specimens:

1. First-void urine specimens (1 mL of first 20 mL urine) are centrifuged 14,000 rpm for 10 min, and the pellet resuspended in buffer. Boil specimens by heating to 95–100°C for 15 min to release DNA.
2. Prepare sufficient LCR reaction mixture for the desired number of specimens and controls (100 µL for each specimen).
3. When specimens have cooled to ambient temperature, add 100 µL of sample to a tube containing 100 µL LCR reaction mixture.
4. Place tubes in thermal cycler, and program for 40 cycles of 1 s at 93°C, 1 s 59°C, 70 s at 62°C (the times and temperatures will vary for LCR or gapped LCR and T_{ms} of primers).
5. Ligated product can be detected by gel electrophoresis and autoradiography, or in the case of Chlamydia LCx, by chemiluminescence following capture of amplified product onto microparticles using immobilized antibodies directed against one of the haptens. The other end of the LCR product containing a second hapten is recognized by a second antibody conjugated to a reported enzyme, such as alkaline phosphatase. Only ligated product with both haptens covalently attached will generate a chemiluminescent signal. For LCx™. The amplification tubes are placed into the reaction wedges and then loaded into the carousel. The LCx analyzer prints the results which are expressed as counts per second per second, and positives are defined using run calibrators that establish the cutoff (*see* **Note 4**)

3.4.4. Transcription-Mediated Amplification (TMA)

Two transcription-based amplification assays that are nearly identical have been developed for *C. trachomatis*. NASBA uses three separate enzymes, AMV reverse transcriptase (RT), RNase H, and T7 polymerase, whereas TMA uses two enzymes, an RT enzyme with RNA-dependent DNA polymerase activity and RNase H activity and a second T7 polymerase enzyme with DNA-dependent RNA polymerase activity. Organon Teknika, which owns the exclusive rights to NASBA, has developed an in-house NASBA for *C. trachomatis*, but has not yet brought it to clinical trials. GEN-PROBE's TMA has completed clinical trials and awaits licensing in the US by the FDA. TMA amplifies a specific 16S rRNA target via DNA intermediates in an isothermal

single-step amplification reaction. In TMA, nucleic acids are first melted by heating to 95°C for 10 min and then lowered to 42°C for reverse transcription, making a cDNA copy of the RNA target using a "chimeric" primer that has sequence complementarity with the target and a sequence for T7 RNA polymerase binding. Following RNase digestion of the target by RT, a second primer anneals at the 3' end of cDNA, and the DNA polymerase activity of RT fills in the complementary second strand, producing a double-stranded DNA that is then transcribed by T7 polymerase generating up to 10^8 copies of RNA amplicon. GEN-PROBE'S TMA detects amplified RNA by a hybridization protection assay (HPA) involving hybridization with a chemiluminescent single-stranded DNA probe and detection with an enzyme-labeled anti DNA:RNA duplex antibody.

The following generic protocol can be used for NASBA amplification of RNA:

1. Pipet 5 µL of nucleic acid specimen into a microfuge tube
2. Prepare buffer and primer mix in a "clean area" with dedicated pipets free of template (*see* **Note 4**)
3. Add 18 µL of premix to each reaction tube containing 5 mL of template
4. Incubate at 65°C in a heat block for 7 min.
5. Cool tubes to 41°C in a heat block for another 7 min
6. Prepare enzyme mix during the above incubation
7. Add 2 µL of enzyme mix to reaction tubes at 41°C (one at a time in heating block)
8. Incubate tubes for 90 min in 41°C heat block in a containment hood
9. Remove tubes outside of "clean area," spin down in a microfuge, and store at –20°C for analysis by agarose-gel electrophoresis and Southern hybridization

3.4.5. Q-β Replicase-Amplified Hybridization (QBRAH)

Gene-Trak has developed Q-β replicase for *C. trachomatis* detection *(32,33)*. The assay is a 4-h test involving capture and release of a modified Q-β phage containing a specific *C. trachomatis* 16S rRNA target sequence followed by amplification of the target probe with Q-β replicase in the presence of propidium iodide. Q-β replicase requires one Chlamydia capture probe immobilized to paramagnetic beads and a replicatable recombinant midi-variant (MDV) RNA containing a second Chlamydia-specific sequence complementary to 16S RNA target inserted between nucleotides 63 and 64 of the RNA plus strand detector probe.

1. Extract RNA using 0.4 mL sample processing buffer Remove a 100-µL portion of the processed sample for Q-β replicase amplification.
2. Mix the capture probe and the MDV detector probe at a final concentration of 300 ng/mL each in probe dilution buffer
3. Combine 66.6 L of specimen and 33.3 mL of probe mixture and hybridize for 30 min at 37°C.

4 Add 100 µL of streptavidin paramagnetic beads, and incubate for 5 min at 37°C.
5 Wash beads twice with low-salt buffer.
6 Elute target–detector complex from beads with 100 µL 3 *M* GuSCN release buffer for 5 min, and transfer the target–detector probe complex to a clean tube.
7 Add 50 µL of 300 mg/mL poly A-tailed capture probe, and incubate for 30 min at 37°C
8 Add 250 µL of oligo-dT magnetic particles, and incubate for 5 min at 37°C
9 Separate target–detection complex with magnetic separator, wash three times, and elute the probe detector complex as before.
10 Transfer the complex to a fresh tube and add 100 µL of 0.12% oligo-dT beads in 200 µL 8 *M* GuHCl detection buffer. Capture and wash a fourth time.
11. Combine a 100-µL portion of the complex with 100 µL of Q-β replicase buffer, and incubate in the 37°C heat block of the kinetic fluorimeter.

An aliquot of the amplification reaction is assayed in a Gene-Trak kinetic fluorescence reader, which monitors production of RNA. Q-β replicase has a sensitivity of 1000 target molecules.

3.5. Discussion

Plasmid DNA amplification has become the most popular target because of its inherent theoretically higher sensitivity, which is owing to the presence of 7–10 copies/*C trachomatis* elementary body. Cryptic plasmid PCR has received extensive evaluation in female swab and male urine specimens. In both of these specimens, the sensitivity of the assay has been reported to be 20% to 30% higher than culture or antigen detection methods. Because this technology is more sensitive than the previous reference methods, several approaches to confirming the extra positives have been developed. This has usually been done by performing a second PCR whose primers are directed against a totally different gene or to a different fragment of the same gene *(28,29)*. PCR rarely demonstrates a sensitivity of 100% because of inhibitors of amplification found in clinical specimens. The rate of appearance of these substances probably varies according to specimen type and may also be different according to gender. Inhibitors of *Taq* polymerase have been found that disappear on storage and can be removed by a number of methods, including dilution, heating or centrifugation *(34)*. Internal control reagents to identify inhibited specimens are available for incorporation into the Roche Amplicor Chlamydia assay so that negative PCR results can be verified as true negatives. LCR testing of female cervical specimens has ranged in sensitivity from 87 to 97%, and female FVU specimens have identified as many infected as cervical cultures in the limited number of studies reported thus far *(22,24,25)*. LCR also has proved to be an effective assay when performed on male FVU. The test has identified >90% infected men, and the presence or absence of symptoms of urethritis has not

affected the positivity rate *(22,23)*. The exquisite sensitivity of amplified DNA probe technology provides a 20–30% increase in the number of infected patients identified. Inhibitors also play a role with LCR, but the rate seems to be lower than for PCR *(35,36)*. Substances in urine inhibitory to PCR and LCR include nitrites, crystals, and beta-HCG *(37)*. Storing urine specimens overnight at 4°C, diluting 1:10 or freezing at –7°C was found to remove the inhibitory activity from at least 85% of the specimens. Although recent publications on TMA and NASBA suggest that these techniques offer sensitivity and specificity comparable to PCR and LCR, further evaluation in populations with different disease prevalence are needed *(38,39)*.

4. Notes

All amplification techniques have pitfalls and limitations.

1. One of the most significant problems with both PCR and LCR is the possibility of false-positive results owing to amplicon carryover contamination. The use of plugged pipet tips and two or three defined work areas separating preamplification and postamplification steps has been widely adopted by most users, and helps to eliminate contamination. Amplicor™ uses uracil-*N*-glycosylase to digest any amplicons that may be present in PCR reaction tubes. LCx uses a chemical inactivation step in the final step of the LCx analyzer to destroy amplicons. The manual Amplicor™ test was susceptible to errors generated by the operator splashing the contents of wells during the EIA detection step. This, however, is not a problem with the automated Cobas Amplicor™ and LCx analyzer, where reagents are added by a robotic pipeter. The LCx analyzer can give low-level diluent error messages and canceled runs if the instrument's routine maintenance schedule is not meticulously followed.
2. The LCx is limited to a run size of 20 samples/run (including controls) necessitating multiple runs The Perkin Elmer 480 or 9600 thermal cyclers are used with 48–96 specimens
3. All amplification technologies, particularly in-house assays, should employ several positive controls or calibrators to show the analytical sensitivity of each run In-house PCR assays should not rely on agarose gels to detect specific products, but instead should confirm the specificity of amplification by Southern blot hybridization or using oligo probes in a microtiter plate hybridization assay. The latter method facilitates the testing of a larger number of specimens.
4. All three commercial amplification tests have notes on procedural limitations, interfering substances, and troubleshooting in the package insert. Most problems can be avoided if the instructions are properly followed. For LCx, the instrument manual contains a list of error codes and a table of what to do for each error message. For example, each reagent pack is bar code-read by the instrument, and a predetermined number of tests are permitted for each reagent pack so that reagents do not run dry during a run. For TMA, the package insert includes a section on decontaminating the work area and luminometer racks. The package

insert also includes a laboratory monitoring procedure for detecting contaminated work areas. The Leader 450i luminometer operating manual contains a troubleshooting section that tells the operator what to do if the instrument gives an error message.

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4

Haemophilus ducreyi Detection by PCR

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1. Introduction

Genital ulcers are typically caused by one of three organisms, *Haemophilus ducreyi*, *Treponema pallidum*, or herpes simplex virus, which cause chancroid, syphilis, and genital herpes, respectively. Although traditionally these diseases have been differentiated by their clinical presentation, there is considerable overlap in their clinical manifestations *(1)*.

The diagnosis of chancroid is typically based on the isolation of *H. ducreyi* from the genital ulcer, and as a result, this disease is probably grossly underdiagnosed. In several studies, the sensitivity of culture of *H. ducreyi* from suspected chancroid cases in experienced laboratories ranged from 56 to 90% *(2)*. However, the sensitivity of culture can only be estimated, since there is no gold standard on which to base the diagnosis of chancroid. Overgrowth of culture plates with other bacteria, isolation of other *Haemophilus* spp. from the genital ulcer, and difficulties in identification of *H. ducreyi* are additional obstacles in the detection of *H ducreyi*. Diagnosis of chancroid may be even more difficult in the US; Shulte et al. *(3)* have shown that <14% of STD clinics in 32 states, the District of Columbia, and Puerto Rico have the appropriate media for *H ducreyi* culture available. Owing to these problems with culture of this organism, alternative methods of detection are urgently needed.

The development of polymerase chain reaction (PCR) techniques for detection of *H. ducreyi* directly in patient specimens bypasses the difficulties experienced with culture of this pathogen. These techniques require the identification of DNA primers that are specific for the bacteria to be detected. Patient specimens are collected in sample transport medium and, if necessary, stored frozen until analysis, then treated, and subjected to PCR amplification using a thermocycler. Amplicons specific to *H. ducreyi* are then detected by hybridization.

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Several groups have reported PCR techniques for the detection of *H. ducreyi* in genital ulcer specimens. These PCR assays vary in the sample transport media used, treatment of the specimen to remove PCR inhibitors and make the DNA accessible for amplification, specific primers and amplification conditions used, and detection techniques (*see* **Note 1**). The different PCR assays for *H ducreyi* are listed in **Table 1** and are discussed below.

Chui et al. *(4)* used primers based on conserved eubacterial 16S rRNA gene sequences to amplify a 303-bp sequence from members of the *Pasteurellaceae* and *Enterobacteriaceae*. Using two *H ducreyi*-specific probes internal to this sequence, they obtained 100% sensitivity with 51 strains from six continents isolated over a 15-yr period. The clinical utility of PCR was compared with that of culture, using 100 clinical specimens from men with genital ulcers consistent with a clinical diagnosis of chancroid. Swab specimens were transported to the laboratory in phosphate-buffered saline (PBS) containing chenodeoxycholate (1 mg/mL). After extraction, the DNA was amplified by means of a PCR protocol that involved 25 cycles of amplification, followed by Southern blot hybridization to detect the PCR products. This procedure yielded a sensitivity and specificity of 83 and 67% relative to culture, respectively. After three rounds of 25 cycles, the sensitivity and specificity were 98 and 51%, respectively.

rRNA primers were also used for detection of *H ducreyi* in a limited number of ulcer specimens from patients in Tanzania *(5)*. In this study, specimens were collected in PBS, centrifuged to pellet the bacteria that were subsequently washed in Tris buffer, and then chloroform-extracted. Amplification was achieved by first using the universal rRNA primers described by Chui et al *(4)*, and then amplifying with two internal primers, one labeled with biotin, and the other labeled with digoxigenin. The PCR products were then captured on avidin-coated microtiter plates and detected by a colorometric reaction using antidigoxigenin antibody-alkaline phosphatase conjugate. In this study, the sensitivity and specificity relative to culture were 100% (4/4) and 76% (16/21), respectively.

Johnson et al. *(6)* developed a PCR assay using primer sequences which amplified a 1.1-kb anonymous sequence from 30 strains of *H. ducreyi* but not 25 strains of other organisms. For genital ulcer specimens, samples were collected in transport medium GA without added agar *(7)*, DNA was extracted by the procedure of Chui et al. *(4)* and then subjected to 25 cycles of amplification. DNA was detected by hybridization using Southern blots and dot blots. Their assay had a sensitivity of 64% (39/61) and a specificity of 50% (71/143) compared to culture. This PCR technique was subsequently modified by collecting the patient specimens in the sample transport medium of Chui et al. *(4)*, extracting the DNA as described by this group *(4)* and then dialyzing the DNA

Table 1
Techniques Used for the Detection of *H. ducreyi* DNA in Consecutive Patient Specimens by PCR Amplification

Gene amplified	Collection medium	Sample preparation	Detection	Ref
rRNA	1 mg/mL chenodeoxycholate in PBS	SDS added; phenol, chloroform extraction; ethanol precipitation	Hybridization of Southern blots with *H. ducreyi*-specific probe	*(4)*
rRNA	PBS	Centrifuged to collect bacteria, washed in 50 m*M* Tris (pH 8.3), extracted with chloroform	Nested PCR; incorporation of biotinylated dNTPs, capture in microwell plates coated with avidin, colorimetric detection	*(5)*
Anonymous 1.1-kb fragment	Transport medium GA **ref. 7**	SDS added, phenol, chloroform extracted, ethanol-precipitated	Hybridization of Southern and dot blots	*(6)*
Anonymous 1.1-kb fragment	1 mg/mL chenodeoxycholate in PBS	SDS added, phenol, chloroform extracted, ethanol-precipitated, dialyzed	Hybridization of dot blots	*(8)*
groEL	Specimen transport medium (Digene Diagnostics, Inc. Silver Spring, MD)	Proteinase K added, purification and concentration of DNA using glassmilk	Hybridization of Southern blots	*(9)*
rRNA/confirmed by groEL, biotinylated oligonucleotide primers	AMPLICOR specimen transport medium (Roche Molecular Systems, Branchburg, NJ)	Dilution of specimen 1:2 in AMPLICOR specimen diluent (Roche)	Colorometric detection using Streptavidin HRP system using microtiter plates coated with capture probe–BSA complex	*(10)*
rRNA/confirmed by groEL, biotinylated oligonucleotide primers	1 mg/mL chenodexoycholate in PBS	Dilution of specimen 1:10 in AMPLICOR specimen diluent (Roche)	Same as above	*(10)*

to get rid of inhibitors *(8)*. These modifications improved the sensitivity and specificity of *H. ducreyi* PCR relative to culture to 100% (25/25) and 84% (51/61), respectively.

Parsons et al. *(9)* determined that primers based on the *H ducreyi gro*EL sequences were suitable for PCR detection. These primers amplified all 139 isolates of *H. ducreyi* tested, but not isolates of 25 other bacteria. Using a commercially available specimen transport medium (Digene Diagnostics, Inc., Silver Spring, MD), incubation of specimens with Proteinase K followed by ethanol precipitation, 40 cycles of amplification, and detection by hybridization of Southern blots, these researchers achieved a sensitivity of 89% (59/66) and a specificity of 79% (76/96) compared to culture.

Finally, a multiplex PCR assay has been developed at Roche Molecular Systems that will simultaneously detect *H. ducreyi*, *T pallidum*, and herpes simplex virus in genital ulcer specimens *(10)*. *H. ducreyi* primer sequences were based on regions of rRNA specific for this microorganism, the assay employed 35 cycles of amplification, and detection was based on oligonucleotide capture probes and a colorimetric detection system. Culture-negative specimens from which *H. ducreyi* DNA was amplified with the rRNA primers were confirmed as true positives using primers specific for *H. ducreyi* groEL. Orle et al. *(10)* further simplified sample treatment, successfully amplifying DNA after collection in Roche AMPLICOR Specimen Transport Medium, by only heating at 100°C for 10 min and diluting 1:2 in AMPLICOR Specimen Diluent before PCR amplification. Similar results were obtained when the samples were collected in chenodeoxycholate (1 mg/mL) in PBS and diluted 1:10 in AMPLICOR Specimen Diluent. The sensitivity and specificity of detection of *H. ducreyi* by this PCR technique compared to culture were 98% (45/46) and 79% (227/289), respectively. Unfortunately, this multiplex PCR test for genital ulcer disease is not commercially available at this time.

In summary, several different laboratories have developed protocols for the detection of *H. ducreyi* by PCR. Because we have the most experience with the primers developed by CDC for amplification of *H. ducreyi* DNA *(6)*, we will describe their use for detection of *H ducreyi* by PCR from both genital ulcer specimens and from urine specimens.

Collection of first-void urine from either men or women has been shown to be a noninvasive and appropriate alternative specimen for the diagnosis of sexually transmitted infections caused by *Neisseria gonorrhoeae (13)* and *Chlamydia trachomatis (14)*. More recently, Trees et al. *(20)* have shown that urine specimens could be used for PCR diagnosis of vulvovaginal ulcers. In a preliminary study, these investigators found that PCR analysis of first-void urine specimens from women with vulvovaginal ulcers had a sensitivity of 88% and a specificity of 100% when compared with PCR analysis of the

corresponding ulcer specimen. Thus, first-void urine specimens may be useful for screening purposes when pelvic examinations cannot be readily performed.

2. Materials

2.1. Preparation of Stock Solutions

1. Proteinase K solution. dissolve 200 mg of proteinase K in 10 mL distilled water, mix, and freeze in 0.5-mL aliquots.
2. PK digest solution: Mix 250 µL of proteinase K (20 mg/mL) and 50 µL of dithiothreitol (DTT, 1 *M*) in a total volume of 25 mL distilled water. Final concentrations are 200 µg/mL proteinase K and 2 m*M* DTT; 1 *M* DTT is prepared by dissolving 1.54 g of DTT in 10 mL distilled water and freezing (–20°C) in 1-mL aliquots.
3. Ammonium acetate/ethanol solution: combine 67.5 mL of 5 *M* ammonium acetate and 335 mL of 95% ethanol. Store in a –20°C freezer.
4. 10X PCR buffer: to prepare 100 mL of 10X buffer, use the following procedure.

Reagent	Volume, mL	Final concentration in 10X buffer
1 *M* Tris, pH 8.3	10	100 m*M*
1 *M* KCl	50	500 m*M*
1 *M* $MgCl_2$	4.0	40 m*M*
Distilled water	36	

Dispense in 10-mL aliquots, and freeze at –20°C.

5. Primer mix. dilute the two primers so the diluted solution contains 180 pmoles/20 µL of each in water, mix, and freeze in 1-mL aliquots.

   Primers are often sold in powdered form (e.g., GIBCO/BRL) and can be reconstituted to the desired concentration. For example, if the tube contains 50 nmoles of primer, add 556 µL dH_2O, thereby making the final concentration of primer 90 pmoles/µL. Next, add 50 µL of primer 1 and 50 µL of primer 2 to 400 µL of dH_2O, giving a final concentration of 9 pmoles/µL or 180 pmoles of each primer in the 20 µL added to the mastermix (**Subheading 3.1.3.**)

   Primers.

   SJ1A: 5'CCCCGACACTTTTACACGCGCT3'

   SJ2A: 5'GCCAGCCCAGTGACGCCGATGCC3'
6. dNTP mix. mix 250 µL of each dNTP (100 m*M*, Amersham Pharmacia Biotech [Piscataway, NJ]) Add 1.5 mL water. The final concentration of each dNTP in this solution is 10 m*M*
7. Gel-loading solution. prepare a solution of 0.25% bromophenol blue and 15% Ficoll (type 400, Pharmacia).
8. 50X TAE: for 1 L, add the following to a final volume of 1 L distilled water.

   242 g Tris HCl.

   57.1 g sodium acetate.

   37.2 g $Na_2EDTA \cdot 2\ H_2O$

   This is diluted 1/50 before use (20 mL 50X TAE plus 980 mL distilled water)

9. 20X SSC. for 1 L, add the following to a final volume of 1 L distilled water
   175 g NaCl
   88 g Sodium citrate.
10. Salmon sperm DNA (10 mg/mL): add 100 mg of salmon sperm DNA (Sigma) to 10 mL distilled water. Let dissolve overnight in a refrigerator. Sonicate in 3-mL batches until DNA loses its viscocity (ca 3 min) Divide solution in 1-mL aliquots, boil for 10 min, and freeze at –20°C until use.
11. Denhart's solution· add the following to a final volume of 500 mL distilled water:
    5 g Ficoll.
    5 g Polyvinylpyrolidine.
    5 g Albumin, bovine.
    Freeze in 100-mL aliquots. Store working solution at 4°C.
12. Acrylamide solution (30/0.8%): mix 176 g acrylamide and 4 g *bis*-acrylamide in a total volume of 500 mL. Store at 4°C.
13. Denaturization buffer (0.5 *M* NaOH, 1.5 *M* NaCl): add 40 g of NaOH and 175 g NaCl to a 2-L flask. Add distilled water to equal 2 L total volume. Mix and store at ambient temperature.
14. Neutralization buffer· 1 *M* Tris, 1 5 *M* NaCl. Add 280 g Tris-HCl, 26 g of Tris-base, and 175 g NaCl to a 2-L flask Add distilled water to equal 2 L total volume Store at ambient temperature
15. MarFarland #1 turbidity standard (**ref.** *11*). Add 0.1 mL of a 1% solution of barium chloride to 9.9 mL of 1% H_2SO_4. Vortex before use.

2.2. Equipment and Supplies

1. Heating block with three 20-well modular blocks.
2. Microcentrifuge.
3. Thermal cycler
4. Polyacrylamide/agarose gel electrophoresis apparatus with power supply.
5. UV transilluminator
6. Photography equipment and supplies.
7. Biological safety cabinet with UV light in room separate from product analysis Two biological safety cabinets, one for sample preparation, one for the setup of PCR reactions, are preferable.
8. Micropipetters and aerosol-free tips. we use three complete sets of micropipetters (20, 200, and 1000 µL): one set for specimen treatment, one to set up the PCR reactions, and one set for all processes done after PCR
9. Shaking water bath.
10. Repipetter (optional).
11. Seal-a-Meal apparatus (obtain from local grocery store).
12. Equipment needed for working safely with radioisotopes-plexiglas shields, disposal boxes, survey meter, and so forth.
13. Dacron-tipped swabs.
14. Nylon membranes (e g., maximum-strength Nytran Plus, Schleicher and Schuell, Keene, NH)

15 Hybridization bags (Gibco BRL) and random primers DNA-labeling system (Gibco BRL)
16. Disposable gloves.
17 Digene specimen transport medium (STM, Digene, Inc., Silver Spring, MD). **Note:** Kits including this STM along with dacron swabs are available from Digene, Inc. under the name ViraPap Collection Kit.
18. Glass matrix (GlasPac· National Scientific Supply Company, San Rafel, CA).
19 HCV Monitor lysis buffer (Roche Diagnostics, Nutley, NJ).
20. Nytran filters.
21 Autoradiography supplies.

3. Methods

3.1. PCR Amplification of H. ducreyi DNA from Genital Ulcer Specimens

3.1.1. Specimen Collection

1 Collect the specimen from an ulcer with a dry dacron-tipped swab.
2. Place the swab in a tube containing Digene STM, break off shaft, leaving the swab in the medium, and close cap.
3 Freeze the specimens at –70 or –20°C until they can be analyzed.

3.1.2. Specimen Processing

All procedures, including preparation of stock solutions, sample preparation, and PCR should be performed in a "clean laboratory," i.e., one that is not used for growth of *H ducreyi* or for PCR product analysis. Any reagents used for PCR should be stored in the "clean laboratory" and should never enter the laboratory used for product analysis. People entering the "PCR-clean laboratory" should wear special lab coats stored in this lab, and should wear disposable gloves when working with the supplies and equipment in this lab. Any deviations from these procedures may result in DNA contamination and might result in false-positive PCR tests (*see* **Notes 3** and **4**).

1 Thaw the specimen tubes, and vortex with swab in place for 5 s. Transfer 200 µL of the specimen to a 1 5-mL microcentrifuge tube. Refreeze the specimen tube with swab in place.

   Also include positive and negative controls for specimen preparation and amplification with each run to control for sensitivity and contamination For specimen preparation, the positive controls consist of 10^5 and 10^3 CFU of *H. ducreyi* cells suspended in 1 mL of collection buffer, and the negative control consists of collection buffer only. Each of these is processed along with the specimens The two positive controls test the sensitivity of the assay for 1000 and 10 CFU of *H ducreyi*/10 µL contained in the PCR reaction.
2 Heat all tubes at 100°C for 10 min.

3. Centrifuge the tubes for 10 s in the microfuge to spin down the condensation on the inside walls of the tube
4. Add 100 µL of "proteinase K digest" stock solution. Incubate the specimen in the 37°C water bath for 1 h.
5. Add 800 µL of ammonium acetate/ethanol solution, mix by inversion, place on ice for 15 min, and then centrifuge the tubes for 30 min (microcentrifuge, 14,000 × *g*).
6. Pour off the supernatant, and heat the tubes with caps open at 65°C for 30 min in the heating block. This step will dry out the tubes and inactivate the proteinase K.
7. Resuspend the dried pellets in 100 µL water. At this point, the samples can be frozen or used immediately for PCR.

3.1.3. PCR

1. Preparation of master mix: calculate the number of tubes needed for PCR, and prepare the appropriate amount of master mix. Note that the amount of mixture needed for 48 specimens was really calculated for 49 to allow for loss during pipeting. Each patient specimen is analyzed using 2 and 10 µL of the processed specimen.

   The amount of the following reagents (in µL) needed to make master mix is:

	1 Specimen		48 Specimens	
	processed specimen volume		processed specimen volume	
Reagent	10 µL	2 µL	10 µL	2 µL
Distilled water	57.5	65.5	2817.5	3209.5
10X buffer	10.0	10.0	490	490
dNTP mix	2	2	98	98
Primer mix	20	20	980	980
Taq polymerase (5 U/µL)	0.5	0.5	24.5	24.5

2. Into two 0.65-mL microcentrifuge tubes (e.g., Intermountain Scientific, Kaysville, UT), add 100 µL mineral oil. For multiple samples, this can easily be accomplished with a repipetter.
3. Add 90 µL of master mix/tube for the 10 µL vol of treated patient sample, add 98 µL of master mix to the tube for 2-µL vol of treated patient sample.
4. Add 10 µL of treated patient sample to one tube and 2 µL sample to the other. The total volume in each tube should be 200 µL (100 µL of sample and 100 µL of mineral oil). Each tube now contains: 10 m*M* Tris, 50 m*M* KCl, 4 m*M* MgCl, 180 pmoles of each primer, 200 µ*M* of each dNTP, 2.5 U Taq, and either 2 µL or 10 µL of treated patient sample.
5. Spin tube for 10 s in microcentrifuge to collect the sample in the bottom of the tube below the mineral oil.
6. Quickly place the tubes in the thermocycler, which has been preheated to 85°C.

7. Amplify the samples in a thermocycler that has been programmed to perform 30 cycles of 1.5 min at 95°C, 2 0 min at 66°C, 4.0 min at 72°C, followed by a final cycle at 72°C for 10 min linked to a soak cycle of 15°C.

3.1.4. Detection of PCR Products

After amplification, the 1100-bp amplification product is detected by Southern blotting.

1. A 16-µL vol of each sample and control is mixed with 4 µL of 5X gel-loading solution, deposited in the wells of an agarose gel, and subjected to electrophoresis using TAE buffer.
2. Molecular-weight markers (e g , 1-kb ladder or λ DNA cut with *Hin*dIII) are loaded into one well of each row of the agarose gel to position the size of the amplification product.
3. The gels are stained with ethidium bromide, photographed, then alkali-denatured and neutralized as described by Southern *(12)* and transferred to Nylon membranes.
4. Dry and bake the membranes at 80°C for 2 h.
5. Prepare hybridization buffer as described below, adding the reagents in the order listed.

	Amount of reagent to add for a given number of blots, in mL		
	Number of blots		
	1	2	4
Distilled water	1.5	3.0	6.0
Formamide	5.0	10.0	20 0
20X SSC	3.5	7.0	14.0
20% SDS	0.05	0 1	0.2
0.5 *M* EDTA	0.01	0 02	0 04
Denhart's solution	2.5	5.0	10 0
Boiled salmon sperm DNA (10 mg/mL dH_2O, Sigma)	0.1	0 2	0 4

6. Put nylon membrane into hybridization bag, add appropriate amount of hybridization buffer, and incubate at 37°C for 1–2 h.
7. Cut open a corner of the bag, add 100 µL radiolabeled probe/10 mL hybridization solution, and incubate overnight at 37°C
8. The radiolabeled probe is generated by PCR using plasmid DNA from pHDH1A4S5H (obtained from Steve Johnson, CDC, Atlanta, GA), forward and reverse primers (we use CAGGGTTTTCCCAGTCACGAC and AGCGGATAACAATTTCAC ACAGGG, but any commercially available forward and reverse primers [e g, Gibco BRL] would amplify this fragment), and thermocycling parameters of 64°C, 2 min, 72°C, 2 min, and 95°C, 2 min, 30 cycles.

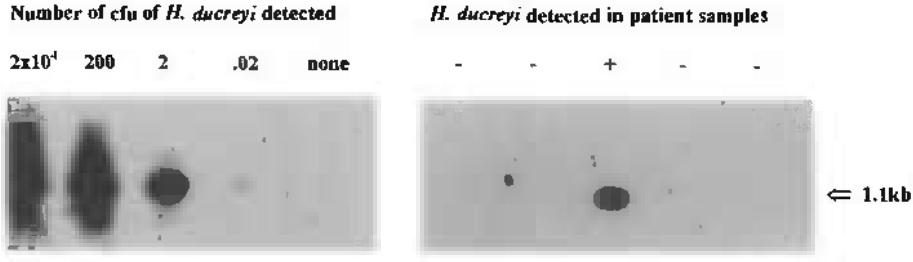

Fig 1. Analysis of sensitivity of PCR for detection of *H. ducreyi* DNA in clinical specimens. **(A)** Serial 10-fold dilutions of *H. ducreyi* strain CIP542 showing the sensitivity of detection as 0.02 CFUs. The number of bacteria detected by PCR is more sensitive than culture, even under laboratory conditions, probably because PCR detects the DNA from individual organisms even if they are clumped or nonviable. **(B)** Genital ulcer specimens tested for *H. ducreyi* DNA by the PCR reaction. A 1.1-kb fragment of DNA was amplified from the sample from one patient indicating the presence of *H. ducreyi* DNA.

9. After synthesis by PCR, the probe is purified by agarose-gel electrophoresis and the Gene Clean II kit according to the directions of the manufacturer (Bio101, LaJolla, CA).
10. The purified probe DNA is labeled with α ^{32}P-dATP ([800 Ci/mM] New England Nuclear, Boston, MA) using a DNA labeling kit according to the directions of the manufacturer (Gibco BRL). The labeled probe is heated to 100°C for 10 min, cooled on ice, and then diluted to a total volume of 1.0 mL.
11. Rinse the membrane three times (about 200 mL each time) in wash solution (5X SSC, 0.1% SDS), and then incubate with shaking at 60°C in remaining wash solution (ca. 400 mL) for 1 h.
12. Dry the blots and expose them to X-Omat AR film (Eastman Kodak, Rochester, NY) overnight at –70°C in an X-ray exposure holder with an intensifying screen. A 1.1 kb band that hybridizes with the probe DNA indicates a positive specimen. This technique is capable of detecting <2 CFUs (**Fig. 1**).

3.2. *Amplification of* H. ducreyi *DNA from Urine Specimens (Adapted from Stacy-Phipps et al. [13])*

1. Warm HCV MONITOR™ lysis buffer in a 37°C water bath, and mix by inversion to dissolve any precipitate.
2. Thaw urine at room temperature.
3. Using a plugged pipet tip, add 1.0 mL urine to a microcentrifuge tube and centrifuge for 5 min at 14,000 × *g*.
4. Remove supernatant to a fresh microcentrifuge tube, add 0.6 mL HCV MONITOR™ lysis buffer to the pellet, vortex, and incubate at 65°C for 10 min.

5 Add 25 µL GlasPac matrix to bind the DNA, vortex, and incubate at room temperature for 15 min.
6 Centrifuge for 1 min at 14,000 × *g*, remove, and discard supernatant.
7. Resuspend GlasPac matrix in 1 mL wash buffer, vortex, centrifuge for 1 min at 14,000 × *g*, and remove supernatant
8. Repeat wash step two more times.
9 After last wash step, remove as much supernatant as possible, and dry pellet for 15 min at room temperature with top of tube open.
10. Add 225 µL TE buffer, vortex to resuspend pellet, and incubate 5 for min at 50°C.
11. Centrifuge for 2 min at 14,000 × *g*, and transfer 200 µL of sample to a clean microcentrifuge tube, being careful not to disturb the GlasPac pellet.
12. Fifty microliters of the processed specimen are used for PCR analysis.

3.3. Detection of PCR Inhibitors and Specimen Adequacy by β-Globin Amplification (14)

Negative specimens are reamplified with β-globin primers using an aliquot of the same treated specimen that was used for *H. ducreyi* PCR.

1. Prepare primer mix the same as in *H. ducreyi* PCR section (**Subheading 2.1.5.**), except with β-globin primers (synthesized commercially, e.g., Gibco BRL):
   PCO4 primer: GAAGAGCCAAGGACAGGTAC.
   GH20 primer: GCCAGCCAGTGACGCCGATGCC.
2 Stock reagents are the same as for *H. ducreyi* PCR.
3. Prepare master mix, and perform PCR as described in **Subheading 3.1.3.**, except use amplification conditions of 94°C, 1.5 min, 55°C, 2 min, 72°C, 4 min, 30 cycles.
4. Assemble acrylamide gel apparatus.
5. Plug the bottom and sides of the gel with 1% agarose in TAE (or use a gel caster when pouring the gel).
6. Make acrylamide solution (30/0.8%), and mix the following:

Acrylamide 30/.8	4.1 mL
50X TAE	315 µL
Distilled water	11.5 mL
10% APS*	200 µL

*Dissolve 100 mg ammonium persulfate (APS) in 1 mL distilled water, and store at 4°C. This solution should be prepared fresh or used within 1 wk
7. Add 25 µL TEMED, pour liquid into gel holder, add comb, and let solidify.
8 When gel has solidified, remove the comb, add 1X TAE running buffer, add samples to wells along with a well containing mol-weight markers (e.g., λ cut with *Hin*dIII, Gibco BRL or 1-kb ladder, Stratagene), and apply 100 V until the blue dye reaches the bottom of the gel.
9. Stain the gel in ethidium bromide, expose to UV light, and take a picture An amplification product of 268 bp indicates the absence of inhibitors and that the specimen contains human DNA consistent with a patient specimen (**Fig. 2**).

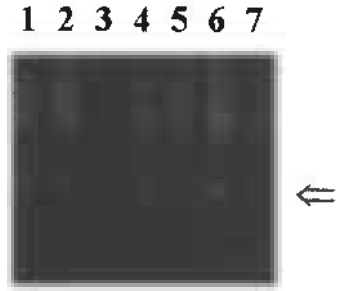

Fig. 2. Analysis of adequacy of specimen for PCR amplification by β-globin PCR. Samples were treated and subjected to PCR analysis using the β-globin primers, and then subjected to electrophoresis on an acrylamide gel. All samples in wells, except sample # 3, were amplified by this method as indicated by the presence of a 268-bp fragment (indicated by arrow).

3.4. Identification of H. ducreyi Isolates by Taxonomic Spot Blots

H. ducreyi isolates are identified based on the results of a limited set of tests. Traditional tests used for the identification of other *Haemophilus* species, e.g., catalase, sugar fermentation, hemolysis of horse blood, and enhanced growth in CO_2 ***(15)***, are not easily employed with *H. ducreyi*, either because they are not differential for *H. ducreyi* or because they require special media for growth. One can also confirm the identity of isolates as *H. ducreyi* by molecular methods. Using the "taxonomic spot blot test" ***(16)***, organisms can be classified as *H. ducreyi* based on the homology of their DNA to whole-cell DNA from the type strain of *H. ducreyi*. In this test, isolates are suspended in water and adjusted to an OD_{600} of 1.0 (McFarland #1 standard), spotted onto Nytran, treated to lyse the bacteria, and then subjected to hybridization with radiolabeled whole-cell DNA from *H. ducreyi* strain CIP542. Isolates of *H. ducreyi* will hybridize to the whole-cell DNA probe, resulting in a dark dot after exposure to X-ray film (**Fig. 3**). DNA from isolates that are not *H. ducreyi* will exhibit background reactions in this test (**Fig. 3**). With the inclusion of positive and negative controls with each test, isolates can be easily identified as *H. ducreyi* or not. The taxonomic spot-blot test is particularly convincing, because it uses a variation of the traditional method, whole-cell DNA homology, to classify organisms into species.

1. Grow suspected *H. ducreyi* isolates overnight on solid medium plates. We use CM agar plates ***(16)***, but any agar medium on which they will grow will suffice.
2. Suspend the growth in water so that it is the approximate turbidity of the MacFarland #1 standard. Include positive and negative control cultures, *H. ducreyi* strain CIP542 (ATCC #39940) and *Haemophilus influenzae* type strain (ATCC

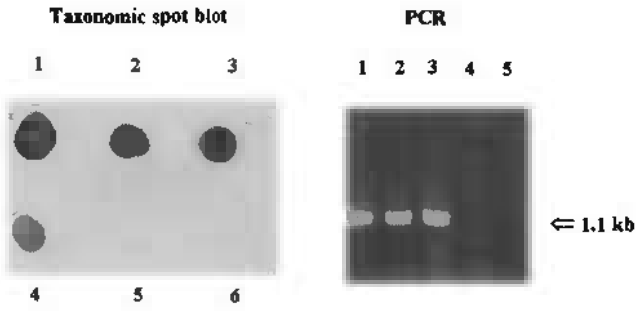

Fig. 3. Identification of *H. ducreyi* isolates by molecular methods: taxonomic spot-blot test and PCR ID. Taxonomic spot-blot test. Samples (1) HMC12, (2) HMC13, and (3) HMC14, presumptive *H. ducreyi* isolates (4) *H. ducreyi* strain CIP542, the positive control, (5) *H. influenzae* strain Rd, (6) *H. influenzae* strain ATCC 33940, the negative control. All presumptive *H. ducreyi* isolates were confirmed as *H. ducreyi*. PCR ID. Bacterial strains were subjected to the PCR with *H. ducreyi*-specific primers. PCR amplicons were visualized on ethidium bromide-stained agarose gels using UV light. Bacteria in wells were: *H. ducreyi* strains (a) HMC15, (b) HMC16, (c) HMC17, (d) *H. influenzae* strains Rd, and (e) *H. influenzae* strain ATCC 33940. All presumptive *H. ducreyi* isolates were confirmed as *H. ducreyi*.

#33391), respectively, grown and suspended as above, except chocolate agar plates are used for growth of *H. influenzae*.

3. Place 10-µL aliquots of the bacterial suspension on Nytran Filters or filter onto Nytran filters using a dot-blot apparatus (e.g., Bio-Dot, Bio-Rad, Hercules, CA).
4. Place the filters on paper towels wetted with the following solutions for the indicated amounts of time: denaturization solution, 10 min; neutralization solution, 1 min, three changes, and then 10 min, no changes. Dry.
5. Bake the filters at 80°C for 2 h.
6. Radiolabel whole-cell DNA from *H. ducreyi* strain CIP542 (isolated by standard techniques *[12]*) by the primer extension method. This is available as a kit and should be used according to the directions supplied by the manufacturer (Gibco BRL).
7. Hybridize the blots with 50 µL of the radiolabeled probe, wash, and expose to film as described in **Subheading 3.1.4.**

   Compare the intensity of the spots on the film with *H. ducreyi* CIP542 and *H. influenzae*. *H. ducreyi* isolates will display a dark spot on the film similar to CIP542 and different from *H. influenzae*, which will only show a background reaction.

3.5. Identification of H. ducreyi Isolates by PCR

An alternative method for identification of *H. ducreyi*, PCR amplification, utilizes the specificity of selected primer sequences for *H. ducreyi* DNA. In this

test, *H. ducreyi* strains are adjusted to an OD_{600} of 1.0, diluted 1:100, then treated with proteinase K, and ethanol-precipitated to purify the DNA as described in **Subheading 3.1.2.** The treated samples are then subjected to PCR using *H. ducreyi* specific primers (**Subheading 3.1.3.**) and analyzed by agarose-gel electrophoresis. Only *H. ducreyi* isolates show a band of the appropriate size (1.1 kb) when stained with ethidium bromide and viewed under UV light (**Fig. 3**).

3.6. Summary

Despite the importance of chancroid and its association with the heterosexual transmission of HIV, much needs to be learned about this disease. The pathogenesis of disease and virulence factors that allow *H. ducreyi* to cause genital ulcers and lymphadenitis are not well understood. Clinical diagnosis is inaccurate because chancroidal ulcers frequently have a clinical appearance similar to herpetic or syphilitic ulcers. In addition, herpetic and syphilitic ulcers may resemble chancroidal ulcers, particularly in HIV-positive individuals. Because culture of *H. ducreyi* from genital ulcers is insensitive, alternative techniques are needed to enhance the diagnosis and surveillance of this disease. Molecular techniques have been developed that can detect the presense of *H. ducreyi* DNA in clinical specimens without the need to culture this fastidious organism.

PCR has been used to determine the relative prevalence of *H. ducreyi* as a cause of genital ulcer disease and to establish that the prevalence of chancroid varies widely in different geographic locales (**Table 2**). Chancroid is a major cause of genital ulcers in many developing countries, including Morocco, Senegal, Kenya, and Lesotho, but not in Peru (**Table 2**). In addition, chancroid is relatively uncommon in the US, although sustained outbreaks have occurred there, for example in New Orleans, LA and Jackson, MS, particularly among those who exchange sex for drugs or money *(6,8,17)*. PCR was used to monitor the presense of chancroid in New Orleans from 1992 to 1994 (**Table 2**). The ability to determine the etiology of genital ulcer disease in 69–94% of the genital ulcers in these studies is consistent with the sensitivity of PCR. The detection of *H. ducreyi* alone vs coninfection (only 1–15% had coinfections of *H. ducreyi* with either *T. pallidum* or herpes simplex virus) is consistent with the lack of PCR contamination between tubes. PCR techniques offer an opportunity to determine the etiology of genital ulcer disease, particularly where culture is not available. Establishing the relative prevalence of the major etiologic agents of genital ulcer disease allows the development of algorithms for the diagnosis and treatment of genital ulcers in different geographic sites.

4. Notes

1. A consideration when analyzing patient material by PCR is sample adequacy. Inadequate specimens may either be owing to the presence of PCR inhibitors in the specimen or to inadequate collection techniques. PCR inhibitors can be detected either by the addition of a known amount of DNA and testing for amplification or by testing for amplification by β-globin primers. β-globin sequences are present in all human tissues, so the ability to amplify with β-globin-specific primers is indicative of an adequate specimen.
2. PCR results were compared to those of culture as the "gold standard" when determining the sensitivity and specificity of these assays. However, since *H ducreyi* is often not isolated from chancroidal lesions, low specificity may only reflect the greater sensitivity of PCR compared to that of culture Thus, it is difficult to distinguish between increased sensitivity of PCR compared to culture vs false-positive results owing to the amplification of sequences other than those of *H ducreyi* in the genital ulcers. For these reasons, it is satisfying when studies that report PCR analysis of all three genital ulcer pathogens from each ulcer specimen show little overlap
3. One of the most important considerations for all PCR amplification protocols is avoiding contamination, which will lead to false-positive results. Because PCR is capable of detecting one copy of the target DNA, airborne and other sources of contamination must be avoided. Thus, a still air hood in a room separate from the area used for PCR product analysis and growth of the organism is essential. The importance of separating the storage of specimens, specimen treatment, and PCR analysis from product analysis in separate rooms cannot be overemphasized All equipment and supplies for PCR should be stored only in the "PCR-clean room." The counter of the hood in the "PCR-clean room" should be washed with 10% bleach and exposed to the internal UV light before each use. Negative controls (containing medium alone) for processing of patient specimens should be included in each run. However, in spite of all these precautions, PCR contamination may still occur, and the appropriate controls are essential
4. The likelihood of PCR contamination increases with the number of manipulations prior to the actual PCR step. Thus, protocols with extensive treatment regimens, such as repeated phenol and chloroform extractions and centrifugations, have more opportunity to become contaminated. The addition of uracil-*N*-glycosylase (AmpErase, Roche Molecular Systems) and the substitution of dUTP for dTTP can be used to prevent amplicon carryover contamination ***(10)***. To control for the possibility of contamination, we perform an additional sample preparation and PCR analysis on each positive specimen, and only consider the specimen positive if it is positive by these two separate analyses.

Note Added in Proof

We have found that collection in AMPLICOR™ STM and dilution 1:2 into AMPLICOR sample diluent as described ***(10)*** can be substituted for the sample preparation procedure described in this chapter.

Table 2
Prevalence of *H. ducreyi* DNA in Genital Ulcer Specimens Obtained from Different Geographic Locations as Determined by PCR

Location	Year	Number positive (percent positive)				PCR technique	Ref.
		Number analyzed	*H. ducreyi* alone[a]	*H. ducreyi* coinfection[a]	Unknown etiology		
US							
New Orleans, LA	1992	101[e]	35 (35)	2 (2)	21 (31)	Multiplex PCR	Orle et al *(10)*
New Orleans, LA	1993	97[e]	17 (18)	2 (2)	13 (13)	Multiplex PCR	Orle et al. *(10)*
New Orleans, LA	1994	100[e]	9 (9)	0	29 (29)	Multiplex PCR	Orle et al. *(10)*
Atlanta, GA	1995–1996	95[e]	3 (3)	4 (4)	7 (7)	Multiplex PCR	Fleming et al , unpublished
Jackson, MS	1994–1995	143[e]	47 (33)	9 (6)	29 (20)	Multiplex PCR and CDC PCR	Mertz et al , submitted
Africa							
Senegal	1992	39[e]	22 (56)	3 (8)	11 (28)	CDC PCR and PCR with groEL primers	Totten et al., unpublished
Nairobi, Kenya	1993[c]	100	62 (62)	ND[c]	ND	PCR with rRNA primers	Chui et al *(4)*
Nairobi, Kenya	1995[c]	152	79 (52)	ND	ND	PCR with groEL primers	Parsons et al *(9)*
Maseru, Lesotho	1993–1994	100[e]	41 (41)	17 (17)	6 (6)	Multiplex PCR plus PCR for *Chlamydia trachomatis*	Morse et al *(21)*

Tanzania	1994	85	9 (11)	ND[b]	ND		West et al. *(5)*
Central African Republic	1994	66[e]	10 (15)	7 (11)	15 (23)	Multiplex PCR	Lewis et al., unpublished
Morocco, multiple sites[d]	1995–1996	46[e]	23 (49)	1 (2)	15 (34)	CDC PCR	Ryan et al , unpublished
Other							
Nassau, Bahamas	1992	47[e]	6 (13)	ND	ND	CDC-PCR	Bauwens et al., unpublished
Lima, Peru	1995–1996	80[e]	2 (3)	ND	ND	CDC PCR	Totten et al., unpublished
Santa Domingo, Dominican Republic	1995	43[e]	10 (23%)	ND	ND	CDC PCR	Totten et al., unpublished

[a]PCR assays specific for *H. ducreyi*, *T pallidum*, and herpes simplex virus were performed when indicated The multiplex PCR can determine the presence of *H. ducreyi* alone or together with HSV or *T. pallidum* For specimens from Morocco and Senegal, standard primers for *T. pallidum* and HSV were used to detect the presense of these pathogens in addition to *H ducreyi*

[b]ND, not determined

[c]Date paper was published Dates of specimen collection were not stated.

[d]Samples tested were from Tangiers, Marrekesh, and Agader were tested

[e]Samples were from consecutive patients.

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Detection of *Treponema pallidum*, *Haemophilus ducreyi*, and Herpes Simplex Virus by Multiplex PCR

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1. Introduction

The three major causes of genital ulcer disease (GUD) are herpes simplex virus (HSV), *Treponema pallidum*, and *Haemophilus ducreyi*. Although techniques exist for the laboratory diagnosis of all three organisms, constraints of cost, availability of equipment and expertise, and the lack of sensitivity and specificity of available tests, result in clinical presentation being primarily used for the diagnosis of GUD both in the United States and in developing countries. Due to the overlapping clinical presentation of the three diseases caused by these etiologic agents, and due to coinfection, these diseases are often misdiagnosed *(1)*. It is now recognized that not only is GUD a cofactor in HIV transmission, but also that treatment of sexually transmitted diseases can reduce the incidence of HIV *(2–4)*, thus efficient and early diagnosis and treatment of GUD is of utmost importance.

PCR assays have been developed for the detection of all three target pathogens that show superior sensitivity and specificity compared to standard laboratory diagnostic tests. *T. pallidum* DNA has been successfully detected from swabs of genital ulcers, as well as in specimens of cerebrospinal fluid, amniotic fluid, and fetal and neonatal sera *(5–9)*. Fulminant and asymptomatic shedding of HSV into genital specimens and cerebrospinal fluid was detected by PCR with an increased sensitivity compared to that of culture *(10–14)*. The exquisite sensitivity of PCR offered a further advantage over culture by successfully identifying HSV in crusted-over lesions, as well as before and after lesions appeared *(11)*. *H. ducreyi* DNA has been detected in genital ulcer specimens by PCR, also with a greater sensitivity than culture *(15–18)*.

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We developed a multiplex GUD PCR assay that simultaneously amplifies DNA from all three of the major GUD etiologic agents. The products are subsequently differentiated and detected in three separate microwells using oligonucleotide capture probes and the colorimetric Roche AMPLICOR™ detection format *(19)*.

Various studies utilizing ulcer swab specimens from the United States and Africa have been performed using this GUD multiplex PCR assay (*see* **Table 1**). The multiplex assay showed excellent sensitivity and specificity for the detection of all three targets when compared to standard clinical diagnostics (darkfield microscopy, RPR, VDRL, herpes viral culture, and bacterial culture for *H. ducreyi*). The true presence of infection in specimens with positive PCR results and negative reference results was confirmed with independent PCR assays targeting an alternate gene *(19)*. Using this definition of true presence of infection, the resolved sensitivities were 91–100%, and the resolved specificities were nearly 100% for all three targets *(19,20)*.

In addition to analyzing performance characteristics of the GUD assay compared to reference diagnostics, the multiplex GUD PCR assay has been utilized to confirm an *H. ducreyi* outbreak in the US *(26)* and for a surveillance study of the occurrence of *H ducreyi* in 10 major American cities *(22)*. The assay has also been used to investigate diagnostic treatment algorithms in Africa, and to establish the prevalence of each GUD agent among a particular population in specific geographic areas to augment and update the current local syndromic management protocol *(20,23,24)*.

The assay, as described by Orle et al. *(19)*, utilizes swabs of genital ulcers collected in AMPLICOR STD Specimen Transport Medium. Specimen preparation consists of a simple dilution of this medium with AMPLICOR Specimen Diluent prior to PCR target amplification. An 50-µL aliquot of the prepared specimen is added to an 50-µL aliquot of a 2X formulation of a reaction mixture, which contains six biotinylated primers resulting in the production of the three distinct biotinylated amplicons, if target DNA for all of the three pathogens is present. The reaction also contains the enzyme uracil-*N*-glycosylase (UNG) and dUTP substituted for dTTP to allow for destruction of prior amplification products that may be present as contaminants.

Amplification is performed in a thermal cycler with a capacity for 96 reaction tubes in an 8 × 12 array, allowing for easy transfer of PCR product to separate 8-well microwell plate strips containing the immobilized oligonucleotide capture probes specific for each of the GUD targets. The biotinylated amplicons are hybridized to the probes, unbound material is washed away, and an avidin-horseradish peroxidase conjugate is allowed to bind to the captured biotin-labeled amplicon. A substrate solution containing hydrogen peroxide

Table 1
Performance of GUD Multiplex PCR Assay

Loaction	No specimens	Prevalence[a] %					Resolved sensitivity of PCR vs reference diagnostic %[b]			Sensitivity of reference diagnostic vs PCR %			
		HD	HSV	TP	Multiple agents	No etiology	HD[c]	HSV[d]	TP[e]	HD	HSV	TP	Ref
New Orleans, LA	298	22	34	25	2	21	98	100	91	100	72	81	***19***
Lesotho	100	56	23	23	13	9	95	93	ND[f]	75	60	ND	***20***
Jackson, MS	158	38	32	18	8	20	ND	100	83	ND	73	34	***21***
10 cities[g], US	516	3	65	13	3	22	ND	ND	89[h]	ND	ND	54[h]	***22***
Abidjan, Ivory Coast	102	47	33	0	7	26	97[h]	96[h]	ND	67[h]	85[h]	ND	***23***
Nairobi, Kenya	215	35	20	269	4	25	77	ND	ND	88	ND	ND	***24***
Fulton County, GA	95	7	77	17	8	7	ND	100[h]	100[h]	ND	59[h]	60[h]	***25***

HD, *H ducreyi*, HSV, herpes simplex virus, TP, *T pallidum*

[a]Prevalence defined by results of GUD multiplex PCR assay.

[b]The presence of infection defined as positive by reference diagnostic test or positive by PCR assay and independent PCR assay targeting a differen gene ***(19)***

[c]*H. ducreyi* culture

[d]Herpes simplex virus culture

[e]Darkfield microscopic detection of *T. pallidum*

[f]ND, reference diagnostic test not performed

[g]Birmingham, Chicago, Cincinnati, Dallas, Houston, Los Angeles, Memphis, New York City, Philadelphia, and St Louis

[h] Confirmatory PCR test targeting another gene was not performed; sensitivity of PCR and culture were calculated in reference to each other

and 3,3',5,5'-tetramethylbenzidine (TMB) is added to the wells. The bound horseradish peroxidase oxidizes the TMB to form a colored complex, sulfuric acid is added to stop the reaction, and the optical density at 450 nm is measured to identify the presence or absence of each of the GUD PCR targets in the reaction.

The protocols presented here use the AMPLICOR *Chlamydia trachomatis* Swab Specimen Collection and Transport Kit containing STD Specimen Transport Medium and Specimen Diluent for sample collection and processing, and the reagents of the AMPLICOR *Chlamydia trachomatis* Detection Kit for detection of amplified GUD DNA. Users can prepare the three GUD-specific microwell plates using the protocol provided. We have also provided an alternate protocol using commercially available research reagents for the detection and identification of the amplified products, as well as protocols for processing specimens collected in other transport media. In general, the preferred protocol using the AMPLICOR *Chlamydia trachomatis* STD Swab Specimen Collection and Transport Kit and the AMPLICOR *Chlamydia trachomatis* Detection Kit will produce results comparable to our published results using immobilized BSA-conjugated oligonucleotide capture probes.

2. Materials

2.1. Solutions

1. TE buffer: 10 m*M* Tris-HCl, 0.1 *M* EDTA, pH 8.0
2. 1 *M* Ammonium acetate (made fresh)
3. Wash buffer: 2.68 m*M* KCl, 137 m*M* NaCl, 1.47 m*M* KH_2PO_4, 8.03 m*M* Na_2HPO_4, 1 m*M* EDTA.
4. 20X SSPE: 3.6 *M* NaCl, 0.2 *M* Na_3PO_4, 0.11 *M* NaOH, 0.02 *M* EDTA, pH 7.4.
5. GUD Multiplex PCR assay: Recipe for 2X reaction mixture (for one reaction)

Component	Final 2X Concentration
Tris-HCl, pH 8.3	20 m*M*
KCl	100 m*M*
$MgCl_2$	3 m*M*
dATP	400 μ*M*
dCTP	400 μ*M*
dGTP	400 μ*M*
dUTP	400 μ*M*
Primers	25 pmol each
AmpliTaq DNA polymerase (Perkin-Elmer)	10 U
UNG uracil-*N*-glycosylase (Perkin-Elmer)	1 U
Glycerol	25%
Water	q.s. to 50 μL
Total	50 μL

2.2. Supplies

1. MicroAmp® 9600 PCR reaction tubes, caps, base, and tray retainer set (Perkin-Elmer, Foster City, CA) or equivalent from other sources.
2. Extended plugged (aerosol barrier) 300 µL pipet tips
3. Plugged (aerosol barrier) pipet tips.
4. Two-milliliter polypropylene screw cap tubes, sterile, nonsiliconized, conical
5. AMPLICOR STD Swab Specimen Collection and Transport Kit (Roche Diagnostic Systems, Branchburg, NJ).
6. AMPLICOR STD Swab Specimen Preparation Kit (Roche Diagnostic Systems)
7. AMPLICOR *Chlamydia trachomatis* Detection Kit (Roche Diagnostic Systems)
8. Microwell plates: EIA/RIA Strip Plate (Costar, Cambridge, MA)

2.3. Equipment

1. Geneamp PCR System 9600 Perkin-Elmer thermal cycler.
2. Vortex mixer.
3. 37°C incubator (not hot air shaker).
4. Microwell plate reader for 450 nm
5. Microwell plate washer (Optional- May wash plates manually).
6. Refrigerated microcentrifuge (max RCF 12,500–16,000g).
7. Heat block
8. Multichannel pipettor

3. Methods

This protocol utilizes microwell plates coated with oligonucleotide capture probes by the user and AMPLICOR *Chlamydia trachomatis* detection reagents.

3.1. Primers and Probes

Primer and probe oligonucleotide sequences are provided in **Tables 2** and **3**. Stock solutions are made in TE buffer. Primers and probes should be stored as stock solutions at concentrations of 50 µ*M* and 200 µg/mL, respectively. It is prudent to divide the solutions into aliquots that are frozen until needed. Avoid freezing and thawing the aliquots.

3.2. Microwell Probe Coating

1. One hundred microliters of a solution of 1 *M* ammonium acetate (made fresh) containing the probe is added into each well of a microwell plate. For probes KO17 and KS54, 50 ng/100 µL is used, and for probe KO15, 200 ng/100 µL (*see* **Note 1**)
2. Use a separate plate for each probe. Color code the end of the strips to differentiate each probe.
3. Cover plate with microwell cover or plastic.
4. Incubate plate at 37°C for 10–20 h and then wash each well twice with 300 µL of wash buffer.

Table 2
GUD Multiplex PCR Primer Sequences

Organism	Primers	Sequence
T pallidum	KO3A	5'-biotinyl-GAAGTTTGTCCCAGTTGCGGTT
	KO4	5'-biotinyl-CAGAGCCATCAGCCCTTTTCA
		Amplifies 260 bp fragment of the 47-kDa membrane immunogen gene
Herpes simplex virus types 1 and 2	KS30	5'-biotinyl-TTCAAGGCCACCATGTACTACAAAGACGT
	KS31	5'-biotinyl-GCCGTAAAACGGGGACATGTACACAAAGT
		Amplifies 431 bp fragment of the Glycoprotein B gene
H ducreyi	KO7	5'-biotinyl-CAAGTCGAACGGTAGCACGAAG
	KO8	5'-biotinyl-TTCTGTGACTAACGTCAATCAATTTTG
		Amplifies 439 bp fragment of the 16S rRNA gene

Table 3
GUD Multiplex PCR Probes

Organism	Probe	Sequence
T pallidum	KO17	5'-CGGGCTCTCCATGCTGCTTACCTTA
Herpes simplex virus types 1 and 2	KS54	5'-GGTCTCGTGGTCGTCCCGGTGAAA
H. ducreyi	KO15	5'-CCGAAGGTCCCACCCTTTAATCCGA

5. Remove all excess wash buffer by patting plate onto paper towel
6. Air dry plates for at least 2 h at room temperature, then store dry plates at 4°C in a sealed bag (such as "Seal-A-Meal" or "Zip-Loc") containing a desiccant pouch (SORB-IT®, Belen, NM).
7. Plates are stable when stored dry at 4°C for at least 1 mo.

3.3. Specimen Collection Using the AMPLICOR Chlamydia trachomatis *STD Swab Specimen Collection and Transport Kit*

1. The ulcer is first cleaned with one of the large sterile swabs moistened with saline.
2. The other large swab is used to swab the ulcer
3. The ulcer swab is vigorously agitated for 15 s in the collection tube containing 1 mL of AMPLICOR Specimen Transport Medium, the liquid is then expressed against the side of the tube, and the swab discarded (*see* **Note 2**)
4. Specimens can be stored at ambient temperature for 24 h, 4°C for 1 wk, or stored frozen at –70°C for extended periods of time

3.4. Specimen Preparation

All pipetting must be performed using plugged pipet tips or positive displacement pipettes. Specimens must be prepared for PCR in an area free of amplified DNA.

1 Allow clinical swab specimens to thaw and come to room temperature.
2 Using an elongated pipet tip, carefully remove 60 µL of the specimen and transfer to a microfuge tube containing 60 µL of AMPLICOR Specimen Diluent
3. Cap the tube and vortex 5–10 s; incubate at room temperature for 10–60 min.
4. Following preparation for PCR, "processed" specimen can be stored at 4°C for up to 7 d. For longer periods of time, the samples must be stored at –20°C, or –70°C

3.5. Controls

1. Negative control for amplification: At least two replicates in every amplification run of a "no DNA control" that consist of Specimen Transport Medium mixed with a equal volume of Specimen Diluent.
2 Negative control for specimen processing: At least two replicates of a mock clinical specimen consisting of Specimen Transport Medium that was processed along with the clinical specimens.
3. Positive controls Each target DNA at a concentration of approximately 10–100 gene copies per reaction either combined or individually. This input level will result in positive signals of A_{450} above 1.0.

3.6. Amplification

1. Fill a tray with empty PCR reaction tubes as needed.
2 Prepare 2X GUD multiplex PCR reaction mixture according to the recipe in **Subheading 2.1.5.** using plugged tips. Mix well by inverting 10–15 times Make enough mix for two amplification reactions per specimen, plus 10% for loss owing to pipetting
3. Aliquot 50 µL of 2X GUD multiplex PCR reaction mixture into each MicroAmp tube with a plugged tip or repeater pipet.
4. Transfer 50 µL of processed specimen into reaction tube containing GUD multiplex PCR reaction mixture using a fresh tip for each specimen. Do not attempt to mix.
5. Add controls (both positive and negative) to the tray after all clinical specimens have been pipetted
6. Seal all tubes.
7 Program the thermal cycler for amplification 15 min prior to use using the following parameters:

Hold program	2 min at 50°C
Hold program	5 min at 95°C
Cycle program	35 cycles of: 20 s at 95°C, 20 s at 62°C, 20 s at 72°C

Hold program at 72°C (at least 10 min, but less than 2 h)
(The program takes approximately 1.5 h)

8 After completion of thermal cycler program, carefully remove caps to avoid aerosolizing the PCR reactions.
9 **Immediately**, pipet 100 µL AMPLICOR Denaturation Solution (#1) into each reaction tube using a multichannel pipettor and plugged tips to inactivate any residual UNG activity Incubate for 10 min at room temperature to allow complete denaturation.
10. Store denatured, amplified samples at room temperature if the microwell plate detection will be performed within 1-2 h. If not, store the samples at 2–8°C until the plate detection is performed Amplicons may be stored for up to 1 wk at 2–8°C, or frozen for longer periods of time.

3.7. Detection

Use gloves when handling microwell plates.

1 Warm all reagents to room temperature.
2 Prepare Working Wash Solution by diluting AMPLICOR Wash Concentrate 10-fold (add 1 vol of concentrate to 9 vol of distilled or deionized water) Mix well.
3. Remove the appropriate number of 8-well microwell plate strips from their packages (*H ducreyi*, HSV, and *T. pallidum*), and set into the microwell plate frame (**Note:** Three detection microwells are needed for each PCR reaction) Return unused strips to pouch and reseal making sure the desiccant pillow remains in the pouch Microwell strips must be handled carefully to avoid breakage
4. Add 100 µL AMPLICOR *Chlamydia trachomatis* Hybridization Buffer (#2) to each well to be tested using a multichannel pipettor.
5. If the amplification samples were stored at 2–8°C, incubate them at 37°C for 2–4 min in order to reduce viscosity.
6. Using plugged tips, pipet 25 µL of each denatured sample to the appropriate well of each of the three probes. Do not mix Once plate is completely filled, gently tap the plates 10–15 times until the color changes from blue to yellow.
7. Cover plates, incubate for 1 h at 37°C.
8 Wash plates 5 times using a Microwell Plate Washer. Use the prepared Working Wash Solution (10X concentrate diluted 10-fold with water) for washing the plates and the following procedure·
   a. Aspirate contents of wells;
   b Fill each well to top with Working Wash Solution (350–450 µL), soak 10 s, aspirate dry;
   c Repeat **step b** four additional times,
   d. Tap the plates dry.
9. Add 100 µL AMPLICOR Avidin-HRP conjugate (#3) to each well. Cover plate; incubate for 15 min at 37°C.
10 Wash plates as described in **step 8**
11. Prepare Working Substrate by mixing 8.0 mL of AMPLICOR Substrate A (#4A) and 2.0 mL AMPLICOR Substrate B (#4B) for each full microwell plate. Prepare this reagent no more than 3 h before use and protect from exposure to direct light

12 Pipet 100 µL of prepared Working Substrate reagent into each well being tested.
13 Allow color to develop for 10 min at room temperature in the dark
14. Add 100 µL of AMPLICOR Stop Reagent (#5) to each well
15. Measure the optical density of the microwell plates at 450 nm within 1 h of adding Stop Reagent.

3.8. Analysis of Results

1 Absorbance values below 0.25 are considered negative.
2. Negative control reactions must have values below the 0.25 cut-off value for a run of amplifications to be valid (normally below 0.1).
3. Positive control reactions should produce signals of A_{450} of 1.0 or above.
4. Specimens are scored as positive for a particular pathogen, if positive signals were obtained from duplicate reactions.
5. Initial PCR results between 0.25–0.80 A_{450} are considered equivocal and should be reassayed on the microwell detection assay.
6 Specimens that produce split PCR results between the duplicate amplification reactions (one negative and one positive) should be reamplified in duplicate.
7. Specimens with negative signals for all three targets should be reamplified in the presence of 20–100 copies of one of the target DNAs to monitor for inhibition. Inhibition is defined as a A_{450} signal below 0.25 for the spiked control DNA in the absence of a positive signal for any of the three GUD targets. A new aliquot of these inhibitory specimens should be extracted with phenol:chloroform and reanalyzed by PCR (*see* **Subheading 3.9.** for extraction protocol)
8 Specimens are scored for the presence of GUD target DNA following the removal of inhibitors.

3.9. Phenol-Chloroform Extractions

In every set of phenol extractions, include at least one "mock" clinical specimen consisting solely of AMPLICOR Specimen Transport Medium as well as a positive control in AMPLICOR Specimen Transport Medium.

1. Thaw clinical specimens, and bring to room temperature.
2. Using a plugged, elongated, pipet tip, add 0.1 mL specimen to a 2-mL polypropylene screw cap tube that has 0.4 mL AMPLICOR Specimen Transport Medium containing 1 µg/mL calf thymus DNA and vortex.
3. Heat at 95°C for 10 min, then let cool to room temperature. Pulse-centrifuge to collect condensation.
4. Add 0 5 mL phenol:chloroform:isoamyl alcohol (25:24:1) using a separate pipet tip for each sample, and mix.
5. Centrifuge 10 min at 16,000*g* in a microfuge.
6. Transfer the upper aqueous layer (using a plugged pipet tip or sterile, fine-tipped transfer pipet) to a fresh microcentrifuge tube containing 0 5 mL chloroform:isoamyl alcohol (24:1) and mix
7. Centrifuge 10 min at 16,000*g*.

8 Transfer aqueous layer to a fresh tube containing 50 µL 3 *M* sodium acetate, pH 5.2
9 Mix, and add 1 0 mL cold 100% ethanol. Precipitate DNA overnight at –20°C. (Samples can be stored indefinitely at this point.)
10 Centrifuge 20 min at 16,000g in a refrigerated microcentrifuge at 4°C. Tubes should be placed in rotor with a particular orientation or marked as to where the pellet should occur
11. Completely remove supernatant being careful to not disturb the pellet (which may not be visible), and remove residual ethanol by inverting tube onto a fresh gauze pad.
12. Air dry pellet by incubating the open tube in a heat block at 55°C until dry (5–15 min).
13 Resuspend pellet in 120 µL of a 1 1 mixture of AMPLICOR Specimen Transport Medium and AMPLICOR Specimen Diluent Heat at 55°C for 10 min, then let cool Pulse-centrifuge prior to opening tubes (Specimens can be stored overnight at 4°C prior to PCR analysis, if necessary.)

3.10. Alternate Microwell Detection Protocol (Without Using AMPLICOR Chlamydia trachomatis Detection Reagents)

The alternate detection protocol using commercially available research reagents may need optimizing owing to lot to lot variation of the reagents. Higher background signals which can reduce sensitivity up to 10-fold compared to using the AMPLICOR *Chlamydia trachomatis* detection reagents have been observed (*see* **Notes 3** and **4**). However, the alternate protocol maybe adequate for temporal and geographic GUD surveillance and prevalence studies.

1. Heat denature amplified product by incubation in the thermal cycler at 95°C for 5 min.
2 Immediately remove 15 µL of denatured amplicon and add into each well containing 100 µL of 5X SSPE containing 0.1% SDS, 30% v/v formamide, and then incubate at 37°C for 1 h (Immediately store any remaining amplicon at –20°C)
3. Wash wells twice with 2X SSPE containing 0.1% SDS. For the first wash, quick rinse with buffer at room temperature For the second wash, allow the buffer to soak in the wells for 10 min at 37°C. All washes use 300 µL buffer per well. Remove residual liquid by patting microwell plate onto paper towel before adding next reagent
4. Add 100 µL streptavidin-horseradish peroxidase to each well. Dilute 50 µL streptavidin-horseradish peroxidase conjugate (Vector Laboratories, Burlingame, CA) in 10 mL of 2X SSPE containing 0.1% SDS. Incubate 15 min at 37°C
5 Wash four times with 2X SSPE containing 0 1% SDS at room temperature with all washes using 300 µL buffer per well, and with a 15-s soak between washes
6. Wash once with 2X SSPE at room temperature as described above
7. Add 100 µL 3,3',5,5'-tetramethylbenzidine substrate to each well. TMB Substrate is prepared according to manufacturer's instructions (TMB Substrate Kit, Vector Laboratories). Incubate for 10 min at room temperature in the dark.
8. Stop the reactions by adding 50 µL 1 *N* sulfuric acid to each well.
9. Measure the optical density of the microwell plates at 450 nm within 1 h.

4. Notes

1. The 1 *M* ammonium acetate solution should be made fresh on the day of microwell coating as the solution is unstable. The probes should be added to the ammonium acetate solution to the final concentrations as stated in **Subheading 3.2.1.**
2. Under **Subheading 3.3.3.**, the swab should not be left in the AMPLICOR Specimen Transport Medium after collection as this may lead to inhibition of the amplification reaction. The swab should be vigorously agitated in the collection tube containing the SpecimenTransport Medium for approx 30 s, rotated against the side of the tube to express as much liquid as possible and then discarded.
3. Because of higher backgrounds using this protocol (**Subheading 3.10.**), cut-off values for negative signals will be higher. The cut-off value may need to be 0.30–0.50 A_{450} units, depending on signals for negative controls. In general, the signals from the positive controls should be at least two standard deviations above the signals from the negative controls.
4. Alternate protocol for removing PCR inhibitors: to overcome inhibition, specimens in AMPLICOR Specimen Transport Medium can be diluted 1:5 or 1:10 in Specimen Transport Medium prior to processing. With a 1:5 dilution, few positive signals are lost, however, positive signals can be lost when specimens are diluted 1:10.
5. Ulcer swab specimens collected in 2-SP Culture Transport Medium, Bartels Chlamydia EIA Transport Medium, distilled water, and Viral Transport Medium have been used successfully after first diluting two- to five-fold with Specimen Transport Medium followed by a two-fold dilution in Specimen Diluent.
6. PBS containing 1 mg/mL chenodeoxycholate has been successfully used as transport medium. Proceed with amplification after diluting 10-fold in Specimen Diluent, with the modification that the magnesium chloride must be omitted from GUD multiplex PCR reaction mixture.
7. Specimen Transport Medium and Specimen Diluent in 50 mL quantities can be purchased in bulk from Roche Diagnostic Systems.

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6

Detection of Genital Mycoplasmas by PCR

Claire B. Gilroy and David Taylor-Robinson

1. Introduction

Mycoplasmas are the smallest prokaryotes capable of self-replication. They belong to the class Mollicutes (meaning soft-skin) and have evolved regressively, by genome reduction, from Gram-positive bacterial ancestors, namely certain clostridia *(1)*. The taxonomy of the class Mollicutes containing four orders, five families, and eight genera, is shown in **Table 1** *(2)*. The term 'mollicute' is sometimes used trivially to describe any organism in the class. The term 'mycoplasma' might be used best to describe any member of the genus *Mycoplasma*, but is also used, as in this chapter, in a trivial way to refer to any organism in the class.

Genetic information is provided by a genome that may be as small as 580 kb, namely that of *Mycoplasma genitalium*, and is estimated to code for less than 500 genes *(3)*. Some of the key differences between mycoplasmas and eubacteria are outlined in **Table 2** *(4)*. Keeping the number of structural elements, metabolic pathways, and components of protein synthesis to an essential minimum, places mycoplasmas closest to the concept of 'minimum cells' *(5)*. They have adopted a parasitic mode of life, securing from the host the many nutrients which they cannot synthesize and are fastidious in their growth requirements owing to the small genome. Replication in broth media varies from one hour for some ureaplasmas to six hours for *Mycoplasma pneumoniae* In recent years, the input of molecular biology and genetics has benefited studies of mycoplasmas, perhaps more than those of any other group of organisms. The need for a molecular approach has been felt particularly as classic genetics could not be applied to most mycoplasmas because of the difficulty in cultivation, as well as the use of the UGA codon to encode tryptophan instead of the

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Table 1
Taxonomy and Properties of Mycoplasmas (Class *Mollicutes*)[a]

Classification	Current no. of recognized species	Genome size (kbp)	Habitat
Order I: Mycoplasmatales			
Family I: Mycoplasmataceae			
Genus I: *Mycoplasma*	100	580–1300	Humans, animals, plants, insects
Genus II: *Ureaplasma*	6	730–1160	Humans, animals
Order II: Entomoplasmatales			
Family I: Entomoplasmataceae			
Genus I. *Entomoplasma*	5	790–1140	Plants, insects
Genus II: *Mesoplasma*	12	870–1100	Plants, insects
Family II: Spiroplasmataceae			
Genus I: *Spiroplasma*	17	940–2240	Arthropods (including insects), plants
Order III Acholeplasmatales			
Family I: Acholeplasmataceae			
Genus I: *Acholeplasma*	13	Approx 1600	Animals, plants, insects
Order IV: Anaeroplasmatales			
Family I: Anaeroplasmataceae			
Genus I. *Anaeroplasma*	4	Approx 1600	Bovine-ovine rumen
Genus II. *Asteroleplasma*	1	Approx 1600	Bovine-ovine rumen
Uncultivated, unclassified MLO's		500–1185	Plants, insects

[a]Adapted from ref. *2*

Table 2
Properties Distinguishing Mycoplasmas from Eubacteria[a]

Property	Mycoplasmas	Eubacteria
Cell wall	Absent	Present
Peptidoglycan	Absent	Present
Genome size	500–1700	>1500
Mol% G + C content of genome	23–41	25–75
No. of detectable cell proteins	Approx 400	1000
No. of rRNA operons	1–2	1–10
5S rRNA length (nucleotides)	104–113	>114
No. of *tuf* genes	1	1 or 2
RNA polymerase	Rifampin resistant	Rifampin sensitive
UGA codon usage	Trytophan codon in *Mycoplasma*, *Spiroplasma* and *Ureaplasma* spp but not in *Acholeplasma* spp.	Stop codon
DNA polymerase complex	One enzyme in *Mycoplasma* and *Ureaplasma* spp., three in *Acholeplasma* and *Spiroplasma* spp.	Three enzymes

[a]Adapted from ref. *4*

(almost) universal STOP signal. Conjugation and/or cell fusion techniques are emerging, as are vectors suitable for mycoplasmal gene expression. Tools used for the investigation of nucleic acids, genes, and proteins have been applied not only to the detection and characterization of mycoplasmas, but also to studying their taxonomic and phylogenetic properties *(6)*.

Species of *Mycoplasma* that have been isolated to date from humans are listed in **Table 3** *(7)*. *M. buccale, M. faucium, M. lipophilum, M orale, M. salivarium,* and possibly *M. fermentans* are considered to be representative of the normal human oropharyngeal flora *(8)*; *M salivarium* resides primarily in the gingival crevices. Mycoplasmas are also common inhabitants of the urogenital tract. *M. hominis, M. fermentans*, and *Ureaplasma urealyticum* have all been isolated from the urogenital tract of healthy individuals and therefore might be considered normal flora. However, some of these species are found both in healthy individuals and in association with disease which complicates enormously attempts to clarify their etiological role in disease. Factors to be considered in assessing their pathogenic potential include site(s) of colonization in the genital tract, the number of organisms present, and perhaps strain differences.

As many mycoplasmas are inhabitants of the mucosal membranes of the oro-respiratory, urogenital, or gastrointestinal tracts, direct host to host transmission of organisms occurs through oral to oral, genital to genital, or oral to genital contact. Certain mycoplasmas that are part of the normal flora of the oropharynx and lower genital tract are most likely acquired by oral to oral and by genital to genital contact, respectively. However, sexual practices have also resulted in apparent alterations in the host tissue location of mycoplasmas such that some commonly found in the oropharynx are to be found in the ano/urogenital tract and vice versa. Sexually transmitted human diseases for which a mycoplasmal involvement has been reasonably assured are mentioned below together with other diseases that are not considered to be sexually transmitted, but that are caused by mycoplasmas usually residing in the urogenital tract.

Several groups *(9–12)* have provided evidence to indicate that *M. genitalium* is a cause of nongonococcal urethritis (NGU) in men. In addition, *U. urealyticum* has been attributed as a cause of epididymitis *(13)* and *M. hominis* as a cause of pelvic inflammatory disease in women *(14)*. Further details of these associations are shown in **Table 4** *(9–11,13–17)* and in the references mentioned therein.

There are several conditions that are not in the usual sense considered to be sexually transmitted but are caused sometimes by mycoplasmas that, by virtue of their dominant urogenital tract colonization, are sexually transmitted. Several examples are outlined in **Table 5** *(18–22)*.

Table 3
Primary Sites of Colonization, Pathogenicity, and Metabolism of Mycoplasmas of Human Origin[a]

Species	Primary site of colonization		Pathogenicity	Metabolism of	
	Oropharynx	Genitourinary tract		Glucose	Arginine
M buccale	+	–	–	–	+
M faucium	+	–	–	–	+
M fermentans	+	?+	?	+	+
M genitalium	–	+	+	+	–
M hominis	–	+	+	–	+
M lipophilum	+	–	–	–	+
M. orale	+	–	–	–	+
M. penetrans	–	+	?	+	+
M pneumoniae	+	–	+	+	–
M. pirum	?	?+	?	+	+
M primatum	–	+	–	–	+
M. salivarium	+	–	–	–	+
M spermatophilum	–	+	–	–	+
U. urealyticum[b]	–	+	+	–	–

[a]Adapted and updated from **ref. 7**
[b]Metabolizes urea

Table 4
Sexually Transmitted Diseases Attributed to Mycoplasmas

Disease	Mycoplasma	Strength of the association	Reference
NGU	*M. genitalium*	+++	*(9–11,15)*
	U urealyticum	++	*(16)*
Epididymitis	*U urealyticum*	+	*(13)*
Pelvic inflammatory disease	*M hominis*	+	*(14)*
Sexually acquired reactive arthritis	*U urealyticum*	++	*(17)*

++++, Overwhelming, +++, Strong, ++, Moderate, +, Weak

Table 5
Other Diseases Attributed to Sexually Transmitted Mycoplasmas

Disease	Mycoplasma	Strength of association[a]	Reference
Pyelonephritis	*M. hominis*	++	*(18)*
Post-partum and post-abortion fever	*M hominis* *U. urealyticum*	+++	*(19)*
Arthritis in hypogammaglobulinaemia and in patients on immunosuppression	*M. hominis* *U. urealyticum*	++++	*(20,21)*
Pneumonia, chronic lung disease in very low birth weight infants	*U urealyticum*	++	*(22)*

[a]As for **Table 4**

1.2. Diagnosis

Diagnostic techniques for mycoplasmas have undergone a major revolution since the methodologies that were published over a decade ago *(23,24)*. The use of improved media and the application of new molecular techniques for the detection and identification of such organisms, has seen a significant expansion in the range of hosts recognized as being colonized or infected with these organisms and in the number of newly characterized *Mycoplasma* species.

1.2.1. Culture

Historically, culture has been, and probably still is, the technique used most widely for the detection of mycoplasmas, but the advent of DNA probes and more recently the polymerase chain reaction (PCR) has seen considerable advances

Many of the current culture media formulations for mycoplasmas are based entirely, or with only minor alterations, on the medium described originally by Derrick Edward *(25)* This is basically the medium that resulted in the cultivation of *M. pneumoniae* in the early 1960s *(26)* and that also supports growth of ureaplasmas, which some regard as fastidious to the extent that several modifications of the Edward medium have been described for them *(26,27)*. In the early 1970s, spiroplasmas were discovered in various diseased plants and insect hosts, and more intense efforts were required to develop culture media for other more fastidious spiroplasmas from arthropod hosts. These efforts culminated in the development of the medium formulation designated SP-4. The application of this medium for primary isolation and maintenance of a variety of new and fastidious mycoplasmas of human origin since 1979, such as *M. genitalium (28)* and *M penetrans (29)*, has affirmed its overall value in meeting the nutritional needs of many different mycoplasmas. SP-4 also enhances primary isolation of other mycoplasmas of human origin, particularly *M fermentans (30)* and *M. pneumoniae (31)*. However, while medium formulations are important, the quality of the components may be even more so. The development of a successful medium is through trial and error and components need to be pretested for their abilities to support growth. Quality control with a fastidious isolate is important.

Inoculation of specimens into liquid medium, which is then diluted serially, followed by subculture to liquid or agar media provides the most sensitive method for the isolation of most mycoplasmas. Jensen et al. *(32)* reported recently an alternative method in which they passaged 11 *M genitalium*-PCR positive (primers from **ref. 33**) urethral NGU specimens in Vero cell cultures that were monitored with a PCR assay. Vero cell-passaged material was inoculated into acellular medium when the PCR product was strongly positive. This technique resulted in the isolation of four strains of *M. genitalium* and may prove to be applicable for the isolation of other fastidious mycoplasmas.

1.2.2. DNA Probes

The first DNA probes applied to the diagnosis of mycoplasmal infections consisted of ribosomal RNA (rRNA) genes of *M. capricolum* cloned into the *Escherichia coli* plasmid pBR325. The recombinant plasmid was named pMC5 ***(34)*** and has become one of the most popular DNA probes in mycoplasmal studies. Using Southern blot hybridization with labeled pMC5 as a probe ***(35)***, the highly conserved rRNA genes were effective in detecting and identifying mycoplasmas contaminating cell cultures.

To provide more specific probes, synthetic oligonucleotides 20–40 nucleotides in length, complementary to variable species-specific regions of mycoplasmal 16S rRNA genes, can be applied. Although the majority of rRNA probes has been designed for detecting contamination of tissue cell cultures, a number of probes for detecting mycoplasmas of human origin has also been reported ***(36–39)***. Another class of DNA probes consists of chromosomal segments specific for a certain mycoplasmal species. These segments are derived from a genomic library of the specific mycoplasma Such species-specific probes have been developed for *M. pneumoniae* and *M. genitalium* ***(40)***. Dot-blot hybridization with these probes, labeled either radioactively or with a variety of nonradioactive molecules (i.e., digoxigenin or biotin) has enabled 10^4–10^5 colony-forming units (cfus) to be detected, a level of sensitivity that often is insufficient for use in a clinical laboratory ***(41,42)***.

1.2.3. PCR

The introduction of PCR technology in the late 1980s diverted attention from all the previously developed DNA probes and kits. PCR tests are several orders of magnitude more sensitive than those based on direct hybridization with a DNA probe. The high sensitivity of the PCR is of value when the number of organisms in a clinical specimen is small, or when there is otherwise difficulty in culturing. PCR is fast, a single DNA sequence being copied over a billion times within three hours. The first reports of the application of PCR assays to the diagnosis of mycoplasmal infections appeared in 1989 ***(43,44)***. Since then, the number of reports of the use of PCR as a tool in mycoplasmal diagnosis has been rising at an exponential rate. In particular, use of PCR technology has increased immensely the detection of certain mycoplasmas, *M. genitalium* being a good example. This mycoplasma was isolated in 1980 from urethral specimens taken from two of 13 men with acute NGU ***(28)***. However, although five strains of *M. genitalium* had been isolated from extragenital sites, together with *M. pneumoniae*, no other isolates from the urogenital tract had been reported, until recently ***(32)***, despite several attempts at recovery ***(45,46)***. Nevertheless, the undoubted existence of *M. genitalium* in the urogenital tract was

shown by the use of PCR technology. The detection of other mycoplasmas, may also benefit from the use of PCR technology rather than culture. Thus, although *U. urealyticum* often can be isolated with ease, it may be difficult if not impossible to culture from certain specimens such as amniotic fluids, endotracheal aspirates of newborns *(47)*, and synovial fluid. Fluid from a patient with septic arthritis was shown to be positive only by use of a PCR assay despite numerous attempts to culture *(17)*. The PCRs (target DNA, primers, probes) outlined in **Table 6** *(33,43,44,47–59)* are those for mycoplasmal species that have been found in humans.

None of the technical aspects appears to be unique to mycoplasmal PCRs and this chapter will explain in detail the protocol used to detect *M. genitalium* in urogenital samples *(9,51,52)*. The preparation of DNA from urogenital samples is the same for all mycoplasmas and the PCRs differ mainly in the choice of primers and thermal cycles. The details of these are provided in **Table 6**, to which the reader is referred in order to select the appropriate primers for the particular genital mycoplasma they wish to study.

2. Materials

1. Swab sample (*see* **Note 1**).
2. Urine sample.
3. InstaGene Matrix (Bio-Rad, Hemel Hempstead, Hertfordshire, UK)
4. Proteinase K solution (20 mg/mL)
5. 10 % SDS (sodium dodecyl sulphate)
6. 10% CTAB (hexadecyltrimethyl ammonium bromide) in 0.7 *M* NaCl (*see* **Note 2**)
7. Chloroform/isoamyl alcohol (25:1)
8. 5 *M* NaCl.
9. Isopropanol
10. Ribonuclease A made DNAse-free by heat treatment (80°C for 10 min)
11. PCR buffer ; 1 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.4, 0.01% gelatin, 0.05% Nonidet-P40 (Sigma, Poole, Dorset, UK), 0.2 m*M* dNTPs (Pharmacia, St. Albans, Hertfordshire, UK) and 2.5 U of Taq DNA polymerase (Gibco Life Technologies, Paisley, Scotland).
12. Hybridization buffer: 5X SSC, blocking reagent 1%, *N*-lauroylsarcosine 0 1%, SDS 0.2%.
13. Washing buffer: Tris-Cl 100 m*M*, pH 7.5, NaCl 150 m*M*
14. Boerhinger Mannheim (Lewes, East Sussex, UK) Digoxigenin DNA Labeling and Detection Kit.
15. Thermal cycler (*see* **Note 3**).
16. Gel electrophoresis equipment and power supply
17. Hybridization incubator.
18. Orbital shaker.
19. Bag sealer and lengths of polythene layflat tubing to make bags

20 Luminograph cassettes and film.
21 Autodeveloper

3. Methods

3.1. Swab Sample Preparation

1. A nasopharyngeal swab is passed 2–4 cm into the male urethra, removed and expressed in 500 µL of phosphate-buffered saline (PBS) in a 1.5 mL microcentrifuge tube (*see* **Note 4**)
2. Centrifuge the tube at 13,000*g* for 10 min to collect the cellular material
3 Carefully decant the supernatant and add 500 µL of PBS and thoroughly resuspend the pellet to wash it and centrifuge as above.
4. Carefully remove all the supernatant without disturbing the pellet and add 50 µL of sterile deionized water (dH_2O) and resuspend the pellet.
5. Add 100 µL of BioRad InstaGene matrix (*see* **Note 5**) and vortex for 10 s to mix thoroughly and incubate in a waterbath at 56°C for 30 min
6. Vortex for 10 s and incubate at 80°C for 10 min
7 Allow the sample to cool to room temperature, then either store it at –20°C or centrifuge it briefly at 6000*g* for 2 min (*see* **Note 6**) and take an aliquot (usually 20 µL) to be used in the PCR assay.

3.2. Urine Sample Preparation

1. Collect a 15–20 mL first pass urine sample (after the patient has had a swab sample taken) into a sterile container, such as a polystyrene universal bottle
2 Centrifuge at 1600*g* for 15 min to pellet the cellular material
3 Carefully decant the supernatant and add back 2 mL to resuspend the pellet Transfer 1 mL of the resuspension to each of two-1.5 mL microcentrifuge tubes One is to be stored, the contents of the other are for DNA extraction ***(60)***.
4 Centrifuge the sample at 13,000*g* for 10 min and carefully remove the supernatant and add 500 µL of PBS. Vortex the tube to wash the pellet thoroughly. Centrifuge as before.
5 Resuspend the pellet in 567 µL of TE buffer by repeated pipetting ***(60)***.
6. Add 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K to give a final concentration of 100 µg/mL of proteinase K in 0.5% SDS. Mix thoroughly and incubate at 37°C for one hour.
7 Add 100 µL of 5 *M* NaCl and mix thoroughly.
8. Add 80 µL of CTAB/NaCl solution and mix thoroughly and incubate at 65°C for 10 min.
9. Add an equal volume (0.7–0.8 mL) of chloroform/isoamyl alcohol, extract thoroughly, and centrifuge for 5 min in a microcentrifuge.
10. Remove the aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind.
11 Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and centrifuge for 5 min.

Table 6
Published PCRs for Mycoplasmas Isolated from Humans

Mycoplasma	Target DNA	Primers	Probe	Ref
M fermentans	Insertion-sequence like element	RW004 GGACTATTGTCTAAACAATTTCCC RW005 GTTATTCGATTTCTAAATCGCCT	RW006 GCTGTGGCCATTCTCTTCTACGTT	*(48)*
		RW003 TTCTCCTGTAGTTTGATTTTGCC RW004 same as above RW006 same as probe RW006	RW005 same as primer RW005	*(49)*
	16S rRNA gene	RNAF1 CAGTCGATAATTTCAAATACTC RNAF2 GGTACCGTCAAAACAAAAT	RNAF3 ATGAAGATTACGGAAAAGAGCNTTTCTTCG CTGGA	*(50)*
M genitalium	MgPa gene[a]	Mg1 TGTCTATGACCAGTATGTAC Mg2 CTGCTTTGGTCAAGACATCA Mg3 GTAATTAGTTACTCAGTAGA	Probe made using primers Mg4 ATCAAACCCTGCTTGTAATG Mg5 ACTTGTTCCTATAGTAGTGAT	*(51)*
		MG1 AGTTGATGAAACCTTAACCCCTTGG MG2 CCGTTGAGGGGTTTTCCATTTTTGC	MG3 GACCATCAAGGTATTTCTCAACAGC	*(33)*
		MG1 same as *(33)* MG2 same as *(33)*	Same probe as *(33)*	*(52)*
		Mg1a GGTTAACTTACCACTGGCCTTTGATC Mg2 same as *(51)* Mg3 same as *(51)*	Same probe as *(51)*	
		G3A GCTTTAAACCTGGTAACCAGATTGACT G3B GAGCGTTAGAGATCCCTGTTCTGTTA	3A3B CCTTTGATTGTAACTGTT	*(53)*
M hominis	16S rRNA gene	MYCHOMP ATACATGCATGTCGAAGCGAG MYCHOMN CATCTTTTAGTGGCGCCTTAC	MYCHOMS CGCATGGAACCGCATGGTTCCGTTG	*(54)*
M penetrans	16S rRNA gene	MYCPENTP CATGCAAGTCGGACGAAGCA MYCPENTN AGCATTTCCTCTTCTTACAA	MYCPENETS[b] CATGAGAAAATGTTTAAAGTTCGTTTG	*(54)*
M pirum	16S rRNA gene	MYCPIRP TACATGCAAGTCGATCGGAT MYCPIRN CATCCTATAGCGGTCCAAAC	MYCPIRS1 CAAATGTACTATCGCATGAGAAACATTT	*(55)*

M pneumoniae	P1 adhesin gene[c]	CAATGCCATCAACCCGCGCTTAACC CGTGGTTTGTTGACTGCCACTGCCG	np[d]	*(44)*
	tuf gene[e]	Mpn38 TACTCGTTACGACCAAATCGATAAG Mpn39 GTTCAACTGTAATCGAGGTATTG	Mpn46 TCCACGTGAGCGGAGTTAA	*(56)*
	Cloned fragment	GAAGCTTATGGTACAGGTTGG ATTACCATCCTTGTTGTAAGG	CGTAAGCTATCAGCTACATGGAGG	*(43)*
U urealyticum	Urease gene	U5 CAATCTGCTCGTGAAGTATTAC U4 ACGACGTCCATAAGCAACT	np	*(46)*
	Urease gene	14b CCAGGAAAAGTAGTACCAGGAGC C72b CTCCTAATCTAACGCTATCACC	np	*(57)*

[a]Gene for the attachment protein designated MgPa by Hu et al *(58)*
[b]Published sequence is incorrect. The correct sequence is given in the table
[c]Gene for the attachment protein designated P1 by Hu et al *(59)*
[d]np, no probe sequence was given in the reference
[e]*tuf* gene encodes the Elongation factor protein Tu.

12 Transfer the supernatant to a fresh tube. Add 0.6 vol of isopropanol to precipitate the DNA. Centrifuge at 13,000*g* for 5 min to collect the DNA.
13 Wash the DNA with 70% ethanol to remove residual CTAB and recentrifuge for 5 min to re-pellet. Carefully remove the supernatant and briefly dry the pellet.
14. Dissolve the pellet in 100 µL of dH_2O. DNA solution can be stored at –20°C until required or an aliquot (usually 10 µL) is taken for the PCR assay.

3.3. PCR Assay

1. Prepare the PCR buffer master mix including primers MG1a and MG2 (*see* **Table 6**), in a clean area separated geographically from any post-PCR product analysis, and transfer aliquots of 80–90 µL (that is, 100 µL minus the amount of sample, usually 10–20 µL) into 0.5 mL microcentrifuge tubes. Overlay the PCR buffer with 75–100 µL of mineral oil (*see* **Note 7**)
2 Transfer the PCR tubes to an area dedicated to the addition of target DNA from the various urogenital samples. Add the aliquots of sample DNA (*see* **Note 8**)
3 Transfer the PCR tubes to the thermal cycler and execute the required thermal profile.
4 After the amplification, transfer the tubes to an area of the laboratory that is dedicated to handling first round amplified PCR products, that is as far away from the area for preparation of the PCR buffer master mix as possible. The *M. genitalium* assay is a semi-nested PCR, so 1/50th of the first round product is added to fresh PCR buffer (98 µL), which has been overlaid with mineral oil as before and amplified using the same thermal profile as before (*see* **Note 9**)

3.4. Detection of the Amplified Product

1 Mix 10–15 µL of second round amplified product with electrophoresis loading buffer and electrophorese on a horizontal agarose gel system using TBE buffer at about 8 V/cm until the bromophenol dye front is approx 1 cm from the end of the gel (both the gel and the TBE running buffer contain 5 µg/mL ethidium bromide).
2 Visualize the products under UV illumination.
3 Positive samples are identified as having a band at the same level as the two positive controls. However, in order to confirm that they are positive, the DNA is transferred on to a nylon membrane (Amersham Hybond-N) by Southern transfer *(61)* and hybridized with a specific probe.

3.5. Preparation, Hybridization, and Detection of Probe

1 The probe is 100 bp long and is the amplified product of primers MG4 and MG5 (**Table 6**), produced using the same thermal profile as the *M. genitalium* PCR. Remove the excess unincorporated primers using a proprietary DNA clean-up system from Promega.
2 Label the probe by using a digoxigenin labeling kit from Boerhinger Mannheim. Denature the probe by heating at 100°C for 8 min and immediately quench on ice for approx 3 min. Into a 0.5 mL microcentrifuge tube on ice, put 2 µL of nucleotide mix, 2 µL of hexanucleotide primer mix, 5–10 µL of probe DNA, 5–10 µL

of dH_2O (final volume is 20 µL for the reaction), and lastly add 1 µL Klenow polymerase. Centrifuge the tube briefly in a microcentrifuge to bring all the components together and incubate at 37°C for at least 1 h, preferably overnight.

3 Stop the reaction by adding 1 µL of 0.2 *M* EDTA. To precipitate the DNA, add 2 µL of 4 *M* LiCl, mix thoroughly, add 75 µL of ice-cold 100% ethanol, and keep at –20°C for 2 h or –70°C for at least 30 min.

4. Centrifuge the tube at 13,000*g* for 10 min to precipitate the DNA. Carefully remove the supernatant (*see* **Note 10**) and wash with ice-cold 70% ethanol and recentrifuge as before.

5 Dissolve the pellet in 50 µL of dH_2O for at least 1 h at room temperature The probe is now ready for use, or it can be stored at –20°C for more than six months We have used probes successfully that have been stored for over a year

6 Prehybridize the membrane (from **Subheading 3.4., step 3**) in 50 mL of hybridization buffer at 42°C for 1 h in a rotating glass drum in a Techne hybridization incubator.

7. Denature 10 µL of the probe by heating at 100°C for 8 min and then quench immediately on ice for approx 3 min, and transfer to 10 mL of fresh hybridization buffer preheated to 42°C

8 Decant the hybridization buffer from the prehybridized membrane and add the 10 mL of the hybridization buffer containing the probe. Leave to hybridize overnight at 42°C

9. Decant the probe solution into a 50 mL polypropylene centrifuge tube or other suitable container for storage as the solution can be reused up to four times if stored at –20°C, and denatured in between each use

10. Wash the membrane at various stringencies, usually twice at room temperature for 5 min with 2X SSC and 0.1% SDS, and then twice at 42°C for 15 min with 0.1X SSC and 0.1% SDS.

11 Transfer the membrane to washing buffer for 2–3 min All of the following procedures are undertaken with materials from the Boerhinger Mannheim Dioxigenin DNA Labeling and Detection Kit in medium sized sandwich boxes containing, approx 100 mL of each solution at room temperature with gentle shaking on an orbital shaker unless specified otherwise.

12 Transfer the membrane to a blocking buffer and block for one hour.

13 Transfer the membrane to some layflat tubing and using a bag sealer make a bag to surround the membrane with approx 1 cm margins. Leave one side open and add 20 mL of blocking buffer containing antidigoxigenin antibody (Fab fragment) diluted to 1 in 10,000. Incubate for one hour with vigorous shaking (tape the bag to the orbital shaker platform)

14 Transfer the membrane to a sandwich box and wash twice for 15 min with washing buffer.

15. Remove the washing buffer and add equilibration buffer (*see* **Note 11**) for approx 3 min.

16. Remove the equilibration buffer, make another bag as before, and add 10 mL of fresh equilibration buffer containing CSPD diluted 1 in 10. This is the luminescent substrate Incubate for 10 min with vigorous shaking.

17 Carefully remove the CSPD solution to a suitable storage container (*see* **Note 12**) Remove the membrane from the bag with forceps and gently touch one of the bottom corners on blotting paper to remove excess CSPD, but do not allow the membrane to dry out Place the membrane in another bag and incubate at 37°C for 10 min

18 Tape the bag to the inside of a luminography/autoradiography cassette and in a dark room place a sheet of luminography/autoradiography film over it, close the cassette, and expose for 15 min Develop the film in an autodeveloper or other suitable equipment. Alter the exposure times to suit the intensity of the image required

3.6. Discussion

The use of the PCR technique has enhanced studies involving mycoplasmas and has been essential for the study of *M genitalium*. In our hands this assay has proved to be at least 1000 times more sensitive than culture (unpublished data) and much faster. The assay is not difficult to perform but requires care and attention to detail especially to avoid potential contamination.

Specimen preparation for PCR testing is an important parameter that should be optimized. Since mycoplasmas do not have a cell wall, boiling the sample after concentration of the organisms by centrifugation can be sufficient to make their DNA accessible. This may be adequate for testing cell culture samples, though treatment of the samples with detergents and proteinase K is preferable *(62)*. For urine specimens, Wang and Lo *(48)* recommend treatment of the urine sediment with proteinase K followed by heating at 95°C for 10 min. However, some samples contain undefined inhibitors of the PCR assay that reduce the efficiency of amplification. To try to overcome this, DNA extraction has to be employed *(9)*. For research purposes this is not a problem, but for clinical laboratories it is a serious drawback that may hamper the adoption of PCR for routine use. The existence of PCR inhibitors may be ruled out by the use of internal controls, of which the β-globin gene or HLA genes are good examples. In addition, they serve as an indicator of the presence of human cells in the sample. In many cases, the effect of PCR inhibitors in specimens may be abolished or reduced simply by diluting the specimens *(47)*.

The results of the PCR assay are relatively easy to interpret, especially if the products have been transferred onto nylon membrane and hybridized. The probe is very specific and a band will be produced only for a positive sample or for the positive controls.

Apart from its value in the detection of mycoplasmas, the PCR is a very powerful molecular tool and its use in other areas is expanding rapidly. One of the areas to benefit from the advent of *in situ* PCR technology is pathogen localization within host tissues *(63–65)*. The adhesion of mycoplasmas to host cells is a prerequisite for colonization and for infection. Most mycoplasmas

adhere tenaciously to the epithelial linings of the urogenital and/or respiratory tracts, and may be considered to be surface pathogens. However, there is evidence for the intracellular location of some mycoplasmas, notably *M. fermentans, M. penetrans*, and *M. genitalium* **(66–68)**, and invasion of tissues may occur particularly in immunocompromised patients, who, as a group, are increasing in incidence. The future use of an *in situ* PCR could help to determine the exact location of these three species in the tissues or alternatively it could be used to locate the area of attachment of the mycoplasmas to the host cells. Indeed, some of the research to establish virulence factors in mycoplasmas has involved the use of molecular methods to examine adhesins, the best defined being those of *M. pneumoniae* and *M genitalium* **(69,70)**.

4. Notes

1. Sample collection. Swabs should be expressed immediately in PBS and should not be allowed to dry Taking the PBS to the location of the patient is a sound policy. The swab should not be broken off into the PBS as the swab stick may contain inhibitors. It should be agitated in the PBS, expressed against the side of the container (usually a 1.5–2 mL microcentrifuge tube), and then discarded.
2. Dissolve 4.1 g of NaCl in 80 mL of dH_2O and slowly add 10 g of CTAB while heating and stirring If necessary, heat to 65°C to dissolve. Adjust final volume to 100 mL
3. Sometimes the make and model of the thermal cycler are provided in a publication and if they are the same as those being used then the thermal cycling profiles can be used directly without any modification. However, if the thermal cyclers differ then a period of optimization is necessary to achieve the desired result.
4. The swab can be prepared in the same manner as a urine sample. Preparation of the latter is described in **Subheading 3.2.**, but this is a lengthy process. Furthermore, the amount of material on a swab is generally much less than provided by a urine sample so that the losses that occur during the DNA extraction can seriously affect the amount of DNA left at the end of the process The use of the InstaGene matrix greatly reduces the number of steps involved and thus less DNA is likely to be lost
5. The InstaGene matrix is a DNA binding slurry suspended in an appropriate buffer. As it is a slurry it must be constantly shaken or stirred so that the suspension is homogenous. A small magnetic stirrer bar is provided with each bottle of InstaGene so the bottle can be placed on a magnetic stirrer and the slurry kept in suspension.
6. The InstaGene matrix is left in the tube since it does not interfere with the PCR However, it can be removed if this is more convenient.
7. Prepare enough for $n + 1$ aliquots of PCR buffer, where n is the number of samples plus their 'DNA preparation' controls' plus one negative 'reaction' control and two positive 'reaction' controls. This ensures that there will be sufficient reaction mixture The oil (three or four drops) is added with a pastet. Oil overlay is not necessary for some brands of thermal eyelets

8 If the PCR buffer is to be overlaid with mineral oil it is important to add the sample DNA below the oil Thus, the pipet tip is placed below the oil before the aliquot is dispensed. Contamination is always a problem with PCR technology and this can be reduced by frequent glove changes; at this stage, change gloves after every 'preparation' negative control, usually every five or six tubes.
9. Contamination is even more of a problem when handling amplified PCR product so change gloves after every sample. This can be very tedious but it is one way of ensuring there is no carry-over of DNA from one tube to the next.
10. At this stage the pellet is often invisible so that it is advisable to mark the tube before it is removed from the rotor in the centrifuge in order to determine the relative position of the pellet
11. This is an equilibration step. The luminescent substrate is made up in buffer 3 and is pH 9.5 The washing buffer has a lower pH value so the membrane needs to be equilibrated, otherwise a precipitate will form, which will greatly increase the level of background.
12 The CSPD solution can also be reused up to four times. If a precipitate forms it can be removed by centrifuging.

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7

Hepatitis B Virus

Detection and Quantitation by Membrane and Liquid Hybridization, Branched DNA Signal Amplification, Hybrid Capture, and PCR Methods

Mel Krajden

1. Introduction

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family and has a (3200-bp) partially double-stranded circular DNA genome *(1)*. This virus causes subclinical, acute self-limited, chronic, and fulminant hepatic infections. Chronic HBV infection is particularly important because of the clinical manifestations which include: cirrhosis, hepatic failure, hepatocellular carcinoma, as well as extrahepatic manifestations, such as vasculitis and membranoproliferative glomerulonephritis. It is estimated that there are currently 350 million chronic HBV carriers worldwide *(2)*.

In unvaccinated populations, 2–10% of infected individuals become long-term carriers. In these populations, vertical transmission is very important because 70–90% of infants born to mothers with chronic HBV infection will become chronic carriers. Infection in children under 4 yr of age is also associated with a higher risk (26–30%) of chronic infection. Although sexual transmission is important because of the high risk of transmission, the risk of a given individual who is in a sexually active age group becoming a chronic carrier is <5%. These modes of transmission are very important in maintaining the pool of chronic carriers worldwide *(1,2)*. Other modes of transmission include intravenous drug use, horizontal transmission within households and institutions, nosocomial and other parenteral forms of transmission, including rare cases of HBV infection associated with screened blood products and organs *(1)*.

It is also very important to recognize that the clinical course of HBV infection reflects the complex interplay between the host's immune response which

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attempts to control or eliminate the infection *(3,4)*, and virus-specific virulence factors *(5–7)*.

This chapter will focus on the strengths and weaknesses of laboratory assays to quantify HBV DNA, which has been shown to be a marker of active HBV replication *(8,9)*. Hepatitis B viral load testing has also been shown to be an important surrogate marker to assess the in vivo therapeutic response of chronic HBV to antiviral agents, such as interferon and lamivudine *(10–12)*. The ultimate goal of treating chronic carriers is to prevent or minimize the risk of developing the clinical complications of chronic HBV infection. Effective treatment of chronic carriers may also decrease the risk of HBV transmission. This chapter will demonstrate how hepatitis B viral load testing has and will continue to play an important role in our understanding of HBV pathogenesis. For readers interested in the diagnosis of acute HBV infection, the reviews by Hoofnagle and Bisceglie *(13)* and Hollinger are highly recommended *(1)*.

Although advances in care and treatment of HBV are laudable objectives, vaccination against HBV has been shown to be extremely effective in preventing HBV infection and the risk of becoming an HBV carrier. Prevention HBV infection by vaccination must therefore be a key element of any public health program! Until effective vaccination programs have eradicated HBV disease, monitoring of viral load will play an increasingly important role in assessing treatment interventions. Another important dimension that is beyond the scope of this chapter is how to deliver effectively both vaccination and therapeutic interventions to the population at risk *(2)*.

1.1. Hybridization-Based Detection of HBV DNA

Figure 1 illustrates in simplistic form, the principle(s) involved in membrane hybridization and three commercial HBV DNA detection assays, the Abbott Hepatitis B Viral DNA Assay (Abbott) (Abbott Laboratories, Abbott Park, IL), the CHIRON Quantiplex™ HBV-DNA (Chiron bDNA) (Chiron Corporation, Emeryville, CA), and the Digene Hybrid Capture™ System HBV DNA (Digene) assays (Digene Diagnostics, Inc., Beltsville, MD). All of the above assays are based on molecular hybridization and do not involve direct nucleic acid amplification using, for example, the polymerase chain reaction (PCR). Viral DNA in serum is first extracted and denatured using various assay specific protocols (*see* **Notes 1** and **2**). This is followed by hybridization with assay specific probe(s) and quantification of the detected HBV target DNA (**Fig. 1**).

1. For membrane hybridization, HBV DNA from the patient's sera is bound to the membrane surface and hybridized with isotopically or nonisotopically labeled HBV DNA probes. The intensity of the hybridized signal is generally measured by autoradiography or by direct counting of the hybridized signal by phospholuminometers. Standard curves derived from known standards and controls allow quantitative assessments of the amount of HBV DNA in the sample.

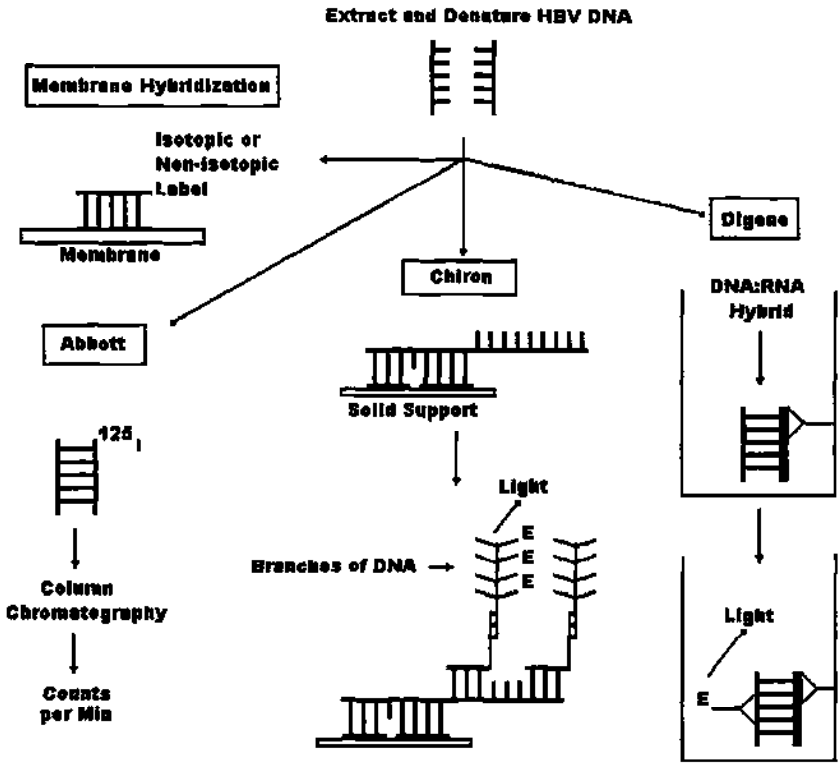

Fig. 1. Demonstrates in simplistic form the principles involved in membrane hybridization, the Abbott, Chiron bDNA, and Digene HBV DNA assays (E = the enzyme alkaline phosphatase)

2. In the Abbott assay, ^{125}I-labeled full-length genomic HBV DNA is hybridized with patient's HBV DNA in solution. Hybridized HBV DNA is then separated from the unhybridized probe by Sepharose column chromatography. The amount of patient specific HBV DNA is measured by the amount of hybridized ^{125}I-labeled probe eluted from the column. The isotopic signal is then compared with the signals generated from known positive and negative controls.
3. The Chiron bDNA assay involves the initial capture of HBV DNA from the patient's serum to a solid phase. Multiple HBV DNA specific probes are then hybridized along the HBV genome. These probes are, in turn, hybridized to branches of DNA (hence the name bDNA), which then bind other probes tagged with alkaline phosphatase On exposure to substrate, light is released in proportion to the amount of target bound to the solid phase. In bDNA assays, the target HBV DNA is not directly amplified (as in PCR assays, where the actual target is amplified in vitro). Rather only the signal from the bound target is amplified (hence the term signal amplification).

4 The Digene assay is based on hybridization of full-length HBV RNA to HBV DNA in the sample The resulting HBV DNA:RNA hybrid is captured in tubes coated with antibody, which specifically binds to DNA·RNA hybrids. Anti-DNA.RNA antibody labeled with alkaline phosphatase is then used to cleave a substrate that generates light in relation to the amount of captured DNA RNA hybrids

1.2. Membrane Hybridization

There are a number of inherent limitations associated with membrane hybridization that render it unsuitable for routine clinical use. These include:

1 The difficulty in accurately measuring the output signal to ensure that the signal generated from hybridized probe truly reflects the amount of HBV DNA bound to the membrane Measuring the intensity of signal by radioautography may not reflect true signal intensity because of the reciprocity effects of X-ray film *(14)* This limitation can be overcome by using phospholuminometers, which are expensive.
2 Part of the target is bound to the membrane and is therefore unavailable for hybridization with the probe, which can limit the dynamic range of the assay *(14,15)*
3. The possibility that certain samples, possibly owing to incomplete nucleic acid extraction, may nonspecifically trap the probe on the membrane and generate false-positive signals.

Given the above limitations, it is not surprising that membrane hybridization technology, although very powerful, has not been adopted for commercial quantitative HBV DNA detection assays.

Since HBV viral load determinations are useful for both quantifying HBV replication *(8,9)* and assessing antiviral efficacy, there is clearly a need for accurate and reproducible HBV DNA assays. Because of inherent difficulties in standardizing in-house quantitative DNA detection assays and the clinical need for assays that are accurate and reproducible between test centers, HBV DNA detection assays have been largely relegated to the commercial arena. The reader will be introduced to step-by-step procedures for three commercial quantitative HBV DNA hybridization assays followed by an analysis of their strengths, limitations, and clinical implications (*see* **Notes 7–14**).

2. Materials

2.1. Chiron bDNA

1 EDP electronic pipetter, with 250- and 1000-µL barrels.
2. Pipetters, 5–40, 40–200, and 200–1000 µL, and disposable tips.
3 Vortex mixer
4. Thermolyne™ Maxi-Mix III with foam insert.
5. Powder-free gloves.
6. Water bath at 37°C.

7. Two Stat-matic plate washers.
8. Wash station with 4-L flask for wash discard.
9. 70% ETOH and bleach

2.2. Digene Hybrid Capture

1. Water bath at 65°C.
2. Vortex mixer.
3. Pipetter to deliver 50 and 80 µL and disposable tips.
4. Repeater pipetter and tips to deliver 1.25 and 12.5 mL (optional)
5. Rotary shaker capable of achieving 1100 rpm.
6. Decanting rack.
7. Wash bottle and absorbent paper.
8. 4000-mL flask, 2000-mL cylinder, and stirrer-mixer to prepare wash solution.

2.3. Abbott HBV DNA

1. Micropipetters to deliver 1,400, 100, 70, 20, and 10 µL with tips.
2. Vortex mixer
3. Microcentrifuge.
4. Water bath at 65°C
5. Boiling water bath
6. Column rack for holding columns.
7. γ-Counter capable of efficiently counting ^{125}I. Counter efficiency should be at least 60%, preferably 80%, or more.

3. Methods

3.1. Chiron Assay Preparation

1. The day before the assay.
   a. Turn on plate heaters and allow temperature to stabilize
   b. Write up the plate assay map
   c. Arrange specimens in a rack and leave at –20°C.
2. On the day of the assay:
   a. Thaw specimens at 4°C or in cold water
   b. When thawed, vortex and place on ice.
   c. Record the temperature of the upper and lower chambers of both plate heaters using the external digital thermometer.
   d. Turn on the computer, printer, and luminometer.

   You may use the HBV-DNA assay checklist with the following notes:
   i. Refer to the plate setup diagram in the Chiron package insert
   ii. Standards and controls are tested in duplicate in the first row
   iii. Specimens are tested in duplicate in the remaining rows, with a maximum of 42 specimens. (No less than a half plate may be run at any one time, and a maximum of 18 specimens.)

Quick reference The manufacturer's protocol always supercedes this quick reference guide

e. Allow lysis diluent, reagent, wash A, wash B, capture wells, denaturing reagent, neutralizing reagent, plate sealers to reach room temperature (box 1).
f. Remove target probes, standards, and controls (box 2).
g Lysis diluent changes from blue/green at 4°C to yellow at room temperature.
h Thaw specimens, target probes, standards and controls, vortex, and then place on ice.
i Place capture wells in plate holder. Set up plate map
j Prepare lysis working reagent (900 µL of lysis diluent + 300 µL of lysis reagent, color changes yellow to orange) Keep at room temperature
k Add 10 µL of lysis working reagent to each well with EDP (250 µL barrel)
l Add 10 µL of specimen, standard, or control to the appropriate well (color changes for orange to yellow/green).
m Seal plate and shake for 30 s.
n Insert PAD (heater liner) in 63°C heater, and incubate plates at 63°C for 30 min
o. Within 10 min before use, prepare target probes working reagent (1200 µL denaturing reagent + 10 µL target probes), vortex, and keep at room temperature
p. Remove plate from heater, and immediately add 10 µL of target probes working reagent (color change yellow/green to purple).
q. Seal plate, shake for 30 s, and then incubate at 63°C for 30 min.
r. Remove plate from heater Immediately add 10 µL neutralizing reagent to each well (color change purple to yellow)
s Seal plate, shake for 30 min, and then incubate at 63°C for 16–20 h

3 Day 2:

a. Place amplifier diluent and label diluent at 37°C for 10–15 min (box 1, time step). Leave label diluent at 37°C until use.
b Thaw at room temperature amplifier and label probe. Place label probe at 4°C until use
c. Set up wash station.
d. Cool plate at room temperature for 10 min.
e. Prepare amplifier working reagent (vortex 4 4 mL amplifier diluent + 33 µL amplifier), and keep at 37°C.
f. Aspirate wells, and wash two times with 400 µL wash A. Add 40 µL amplifier working reagent to each well. Seal plate, shake for 30 s, and incubate for 30 min at 53°C
g Cool plate for 10 min Prepare label working reagent 4.4 mL + 10 µL, vortex, and keep at 37°C. Wash plate two times with 400 µL wash A.
h Add 40 µL of label working reagent. Seal plate, shake for 30 s, and incubate for 15 min at 53°C.
i. Set up data reduction software and luminometer.
j. Cool plate at room temperature for 10 min. Aspirate all wells. Wash two times with wash A and three times with wash B (400 µL/wash).

k. Add 30 µL of substrate to each well.
l. Seal plate, shake for 30 s, and incubate at 37°C in luminometer for 25 min Then read on luminometer.

4 Repeats and dilutions·
a. The computer printout will flag specimens that need to be repeated and/or diluted, i.e., specimens above assay cutoff with CV% > 20% and specimens below assay cutoff with CV% > 25% (*see* **Note 4**).
b. Repeat once after centrifuging specimen for 2 min at 3000 rpm in a microcentrifuge (Eppendorf)
c Specimens with quantitation value above the upper limit of the standard curve
d Dilute/repeat according to the following chart, in human serum negative for HBV DNA by the Chiron assay (stored in 1-mL aliquots).
e. Luminescence value of undiluted sample versus recommended specimen dilution

Luminescence	Dilution
>900	1 200
600–900	1:50
400–600	1:25
300–400	1·10

f. Specimens for which quantitation values for the duplicates are above and below the cutoff value are repeated undiluted on next assay run.

5. Assay validation:
a The quantitation of the positive control must fall within the range specified in the product insert supplement, for the lot number of kit you are using The CV% for the positive control must be ≤20%.
b Standards A–D· The CV% for standards A–D must be ≤20%.
c. Negative control· The CV% for the negative control must be ≤25%. If any of the standards (A–D) and/or the controls do not fall within the specified ranges, the assay is invalid and must be repeated If the negative control CV% is >25%, contact Chiron Technical Service for advice. If Technical Service approves the assay, please request verification in writing with date and signature for quality-assurance purposes.

6 Assay quality control· For each assay, complete the information on the Chiron HBV DNA assay quality-control sheet: date; kit lot number; CV% for standards A–D; positive control range from product insert supplement; positive control value from assay printout, initials of technologist performing the assay

7. Reporting:
a. Positive results: should be reported quantitatively in pg/mL (results can be reported in MEq if desired)
b. Negative results: report as <2.5 pg/m.
c. High CV% results: specimens with a high CV%, after repeat testing one to two times should be reported as: high CV%, unable to provide valid result.
d Intermediate results· if after two consecutive assay runs, the specimen remains indeterminate, report as: indeterminate.

3.2. Digene Hybrid Capture Assay Preparation

1 The day before the assay:
   a Assemble work list
   b. Remove specimens to be tested from the –20°C freezer, and thaw overnight at 4°C
2 On the day of the assay
   a Equilibrate 65°C water bath
   b Allow specimens and HBV-DNA kit to reach room temperature
   c Record the ambient room temperature.
   d Run the background quality-control check on the DCR-1 luminometer

Quick reference. The manufacturer's protocol always supersedes this quick reference guide
   a. Allow specimens and reagents to reach room temperature.
   b. Label hybridization tubes for controls, standards, and specimens. Negative control and standards are tested in duplicate, and specimens are tested individually
   c. Add 50 µL of control, standard, or specimen to tube.
   d Using a repeater pipet with disposable tips, add 25 µL of sample diluent and 25 µL of sample preparation reagent to each tube
   e. Cap tubes and place in hybridization rack on a rotary shaker at 1100 ± 100 rpm for 30 s. Tube contents should turn light green or blue.
   f. Incubate at 65 ± 2°C for 20 ± 5 min in the water bath
   g Prepare HBV probe mix: 1 tube probe diluent + 80 µL HBV probe Vortex to mix (Store the diluted probe at –20°C after preparation and initial use, and equilibrate to room temperature before use)
   h Remove hybridization tubes from water bath Uncap tubes individually Add 50 µL of denaturation reagent.
   i. Recap tubes Mix on rotary shaker for 30 s (1100 ± 100 rpm). Tubes' contents should turn light pink.
   j. Incubate at 65 ± 2°C for 30 ± 5 min in water bath
   k Remove from water bath, and uncap tubes individually Add 50 µL of HBV probe mixture into each tube
   l Tighten caps Mix on multitube rotary shaker (1100 ± 100 rpm) at 20–25°C for 3 ± 2 min. Tubes should turn a blue-green
   m Incubate at 65 ± 2°C in the water bath for 60 ± 5 min
   n Label required number of capture tubes.
   o Transfer contents of hybridization tube to corresponding capture tube, using a clean 1-mL plastic pipet.
   p. Cover with Parafilm Shake on rotary shaker (1100 ± 100 rpm) at 20–25°C for 60 ± 5 min. Prepare wash buffer.
   q. When capture step is complete, decant the tubes by inverting rack over sink and shaking out contents Blot tubes two to three times on absorbent paper
   r. Pipet 250 µL of detection reagent 1 into each tube. Shake rack by hand, several times to verify that all tubes are filled accurately.

s Cover with Parafilm. Incubate at 20–25°C for 30 ± 3 min on bench top
t. When incubation is complete, decant contents of tubes by inverting over sink Blot inverted tubes two to three times on absorbent paper.
u. Fill all tubes to overflowing with wash buffer. Decant over sink Repeat four times (five washes). After final wash, remove excess wash buffer by shaking inverted tubes vigorously over sink.
v. Drain inverted capture tubes for 5 min on absorbent paper.
w. Pipet 250 µL of detection reagent 2 into each tube, and add the reagent in the same order in which the tubes will be read.
x Pipet 250 µL of detection reagent 2 into an empty, lean 12 × 75 mm polystyrene tube to serve as the detection reagent 2 blank
y Cover tubes with clean Parafilm. Incubate at 20–25°C for 30 ± 3 min on bench top. Cover the tubes with a box or foil to avoid exposure to light.
z Immediately following incubation, read tubes on the Digene luminometer. Wipe each tube gently with a moist Kimwipe before inserting into instrument.

3 Quality control: Perform luminometer background check Refer to the Digene DCR-1 Luminometer Manual. Use 10 empty tubes counted 5 times for 10 s each Background should be <500 relative light units (RLU)/10 s count Complete the HBV-DNA assay data sheet Include date performed, initials of technologist, room temperature, lot number, and expiry date of kit

Enter the RLU values for the positive standards and negative control into the table on the assay data sheet. Calculate the ratios for assay validation

4. Assay validation.
   a. The positive standards and negative control are tested in duplicate for each test run.
   b. The positive standards and negative control results are used to calculate ratios to validate the assay. Ratios must be within the following ranges·
      i Positive Stn 1 mean RLU ÷ negative Ctrl mean RLU ≥ 1 5
      ii. Positive Stn 2 mean RLU ÷ positive Stn 1 mean RLU ≥ 5 0.
      iii Positive Stn 3 mean RLU ÷ positive Stn 2 mean RLU ≥ 4 0.

      If any values fall outside the above ranges, the assay is invalid, and the specimen results cannot be interpreted.
   c. The detection reagent 2 blank (DR-2 blank) must be.

      DR-2 blank < 5000 RLU

      DR-2 blank < RLU. Values for all controls.

      RLU values above 5000 indicate contamination of the detection reagent 2
5. Reporting·
   a Record all RLU readings and interpretations in pg/mL on the worksheet.
   b. Report results as the numeric value printed on the luminometer tape.
      i. HBV DNA· "X" pg/mL by Digene assay.

      All values <5 pg/mL should be reported as.

      ii HBV DNA <5 pg/mL by Digene assay

      All computer extrapolated values should be reported as HBV DNA >2000 pg/mL by Digene assay (*see* **Note 5**).

3.3. Abbott HBV DNA Assay Preparation

1. On the day of the assay:
   a Bring specimens and kit reagents to room temperature. Vortex specimens before use.
   b. Equilibrate at 65°C in water bath
   c Reconstitute reagent 2: Add 400 µL of solution A, vortex for 5 s, and store at room temperature for 2 h.

   Quick reference. The manufacturer's protocol always supersedes this quick reference guide
   a. Label reaction tubes: three negative controls, two positive controls, and one for each specimen.
   b Add 100 µL of reagent 1 to each tube.
   c. Pipet 100 µL of control or specimen to the appropriate tube.
   d Add 10 µL of reconstituted reagent 2 to each tube. Cap tubes, vortex, and centrifuge for 1–3 s
   e Incubate at room temperature (15–30°C) for 60 ± 2 min. Warm reagent 4 at 65 ± 1°C while tubes are incubating.
   f Add 20 µL of reagent 3 to each tube Cap tubes and vortex
   g Incubate at room temperature (15–30°C) for 30 ± 2 min Turn on the boiling water bath at the beginning of the incubation
   h Prepare columns (18–24 h prior to use) Arrange columns vertically in the rack, remove cap, remove plug using forceps, and recap column.
   i. Approximately 10–15 min before the end of incubation, remove reagent 4 from 65°C water bath, vortex, heat in boiling water bath for 5 ± 0 5 min, remove, and stand at room temperature (15–30°C) for 5–10 min Vortex before use
   j Add 70 µL of reagent 4 to each tube, cap tubes, vortex, and centrifuge for 1–3 s (addition of reagent 4 should be completed within 10 min of being placed at room temperature)
   k. Incubate at 65 ± 1°C for 18 ± 2 h Leave reagent 5 at room temperature (15–30°C) overnight
2 Day two:
   a. Remove top and bottom cap from columns Allow storage buffer to drain completely (approx 30 min).
   b Label counting tubes and place under columns
   c Remove reaction tubes from water bath, and centrifuge for 1–3 s (to precipitate condensate).
   d Immediately transfer contents (300 µL) of each control and specimen tube to the appropriate column.
   e Add 1400 µL (2 × 700 µL) reagent 5 to each column Collect eluate in the counting tube until the column has stopped dripping (total volume collected = 1700 µL).
   f. Cap counting tubes, place in γ-counter, and select HBV DNA program
   g. Count each tube for 10 min.

3. Calculation of results:
   a. γ-Counter is calibrated daily as is the background counts, which are subtracted automatically.
   b. Total counts in 10 min are used for calculations rather than counts per min.
   c. Calculate the mean of the positive controls: (calculated by the computer program). Each positive control value should be ≥0.75 times the positive control (PC) mean and ≤1.25 times the PC mean. If one value is outside the acceptable range, repeat the run (*see* **Note 6**).
   d. Calculate the mean of the negative controls (calculated by the computer program). Each negative control value should be ≥0.5 times the negative control (NC) mean and ≤1.5 times the NC mean. If one value is outside the acceptable range, discard this value and recalculate the mean. If two values are outside the range, repeat the run.
4. Assay validation: (calculate manually and record): The run is valid if the mean of the positive control is ≥3000 counts and the ratio of the mean of the positive control to the mean of the negative control (P/N) is ≥20
   a. Calculate the cutoff value (calculate manually and record): cutoff value = (0.015 × PC mean counts) + NC mean counts.
   b. Calculate the retest range (calculate manually and record): 10% on either side of the cutoff value.
   c. Quantitation of HBV DNA concentration (calculated by the computer program): concentration of the positive control:

$$103\ \text{pg/mL}\ (\text{Sample counts} - \text{NC mean})/(\text{PC mean} - \text{NC mean}) \times 103 = \text{pg HBV DNA/mL} \quad (1)$$

5. Reporting:
   a. Negative: counts < cutoff value, i.e., <1.6 pg/mL.
   b. Positive: counts ≥ cutoff value report value in pg/mL.
      Retest in duplicate to confirm initial test result when counts are within 10% of the cutoff value.
   c. Retests: Specimens with counts < cutoff value are considered negative. Specimens with counts ≥ cutoff value are considered positive.

4. Notes

1. All specimen handling should involve universal precautions. Technologists should have received a course of HBV vaccination and have had a documented serological response to HBV vaccine. Meticulous specimen handling is required to prevent carryover of infectious material as well as noninfectious nucleic acids.
2. A key factor affecting accurate quantification of HBV DNA is sample handling and storage. Unfortunately, there is limited published information on optimal sample handling for detection of HBV DNA in sera. Most specimen handling data are gleaned from published studies involving quantitative detection of nucleic acids from, e.g., hepatitis C virus and HIV *(16)*. Early separation of samples within approx 4 h of collection minimizes the chances that white blood

cells in the sample will degrade and release enzymes capable of destroying the viral nucleic acids

We have recently assessed the stability of HBV DNA in 26 neat sera, which were previously frozen at –70°C prior to being stored for 1–5 d at 4, 25, 37, and 45°C (n = 20), and for 1–21 d at 4, 25, and 37°C (n = 6). All specimens were subsequently refrozen at –70°C prior to undergoing quantitative HBV DNA testing by Chiron bDNA. We found no significant HBV DNA degradation at 4°C over 5 d by logistic regression However, HBV DNA levels did decrease by approx 1.8%/d at 25°C, 3.4%/d at 37°C, and 20%/d at 45°C. Thus, specimen integrity for detection of HBV DNA in serum is preserved in separated serum at 4°C for at least 5 d *(17)*.

3. **Table 1** contrasts some technical and design aspects of the three commercial HBV DNA assays. The Chiron bDNA and Digene assays are best suited for high-volume laboratories because 42 and 52 specimens, respectively, can be processed at once. In contrast, a single Abbott assay can test 20 specimens. The Chiron and Digene assay kits also have longer shelf lives (approx 1 yr), whereas the Abbott assay, which is isotopic, must be used within 1 mo. When testing batches of approx 40 samples, the overall hands on technical time are quite similar, at approx 7 h, despite the fact that the Chiron and the Abbott assays require overnight hybridization steps.
4. The high degree of reproducibility of the Chiron bDNA assay occurs, in part, because the standards, controls, and specimens are tested in duplicate. Testing in duplicate allows the CV% to be calculated permitting detection of technical or equipment errors, ensuring precise quantification of specimens and allowing a precise assay cutoff to be generated. Approximately 0–6% of specimens will have an elevated CV% on initial testing and, therefore, require repeat testing to generate valid results. The Chiron bDNA assay also has the widest dynamic range (2 5 pg/mL to approx 16,000–18,000 pg/mL, where 1 pg is equivalent to 285,000 Eq or copies) allowing more specimens at the low and high end of the dynamic range to be accurately quantified on initial testing. In addition, specimens that quantitate above the dynamic range of the assay are flagged for repeat testing using a defined dilution protocol. Repeat testing of high-end specimens further expands the dynamic range.
5 For the Digene assay only, the controls (high-, mid-, low-range, and negative controls) are tested in duplicate. The manufacturer recommends that specimens be tested individually. Inherently this decreases the ability to detect technical or equipment errors, and this theoretically decreases intra- and interassay result reproducibility The low-end cutoff of the Digene assay has recently been lowered to 5 from 10 pg/mL, which is slightly less sensitive than the Chiron assay whose low-end cutoff is 2.5 pg/mL. Another important point is that the luminometer software provided by the manufacturer extrapolates HBV DNA values, which quantify beyond the high-end cutoff of the assay, i.e., >2000 pg/mL. These extrapolated HBV DNA concentrations are not precise when compared to specimens diluted in HBV DNA negative serum. Thus, specimens that have HBV

Table 1
Technical and Design Aspects of the Three Commercial HBV DNA Hybridization Assays

Issue	Chiron bDNA	Digene	Abbott
Capacity	4 Standards, 1 positive and 1 negative control, 42-specimens kit	4 Controls, 52 samples/kit	5 Controls, 20 samples/kit
Hands-on technical time (assay run time)	6–8 h (20 h)	6–7 h (6–7 h)	4–5 h (20 h)
Dynamic range	3–4 log	3 log	2–3 log
Factors affecting assay reproducibility	All testing performed in duplicate; high-, mid-, and low-range standards; high CV% samples are flagged for retesting, samples quantitating beyond range of the standard curve flagged for dilution and retesting	Controls are tested in duplicate; samples are tested individually; high-, mid-, and low-range controls are used; computer software extrapolates specimen's quantitating below and above dynamic range of the standard curve	A positive control is tested in duplicate with three negative controls; specimens are tested individually; no low- or mid-range standards are used; there is no intrinsic method to determine if specimens are within the linear range of assay; the low-end cutoff is calculated, but cannot be precisely defined

DNA concentrations >2000 pg/mL should be diluted and retested if accurate quantification is required Because of the narrower dynamic range of the Digene assay relative to that of the Chiron bDNA, more samples may require repeat testing after dilution to quantify HBV DNA accurately. Despite these limitations, the Digene assay is reliable, easy to perform, and reasonably accurate

6 The Abbott assay was the first commercially available HBV DNA hybridization assay. Its intrinsic design, however, limits its ability to quantify accurately HBV DNA in specimens. The assay uses duplicate testing of a single positive control (set at 103 pg/mL of HBV DNA) and three negative controls to create its standard curve. No high-, mid-, or low-range standards are included in the assay The lack of standards at various HBV-DNA concentrations limits the ability to set an accurate low-end cutoff and to determine if a strongly positive specimen exceeds the dynamic range of the assay Abbott recommends that specimens be tested individually. As with the Digene assay, this does not allow for CV% determinations As stated by the manufacturer, samples with net counts greater than or equal to the calculated cutoff of 1.6 pg/mL are considered reactive, and specimens with net counts within ±10% of the cutoff value should be retested in duplicate to confirm the initial test result. However, because this is a very low radioactivity assay, the background radioactivity in the counting chamber itself can exceed the background radioactivity of the negative controls limiting the ability to detect low positive specimens accurately In practice, specimens that yield net counts that are within ±10% of the low-end cutoff (generally specimens yielding HBV DNA values of <3–4 pg/mL) are not accurately detected *(18,19)*. These design features also limit the dynamic range of the Abbott assay to 2–3 log

7 Internationally accepted HBV DNA standards, such as those developed by the EUROHEP organization and World Health Organization, permit accurate interassay validation Unfortunately, these standards have not yet been adopted by all manufacturers Chiron has recently revised its own HBV DNA standard such that samples tested prior to March 1996 should be multiplied by 0.65 to convert previously quantified HBV DNA values to the new standard (Chiron technical bulletin, March 10, 1996). The technical aspects of developing such standards, as applied to RNA, have been discussed by Collins et al *(20)*. The acceptance and application of defined nucleic acid standards have progressed rapidly within the field of HIV, where common standards allow accurate interassay comparisons even when using different amplification technologies and multiple test sites *(21)*. **Figure 2**, reproduced from Zaaijer et al. *(15)*, illustrates how specimens tested by membrane hybridization, Chiron bDNA, Abbott, and HBV PCR compare. On the *x*-axis is the log dilution of the "input" copies of HBV DNA using the Eurohep HBV DNA type ad and ay standards On the *y*-axis is the measured amount of HBV DNA or "output" signal generated by each of the assays. This figure illustrates a number of clinically relevant issues pertaining to HBV DNA quantification

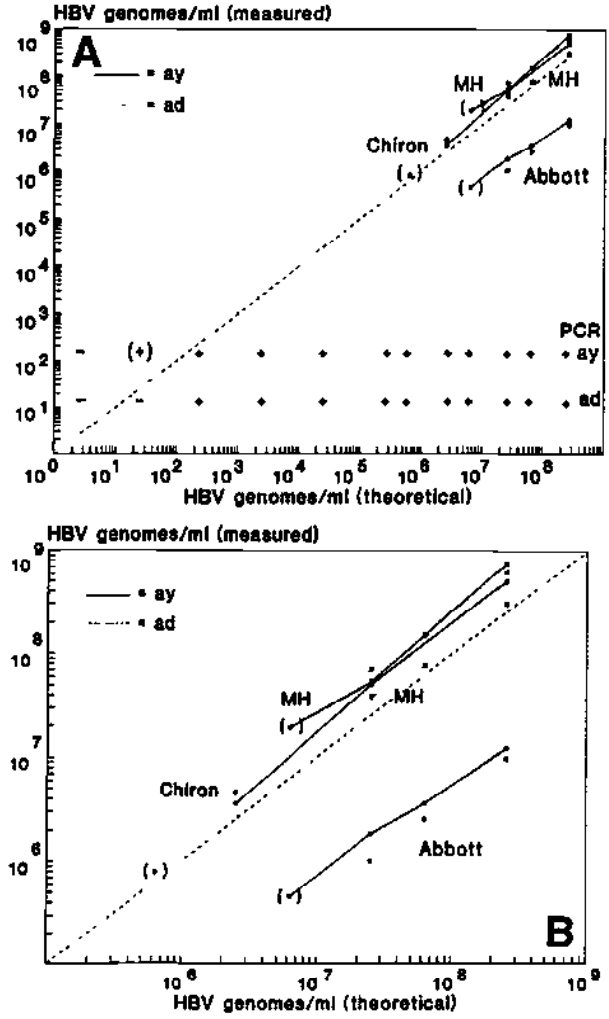

Fig. 2. Reproduced with permission from **ref. *15*** **(A)** Test results produced by PCR and three quantitative hybridization assays with dilutions of the Eurohep HBV DNA type ad and ay standards. The *x*-axis shows theoretical HBV DNA genome copy number in dilutions prepared from the standard plasma The *y*-axis shows HBV DNA genome copy number as reported by the Chiron, Abbott, and membrane hybridization (MH) assays (Indeterminate test results are shown in parentheses) The dotted diagonal line depicts the hypothetical situation of complete agreement of theoretical and measured levels of HBV DNA. **(B)** Test results of three quantitative hybridization assays on dilutions of the Eurohep HBV DNA type ad and ay standards, as shown in panel A

a. Membrane hybridization was the least sensitive assay requiring approx 10^7 copies/mL of HBV DNA for detection *(15)*. It is likely that the sensitivity of membrane hybridization is affected by the volume of serum or plasma used for hybridization. Of particular interest, however, is the fact that the relationship between the input HBV DNA and output signal by membrane hybridization assay as performed by these authors was similar to that of the Chiron bDNA assay. This may be a function of the standards used by the authors

b Chiron bDNA was more sensitive than membrane hybridization requiring a minimum of approx 10^{5-6} copies/mL of HBV DNA for detection The assay also demonstrated a dynamic range of 3–4 log, which was greater than membrane hybridization and the Abbott assay

c Of importance, specimens tested by the Abbott assay generated quantitative values that were approx 19 times less than those reported by the Chiron bDNA and membrane hybridization (**Fig. 2**) The Abbott assay was also less sensitive and had a narrower dynamic range than the Chiron bDNA assay This is consistent with the data reported by Kapke et al *(18)*, as well as our own experience where specimens tested on the Abbott assay yield HBV DNA concentrations in pg/mL, which are approx 10- to 30-fold less than the same specimens tested by Chiron bDNA *(19)*. It is important to note that the two assays correctly identify HBV DNA-positive specimens, at least those that contain amounts of HBV DNA, which fall within their respective dynamic ranges. This suggests that the quantitative differences in HBV DNA reported by the Chiron bDNA and Abbott assays are, at least in part, a reflection of the different standards used for assay calibration *(14,18)*. This clearly illustrates the critical need for improved interassay standardization.

d **Figure 2** also illustrates that current hybridization assays are approx 100,000-fold less sensitive than HBV PCR.

8. Another important issue involves accurate quantification of different HBV subtypes and genotypes. In the study by Zaaijer et al *(15)* (**Fig. 2**), two HBV DNA subtypes were assessed by the various assays with similar detection accuracy Because hybridization assays generally use whole genomic probes or multiple probes targeting multiple genomic regions at once (Chiron bDNA), one would expect that these assays would be minimally affected by minor genotypic sequence variations. In contrast, minor changes in the sequence between the PCR primer and the target (particularly primer-target heterogeneity at the 3'-end of PCR primers) dramatically alter PCR amplification efficiency and quantitative accuracy *(22)*.

9 Detection of HBV DNA in clinical specimens by Chiron bDNA, Digene, Abbott, and membrane hybridization. It is important to understand that patients with chronic HBV infection are assessed using clinical criteria, serum transaminases, hepatic histopathology, and serological markers of HBV infection *(13)*. Quantitative HBV DNA using molecular hybridization assays give additional information by providing a marker of HBV replication *(8,9)* There are currently limited published data comparing the performance of the Chiron bDNA, Digene, Abbott,

and membrane hybridization assays in well-characterized populations. These data are also difficult to interpret because of poor interassay standardization, which results in the dramatic differences in the quantified amount of pg/mL of HBV DNA when the same specimen is tested by the different assays Hendricks et al *(23)* have demonstrated that the analytical detection limit of the Chiron bDNA is about 100,000 Eq/mL (where 285,000 Eq = 1 pg of double-stranded DNA). At this level of assay sensitivity, 94–100% (222/237 and 54/54) of chronically hepatitis B e antigen- (HBeAg) positive patients were Chiron bDNA-positive as compared with 27–31% (50/186 and 5/16) of hepatitis B surface antigen- (HBsAg) positive, HBeAg-negative patients *(23)*. This is consistent with the data obtained by Zaaijer et al. *(15)*, where 73, 67, and 40% of HBeAg-positive and 25, 13, and 8% of HBeAg-negative patients were Chiron bDNA, Abbott, and membrane hybridization positive, respectively. This is also in agreement with Chen et al *(24)*, who demonstrated that 19/27 (70%) of HBeAg-positive patients were Chiron bDNA-positive

Butterworth et al. *(14)* assessed a limited number of patient samples and performed dilutional studies comparing Chiron bDNA, Digene, Abbott, and membrane hybridization. They were able to confirm the work of Zaaijer et al *(15)* demonstrating that the reported values by Abbott assay (in pg/mL) were approx 10-fold less than those reported by Chiron bDNA. Although the Digene assay was somewhat less sensitive than the Chiron bDNA, the quantified pg/mL of HBV DNA for a given specimen more closely approximated those reported by Chiron bDNA Of note, sequential patient samples obtained before, during, and after interferon treatment when tested by the four assays demonstrated similar overall HBV DNA trends over time, despite the fact that the quantified amount of HBV DNA by the Abbott assay in specimens was approx 10-fold less in pg/mL than those reported by Chiron bDNA *(14)* These authors also quantified the HBV DNA controls and standards supplied by each of manufacturers on each of the four assays. They were able to confirm that differences in the measured HBV DNA values reported by each assay were largely owing to differences in the standards used for assay calibration *(14)*. Aspinall et al. *(25)* studied a limited number of patients by Abbott, Digene, and HBV PCR Unfortunately, many of the specimens that were tested were within the low-end detection limits of the Abbott and Digene assays, thus making comparative interpretations between the assays difficult *(26)*.

In an effort to assess and compare the performance of the Chiron bDNA, Digene, and Abbott HBV DNA assays, we have applied a novel method, called the multimeasurement method (MMM), to analyze interassay performance *(19)*. A key assumption of MMM is that all assays, regardless of technique, essentially measure the same analyte, in this case, HBV DNA. Unlike regression methods, which assume that one assay predicts another, MMM simultaneously estimates the true value of the analyte and the curves that associate each assay's results with this true value. Inherent in MMM analysis is a comparison of assay sensitivity, responsiveness, linearity, and precision, as well as the ability to convert

between the measurements of each of the assays. The MMM estimated that Chiron b(DNA) demonstrated greater responsiveness, precision, and linearity over a 3-$\log_{10}$ HBV DNA concentration range than either the Abbott or Digene assays. MMM estimated that Chiron bDNA and Digene assays had equivalent precision, which was greater than that of Abbott assay, and that the limit of quantitation of the Abbott assay is set too low. Because the MMM can create conversion equations directly from a single nomogram, it is possible to convert HBV DNA quantification values between the Chiron bDNA, Abbott, and Digene HBV DNA assays.

10 Hybridization vs nucleic acid amplification (e g , PCR) for detection of HBV DNA. There are two important issues that have precluded widespread application of ultrasensitive nucleic acid amplification, such as PCR, to detect HBV DNA in clinical specimens. The first issue is that although HBV PCR is approx 100,000-fold more sensitive than hybridization assays *(15)*, the optimal assay sensitivity required for clinical care remains undefined The second is that in order to define the clinical utility of HBV PCR assays, these assays need to be highly standardized and quantitative.

Although a number of PCR-based HBV DNA detection assays have been published *(15,27,28)* using in-house or commercial platforms for sample preparation *(29)*, nucleic acid amplification, and detection of amplified target *(30–32)*, these assays remain poorly standardized *(33)*. Because of poor assay standardization, published clinical correlations are difficult to interpret. In general, the vast majority of HBsAg-positive patients are HBV DNA PCR-positive *(15,27,31,33)* Because HBV PCR assays are more sensitive than hybridization assays, they have been particularly useful for detection of active HBV infections associated with low HBV DNA copy number.

For example, ultrasensitive PCR-based detection has been useful to.

a Document active HBV infection in HBeAg-negative surgeons who have transmitted HBV to their patients *(34)*.
b Detect HBV DNA in potential liver transplant recipients who, if HBV DNA-positive, indicates a high risk of HBV reactivation after organ transplantation *(35)*.
c. Detect HBV DNA in some individuals who are HBsAg-negative and HBc-Total antibody-positive (challenging the current dogma that these individuals have truly resolved their HBV infection) *(36,37)*. Similarly it has been demonstrated that organ donors who are HBsAg-negative and HBcTotal-positive can transmit HBV infection to liver transplant recipients *(38)*
d It has also been suggested that in some individuals, HBV DNA may be the sole marker of HBV infection *(39,40)*

It is important to recognize that improvements in nucleic acid amplification technology are occurring concurrently with improvements in direct nucleic acid detection. For example, second-generation HIV assays using bDNA technology detect approx 500 copies of nucleic acid, and assays under development detect approx 50 nucleic acid copies without the need for PCR

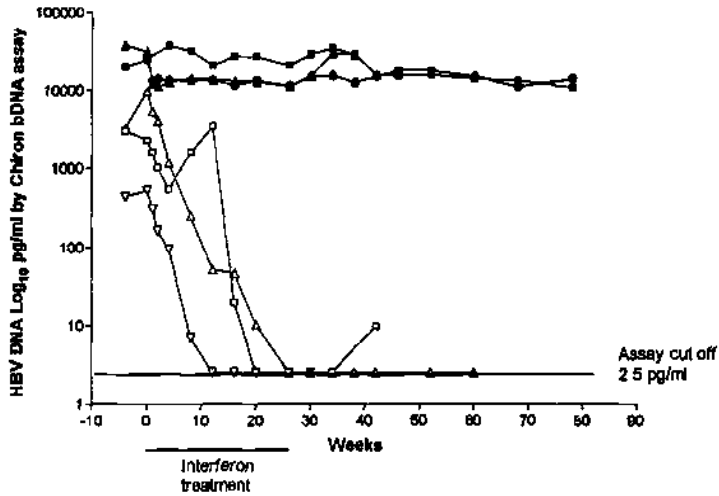

Fig 3 Demonstrates the relationship between HBV DNA in response to interferon treatment in six patients over time

Since submission of this chapter, the Roche AMPLICOR HBV Monitor PCR Test, a standardized commercial PCR assay, has become available We have used the Multi-Measurement Method, a minimal bias, nonlinear regression technique that simultaneously evaluates multiassay linearity, responsiveness, and precision to demonstrate that in comparison to the Chiron and Digene assays the HBV Monitor PCR Test was approximately 3 logs more sensitive and has a lower limit of detection of approximately 10^3 copies/mL of HBV DNA. However, patients with $>10^7$ copies/mL, i.e , specimens obtained from most individuals who are HBeAg positive would require dilution and retesting for accurate HBV DNA quantification *(40a)*.

A second generation Digene HBV DNA assay which has a lower limit of detection of approximately 6000 copies is currently undergoing alpha testing.

As the sensitivity and standardization of nucleic acid-based detection of HBV improves, it will become possible to replace the current myriad of HBV serological assays with nucleic acid-based assays that more accurately reflect active HBV infection

11. Measuring antiviral treatment response in individuals: **Figure 3** illustrates results of serial serum HBV DNA determinations (reported in $\log_{10}$ of the pg/mL of HBV DNA using Chiron bDNA) from six patients undergoing interferon therapy The three interferon nonresponders maintain elevated HBV DNA levels despite treatment. In contrast, interferon responders demonstrate dramatic decreases in HBV DNA within weeks of initiating therapy, generally dropping their serum HBV DNA to below the assay baseline within 16–20 wk. Based on published literature, approx 30–40% of interferon treated patients will have a sustained

response and remain HBV DNA-negative by hybridization assay after completing a course of therapy *(10,11)*. Such treatment is likely cost-effective for HBeAg-positive patients with chronic HBV infection *(12)*.

Pretreatment factors that have been shown to favor interferon response include high serum aminotransferases, evidence of hepatic inflammation, low level of HBV DNA, a recent onset of chronic infection, and lack of coexisting immunosuppression *(10)*. It is important to recognize that optimal nucleic acid, serological and clinical end point(s) for interferon, or other antiviral treatment responses are not completely defined Although a complete and sustained loss of HBV DNA as detected by current-generation HBV hybridization assays is suggestive of a desirable end point, Marcellin et al. *(41)* and Carman et al *(42)* have demonstrated that HBV DNA can still be detected by more sensitive PCR assays in some patients who have lost HBsAg in relation to antiviral treatment.

Of note, one of the patients who initially responded to interferon (as measured by Chiron bDNA) relapsed by about week 40 (**Fig. 3**). Given the fact that HBV DNA hybridization assays are approx 100,000-fold less sensitive than nucleic acid amplification assays, it is important to ask whether detection of HBV DNA by ultrasensitive assays could help predict treatment response or failure at an earlier stage. Early and accurate identification of long-term treatment responders allows one to balance the cost of surrogate marker testing vs the cost of the therapeutic intervention(s) and provide information for cost-effective therapeutic decisions. Similarly, the ability to provide accurate and early detection of, e.g., interferon nonresponders, allows one either to terminate ineffective therapy or to develop and assess novel therapeutic interventions.

It is likely that the most cost-effective way of treating chronic HBV infection will require integration of clinical and laboratory data. Lau et al. *(43)* have looked at a number of statistical models that predict outcome of interferon treatment based on the sex, AST, pretreatment hepatic necroinflammatory score, and HBV DNA quantitation before and after 1 mo of therapy. Such statistical models correctly identified between 77 and 86% of responders and 87 and 92% interferon nonresponders.

Current therapy of chronic HBV infection involves the use of agents that have direct or indirect antiviral activity. Interferon is therefore the "model" agent whose effects are indirect, and owing to its immune enhancing activity In contrast, such agents as Lamivudine and famciclovir directly interfere HBV replication *(44)*. Nucleoside analogs, such as lamivudine, act very rapidly to clear HBV DNA in patient sera within 1–2 wk In fact, Nowak et al. *(8)* have used the changes in HBV DNA in individuals treated with lamivudine to estimate that approx 10^{11} HBV viral particles are released into the serum each day with a half-life of approx 1 d and that the half-life of virus-producing cells is between 10 and 100 d.

Although lamivudine has been shown to be efficacious for short-term treatment of patients infected with HBV *(45)* as well as in patients coinfected with HIV *(46)*, at this point in time, it is unclear how many individuals will be able to maintain elimination of HBV DNA once therapy with lamivudine has been

stopped With further improvements in the standardization of ultrasensitive nucleic acid amplification assays and/or development of more sensitive hybridization assays, one will be able to assess if ultrasensitive assays provide additional prognostic information for the optimization of patient care in patients using antiviral agents that directly affect viral replication or in combination with interferon.

12. Host factors associated with HBV pathogenesis: Thursz et al. *(4)* have shown that the MHC class II allele DRB1*1302 is associated with protection against chronic HBV infection in both children and adults in Gambia. More recently, Thomas et al. *(3)* have suggested that a mutation in codon 52 of the gene that codes for mannose binding protein is associated with chronic HBV infection in Caucasians, but not in individuals of Asian origin. Clearly host immune factors play an important role in the clinical outcome of HBV infection.

    Individuals with acute, self-limited HBV infection have been shown to mount vigorous cytotoxic T-lymphocyte responses against HBV nucleocapsid, envelope antigens, and the viral polymerase *(47,48)* The dogma has been that an effective cellular immune response results in lysis of infected hepatocytes, viral clearance, and resolution of clinical infection. In contrast, individuals who manifest a relatively weak cell-mediated immune response progress to chronic HBV infection, because their cellular immune response causes incomplete lysis of infected hepatocytes permitting ongoing viral replication Persistently infected cells then either induces a chronic cell-mediated immune response with chronic hepatocellular injury, or viral cytotoxicity enhances hepatocyte turnover, resulting in necroinflammation and secondary inflammation from degenerating and regenerating liver cells. In either case, chronic infection culminates in fibrosis, liver failure, and an enhanced risk of hepatocellular carcinoma or extrahepatic manifestations of HBV infection.

    Although the hypotheses regarding why different individuals develop chronic vs acute self-limited HBV infection are intriguing, ultrasensitive HBV DNA assays suggest that it is simplistic to presume that individuals with acute self-limited infection completely clear the virus. For example, liver allografts from hepatitis B core total (HBVcTotal) antibody-positive, hepatitis B virus core IgM (HBVcIgM), and HBsAg-negative donors can transmit HBV to seronegative recipients *(38)*. This demonstrates that these organ donors, who by conventional testing are considered HBV-immune, clearly have replication-competent HBV in their donor graft tissue or serum. Similarly, Rehermann et al. *(37)* have demonstrated the HBV DNA can be detected by PCR in serum and/or peripheral blood mononuclear cells from individuals decades after acute self-limited HBV infection in the presence of strong cytotoxic T-lymphocyte activity. Rare cases of HBV DNA positivity as a sole marker of HBV infection in patients have also been recognized *(36,39,40)*.

13. Viral factors associated with HBV pathogenesis: A number of authors have demonstrated that sequence variations either in the precore *(6,7)* or the core promoter region of HBV *(5)* are associated with fulminant and severe hepatitis. Unfortu-

nately, the correlations between genotypic variants and the clinical manifestations of HBV infection have not been universal *(49–52)*. It is clear that accurate assessments of the role of different viral genotypic and phenotypic variants on the clinical manifestations of HBV infection will require a better understanding of how different hosts handle HBV infection and the characterization of the viral virulence determinants in the context of an individual host's immune makeup

14. Sequence-based assessments of host immune and viral virulence factors and future directions: Although this chapter has focused on the use of HBV DNA quantitation as a marker of antiviral agent efficacy, there are a number of molecular technologies being applied to help advance our understanding of the interplay between host and viral factors in pathogenesis. Correlating gene(s) and gene product(s) expression involved in host immunity and virus-specific virulence determinants will be key in improving our understanding of HBV pathogenesis Although PCR-based sequencing has been applied to sequence host and viral genes, a particularly powerful new technology is hybridization-based sequencing This technology involves generation of high-density oligonucleotide arrays that can be attached to microchips. These oligonucleotide arrays can be designed to detect simultaneously single- or multiple-gene polymorphisms *(53,54)*. We can expect that in the near future, a tube of blood will simultaneously provide information on an individual's viral load as well as sequence-based information relating to host and virus factors. This information will be correlated with prognosis and response profiles to tailor specific therapeutic treatment cocktails.

Molecular technology has only begun to enhance our understanding of the complex interactions between viruses and their host Reliable and reproducible HBV DNA diagnostic assays are tools that provide an in vitro surrogate marker for host and virus interactions. This information combined with a better understanding of factors affecting host and viral response will lead to individualized treatment based on cost and effectiveness. Fundamentally, the ultimate goal of therapy is to eliminate viral replication in the hope that this translates into a decreased risk of extrahepatic and hepatic sequela of HBV infection. The ultimate challenge is to apply sufficient resources to improve HBV diagnosis and treatment to those in need, while implementing widespread and effective public health programs to prevent HBV infection by vaccination.

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8

Molecular Diagnosis of HIV-1 by PCR

Susan A. Fiscus

1. Introduction

Most individuals can be diagnosed as being infected with HIV-1 with an enzyme immunoassay (EIA), which detects antibodies to the virus. Repeatedly positive results must be confirmed, usually by Western blot. These assays have been improved since their introduction in 1985. When used together, the EIA and Western blot are now extremely reliable, with sensitivities of 99.5% and specificities of 99.8% for the diagnosis of HIV-1 *(1)*. These tests are fairly rapid, reproducible across laboratories, well quality-controlled, relatively inexpensive, and form the basis of the protection of the blood supply.

1.1. Diagnosis of Primary HIV Infection

However, these assays are indirect measures of a viral infection and are not suitable for all clinical situations, such as acute HIV infection following sexual or parenteral exposure to HIV. Primary HIV infection ranges from asymptomatic seroconversion to a severe symptomatic illness resembling infectious mononucleosis *(2,3)*. It takes some time (usually 1–3 mo) for detectable antibodies to be produced, and during this seroconversion process or window period, techniques that target the virus and not antibodies have been shown to be more sensitive *(4,5)*. Assays that detect p24 antigen have been used in the past to diagnose primary HIV-1 infection, and have recently been added to the list of assays required for blood and organ donations *(6)*. However, these assays lack sensitivity either because of the small amount of antigen present early in infection or because of the complexing of antigen with antibodies once the immune response has been initiated *(7)*. Disruption of the immune complexes with acid or base has improved the sensitivity of these assays to some extent, although during primary HIV infection, the procedure of immune complex

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disruption, in the absence of antibodies, may actually decrease the sensitivity of the assay. In addition, p24 antigenemia is transient, lasting only a few weeks prior to the formation of antibodies *(8,9)*. Unless the specimen is obtained at the correct time, an infected individual may be negative in the p24 antigen assay.

Using specimens from a variety of sources, such as infected health care workers, seroconverters in high-risk cohorts, and frequent plasma donors, Busch et al. *(10,11)* determined progressive stages of seroconversion and calculated the length of the window period. According to these data, HIV RNA first becomes positive approx 11 d after a person become infectious, whereas p24 antigen and HIV DNA assays become positive approx 16 d after infectiousness. In the era of "hit HIV infection early and hard" *(12)*, it makes sense to determine a diagnosis of HIV infection as soon as possible. Quantitative HIV-1 RNA assays, however, are expensive and perhaps not necessary if one merely wants to determine a diagnosis. At least two manufacturers (Organon-Teknika, Durham, NC and Roche Molecular Systems, Branchburg, NJ) currently market or will soon have available qualitative HIV-1 plasma RNA assays for diagnosing primary HIV-1 infection.

1.2. Diagnosis of Perinatal HIV Infection

A second situation in which molecular diagnosis of HIV infection may be required is in infants exposed perinatally to HIV-1. Only a fraction of these infants are actually infected, although all are seropositive owing to the transplacental transfer of immunoglobulins. Conventional serological assays cannot discriminate between maternal and infant antibodies, and the maternal IgG can persist for as long as 15–18 mo *(13)*. Infected infants often have a shorter disease course than do adults, so it is extremely important to diagnose infection in these children as early as possible so that treatments can be initiated. One must look for evidence of the virus in these infants to determine whether the baby is infected or not. Historically, HIV culture was used to diagnose these infants. Sensitivity of HIV culture ranges from 40 to 50% in newborns, increases to 70 to 95% by age 3 mo, and after 3 mo is close to 100% *(14)*. However, these assays are expensive, labor-intensive, available in only a few research laboratories, and take up to 21–28 d for the report of a negative result *(15)*

Commercially available molecular assays that detect HIV-1 DNA in the peripheral blood mononuclear cells have been reported to be as sensitive as HIV-1 culture in diagnosing perinatal HIV infection and are less expensive, rapid, more suited for routine laboratories, require as little as 0.1 mL of whole anticoagulated blood *(16)*, and can even be performed on dried blood spots from heelsticks *(17)*. There is some evidence that HIV-1 plasma RNA may be even more sensitive, especially in the first weeks of life, in diagnosing perinatal HIV infection *(18)*.

1.3. Quantitative HIV-1 RNA Determination

Early attempts to quantitate the amount of HIV RNA in plasma used in-house PCR-based assays, which, in general, were laborious and lacked proper quality assurance and control *(19–21)*. More reproducible and standardized results can be obtained using commercial assays. At the present time, there are three commercially available assays that quantitate the amount of HIV-1 RNA in a given specimen. In the Roche Monitor assay, plasma is spiked with an internal standard prior to RNA extraction, reverse transcription, and amplification by polymerase chain reaction. Organon-Teknika's NASBA assay employs three internal standards per specimen, a silica bead-based isolation procedure, and isothermic amplification using T7 polymerase, reverse transcriptase, and RNase H. Chiron's branched DNA system relies on signal amplification from a captured HIV-1 RNA as opposed to target amplification and uses external standards. All three give generally similar results for HIV-1 clade B *(22–25)*. The Chiron branched DNA and Organon-Teknika's NASBA can detect virtually all HIV-1 clades, except perhaps subtype O *(26,27)*. At the present time, however, the Roche assay does not recognize subtype A or subtype E well *(28)*, probably because of primer mismatches *(29)*. The commercially available kits are equally expensive for quantitation of HIV-1 RNA in plasma. However, for the purpose of diagnosis, quantitation may not be necessary; one may simply want a qualitative answer. Both Roche and Organon-Teknika are developing qualitative versions of their assays.

Plasma appears to be a better sample than serum for measuring HIV-1 RNA *(30)* and acid citrate dextrose or EDTA, a better anticoagulant than heparin *(31)*. Guanidinium thiocyanate is usually used in sample preparation to inhibit ribonucleases. Once the nucleic acid has been prepared, it can be stored at −70°C or amplified immediately. A quality-assurance program that utilizes a common set of standards has been established by the AIDS Clinical Trials Group to ensure proficiency in performance of the RNA assays and to allow comparisons of data among the three manufacturers' kits *(22,32)*.

1.4. Qualitative HIV-1 DNA Amplification

Commercially available assays for the detection of HIV-1 DNA from infected peripheral blood mononuclear cells are currently available. The Roche Amplicor HIV-1 Test Kit employs biotinylated primers specific for HIV-1 *gag* sequences and thus can be detected by a simple enzyme-linked absorbance assay. Amplified products are incubated in the wells of a 96-well plate coated with complementary sequences, which capture the specific *gag* sequences. The biotin-labeled captured DNA sequences are detected with horseradish peroxidase-labeled Streptavidin, hydrogen peroxide, and TMB.

The Gen-Probe HIV-1 chemiluminescent assay uses a hybridization protection assay in which a chemiluminescent acridinium ester-labeled probe hybridizes with the amplified HIV-1 gag sequences following polymerase chain reaction amplification. After the addition of base to the sample, the probe is hydrolyzed and loses its luminescent property, unless it is hybridized to the target HIV-1 DNA. The amount of probe:target heteroduplex material is proportional to the intensity of the chemiluminescent signal as measured in a luminometer. The AIDS Clinical Trials Group has found these kits to be accurate, sensitive, specific, and easy to use *(34)*. In addition, they have the advantage of being quality-controlled at every step and results from laboratory to laboratory should be fairly standardized and comparable *(35,36)*.

Alternatively, a laboratory can prepare its own reagents and perform PCR. Although there have been many methods described, the one given below has worked well and was adapted from published methods from individuals at Roche Molecular Systems *(37)*. Primers and probes should be selected from highly conserved regions of the genome. A number of primer pairs and their probes specific for certain regions of HIV-1 have been tested with clinical specimens from North America and Europe, and their sequences have been published *(38)*. Some of the primers, however, may not be as efficient in amplifying HIV-1 sequences from Africa or Asia *(39)*. The primers should be 20–30 bp in length to accommodate mismatches better. The substitution of inosine at positions of sequence ambiguity has been reported to improve detection *(40)*. Kwok et al. reported that primers that end in a T work better in many situations *(41)*.

2. Materials

1. Anticoagulated blood collected in tubes containing EDTA (lavender top) or ACD (yellow top), not heparin (green top)
2. Ficoll-Hypaque solution
3. Isotonic phosphate-buffered saline (PBS), containing 1% zap-oglobin.
4. Solution A: 100 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3.
5. Solution B: 10 m*M* Tris-HCl, pH 8.3, 1% Tween 20, 1% Nonidet P-40. Just before use, add proteinase K to a final concentration of 0.02%.
6. PCR master mix: 10 µL of 10X *Taq* buffer (500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 10 µL of 25 m*M* $MgCl_2$, 2 µL each of 10 m*M* dATP, dUTP, dCTP, and dGTP, 2 µL of uracil-*N*-glycosylase (UNG) (2 U), 1 µL of upstream primer 1 (50 pmol), 1 µL of downstream primer 2 (50 pmol), 0.5 µL of *Taq* polymerase (2.5 U), 17.5 µL glass distilled water. Prepare this just before use.
7. Probe mix: 4 µL of 60 m*M* NaCl, 1 µL of 40 m*M* EDTA (pH 8.0), 0.2 pmol of ^{32}P-end-labeled probe (end-labeled with [^{32}P]ATP [6000 mCi/mmol] to an SA of 1.5–3 µCi/pmol with polynucleotide kinase *(39)*, glass-distilled water to 10 µL.
8. Positive HIV-1 control.

9. Negative controls.
10. 40% Acrylamide-*bis*-acrylamide (19:1) *(42)*.
11. TEMED.
12. 10% Ammonium persulfate.
13. Bromophenol blue-xylene cyanol running dye.
14. Chloroform.
15. 2-mL sterile screw-cap tubes.
16. Bench-top swinging bucket—centrifuge at 800*g* for Ficoll-Hypaque separation.
17. Microfuge.
18. Repeater pipet (optional).
19. Micropipets, adjustable volumes 1–1000 µL.
20. Thermal cycler
21. Heat block/water bath.
22. Polyacrylamide gel electrophoresis apparatus with power supply.
23. XAR50 film for autoradiography.

3. Methods

Follow universal precautions, and treat all specimens as though they contain HIV or HBV. Wear appropriate protective gear, including lab coat, mask, gloves, and safety glasses. Avoid the use of sharp objects. Remove lab coat, and wash hands when leaving the lab (*see* **Note 1**).

3.1. Sample Preparation for Leukocytes

1. Perform Ficoll-Hypaque separation of mononuclear cells as described *(34,43)*. Wash the cells twice in PBS, and count the cells. Place approx 2–5 × 10^6 cells in a microfuge tube, microfuge for 3 min, and carefully aspirate the supernatant.
2. Resuspend the pellet in 0.4 mL to solution A, and mix well.
3. Add an equal volume of solution B to which proteinase K has just been added. Vortex to resuspend the pellet.
4. Incubate cells at 60°C for 1 h.
5. Inactivate the proteinase K by heating at 95°C for 10 min.
6. Cool to room temperature and refrigerate.

3.2. Sample Preparation from Whole Blood

Alternatively, whole blood can be used without separating the leukocytes first. In this method, 0.5 mL (0.1 mL for infants <18 mo of age) of well-mixed whole blood (collected in EDTA or ACD tubes) is placed in a 2.0-mL microfuge tube. Add 1 mL of isotonic PBS to which 1% zap-Oglobin has been added. Cap the tube and mix well. Incubate for 5 min at room temperature. Invert tubes 10–15 times to mix, and incubate an additional 10–15 min at room temperature. Microfuge the tubes at maximum speed for 3 min. Remove the supernatant carefully without disturbing the pellet. Add 1 mL of the PBS plus zap-Oglobin, cap, vortex , and microfuge for 3 min. Repeat the wash step one

more time. Carefully aspirate the supernatant. The dry cell pellet can be extracted immediately or frozen at –70°C. The investigator should then proceed with **steps 2–6** of the sample preparation procedure (**Subheading 3.1.**).

3.3. DNA Amplification

1. If 0.5-mL microcentrifuge tubes are used, dispense 50 µL of mineral oil in each tube. Mineral oil is not required for GeneAmp 9600 thermal cyclers
2. Prepare the PCR master mix for the number of samples to be amplified, plus at least two more (one positive and one negative control).
3. With a repeater pipet, dispense 50 µL of master mix to each tube.
4. With a positive displacement pipet or pipet with an ART (aerosol resistant tip), add 50 µL of sample DNA, or add the sample and qs to 50 µL with glass-distilled water
5. Cap each tube after the addition of sample before proceeding to the next tube.
6. Microfuge the tubes for 2 s
7. Place the tubes in the thermal cycler, and cycle as follows. 96-well thermocycler—1 cycle at 50°C for 2 min, 5 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s; 30 cycles at 90°C for 10 s, 60°C for 10 s, and 72°C for 10 s; 1 cycle at 72°C soak. For the GeneAmp 480 or equivalent—1 cycle at 50°C for 2 min; 35 cycles at 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min, 1 cycle at 72°C soak.
8. Remove the samples from the thermal cycler. To prevent reactivation of UNG, analyze the samples immediately or freeze them at –20°C until analysis

3.4. Detection of Amplified Products by Oligomer Hybridization

1. Add 50 µL of mineral oil to 0.5-mL microcentrifuge tubes.
2. Add 10 µL of probe mix
3. Add 30 µL of amplified DNA
4. Mix and microcentrifuge.
5. Denature the DNA at 95°C for 5 min
6. Allow the probe and target sequences to anneal by incubating the tubes at 55°C for 15 min.
7. Add 10 µL bromophenol-blue-xylene cyanol dye mix to each tube.
8. Extract the mineral oil with 100 µL chloroform The dye and sample will migrate to the top.
9. Load 25 µL of the sample onto a 10% polyacrylamide minigel *(42)*.
10. Run in 1X TBE buffer at 100 V until the bromophenol blue dye approaches the bottom of the gel.
11. Cut the unhybridized probe from the gel by slicing just below the xylene cyanol front Dispose of the bottom gel strip in a radioactive waste container.
12. Blot the gel with tissue to remove excess moisture. Wrap the gel in plastic, and expose to XAR 50 film with an intensifying screen for 2 h to overnight at –70°C
13. Develop the autoradiograph. The presence of the probe–target duplex is indicative of HIV infection (*see* **Note 2**).

3.5. Interpretation of Results

To be confident of the results, duplicate amplifications should be performed on duplicate extractions. Discordant results might be owing to low target copy number, sample mix-up, or contamination. Statistically a sample must contain at least five copies of HIV template to have a 99% chance of being reproducibly detected. Reactions containing fewer than five copies on average may appear irreproducible. Sample mix-up can be resolved by amplifying a polymorphic region of HLA-DQα and detecting different alleles by using type-specific probes *(44)*.

Contamination is greatly reduced with the inclusion of dUPT-UNG. However, care must still be taken as with any PCR assay to separate the pre- and postamplification steps physically and to change gloves often when working with the specimens. Clinical negatives, reagent, and low positive controls must be included with each amplification to ensure the validity of the assay.

4. Notes

1 The greatest problem with amplification assays in the clinical laboratory is false-positive reactions that result from contamination. Because of the exquisite sensitivity of the amplification techniques, the inadvertent transfer of a tiny amount of target nucleic acid into a neighboring tube can lead to a false-positive, and thus, an incorrect diagnosis. Meticulous care must be taken to avoid contamination Specific steps that should be used have been described in detail elsewhere *(45–48)*. For instance, amplification reactions should be set up in rooms separated from sample preparation and postamplification testing Dedicated supplies, reagents, and pipets should be provided for procedures before or after PCR amplification and never to be used interchangeably. Reagents should be aliquoted and used only once. Positive displacement pipets or aerosol-resistant pipet tips should be used. Aerosols should be carefully avoided and gloves changed frequently The substitution of dUTP for dTTP in the amplification procedure and the use of UNG greatly reduces the risk of crosscontamination.

2 Multiple bands are sometimes observed in the hybridization assay. These bands are artifacts and may be more prevalent with high copy number input samples. No bands should be observed in the uninfected samples or negative controls. If a specimen has no or little detectable DNA, it may be because of the selection of unsuitable primers.

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III

Research Techniques for Epidemiology and the Management of STDs

9

Genotyping of *Neisseria gonorrhoeae* by Pulse Field Gel Electrophoresis and PCR

Ian Maclean

1. Introduction

Auxotyping (growth of cells on chemically defined media) and serotyping (reaction of cells with a defined set of monoclonal antibodies [MAbs]) are the traditional and most widely used methods for the classification of *Neisseria gonorrhoeae* isolates. As an example, Knapp et al. *(1)* used the combination of these two typing techniques to study the epidemiology of 489 isolates of *N. gonorrhoeae* collected over a 3-mo time period. Separately, 11 different auxotypes and 19 different serotypes were found, but when combined, a total of 57 auxotype/serotype classes were identified. Although this system was and still is very useful for epidemiological studies, some laboratories felt more comfortable using molecular techniques to classify their gonococcal isolates.

The first of the molecular techniques was the use of restriction enzymes to cut the chromosomal DNA to generate a genomic fingerprint *(2)*. The gonococcal DNA was digested with the restriction enzyme *Hin*dIII and the DNA fragments separated on a 4% polyacrylamide gel, stained with ethidium bromide, photographed, and the banding patterns from various isolates compared visually. In this study, sexual contact pairs all had the same genomic fingerprint. A different study of 26 isolates by restriction enzyme fingerprinting proved useful, since it was able to differentiate a number of isolates that had the same auxotype/serotype classification *(3)*. Modifications of the procedure included using different restriction enzymes (*Hin*fI and *Bgl*II) to cut the DNA *(4)* and a laser densitometer to scan the photographs to differentiate between 20 and 30 DNA bands *(4,5)*.

The very success of the restriction digests creates so many bands that even with a laser scanner, analysis can be difficult. For this reason, the use of rRNA

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probes to generate restriction fragment-length polymorphism (RFLP) patterns was used next to differentiate gonococcal isolates *(6)*. In this technique, the chromosomal DNA was again cut by restriction digestion, the fragments separated by gel electrophoresis, but then they were transferred to membrane filters and probed with labeled 16s and 23s rRNA genes, hence the term ribotyping. The digests produced 8–12 bands that hybridized with the rRNA gene probes and allowed the isolates studied to be divided into 9 groups. Some isolates of the same serotype had different RFLP patterns, but the best differentiation of the isolates still needed a combination of ribotyping and serotyping.

The use of ribotyping for differentiation of *N gonorrhoeae* was quickly forgotten, since it was no more useful than generalized restriction enzyme digests, and the technique of pulsed-field gel electrophoresis (PFGE) was coming into general use *(7)*. This procedure again involves the restriction enzyme digestion of chromosomal DNA, but uses an enzyme that cuts the DNA infrequently. This generates a small number of high-mol-wt DNA fragments (30–450 kb), which are resolved under specific electrophoretic conditions. When used to differentiate 48 gonococcal isolates that were from 18 auxotype/serotype groups, 40 different restriction patterns were identified using the enzyme *Nhe*I *(8)* The DNA fragment patterns were stable for the same isolate over a number of subcultures and were reproducible from gel to gel. Li and Dillon *(9)* showed that PFGE allowed greater differentiation of isolates than ribotyping and restriction enzyme analysis.

These reports have all used the molecular techniques to examine isolates that are epidemiologically unrelated. Poh et al. *(10)* used PFGE with the restriction enzymes *Spe*I and *Bgl*II to show that there were 7 subtypes within a group of 19 serovar 1B2 *N gonorrhoeae* isolates from an outbreak in Sydney, Australia. Sexual contact pairs were not identified, so there was no way to link identical and closely related patterns. Hesse et al. *(11)* and Xia et al. *(12)* have shown that confirmed sexual contacts had identical PFGE patterns, suggesting that this method would be useful for epidemiological analysis of *N gonorrhoeae* outbreaks.

The polymerase chain reaction (PCR) has added a new tool for the molecular typing of *N. gonorrhoeae*. This technique allows a specific gene to be targeted and amplified. The PCR product can then be digested with a variety of restriction enzymes, and an RFLP pattern can be generated. The pattern is not as complex as when the whole chromosome is digested and probes are used, so it is much easier to interpret. The two main gene targets from *N gonorrhoeae* have been the *por* and *opa* genes. The *por* gene codes for the Por (PI) protein, and the *opa* gene codes for the Opa (Opacity or PII) protein. Both proteins are components of the outer membrane of *N. gonorrhoeae*.

The *por* gene is a single-copy gene in the gonococcal chromosome and would naturally be expected to have variability, since it codes for the Por protein on which the serotyping system is based. Using a number of different restriction enzymes (*Sac*I, *Hae*II, *Hha*I, *Hpa*II, *Alu*I), the Por RFLP patterns can separate isolates into a large number of groups, but no better than serotyping alone *(13)*. This system was helpful in identifying some isolates of the same serotype that had different Por RFLP patterns. Generating RFLP patterns from PCR products seems to be the most useful when the *opa* gene is targeted *(14)*. Every gonococcal isolate has 11 different *opa* genes, which have both conserved and variable domains. Recombination within this region between the same and different isolates (intragenic and intergenic, respectively) has the potential to generate a large number RFLP patterns when the DNA is restricted. This technique was used to track sexual contact pairs, and it was found that almost 100% of the paired isolates had the same Opa RFLP pattern. Unrelated isolates with the same auxotype, serotype, and Por RFLP pattern had different Opa RFLP patterns, suggesting that this system may be the best for following local outbreaks *(14)*. The authors were unable to estimate at what point along the sexual contact chain the Opa RFLP pattern might change. This system has too much variation to examine large groups of isolates collected over an extended period of time, but has the ability to focus in on isolates that are potentially connected via sexual networks over short time frames.

Two other methods for typing *N. gonorrhoeae* using PCR have been described. Neither appears to be of much value in its ability to differentiate strains beyond the auxotype/serotype system. The first is the technique of generating DNA fragments using single primers of an arbitrary nucleotide sequence *(15)*. The procedure was not consistent from run to run with nonreproducible bands being produced, and some isolates with the same DNA profile had different phenotypic characteristics. The second method is a process called whole-cell repetitive element sequence-based PCR, which uses universal repetitive element sequence primers *(16)* to generate a DNA banding pattern. Although this technique allowed the subgrouping of same auxotype/serotype isolates, it did not have the discriminatory power of PFGE. It also had problems with reproducibility with the intensity of some bands. Although this might seem insignificant, the presence or absence of a particular band could define a new subgroup.

For the purposes of this chapter, the procedures of PFGE and Por/Opa typing will be discussed further as the most reliable and able to provide the information required.

2. Materials

Suitable specimens for all procedures are gonococcal cultures that have been cloned from a single colony (*see* **Notes 1–3**).

2.1. PFGE

1. Cell lysis buffer (buffer A): 0.25 *M* EDTA, pH 9.0, 0.50% sodium lauryl sarcosine (Sarcosyl), 0.50 mg/mL proteinase K (*see* **Note 4**).
2. Tris/EDTA (TE buffer): 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA
3. TBE buffer (agarose-gel buffer): 100 m*M* Tris, pH 8.5, 100 m*M* boric acid, 2 m*M* EDTA

2.2. Por/Opa Gene Amplification/RFLP Analysis

1. Cell lysis buffer (buffer B): 10 m*M* Tris-HCl, pH 8.0, 0.1% Triton-X100, 0.05 mg/mL proteinase K.
2. 10X reaction buffer (buffer C): 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$.
3. *por* gene primers (50 μ*M*):
   Upstream—5'-CAATGAAAAAATCCCTGATTG-3'
   Downstream—5'-TTTGCAGATTAGAATTTGTGG-3'
4. *opa* gene primers (50 μ*M*)
   Upstream—5'-GCGATTATTTCAGAAACATCCG-3'
   Downstream—5'-GCTTCGTGGGTTTTGAAGCG-3'
5. Deoxynucleoside triphosphate solution: Each dNTP is at 2.5 m*M* in a 12.5X stock solution.
6. Gene amplification reaction mix:

DNA	2 μL (lysed cells)
dNTPs	8 μL (final concentration 0.2 m*M*)
10X buffer	10 μL (buffer C)
Upstream primer	1 μL (final concentration 0.5 μ*M*)
Downstream primer	1 μL (final concentration 0.5 μ*M*)
Taq polymerase	0.5 μL (2.5 U)
dH_2O	77.5 μL

7. Restriction enzyme analysis of *por/opa* gene products

Gene product	2 μL
10X restriction enzyme buffer	2 μL (supplied by manufacturer)
Restriction enzyme	0.5 μL (*see* **Note 6**)
dH_2O	15.5 μL

   Most enzymes are incubated at 37°C, but *Taq* restriction enzyme is incubated at 65°C with an oil overlay. Stop both reactions by adding 4 μL of DNA tracking dye.

2.3. Equipment

1. Thermal cycler.
2. Agarose/polyacrylamide gel electrophoresis apparatus
3. Contour-clamped homogeneous electric field (CHEF DR III) apparatus (Bio-Rad, Hercules, CA)
4. UV transilluminator

5. Photography equipment and supplies.
6. Laser scanner (optional).
7. Microfuge
8. Micropipets, adjustable volumes 0.5–1000 µL
9. Heat block/water bath.

3. Methods

3.1. Pulsed-Field Gel Electrophoresis (PFGE)

1. Grow up cells overnight, wash in TE buffer, and adjust to about 10^9 CFU/mL (*see* **Notes 2 and 3**).
2. Add equal volumes of cell suspension and 2% low-melting agarose, and pour into mold.
3. Transfer agarose plugs into lysis buffer A, and incubate at 55°C for 48 h.
4. Wash agarose plugs three times with 15 mL TE buffer to remove proteinase K (*see* **Note 7**).
5. Equilibrate agarose plug in *Spe*I restriction enzyme (RE) buffer for 60 min
6. Add 150 µL of fresh RE buffer with 10 U of enzyme, and incubate at 37°C for 16 h.
7. Wash the agarose plug with 10 mL TE buffer, load it into a well of 1% agarose gel, and seal with 1% agarose.
8. Electrophoresis is done in the CHEF apparatus at 12°C in 0.5X TBE buffer at a constant voltage of 200 V with ramped pulse time (switch time) of 1–15 s for the first 8 h and then 15–25 s for the next 16 h, using various molecular-size markers to facilitate comparisons between gels. This procedure will allow good separation of DNA fragments between 25 and 500 kb (*see* **Notes 8** and **9**).
9. Following electrophoresis, stain the gel in an ethidium bromide (EtBr) solution, visualize, and photograph using a UV transilluminator
10. Fragment patterns can be compared visually, but it is recommended that a scanner or densitometer be used to analyze and catalog the gel patterns (*see* **Note 10**).

3.2. por/opa Gene Amplification

1. Make a 10^7 CFU/mL suspension of cells in buffer B.
2. Incubate for 90 min at 55°C and then boil for 5 min
3. This whole-cell DNA stock solution can be stored at 4°C or frozen for future use.
4. The gene-amplification reaction mixes are set up as listed in **Subheading 2.2.**
5. Por gene amplification cycling conditions are set up as follows:

   95°C 30 s
   47°C 30 s
   72°C 90 s

   for 25 cycles, and then at 72°C for 7 min.
6. Opa gene amplification cycling conditions are as follows:

   95°C 30 s
   65°C 30 s
   72°C 30 s

   for 25 cycles, and then at 72°C for 7 min.

3.3. Analysis of the Gene-Amplification Products

1. For both Por and Opa, 3 µL of the amplification product can be run on a 1.5% agarose gel to look for a DNA fragment of 1075 bp (PorA strain), 1150 bp (PorB strain), and 550 bp for the *opa* gene (*see* **Note 11**).
2. PorA genes are cut with the restriction enzymes *Sac*I, *Hpa*II, and *Hha*I (each separately) and the fragments run on a 1.5% agarose gel for *Sac*I and a 10% polyacrylamide gel for *Hpa*II and *Hha*I (*see* **Note 11**)
3. The *Sac*I restriction site may or may not be present in the PorA genes (a 900-bp fragment results if it is present), and the *Hpa*II enzyme generates fragments from 100 to 600 bp and the *Hha*I enzyme from 250 to 800 bp
4. PorB genes are cut with *Hae*II, *Alu*I, and *Hha*I (each separately) and the resulting fragments run on a 1 5% agarose gel for *Hae*II and *Alu*I and a 10% polyacrylamide gel for *Hha*I
5. The *Hae*II restriction sites may or may not be present in the PorB gene, but when present, DNA fragments of between 100 and 1000 bp are produced. In the case of *Alu*I, the presence or absence of the *Alu*I site is what is important, whereas with *Hha*I, a number of fragments ranging in size from 100 to 1000 bp are produced
6. Opa genes are cut with *Taq*I restriction enzyme, and the fragments separated on a 12% polyacrylamide gel. The fragments range in size from 100 to 550 bp (*see* **Notes 12–14**).

3.4. Discussion

The overall objective of these techniques is to put unknown strains into specific groups. The easiest way to approach the resulting DNA patterns from PFGE is to give each different banding pattern an arbitrary genotype number and start to build up a database of different patterns and genotypes. Strains isolated over long time periods and different geographical areas will most likely have different PFGE patterns. Strains isolated from a local outbreak or known sexual contacts should have the same PFGE pattern. The high cost of the PFGE system dictates that it should be used in a laboratory with a heavy demand for genotyping not just for *N gonorrhoeae*, but with other organisms as well.

The *por*/*opa* genotyping method has the potential to offer more relevant information, because two specific genes have been targeted and not the entire chromosome, as with PFGE. The *por* genotyping follows the serotyping quite closely as might be expected, since both systems look at the same gene/gene product. Each restriction enzyme will have a specific banding pattern, which can be given a letter designation, and the combination of these designations for the three enzymes determines the genotype. Again, just as with the PFGE patterns, a database must be built up to see how many different patterns and genotypes are present in the strains of interest. The *por* genotyping system will not identify as many different genotypes as PFGE when looking at large group of isolates. However, the *opa* gene RFLP will allow the break-

down of Por genotypes into smaller groups. The *opa* typing can be used to follow local outbreaks and sexual contacts as effectively as PFGE. Since most laboratories now use a thermocycler, the *por/opa* genotyping system is probably more cost-effective than the PFGE procedure.

4. Notes

1. To identify if any problems exist, the same specimen should be run a number of times going through the entire procedure to make sure everything is consistent.
2. If the DNA bands are hard to see, then the initial cell concentration should be increased
3. If the DNA bands are very bright and overlap each other, then the cell concentration should be decreased
4. If there is a lot of smearing of the DNA, there could be incomplete protein digestion, so more proteinase K needs to be added or put in fewer numbers of cells
5. Smearing of the DNA could also be because of mechanical shearing owing to excessive handling or manipulation
6. If the banding pattern is not reproducible from run to run with the same strain, it is most likely because of incomplete digestion with the restriction enzymes, in which case more enzyme can be added.
7. The restriction enzyme present could be being degraded by residual proteinase K, in which case the agarose plug needs to be washed more extensively
8. The switch time is what controls the resolution of the DNA fragments
9. Longer switch times will resolve the larger DNA fragments.
10. A recommended general reference for pulsed field is the guide by Birren and Lai *(17)*
11. If the gene amplification does not produce a product or only a small amount, lyse some new cells to make sure the whole-cell DNA is of good quality.
12. Inconsistent banding patterns for the same strain is probably owing to incomplete restriction enzyme digestion
13. In this case, cut back on the amount of DNA, and check that the proper buffers are being used.
14. Do not be alarmed with the *opa* amplification when multiple bands show up, because potentially 11 *opa* genes of different sizes are being amplified from a single strain

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10

Genotyping *Chlamydia trachomatis* by PCR

Deborah Dean

1. Introduction

Strain identification of *Chlamydia trachomatis* has historically been accomplished using serotyping as a phenotypic marker to differentiate chlamydial isolates *(1)*. The target for serotyping is the major outer membrane protein (MOMP) which is the most antigenically diverse and abundant surface protein of the organism. Polyclonal antibodies (PAbs) were initially used for typing and were able to identify serovars D through K and L1, L2, and L3 as primarily genital pathogens, and serovars A, B, Ba, and C as trachoma pathogens. However, these groupings are somewhat imprecise. As immunotyping methods evolved, MOMP-specific monoclonal antibodies (MAbs) were produced that were able to detect additional serovars of the organism *(2,3)*. These include Da, Ia, and L2a *(2)*. MAbs recognize serovar-, subspecies-, and species-specific epitopes *(4,5)* that reflect many of the amino acid variations found among the 18 known serovars of *C. trachomatis*, and are located within three of the four variable sequence regions of MOMP, termed variable segments (VS) 1, 2, 3, and 4. Sequence analysis of the MOMP gene (*ompl*) supports these findings in that VS 1, 2, and 4, in contrast to VS 3, contain the greatest degree of nucleotide sequence variation *(6,7)*.

Immunotyping has enhanced epidemiologic studies of *C. trachomatis* by revealing, for example, an association between specific serovars and proctitis in homosexual males *(8)*, and an association of recurrent chlamydial infection with the same serovars and concurrent gonorrhea infection *(9)*. Refined techniques such as the microtiter plate method have improved the number of low passage isolates that can now be serotyped *(10)*. Yet, this method still necessitates relatively large numbers of viable organisms to reach the detection limit for the MAbs, requires expensive MAbs that have not been standardized across

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different laboratories, and is labor intensive. Further, we now know that immunotyping does not reflect the same degree of variation found at the gene and protein level of this organism *(11–13)*. The time and expense required for producing a specific MAb for each isolate suspected of representing a new serovariant is not an acceptable approach to resolving this issue *(14)*. Because of these limitations, investigators have pursued alternative methods for typing chlamydial organisms.

An important prelude to current day typing of *C trachomatis*, referred to as *ompl* genotyping, involved cell culture propagation of the strain of interest, extraction of chlamydial RNA from the cells, reverse transcription of the mRNA, and manual sequencing of cDNA by the dideoxynucleotide chain termination method *(15)* using ^{32}P *(7)*. Although this method was employed more as a technique for sequencing the VS of *ompl* for each of the known serovars than as a typing method, it provided an accurate means for exploring *ompl* variability and the proteins encoded by each gene. Yet, the technical requirements involved in this method limited its usefulness as a large scale typing method. Thus, investigators focused on techniques that would identify specific nucleotide mutations or sequence differences between prototype and unknown types using other molecular techniques. These approaches were greatly facilitated by the discovery of the polymerase chain reaction (PCR) technique which facilitated the preparation and screening of DNA templates.

Restriction fragment length polymorphism (RFLP) analysis is one of the techniques that was developed. It is based on amplification of *ompl* by PCR and restriction enzyme digest of the product. The digested products are then run on an agarose gel and stained with ethidium bromide. RFLP is capable of differentiating the three species of *Chlamydia* and the 18 known serovars of *C. trachomatis (16–23)*. Four restriction enzymes are required to differentiate isolates to the same degree as immunotyping, yet, subtypes can be detected with additional enzymes. The main limitation of this method is that only those nucleotide changes that occur at restrictions sites can be identified. For known mutations, however, this technique is ideal.

A newer spin on RFLP is mutation-enriched PCR. Here, mismatched primer pairs are used to generate a restriction enzyme recognition site only in the prototype strain. After the PCR product is digested, reamplification with the same primers results in a DNA fragment for the variant sequence alone as the prototype sequence no longer exists *(24)*. However, this method can not pin point the location of the mutation(s). Few other techniques that employ gene mutation or variant analysis as a typing technique have been applied to *C. trachomatis*. Random amplification of polymorphic DNA (RAPD) has recently been used to differentiate chlamydial strains *(25)*. This test is based on *ompl* amplification with four 10-mer primers instead of two as for RFLP. The PCR products

are resolved on an agarose gel stained with ethidium bromide. But, the specificity of this technique is low and certain serotypes can not be differentiated from one another by this method.

Denaturing gradient gel electrophoresis (DGGE) is another technique that has been applied to *C. trachomatis* for detecting mutations *(26)*. PCR is used to amplify the VS of *ompl*. These amplified DNA products are then loaded on a gel with a gradient of increasing concentration of denaturant on the vertical axis of the gel. Specific migration patterns are observed for each serovar group (i.e., F, E, or D) owing to melting of the fragment that occurs in segments (melting domains), and formation of branched fragments that retards the movement of the double-stranded DNA in the gel. Although some *ompl* genotype variants within a serovar group can be observed by migration patterns that are distinct from the reference strain, the problem remains that many variants with few nucleotide sequence changes (yet with biologically important amino acid substitutions) may be missed by this technique. One method to overcome this limitation is to use a GC-clamp *(27)*. However, this requires the synthesis of longer primers and special equipment that may make this prohibitively expense especially for small laboratories.

Arbitrary primer PCR (AP-PCR) has also been used to differentiate chlamydial strain types *(16)*. This is a DNA fingerprinting technique that utilizes short primers with arbitrary sequences that can amplify many sites in the genome of interest *(28)*. A lower annealing temperature is used to decrease the stringency of the reaction, which allows for simultaneous elongation of different segments of template DNA by the same primer. The products are then electrophoresed on denaturing polyacrylamide gels. Length polymorphisms that occur in many different sites can be determined by the presence or absence of the appropriate band at the appropriate location in the gel. This technique is best used in a two-allele system where a visible band or lack of one clearly distinguishes the sequences. Thus, the analysis of the results can be more difficult when examining multiple loci mutations as in the case of *ompl*, unless the focus is on a single serovar group. Further, nonspecific fingerprinting patterns that do not involve *ompl* can result as the entire genome is subjected to arbitrary priming.

Unfortunately, all of the above methods have similar disadvantages in that unknown sequences can only be compared against reference strains and, if they differ, there is no information on the precise locations and number or type of nucleotide changes within the gene segment under analysis. Most importantly, they offer no data on the encoded genetic information.

Some newer technologies hold promise for providing data on the specific location of the mutations for a given region of a given gene, such as *ompl*. These have not yet been applied to chlamydia. One of these techniques utilizes

computer chip technology where different sequences (up to 100,000) can be precisely placed on a "genotyping chip" *(29)*. The DNA sample of interest is then hybridized to the chip. By observing where on the chip the DNA binds, the nucleotide mismatches can be detected and are called 'single nucleotide polymorphisms' (SPNs). The exact location of each mismatch can presumably be determined. This is an exciting step in identifying sequence heterogeneity. However, this approach will need to be assessed by comparison with known prototypes and variant sequences, for sensitivity and specificity, and for cost per sample.

As PCR has facilitated the preparation of DNA templates, this has led to enhanced capabilities for sequencing. Many protocols have now been developed for sequencing PCR products. Thus, sequencing has become a gold standard for the identification of mutations on the genome level as a precise nucleotide sequence can be determined, the reading frame can be established, and the amino acid sequence can be deduced. This has contributed greatly to many aspects of molecular biology and has enhanced diagnostic approaches to genetic disorders. These technologies have been adapted to strain typing for *C. trachomatis*, which is now referred to as *ompl* genotyping. There are two common approaches for sequencing PCR products for *ompl* genotyping which are described in this text: 1) Manual sequencing of single-stranded DNA from asymmetric PCR or from a cloned PCR product; and 2) Molecular cloning of the PCR product prior to automated sequencing or direct automated sequencing of dsDNA PCR product. Both offer the maximum amount of information and are sufficiently straight forward to perform. This makes them highly competitive compared with the above mentioned nonsequencing techniques. Further, with improvements in automated sequencing technology including the capabilities for reading 800–900 base pairs (bp) in a single run, it is now possible to generate high throughput data which has made *ompl* genotyping feasible. The only disadvantage is that most laboratories can not afford the equipment for automated sequencing which means that core laboratory facilities are still required.

ompl genotyping evolved out of a perceived need to know whether the gene varied significantly within trachoma serovars. This was important as usually only one or two serovars would predominate in a given geographic region which made tracking the organism difficult for epidemiologic or transmission studies. Initially, allelic polymorphisms were identified among B/Ba samples from individuals residing in an endemic region of Tunisia *(30)*. Multiple variants of trachoma serovars A and B were also identified in The Gambia *(31)*. *ompl* genotyping was subsequently applied to sexually transmitted strains of *C. trachomatis* where considerable variation has also been observed *(32,33)*. In a recent study, 36 (66%) cervical and endometrial serovars were found to be

ompl variants *(12)* where variant F genotypes were found to be significantly associated with upper genital tract infection, symptomatology, and histopathology whereas E genotypes were found among women with mild, asymptomatic, and mainly lower genital tract infections *(12)*. One of the most recent applications of *ompl* genotyping has been to sequence constant regions of the gene. This approach has been able to distinguish isolates from ocular versus genital tissue that represent the same serovar *(34)*.

Thus, *ompl* genotyping has identified considerable variation within the gene for both ocular and genital specimens *(11,12,30–36)*. Many isolates, even within serovars, can now be individually identified by their unique "nucleotide signature." Further, some newly discovered genotypes encode for surface antigens that are immunologically distinct from the related prototype serovar *(2,11,13)*. However, it is important to keep in mind that newly discovered genotypes may actually represent genotypes that have been circulating in a particular community for a while and were not identified until now because we lacked the appropriate technology.

2. Materials

2.1. Sample Preparation

1. Proteinase K is recommended for samples that have a high protein content. The enzyme is added to a solution containing Non-idet P-40 (NP-40) and Tween-20, which eliminates the need for SDS which can affect the efficiency of PCR *(see below)*. The subsequent DNA purification step requires ultra pure phenol:chloroform:isoamyl alcohol in a ratio of 25:24:1, weight per volume 95% ethanol is required for precipitation of the DNA (**Subheading 2.2.**). The DNA pellet can be resuspended in TE or double distilled water that is free of DNase
2. Phosphate-buffered saline (PBS) or 0.09% normal saline is used for washing specimen pellets prior to resuspension in TE when no prior digestion step is required.

2.2. PCR

1. DNA polymerase is required to catalyze primer extension in the PCR. Both native enzyme that has been purified from the bacteria *Thermus aquaticus* and genetically engineered enzymes are available. There are many commercial polymerases on the market. One or two units of polymerase are required to catalyze the reaction. Excess polymerase may result in nonspecific amplification products
2. A standard buffer for PCR includes 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8 3 at room temperature) and 1.5 m*M* of $MgCl_2$ *(37)*. Since specimens can contain substances that chelate the Mg^{2+}, for example, EDTA and phosphates, the Mg^{2+} concentration often requires adjustment anywhere from 0.05 to 5 m*M*. Thus, it is advisable to resuspend template DNA in TE that contains 10 m*M* Tris-HCl (pH 7.6), 0.1 m*M* EDTA (pH 8.0). The Mg^{2+} concentration may also require modification when new primers or different concentrations of dNTPs are used.

3. Deoxynucleotide triphosphates are required for enzyme extension and are used in a final concentration of 200 μ*M* for each. Commercially available dNTPs require H adjustment to 7.1 to prevent the reaction from dropping below pH 7.1 *(37)*.
4. Oligonucleotide primers initiate the enzymatic extension of the template The optimal concentration of primers is 1 μ*M* which is sufficient for approx 30 cycles. Higher concentrations can result in nonspecific binding to the template and extraneous amplification products. Lower concentrations of primer can significantly alter the efficiency of the reaction.

2.3. Purification of Chlamydial DNA

In order to purify amplified chlamydial DNA from agarose gels, a 6 M sodium iodide solution is used for extracting the DNA from the gel. The DNA that is released is absorbed onto a silica matrix and washed. TE or deionized water is required for eluting the DNA from the silica.

2.4. Sequencing

1 Manual sequencing: Radioactively labeled nucleotides with ^{35}S or ^{32}P can be purchased commercially for use in the sequencing reaction. There are a number of commercial kits on the market that provide the required deoxy- and dideoxynucleotides, buffers, and stop solution for the reaction Alternatively, nonisotopic sequencing can be performed

2 Automated sequencing: Dye-labeled oligonucleotides are required for automated sequencing using fluorescent laser detection. These primers can be purchased from commercial vendors or synthesized independently For the cycle sequencing reaction, d/ddNTPs, buffer (400 m*M* Tris-HCl [pH 9.0], 100 m*M* ammonium sulfate, and 25 m*M* $MgCl_2$), purified DNA template, and DNA polymerase are utilized to incorporate the labeled primers into the double stranded DNA.

2.5. Solutions

1. Chlamydia transport media (2-SP)
2. TE: 10 m*M* Tris-HCl (pH 7 6), 0.1 m*M* EDTA (pH 8.0).
3. DTT: 40 m*M*
4. Lysis buffer: 50 m*M* Tris-HCl (pH 8.3), 1 μg/μL proteinase K, 0 45% NP-40, 0.45% Tween-20 in deionized water.
5. DNA extraction: phenol/chloroform/isoamyl alcohol (ratio of 25:24:2) (v/v)
6. Ethanol 95% or 70% as required.
7. NH4AC: 7.5 *M*.
8. Urine lysis solution: 0.32 *M* sucrose, 10 m*M* Tris-HCl (pH 8.0), 2.0 m*M* $MgCl_2$, 1% Triton X-100, 1 m*M* $CaCl_2$, 0.02 U of micrococcal nuclease.
9 EGTA: 50 m*M*.
10 10X PCR buffer 500 m*M* KCl, 100 m*M* Tris-HCl (pH 8.3 at room temperature), and 25 m*M* of $MgCl_2$.

11. PCR reaction: 10 µL of 10X PCR buffer; 2 µL of dNTP stock; 1 µL of each primer (1 µ*M* stock); 1–2 µL of template DNA (from prepared sample); 1 µL polymerase (1–2 U); and deionized water to make up a final volume 100 µL.
12 Primers that flank VS1, 2, 3, and 4:
   FII 5' ACCACTTGGTGTGACGCTATCAG
   BII 5' CGGAATTGTGCATTTACGTGAG
13 Dye loading buffer: 0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 40% wt/v sucrose in water.
14. Ethidium Bromide.
15 Agarose: ultrapure grade, DNAase free.
16 10X Stoffel PCR buffer. 100 m*M* KCl; 100 m*M* Tris-HCl (pH 8.3 at room temperature); 25 m*M* $MgCl_2$; and 4 µL dNTP stock (10 m*M* of each deoxytriphosphate nucleotide)
17 Primers for nested and asymmetric nested PCR.
   MF21: 5' TGTAAAACGACGGCCAGTGCCCGACCGCG
   TCTTGAAAACAGATGT
   MB4: 5' CTAGATTTCATCTTGTTCAATTGC
   MB22: 5' CACCCACATTCCCAGAGAGCT
   MVF3: 5' TGTAAAACGACGGCCAGTCGTGCAGCTTTGTGGGAATGT
18 Resuspension buffer I. 10 m*M* Tris-HCl (pH 8.3), 1 m*M* EDTA, 0.1 *M* NaCl
19 Resuspension buffer II: 50 m*M* Tris-HCl, pH 5.0, 50 m*M* EDTA, 8% Sucrose, 5% NP-40
20 Lysozyme. 10 mg/µL, freshly made.
21 NaI: 6 *M*.
22 Silica matrix: add 10 g of silica matrix to 100 mL of PBS; allow to stand for two hours prior to use; remove the supernatant and repeat, centrifuge at 2000*g* for 10 min, resuspend the pellet in 100 mL of 3 *M* NaI)
23. Wash solution. 50% ethanol (v/v), 2.0 m*M* EDTA, 50 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.5)
24. 5X reaction buffer: 200 m*M* Tris-HCl (pH 7.5), 100 m*M* $MgCl_2$, 250 m*M* NaCl
25. [α ^{35}S]dATP (~1000 Ci/m*M*)
26. Taq polymerase.
27. Stop solution: 20 m*M* EDTA, 95% deionized formamide, 0 05% bromphenol blue, 0 05% xylene cyanol FF.

2.6. Equipment

1. Laminar flow hood with UV light attachment.
2. Thermocycler with bloc apparatus that accommodates 0.5 mL thin-walled PCR tubes
3. Microcentrifuges with capabilities for 14,000*g*.
4. Walk-in refrigerated room with electrical outlets.
5 Pipettors with capabilities for aspirating 1–20 µL, 20–200 µL, and 200–1000 µL and aerosol barrier pipet tips.
6. Electrophoresis apparatus for running small and large agarose gels.

7. Power pak with adjustable voltage from 50 to 80.
8 UV light box
9. Gel documentation system.
10 Vacuum centrifuge.
11. Manual sequencing apparatus.
12. Automated sequencing apparatus

3. Methods

3.1. Specimen Preparation (see Note 1)

The preparation of the samples is somewhat dependent on the anatomic source which can affect the amount of mucous/protein present in the sample. Sample preparation is one of the most critical steps as without adequate chlamydial DNA, there will be no template for typing the organism. Only one or a few strands of intact DNA are actually needed, yet, excess cells or cellular debris can significantly affect the availability of this DNA in PCR.

3.1 1 Preparation of Ocular or Urogenital Samples with Little or No Mucous

Conjunctival, urethral (male or female), or cervical samples are used and transported in chlamydia transport media (2-SP, or an individually made collection media containing appropriate anti-fungal and anti-bacterial antibiotics: mycostatin, gentamycin, and vancomycin). This procedure is simple and provides sufficient (but crude) DNA for amplification as long as the sample does not contain a lot of protein, blood, or impurities that can bind Mg^{2+} or DNA. This procedure takes approx 50 min.

1. Take 100 µL of sample and centrifuge at 14,000*g* for 10 min in a refrigerated room or in a 4°C centrifuge
2 Discard the supernatant and wash the pellet three times with 500 µL of PBS or 0 9% normal saline by following the centrifugation step in **step 1**
3. Resuspend the pellet in 25 µL of TE
4 Boil the sample for 10 min and place on ice; store at –20°C.

3.1.2. Preparation of Ocular or Urogenital Samples with Mucous/Protein

Conjunctival, urethral (male or female), cervical, or rectal samples can be used, and should be transported in a chlamydial transport buffer as above. This procedure takes ~3.5 h.

1. Take 200 µL of the sample and add 100 µL of a lysis buffer.
2 Incubate at 55°C for 2 h prior to boiling for 10 min to inactivate the proteinase K
3. Add an equal volume of phenol/chloroform/isoamyl alcohol (ratio of 25:24:1 [v/v]), vortex on high speed for 1 min, and centrifuge at 14,000*g* for 1 min,

4. Aspirate off the top layer without disturbing the interface to a siliconized, DNase free tube;
5. Add 2 5 vol of 95% ethanol and 0.5 vol of 7.5 *M* ammonium acetate (NH_4 acetate); let stand on ice for 10 min;
6. Centrifuge in a cold room for 10 min at 14,000*g*, aspirate off supernatant and discard; repeat **steps 5** and **6** with 70% ethanol alone,
7 Resuspend pellet in 50 µL of TE.

3.1.3. Preparation of Urine Samples

Urine samples should be collected in sterile containers and stored up to one week at 4°C. Thereafter, the urine should be stored at –20°C prior to processing. This procedure takes ~1.5 h.

1 If the urine contains precipitate, warm the urine to 37°C until dissolved;
2. Take 10 mL of urine and vortex at high speed for 30 s;
3. Centrifuge at 1500*g* for 10 min and discard the supernatant;
4 Resuspend the pellet in 100 µL of the urine lysis solution containing 0.32 *M* sucrose, 10 m*M* Tris-Cl (pH 8 0), 2 0 m*M* $MgCl_2$, 1% Triton X-100, 1 m*M* $CaCl_2$, and 0.02 U of micrococcal nuclease;
5. Incubate at 37°C for 30 min; add 5 µL of 50 m*M* EGTA, and centrifuge for 10 min at 14,000*g*;
6 Wash pellet with the same solution as in **step 4** (without $CaCl_2$ and nuclease) once; discard the supernatant and resuspend the pellet in 50 µL of TE and 50 µL of 40 m*M* DTT; boil for 10 min.

3.1.4. Preparation of Samples in Commercial Buffers (i.e., Roche PCR Buffer, Abbott LCR Buffer, Behring EIA Buffer, Gen-Probe TMA Buffer)

The source of samples in commercial buffers includes the cervix, urethra, conjunctivae, and urine. A remnant of the sample (prior to actual processing in the assay) is used here. This procedure takes approx 40 min.

1. 200 µL of the remnant sample is boiled for 15 min and then placed on wet ice (in some cases, 30 µL of this boiled sample can be used directly in a 200 µL PCR reaction volume [*see* **Subheading 3.2.2.**]);
2. Add 2.5 vol of 95% ethanol and 0.5 vol of NH_4 acetate; incubate on ice for 10 min;
3. Centrifuge for 10 min at 14,000*g*; aspirate off all ethanol;
4. Wash pellet with 70% ethanol once; resuspend the pellet in 50 µL of TE;

3.2. Amplification of Chlamydial DNA

All PCR reaction protocols are identical except for the DNA extracted from commercial buffers without an ethanol precipitation step. This crude DNA requires a different enzyme and buffer to optimize the amplification as inhibitors are invariably present. When the amount of DNA is known, use 1–50 ng

for optimal results. The forward primer is designed with the universal M13 base pair sequence attached as a tail to the 5' end of the oligonucleotide *(11,12,35)*. This facilitates manual and cycle sequencing where the latter dye-labeled primers that contain the M13 sequence are incorporated into the double stranded DNA template.

3.2.1. Amplification of Chlamydial DNA

This procedures takes ~4.5 h to perform.

1. Combine 10 µL of 10X PCR buffer, 2 µL of dNTP stock, 1 µL of each primer that flank VS1, 2, 3, and 4 of *ompl* [1 µ*M*; F-II 5' ACCACTTGGTGTGACGCTATCAG 3' and B-II 5' CGGAATTGTGCATTTACGTGAG 3'] *(11,12,35)*, template DNA (1–2 µL from prepared sample), and sufficient deionized water to a final volume of 100 µL in a thin walled PCR tube (*see* **Note 2**)
2. Add 1–2 U of thermostable DNA polymerase (depends on polymerase used),
3. Place the tubes in the thermocycling bloc and cycle the reaction as follows: 95°C for 1.5 min followed by 30 cycles of 94°C for 15 s, 55°C for 45 s, and 72°C for 1 min with a final incubation of 7 min at 72°C (*see* **Note 3**)
4. Run 10 µL of the PCR product with 2 µL of 6X dye loading buffer on an ethidium bromide stained, 1 5% agarose gel with appropriate mol wt markers, the appropriated base pair size of the product is 1034 (*see* **Note 4**)

3.2.2. Amplification of Chlamydial DNA from Commercial Buffers (No Ethanol Precipitation)

This procedure takes ~ 4.5 h:

1. Combine 20 µL of 10X Stoffel PCR buffer, 2 µL of each primer as above (1 µ*M*), 30 µL of template DNA, with sufficient deionized water to a final volume of 200 µL in a thin walled PCR tube
2. Add 4 U of *AmpliTaq* DNA polymerase, Stoffel Fragment (Perkin-Elmer/ABI, Foster City, CA) to the PCR tube;
3. Place the tubes in the thermocycling bloc and cycle the reaction as follows: 95°C for 1.5 min followed by 30 cycles at 94°C for 15 s, 50°C for 1 min, and 72°C for 2 min with a final incubation of 10 min at 72°C.
4. Run 20 µL with 4 µL of 6X dye loading buffer on an ethidium bromide stained, 1 5% agarose gel with appropriate molecular weight markers; the product size is 1034 (*see* **Note 5**).

3.2.3. Protocol for Nested and Asymmetric Nested PCR (see **Note 6**)

When there is insufficient amplification product from the first PCR, a nested PCR can be performed to increase the yield of DNA for sequencing. Asymmetric nested PCR is required for manual but not automated sequencing in order to generate a single-stranded template. This procedure takes ~5 h.

1. Add the entire product of the first PCR to a Centricon-30 microconcentrator containing 2 mL of ddH_2O,
2. Centrifuge at 3000*g* until the membrane is almost dry (20–60 min); invert the tube into the conical cup supplied and recentrifuge for 1 min at 1500*g*,
3 Measure the volume (should be ~50 µL);
4 Use 5 µL of the concentrated sample in a 100-µL volume PCR as above with 1 µ*M* each of primer pair: MF21 and MB4 *(11,12,39,40)*; these primers also flank VS1, 2, 3, and 4 of *ompl* but are internal to the F-II and B-II primers; the same cycling parameters as for a 100 µL reaction are used; the product size runs at 864 on an agarose gel
5 For asymmetric PCR that is required for optimal manual sequencing results, a 1:100 ratio of the 5' primer to the 3' primer is used *(38)* and two asymmetric reactions instead of one should be performed, the above primer pair, MF21 and MB4, can also be used but the sample will have to be loaded multiple times on the manual gel to ensure the full read of the template; the first primer pair is MF21 and MB22 that flanks VS1 and VS2, and the second pair is MVF3 and MB4 that flanks VS3 and VS4 (36); the same buffer and PCR cycling parameters are used as described above The product sizes are 411 and 479, respectively.

3.3. Cloning of PCR Products and Preparation for Purification

Once the PCR products have been determined to be of the correct size, each can be individually ligated into a vector; transformed into competent cells, and grown up for subsequent purification. **Step 1** below can be skipped if using a direct PCR cloning kit. The following protocol is adapted from Tiesman and Rizzino *(38)*. This procedure takes ~ 2 d (*see* **Note 7**).

1 In order to remove undesired reagents from the PCR product, add the remaining 90 µL of PCR product to 2 mL of double distilled water already in a centricon-30 column; centrifuge at 3000*g* for 20 to 40 min; invert the column and collect the washed amplified template in a conical tube supplied by the manufacturer,
2 Use an appropriate protocol for ligation into a vector (that contains the M13 sequence that will facilitate subsequent sequencing) and transformation of a suitable cell line (*see* protocols in **ref. *42***);
3 From an overnight culture of one colony in appropriate antibiotics, centrifuge at 5000*g* for 5 min at 4°C,
4. Resuspend the pellet in 5 mL of an ice cold resuspension buffer I,
5. Repeat the centrifugation as in **step 3** and resuspend the pellet in 0.7 mL of ice cold buffer II,
6. Add 50 µL of a newly made solution of 10 mg/mL lysozyme and 2 mg/mL RNase A in 10 m*M* Tris-HCl (pH 8.0) and incubate at 37°C for 10 min;
7 Transfer to a 100°C water bath for 1 minute; centrifuge at 14,000*g* for 10 min at 4°C;
8. Carefully remove the pellet and add 0.7 mL of ice cold isopropanol to the supernatant; precipitate for 5 min at –20°C;

9 Centrifuge at 14,000*g* for 10 min at 4°C and resuspend the pellet in 100 µL of TE
10. At this point, the DNA should be purified prior to sequencing, *see* **Subheading 4.4.** below.

3.4. Purification of Chlamydial DNA from Gels and Plasmid Preps

Once a band of the appropriate size is visualized on the gel, it must be purified from the gel prior to cycle sequencing. This procedure takes ~2.5 h. If a plasmid is to be purified, skip to **step 5** below. This procedure takes ~1.5 h (*see* **Note 8**).

1. Load the remaining 90 µL of PCR product on a 1.5% agarose gel; this can be accomplished by taping together 2 or 3 wells prior to casting the gel;
2. Run the gel at 70 volts to improve resolution of the bands from other nonspecific products;
3 Cut the gel out under long UV light to avoid nicking the DNA template and place the gel slice in a sterile 1.5 mL DNase free tube,
4 Weigh the gel by subtracting the weight of the tube;
5. Add 3 vol of 6 *M* NaI and incubate at 55°C; shake the tube vigorously to dissolve the agarose;
6 Once dissolved, add 5 µL of silica matrix; add 25 µL of silica matrix if using a cloned sample;
7 Put on ice for 10 min and shake the tube frequently to improve the recovery of DNA from the NaI,
8 Centrifuge at 14,000*g* for 30 s and remove all excess NaI from the silica matrix; add 300 µL of ice cold wash solution by resuspending the pellet by pipetting up and down; do not vortex; centrifuge at 14,000*g* for 30 s; repeat the wash step twice,
9 Remove all of the wash solution and elute the DNA in 5 µL of TE at 55°C for 5 min (25 µL of TE if a cloned sample); remove the TE containing the DNA into a siliconized, 500 µL tube; repeat the elution step once. Discard the silica matrix

3.5. Manual Sequencing of Chlamydial DNA (see Notes 9–12)

The purified chlamydial DNA from the gel or plasmid prep can now be sequenced either manually or by automation (*see* **Subheading 3.6.**). The dideoxynucleotide chain-termination method is used for either method. For manual sequencing, the single-stranded template is first annealed to the primer before addition of the radioactive α-labeled dATP along with the three nonlabeled nucleotides. The reaction is then extended in four separate base-specific tubes with the respective chain terminating dideoxynucleotide and *Taq* polymerase. The labeled template is then loaded onto a polyacrylamide-urea gel, run for a designated time frame depending on the length of the DNA to be sequenced, and autoradiographed for 24 to 48 hours. The procedure from sequencing reaction to loading the gel takes approx 45 min. Running the gel varies from 2 to 6 h.

1. Add 0 5 pmol of DNA template to 0.5 pmol of primer (M13 universal primer or the 5' primer used for asymmetric PCR) and 2 µL of a 5X reaction buffer for a total of 10 µL; Denature at 70°C for 2 min and then at 45°C for 15 min.
2. Add to the annealing reaction 0.5 µL [α-^{35}S]dATP (~1000 Ci/mmole), 2 µL of deoxynucleotide mix (1 5 µ*M* dGTP, 1.5 µ*M* dCTP; 1.5 µ*M* dTTP), 2 U of *Taq* polymerase, and ddH_2O for a total volume of 17.5 µL; mix briefly by flicking the side of the tube with a finger, spin down all droplets, and incubate at 42°C for 5 min; remove tubes to a rack at room temperature.
3. Transfer 4 µL of the sequencing reaction solution to each of four tubes labeled with G, A, T, and C containing 4 µL of the appropriate termination mix (20 µ*M* of each deoxynucleotide with 60 µ*M* ddGTP, 800 µ*M* ddATP, 800 µ*M* ddTTP, or 400 µ*M* ddCTP, respectively), incubate at 70°C for 5 min and then allow to cool to room temperature before adding 4 µL of stop solution,
4. Incubate the four tubes at 80°C for 3 min and immediately place on ice just before loading a 5% polyacrylamide-urea gel with 2 µL of sample per well, run the gel for designated time to accommodate the length of read desired for the DNA; store remaining sample at –20°C for up to one week.
5. Dry the gel in a vacuum apparatus for ~1 h prior to autoradiography for 24 to 48 h.

3.6. Automated Sequencing of Chlamydial DNA

Dye-labeled primers are incorporated into the purified PCR or plasmid DNA. Dye terminators can also be used but require a higher concentration of DNA. A ready reaction kit (ABI Prism Dye Primer Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Foster City, CA) that includes all the reagents for the reaction facilitates labeling and sequencing of the template DNA. The labeled template is loaded on a polyacrylamide gel and scanned by a laser. This procedure takes ~3 h up to loading on the sequencing gel. The sequencing gel can be run overnight to save time, and can be set to run for seven to nine hours or indefinitely.

1. Add 1 µL of template (50 ng for PCR DNA, 100 ng for single-stranded DNA from a clone) to 4 µL of a ready reaction mix containing dNTPs and d/ddATP, 0.4 pmol/µL dye primer, 1 µL of 5X cycle sequencing buffer, and 1 µL of *AmpliTaq* Polymerase, FS, in a thin walled PCR tube; add 1 µL of template to 4 µL of a ready reaction mix for d/ddCTP; add 2 µL each to the d/ddGTP and d/ddTTP ready reaction mixes;
2. Place all four tubes in a thermocycler bloc preheated to 96°C; cycle the reaction for 15 cycles of 96°C for 10 s, 55°C for 5 s, and 70°C for 1 min; follow this cycling pattern with 15 cycles of 96°C for 10 s and 70°C for 1 min and hold at 4°C;
3. Transfer the contents of all four tubes to 80 µL of 95% ethanol; place on ice for 10 min;
4. Centrifuge at 14,000*g* for 15 min; pour off the ethanol from the opposite side of the pellet; rinse the pellet with 70% ethanol, recentrifuge for 5 min, and carefully pour off the ethanol from the opposite side of the pellet; the pellet will be invisible

5. Dry the pellet using a vacuum centrifuge with heat for one to five minutes until the pellet is dry but not over-dry (should not see a drop at the site of the pellet),
6 Resuspend the pellet in 6 µL of a solution composed of deionized formamide and 50 m*M* EDTA in a ratio of 5:1 (formamide to EDTA);
7. Load 1.5 µL of sample per well in an automated sequencing machine; follow manufacturer's instructions for making the polyacrylamide gel and for running the equipment with appropriate software;
8 The fluorescent-labeled product is analyzed by the software program, but can be reanalyzed by following the instructions in the software program; print the laser generated images of the sequence and examine the output by semi-automation (*see* **Note 15**).

4. Notes

1. Specimen Preparation: Although the protocol in **Subheading 3.1.1.** works extremely well, occasionally it is worth performing one precipitation with 95% ethanol and 0.5 vol of 7.5 *M* NH_4 acetate This serves to clean up the DNA such that any inhibitors remaining in the washed pellet are removed. For protocols in **Subheading 3.1.2.**, if there is a thick protein interface after the addition of phenol/chloroform/isoamyl and centrifugation, it is important to repeat this step to remove any additional protein which can bind the DNA. For the protocol in **Subheading 3.1.3.**, if there is excess precipitate in the urine, it is important to do a proteinase K digestion after washing the pellet in **step 6**. This should be followed by ethanol precipitation as described. This will digest and eliminate the excess protein that will provide a cleaner DNA sample for amplification. When there is excess mucous in the commercial buffers, it is worthwhile doing one phenol/chloroform/isoamyl extraction followed by ethanol precipitation prior to boiling the sample. If the sample in the commercial buffer is completely clear, 30 µL of boiled sample can be used directly in PCR using a 200 µL volume with *AmpliTaq* polymerase, Stoffel fragment, as described in **Subheading 3.2.**
2 When setting up multiple reactions for PCR, it is more time efficient to make a master mix of reagents that can then be aliquoted into PCR tubes prior to the addition of template and polymerase. This master mix can also be stored at –20°C pre-aliquoted or as a stock solution for up to two months This also ensures that all the reagents are equivalent among each reaction.
3 A modified hot-start technique can be very helpful in optimizing the PCR. We place the DNA polymerase on the side of the PCR tube after all other reagents have been added. Once the tube is placed in the heat bloc which has been preheated to 95°C, the polymerase slides into the reaction mix thus initiating PCR.
4. If there is sufficient sample, determining the exact amount of DNA will facilitate PCR as overloading the reaction with DNA can result in no amplification products. This can be accomplished easily by using a spectrophotometer to measure 10 µL of DNA added to 90 µL of deionized water after calibration of the machine (*see* **ref. *37*** for details of this procedure).

5 Each commercial polymerase has a different efficiency that is somewhat dependent on the buffer that is used. Often, the optimal buffer comes with the polymerase. But, it may be prudent to test the polymerase with different buffers, including one made in your own laboratory which might be more cost effective than purchasing commercially prepared buffers.
6. Regarding nested PCR, it is important to concentrate the first PCR reaction to provide sufficient DNA for reamplification. This can be achieved as described under **Subheading 3.2.3.** A critical step in this protocol is to add the 90 µL of remaining PCR product to 2 mL of water that are already in the column. The water moistens the membrane that filters out excess primers and dNTPs from the first reaction. An alternative to this procedure would be to do an ethanol precipitation with 95% ethanol and 0.5 vol 7.5 *M* NH_4 acetate. Although this is relatively rapid and less expensive as columns do not need to be purchased, excess primers can also be precipitated and carried over into the nested PCR. However, the carry over does not appear to significantly affect the nested PCR as we have not observed extraneous bands at the position of the first PCR.
7. Cloning of PCR Products and Preparation for Purification: A simple solution to cloning the PCR products is to use a kit that facilitates direct cloning without restriction digests of the 5' and 3' ends. There are a number of cloning kits on the market that yield excellent results in a short period of time and, in this regard, can be quite cost effective.
8. Purification of Chlamydial DNA from Gels and Plasmid Preps: A critical step in the purification of chlamydial DNA from agarose gels is to make sure that the slice of gel has been trimmed of all excess gel that is not fluorescing with ethidium bromide This will provide the most concentrated amount of DNA and hasten the time it takes to dissolve the gel in NaI. Also, it is important that the NaI solution is at a pH <7.4 The pH can be adjusted by adding 10% acetic acid in 1 mL of 6 *M* NaI An alternative to the protocol described under **Subheading 3.4.** would be to purchase a commercial DNA purification kit that is especially designed for DNA extraction from gels There are many on the market and the advantage is that all of the reagents are quality controlled
9. Manual Sequencing. Other polymerases can be used for radionucleotide sequencing The T7 sequenase polymerase has been around for a long time but is most efficient when used with higher quantities of DNA templates. A newer polymerase, Thermo Sequenase, works extremely well in cycle sequencing formats with ^{32}P, ^{33}P, and ^{35}S. This polymerase efficiently incorporates both dideoxynucleotides and deoxynucleotides triphosphates fairly evenly during the reaction which generates more uniform band intensities. Another advantage of this polymerase is that only 0 01 µg of DNA are required for the reaction. A Thermo Sequenase cycle sequencing kit is available that can facilitate radionucleotide sequencing (Amersham Life Sciences, Cleveland, OH).
10. Manual Sequencing: The base analog 7-Deaz-2'-deoxyguanosine-5'-triphosphate is an important alternative to dGTP for resolving G + C rich templates to prevent gel compressions *(39)*.

11. In efforts to avoid radioactive substances, it is possible to do manual sequencing using nonisotopic detection methods with very good results. There are a number of referenced protocols for this in the literature *(41,42)*.
12. Kits are also available for sequencing and are time saving as all the reagents have already been prepared, although they are more expensive. Sequenase and *Taq* polymerase are the most common enzymes used in these kits. Most perform extremely well.
13. Techniques for Preventing Aerosol or Carryover Contamination: As a laboratory performs more and more PCR using a specific set of primers, many amplification products are produced that can contaminate specimens and subsequent PCR. Usually, 6×10^6 molecules per μL are generated by PCR. If precautions are not taken to prevent aerosolization and physical carry-over, these molecules can end up everywhere. This problem is most clearly identified when negative controls are positive or when sequences are suspiciously identical to others processed at the same time or immediately following a previous PCR and sequencing run. A number of precautions can be taken to prevent this. First, it is important to have a separate room for preparing and aliquoting reagents for PCR. This area can also be used for setting up PCR prior to adding the template. Ideally this room should be supplied with DNase free tubes, PCR tubes, pipet tips (no need for aerosol pipet tips as this is a clean room), sets of pipettors (p20, p200, and p1000), gloves, racks, and nonrefrigerator buffers and chemicals. None of these supplies should be removed from this room nor should any sample or potentially contaminated substance be introduced. Also, each person working in this area should wear a lab jacket that is removed when they leave the room. To further avoid contamination, it is recommended to make small aliquots of primers, dNTPs, and so on, so that if these reagents become contaminated they can be discarded without incurring significant cost. Also, since these small volumes are used up rapidly there is less of a chance for contamination. A second room should be designated for sample preparation, and loading the prepared PCR tubes with template DNA. These procedures should be performed in a laminar flow hood with UV attachment so that the area can be decontaminated in between users. This area should contain its own centrifuge and set of supplies, including aerosol pipet tips and pipettors which are never removed from the area. A third room should be used for electrophoresis of PCR products, DNA purification from gels, cycle sequencing of purified DNA, and loading of the sequencing gel. A separate set of pipettors should be assigned to gel loading versus DNA purification and cycle sequencing to avoid carry over. Aerosolized tips should also be used to prevent contamination of the pipettors and subsequent carry over. Although this room is the most contaminated as aerosolization is occurring as amplification products are pipetted into wells, pipet tips are ejected, and tubes containing amplified or purified DNA are opened, purification of DNA from a gel is not a problem as the amount of the appropriate template DNA is so high that aerosols are not cycle sequenced in sufficient quantities.

14. Pipettors can be an inconspicuous source of carry over as DNA can contaminate the inside of the shaft. Although some investigators prefer positive displacement pipettors, with careful use of aerosol pipet tips, it is possible to avoid any contamination of the shaft, the extra cost of these pipettors, and the tips required for their use.
15. Managing the Sequence Data: Although sequencing technologies have advanced considerably, the amount of sequence data generated can become overwhelming. Currently, there are software programs available for large-scale sequencing projects. But, these are difficult to adapt to small scale data manipulations, especially for identifying nucleotide differences across several sequences and by comparison with prototype strains for data analysis. Thus, there is a need for the development of software programs that can store data and perform comparisons of sequences from different individuals or anatomic sites. Some companies have developed data entry programs without the capabilities for data analysis, and these would be recommended for use in storing data. Clearly, an area of future development will be the design of user-friendly programs for the above mentioned applications.

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11

Human Papillomavirus Detection by PCR and Typing by Dot-Blot

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1. Introduction

1.1. Human Papillomavirus (HPV)

The papillomaviruses form a nonenveloped virion with an icosahedral capsid structure and contain a double-stranded circular DNA genome of 7800–7900 bp. The HPV genome is organized into three major regions; two protein-coding regions (1) early and (2) late-expressing genes, and (3) a noncoding upstream regulatory region (URR) (**Fig. 1**). The early region is downstream of the URR and consist of six open reading frames (ORFs) (E1, E2, E4, E5, E6, and E7). E1 encodes a DNA binding protein involved in the regulation of viral DNA replication. E2 regulates viral DNA replication and gene expression. E4 is only expressed during the viral infection phase, and important for the maturation and replication of the virus. The function of E5 is less well known and may involve a stimulation of cell proliferation in HPV-infected cells. E5 has also shown a weak transforming activity. The E6 and E7 are coding for two oncoproteins with a high transforming activity. Upon integration into the human genome, E2 and parts of the E1 gene are, in general, deleted. This is followed by high levels of E6 and E7 expression. The late region contains two ORFs termed L1 and L2 that code for the viral capsid proteins *(1)*. Finally, the URR, or LCR long control region, contains binding sites for different transcriptional repressors and activators, and along with E5-7, is highly variable among HPV types.

Infection with HPV is associated with an increased risk for the development of papillomas and dysplasias of the skin and mucosa of humans, and is presently one of the most common STDs in the world. HPV is also the primary risk factor for cervical cancer in women. As the methods for detection and characterization of HPV have become more sensitive and accurate, the number of

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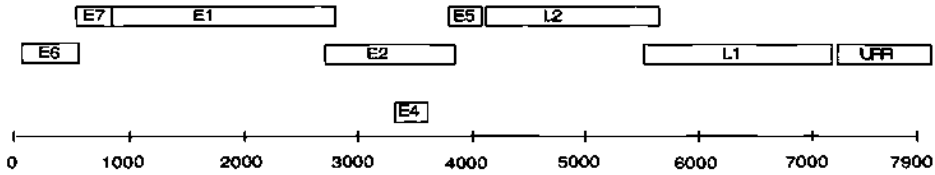

Fig. 1. Genome organization of HPV 16, in linearized form

recognized viral subtypes has reached over 70. Some of these (predominately 6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 66, and 68) are frequently found in the genital tracts of both men and women, and are sexually transmitted. Out of these, types 6 and 11 are associated with *condylomata acuminata* (genital warts), which is a benign atypia, whereas 16, 18, 31, 33, 35, 45, and additional types occurring at lower frequencies are associated with low- and high-grade dysplasias and cervical cancer. In women, these infections are usually located at the border between the squamous and the columnar epithelia of the cervix (the transforming zone). HPV DNA is found in 70–95% of excised biopsies of warts, neoplasias, and tumors of the genital tract. The prevalence of HPV varies locally and between age groups, with the highest prevalence for young, sexually active individuals (16–25 yr) *(2)*.

HPV infection has been diagnosed in a number of ways, based on clinical, cytological, histological, and molecular examinations, the latter becoming increasingly important owing to its higher sensitivity and the potential for detecting the presence of virus before the onset of clinical manifestations. The correlation between HPV type and disease severity makes the specificity of the methods an important factor in the diagnosis. Abnormalities (especially *condylomata acuminata*) in the exterior parts of the female genital tract are usually detected by visual inspection during clinical examination, but the more hidden manifestations in the cervix frequently remain undetected. Histological or cytological examinations under light microscope can reveal HPV infections through the rather subtle and nonstandardized manifestations, such as koilocytosis or dyskeratosis of the squamous cells. However, these changes are not always easy to detect *(3)*. To aid in the diagnosis, a variety of molecular protocols for the detection of HPV have been developed. In the present chapter, we survey the methods available for detection of HPV with special emphasis on the molecular DNA methods.

1.2. Serological Methods for HPV Detection

Serological detection methods, based on serum antibodies mainly against the L1, L2, E6, and E7 HPV proteins, are used to indicate previous or present infection with HPV. The initial assays used bacterially expressed fusion pro-

teins or synthetic peptides as the antigen targets in Western blot assays or enzyme-linked immunosorbent assays (ELISAs), and had limited ability for HPV detection *(4)*. The HPV major capsid protein (L1 with or without the L2 protein) can self-assemble into virus-like particles (VLPs), which are structurally indistinguishable from native virions, and therefore recognized by neutralizing antibodies *(5,6)*. Since high-risk genital HPV virions are less frequent in the high-grade lesions, and cannot be grown either in cell-culture or in animal systems in large amounts, VLPs have proven to be valuable reagents for serological assays *(7,8)*. HPV VLPs have been produced in different eucaryotic expression systems by using recombinant vaccinia virus, baculovirus, semliki forest virus, and yeast *(9)*. Since VPLs lack the potential oncogenic viral genome and generates high titers of neutralizing antibodies, they constitute candidates for prophylactic vaccine *(10)*.

In preinvasive lesions and carcinomas the HPV genome have been found to be integrated, with deletions reported mainly of the 3'-part of the E1 region and the E2 region *(11)*. Concomitant with such deletions is an increased expression of the two oncogenes E6 and E7. E7 in particular has proven to be a good marker for serological assays owing to the elevated levels of E7 antibodies and proteins *(12,13)*. The most common assays for the detection of neutralizing antibodies against HPV early and late proteins are different combinations of ELISAs using antigen or antibodies immobilized to a solid support. ELISA-based assays have been employed for analysis of peptides, antibodies, virions, and recently VLPs. In these assays, microtiter plate wells coated with antigen are covered with patient sera to capture high-affinity antibodies present in the serum *(4,14–16)*. These complexes are then detected by enzyme-conjugated secondary antibodies.

Monoclonal antibodies (MAbs) have been developed as specific probes to identify L1 and for use in ELISA, western blot, and neutralization assays *(14,16–18)*. These MAbs and polyclonal antibodies are commercially available and are labeled with biotin or enzymes, such as horseradish peroxidase and alkaline phosphatase, allowing detection by chromogenic substrates or chemiluminescence (ECL, Amersham). Immunoprecipitation assays have been used for the detection of E7 expression and are performed in solution or immobilized to a solid support, where complexes of antigen/antibody are precipitated in the presence of a specific HPV antibody *(19)*. In general, the immunobased assays for diagnosis have a lower sensitivity than DNA- and RNA-based methods, since not every infected person develops antibodies to HPV16. Also, immunobased methods have a lower specificity, caused by the crossreactivity in the response against closely related HPV types *(7)*. Finally, since today HPV type classification is based on DNA similarity, serological assays may be discordant with DNA classifications.

The alternative strategy is to detect the presence of HPV DNA or RNA. A number of methods have been developed, either based on direct detection of viral nucleic acid or including an amplification step using the polymerase chain reaction (PCR) prior to detection.

1.3. DNA-Based Methods for HPV Detection

1.3.1. Hybridization to Native Viral DNA

Methods without an amplification step rely solely on hybridization with a homologous probe for detection of viral DNA. An example is *in situ* hybridization (**Table 1**), where viral DNA is detected on the microscope slide (smears or slices of a biopsy). The visualization of hybridized probe is through an enzyme-linked secondary molecule (e.g., Streptavidin-phosphatase) binding to the biotinylated probe *(20)*. One benefit of *in situ* hybridization is that it permits not only detection, but also localization of infected cells in a clinical sample. The pattern of hybridization within cells can also be used to indicate whether the virus has been integrated into the human genome or is in episomal form, information that can be of prognostic value *(21)*.

Direct detection of HPV has also been achieved using restriction fragment-length polymorphism (RFLP) and Southern blot technique (**Table 1**) *(22)*. The restricted DNA is separated in a gel matrix (agarose or polyacrylamide) by electrophoresis, transferred to a membrane, and then hybridized to labeled HPV probes. Depending on the conditions used for the hybridization and subsequent washes, the stringency of the detection can be adjusted (low stringency resulting in the detection of a broad spectrum of HPV types and high stringency in detection of specific HPV types). This method can be relatively sensitive and has the advantage of yielding information about the integration/episomal state of the virus *(23)*. However, the Southern blot technique requires fairly large amounts of sample material and is too labor-demanding for large-scale routine use. A simplification of the hybridization method is the dot-blot analysis (**Table 1**), where HPV DNA is applied directly to a membrane and hybridized to type-specific HPV probes *(24)*. The sensitivity of this technique is similar to that of the RFLP Southern blot, and requires sample specimen of the same quality.

An alternate possibility is to use RNA probes for hybridization. The resulting DNA–RNA heteroduplex can be detected using MAbs directed against DNA–RNA hybrids. This is the concept of the commercially available hybrid capture assay marketed as ViraType Plus (Digene Diagnosis, Beltsville). In this assay, the tube walls are coated with anti-DNA–RNA MAbs. DNA–RNA hybrids bound to the tube walls after washing are detected by alkaline phosphatase linked to an anti-DNA–RNA antibody. The signal can then be measured by using a luminometer *(25)*. The sensitivity of all hybridization assays are reliant on an accurate stringency control in the hybridization step.

Table 1
Summary of Non-PCR Based Methods

Method	Applicable material	Sensitivity	Specificity	Main advantage	Main disadvantage
Serology ELISA and immunocyto-chemistry	Virus-like particle (VLP) Blood serum	Lower compared to DNA-based techniques	Low	Possible to detect cleared infections Technically easy to perform	Crossreactivity between HPV types
Serology Immunocyto-chemistry	Antibodies Tissue or exfoliated cells	Lower compared to DNA-based techniques	Low	Possible to detect cleared infections Technically easy to perform	Crossreactivity between HPV types
Electron microscopy	Tissue or exfoliated cells	Low	Very low	Identify virus particle	No identification of specific HPV type Expensive Labor-intensive
RFLP Southern blot	Fresh or frozen samples	High under optimal conditions	High	Reliable, possible to obtain information about state of the virus	Labor-intensive Fresh or frozen material is needed
Dot blot	Fresh or frozen samples	High under optimal conditions	High	Multiple samples can be examined simultaneously	Risk of false-positive results (depending on stringency used) Fresh or frozen material is needed
In situ hybridization	Cervical smears Sliced biopsies on microscopy slides	Approx 20 viruses/cell needed	High, but probes often pooled to separate between low- and high-risk types	Hybridization pattern can give information about physical state Gives information of the localization of HPV DNA in the tissue (biopsy slice)	Extensive experience needed
Hybrid capture assay	Fresh or frozen samples	Intermediate	High, but probes often pooled to separate between low- and high-risk types	Technically easy	RNA probes are unstable and therefore hard to handle Fresh or frozen material is needed

1.3.2. Analysis of PCR-Amplified Viral DNA

By introducing a PCR-based amplification step prior to typing, very limited amounts of viral DNA or RNA can be detected. Amplification-based methods have therefore become the method of choice when the amount of sample material is very limited or when the material has been fixed and stained by reagents affecting DNA quality. Owing to the higher sensitivity provided by PCR, estimates of population prevalence of HPV infections in PCR-based studies have been two to three times higher compared to studies using nonamplification-based techniques *(2)*.

Several of the assays containing a PCR step are modifications of the strategies discussed above. The specificity and sensitivity of the detection will be increased substantially by including the PCR step, since the number of DNA molecules to be used in the typing will be in great excess over the original HPV copy number. These assays all start with a PCR step, which can either be a HPV type-specific PCR or with primers designed to amplify a range of HPV types. For HPV type-specific PCR assays, the detection is accomplished by examination of the size of PCR products on an agarose gel (**Table 2**). The type-specific PCR assays have the advantage of being very sensitive, since only a single oligonucleotide pair is being used in each reaction. However, to use the type-specific PCR assays for large patient cohorts becomes impractical because of the need to screen for a relatively large number of HPV types. The method employing consensus primers, although sufficiently robust for most applications, has the potential drawback of underestimating the proportion of patients infected with more than one HPV type. This is because HPV types occurring at high copy number may outcompete viral types occurring at much lower copy number in the PCR. When PCR products have been generated in a multitype HPV PCR, detection and/or typing can be performed by a number of means, e.g., RFLP, dot-blot, or hybrid capture (**Table 2**).

In analyzing especially demanding samples, like formalin-fixed biopsies and old Papanicolaou smears, a nested PCR has sometimes been employed. In nested PCR, two set of primers are used, the location of the second primer pair being internal to the first set. The PCR product generated by the first primer pair is diluted and subjected to a second round of PCR using the internal primers. By this procedure, the sensitivity and specificity are increased further. The drawback of the nested PCR is the increased risk of false positives owing to contamination. This can be overcome by a system utilizing ramp-specific primers that allows a nested PCR to be performed without any transfer of PCR products (one-tube nested PCR *[26]*).

A combination of *in situ* hybridization and PCR is the *in situ* PCR *(27)*. In this method, amplification is performed directly on the specimen slide. The

Table 2
Summary of PCR-Based Methods for Detection and Typing of HPV

Method	Applicable material	Sensitivity	Specificity	Main advantage	Main disadvantage
PCR dot blot	Cervical smears Formalin-fixed biopsies Fresh/frozen samples	Very high	Very high	Possible to analyze multiple samples simultaneously	Membranes have to be hybridized multiple times for complete typing
PCR RFLP	Fresh/frozen samples	Very high	Intermediate	Easy and rather cheap	Not all types possible to resolve
PCR Southern blot	Cervical smears Formalin-fixed biopsies Fresh/frozen samples	Very high	High when combined with restriction cleavage	Reliable method	Labor-intensive and time-consuming method
In situ PCR	Sliced biopsies on microscopy slides	Very high	Possibly very high, but remains to be evaluated	Yields information of tissue localization of HPV DNA	Method still under development
Solution hybridization assay for PCR products (SHARP)	Cervical smears Fresh/frozen samples	Very high	High, but probes often pooled to separate between high- and low-risk types	No electrophoresis step needed	RNA probes are unstable
Type-specific PCR	Cervical smears Formalin-fixed biopsies Fresh/frozen samples	Very high	Very high	Able to detect most of the subtypes given enough PCRs are performed	A number of PCRs are needed for each sample
SSCP	Cervical smears Formalin-fixed biopsies Fresh/frozen samples	Very high	Very high	Able to detect one single-basepair mutation	Labor-intensive
Sequencing	Cervical smears Formalin-fixed biopsies Fresh/frozen samples	Very high	Very high	Able to detect the specific DNA sequence	Expensive Labor-intensive

products are subsequently detected by hybridization. The main advantage of this procedure is that the sensitivity and specificity of PCR are combined with the positional information provided by *in situ* hybridization. However, further development of the method is required before *in situ* PCR can be used for large-scale routine diagnosis.

The solution hybridization assay for PCR products (SHARP) (Digene Diagnostics, Inc., Silver Spring, MD) is an extension of the hybrid capture assay based on the same DNA–RNA hybrid concept, but includes a PCR step to generate an HPV PCR product with a biotinylated PCR primer, and Streptavidin-coated wells for capture of hybrids (**Table 2**) *(28)*.

To distinguish DNA HPV types based on sequence variation in the PCR product, a number of methods are available, such as dot-blot hybridization, restriction fragment analysis, single-strand conformation polymorphism (SSCP), and DNA sequencing (**Table 2**). The dot-blot method will be discussed in detail under **Subheading 3.** The SSCP analysis is based on the principle that single-stranded DNA will form a secondary structure determined by its nucleotide sequence. Single-base differences may change this conformation and shift the mobility through the nondenaturing polyacrylamide gel *(29)*. Direct sequence analysis with terminating fluorescent dye-labeled dideoxynucleotides and analyzed on an instrument for automated DNA sequencing, is an alternative *(29)* However, the throughput of this method may still be somewhat limited. In summary, a large number of techniques have been developed for detection, typing, and sequence determination of HPV in clinical samples, each having its own advantages and disadvantages. The following sections focus on a PCR-based assay developed and employed by us for large-scale analysis of HPV from a variety of sources of materials.

2. Materials

2.1. Specimens

The normal range of materials for HPV detection include:

1. Formalin-fixed biopsies: Fixation of fresh biopsy material in 10% formalin and embedding in paraffin is the most common way to preserve tissue. This permits storage at room temperature. However, this type of preservation is not recommended if DNA analysis is to be performed.
2. Nonfixed, fresh or frozen tissue (biopsies, scrapes, and exfoliated cells): To allow PCR amplification of large DNA fragments, fresh tissue should be extracted immediately or be kept at –70°C for longer periods of time.
3. Papanicolaou-stained smears: Smears are usually fixed in ethanol and then stained with Papanicolaou stains. These include hematoxylin, which stains the nuclei darkblue and orange G and eosin-alcohol, which gives an orange and reddish color to the cytoplasm

Following is a description of the equipment that we find most useful in dealing with the materials described above, and the procedures used for extraction, amplification, and typing.

2.2. Solutions

1. Denaturing solution: 0.4 m*M* NaOH, 25 m*M* EDTA.
2. Digestion solution for formalin-fixed material: 0.2 *M* Tris-HCl, pH 8 0 1% SDS, 1 mg/mL proteinase K, 10 m*M* EDTA.
3. dNTP: 25 m*M* of each nucleotide (dATP, dCTP, dGTP, TTP) in stock
4. EB buffer: Equal parts of lysis buffer and suspension buffer with 25 µL of proteinase K/mL.
5. ECL Chemiluminescence kit, Amersham, UK.
6. Ethidium bromide. Dissolve in sterile water 10 mg/mL.
7. Hybridization buffer: 2X SSPE, 0.5% SDS.
8. Loading buffer: 15% Ficoll 400, 0.2% bromphenol blue, 50 m*M* EDTA.
9. Lysis buffer 1% Sarcosyl, 8 *M* urea, 20 m*M* EDTA, 0.4 *M* NaCl, 200 m*M* Tris-HCl, pH 8.0.
10. Oil. Light white mineral oil (Sigma).
11. 1X PBS: 136 75 m*M* NaCl, 2.68 m*M* KCl, 10.14 m*M* Na_2HPO_4, 1.76 m*M* KH_2PO_4
12. TE-low: 10 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA.
13. Primers: Dissolve the primers in TE-low (500 m*M* in stock). Dilute the stock into a 10-µ*M* working solution.
14. Proteinase K: 20 mg/mL dissolve in water; aliquot it into smaller amounts (1 mL).
15. Purified BSA: 10 mg/mL.
16. Saturated ammonium acetate: Dissolve the powder in sterile water until the solution reaches its saturation.
17. 1X SSPE: 0.18 *M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* EDTA, pH 7 7
18. 20X SSC: 3 *M* NaCl, 0.2 *M* Na citrate.
19. Stringency buffer: 1X SSPE, 0.1% SDS.
20. Suspension buffer: 10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0.
21. 1X TEB: 0.089 *M* Tris-borate, 0.089 *M* boric acid, 0.0024 *M* EDTA.
22. 10X *Taq* polymerase buffer: 500 m*M* KCl, 15 m*M* MgCl, 100 m*M* Tris-HCl, pH 8 3. Prepare fresh buffer each 6 mo. Store at –20°C.

2.3. Equipment and Supplies

2.3.1. Extraction

1. Fume hood.
2. Incubator.
3. Humidified chamber (a box filled with water or lined with wet paper towel).
4. Water bath.
5. Micropipets.
6. Aerosol-resistant micro-pipet tips (0–40, 0–200, 200–1000 µL).
7. Sterile tubes (50-, 1.5-, and 0.5-mL test tubes).

2.3.2. PCR

1 Thermal cycler (Perkin Elmer Cetus TC1 or 9600)
2 Aerosol-resistant micro-pipet tips (0–40, 0–200, 200–1000 µL).
3. Micropipets.
4. PCR reaction tubes with caps.
5 NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) (*See* **Note 4.**)

2.3.3. Typing

1. Bio-Dot microfiltration apparatus (Bio-Rad).
2 Nylon membrane (BioDyne B membranes, Pall Corp).
3. Microtest tubes.
4 Vacuum pump
5 Streptavidin–POD conjugate.
6. Hybridization oven/shaker.
7. ECL kit (Amersham).
8 X-ray film.

3. Methods

3.1. Extraction of DNA (see Note 1)

3.1.1. Formalin-Fixed Biopsies

This is a modification of the protocol by Kösel and Graeber *(30)*.

1. Put the paraffin-embedded tissue in 1 mL xylene for 2 h at room temperature, with occasional mixing by squeezing the pellet with a plastic stick.
2. Discard the xylene
3 Wash with 95% ethanol, and incubate for 5 min at room temperature.
4. Repeat the washing step with 70% ethanol.
5. Dry the material for 20 min in 50°C.
6. Add 200 µL digestion solution.
7. Incubate for 3 h at 50°C.
8. Transfer to a second incubation for 14 h at 30°C.
9 Inactivate the proteinase K by incubation for 10 min at 95°C.
10 Centrifuge at 21,000*g* for 5 min to pellet undissolved matter
11. Transfer the supernatant into a new tube.
12. Add the same volume phenol/chloroform, mix, and centrifuge at 21,000*g* for 3 min. Transfer the DNA phase to a new tube.
13. Repeat **step 12**
14 Add 0.1x the remaining volume of 3 *M* NaAc, pH 4.8, and 2 5 vol of 95% ethanol to precipitate DNA.
15 Incubate for 1 h at –20°C.
16 Centrifuge the tube for 30 min at 21,000*g*, remove liquid, and dry pellet under vacuum for 5 min.
17. Dissolve pellet in 100 µL TE-low. Use 5 µL for PCR amplification (*see* **Note 1**)

3.1.2. Nonfixed, Fresh, and Frozen Tissue

This is a modification of the protocol by Higuchi that is appropriate for biopsies, scrapes, and exfoliated cells *(31)*.

1. Put the tissue (or wad from the cotton tops) in 0.5 mL of PCR buffer with nonionic detergents
2. Add 1.5 µL proteinase K (final concentration, 6.0 µg/mL).
3. Incubate at 55–60°C for 1 h or until the tissue has been degraded. For the cotton top wads, 1 h is enough Then transfer the supernatant to a new tube
4. Add 0 5 mL phenol.
5. Centrifuge for 3 min at 21,000*g*
6. Move the DNA phase (upper phase) to a new tube.
7. Repeat **steps 4–6** if the interphase is cloudy.
8. Add the same volume of chloroform, mix, and centrifuge for 3 min at 21,000*g*
9. Transfer the DNA phase to a new tube
10. Add 0.1x remaining volume of 3 *M* NaAc, pH 4.8
11. Precipitate the DNA by adding 2.5x the remaining volume of 99.9% ethanol.
12. Incubate for 30 min at –20°C.
13. Centrifuge the tube for 30 min at 21,000*g*, and remove liquid
14. Wash pellet once with 1 mL of 70% ethanol.
15. Centrifuge for 5 min
16. Dry pellet under vacuum
17. Dissolve in 100 µL TE-low (*see* **Note 1**).

3.1.3. Papanicolaou-Stained Smears

This is a modification of the protocol described by Chua and Hjerpe *(32)*.

1. To remove cover slips, soak slides in xylene for 5–6 d
2. To destain, soak slides in 99.9% ethanol for a minimum of 30 min.
3. Allow smears to dry at room temperature
4. Add 150 µL of EB, and mix onto each slide.
5. Incubate at 37° C for 20 min in a humidified chamber.
6. Dislodge cells from surface of glass with pipet tip, and transfer the cell suspension to a microfuge tube
7. Repeat **steps 5–7**. Pool cell suspensions into the same tube.
8. Add 10 µL proteinase K to each tube.
9. Incubate at 60°C for at least 1 h
10. Add 100 µL saturated ammonium acetate.
11. Mix for 30 s, and centrifuge at 21,000*g* for 5 min.
12. Transfer supernatant to a new tube. Discard pellet.
13. Add 1 2 mL of 99 9% ethanol
14. Incubate at –20° C for at least 30 min
15. Centrifuge at 21,000*g* for 5–30 min Discard supernatant, and retain pellet

16. Wash pellet once with 1.5 mL of 70% ethanol.
17. Centrifuge for 5 min.
18. Dry pellet in a fume hood.
19. Dissolve in 200 µL TE-low (*see* **Note 1**).

3.2. PCR Amplification (see Note 2)

The design of an optimal HPV PCR primer system for use in clinical and epidemiological studies relies on a number of considerations. Such a system should be able to detect HPV in a variety of clinical materials, i.e., the size of the PCR product has to be minimized, and it has to be targeted to a region conserved among all the HPV DNA sequences, in order to allow the amplification of multiple HPV types and, finally, the region between the primers has to be variable enough to design type-specific probes. The L1 and E1 are the two most conserved regions among HPV types and suitable for the design of an efficient amplification system. However, there are reports of systems designed for the less-conserved ORF regions in E6/E7 *(33)*. Since many regions in the HPV genome have been found deleted when integrated this further delimits the regions most suitable as targets *(34–37)*.

Several amplification systems targeting different portions of the HPV genome have been described. Among the most frequently used ones for the L1 region are MY09/MY11 system using the degenerate primers, and the GP5+/GP6+ system, using a pair of consensus primers *(38,39)*. The GP and MY primers can be combined to give a nested PCR with a very high sensitivity *(32)*. The MY09/MY11 primers are degenerate primers with 8–16 variants for each strand and therefore detect a wide range of HPV DNA types in comparison with GP5+/GP6+, which is a single primer pair made from the consensus sequence of an alignment of HPV types. There is a substantial difference between the amplicons in length: 450 bp for MY09/MY11 compared to 150 bp for the GP pair, which may be important when dealing with materials, such as formalin-fixed and stained tissues. The deletions on integration do not involve the regions where the MY09/11, GP5+/6+, and E1 350L/547R primer-pairs described below are located *(26,38,39)*.

The most frequently used primers and probes for the L1 ORF have the following sequences:

MY09 5'-CGTCCMARRGGAWACTGATC-3',
MY11 5'-GCMCAGGGWCATAAYAATGG-3';
GP5+ 5'-TTTGTTACTGTGGTAGATACTAC-3',
GP6+ 5'-GAAAAATAAACTGTAAATCATATT C-3'
(R = A or G; Y = T or C; W = T or A; M = A or C.)

We have previously described a set of E1 degenerate primers designed by aligning the 19 most common genital HPV genome sequences *(26)*. The 5'-end in the E1 ORF region has a region conserved among all HPV DNA types providing an excellent site for a primer *(40)*. The E1 degenerate primers generates a 180-bp long product. The upstream E1 350L primer is conserved at 13 positions in the 3'-end, whereas the downstream primer is a degenerate primer *(41)*.

There are two alternate nested amplification systems for the E1 and L1 reading frames, both equally sensitive and specific. The E1 primer pair has been tested against the MY09/MY11 primer set for sensitivity on cervical smears and cervical cancer biopsy material with mixed infections, and for the specificity by examining the amount of nonspecific DNA background visualized on an ethidium bromide-stained gel. The E1 primer pair showed both higher sensitivity and specificity, compared to the MY01/MY11 *(26)*.

The degenerate primers used for the E1 ORF have the following sequences *(26)*:

E1 301L, (E11088) 5'-GRCWGCMARKGCRTTGTTYMMTRYRCAGGA-3';
E1 847R, (E11539) 5'-CCAATCDSWACABSTKSWTTTATYRCTYTKAAA-3';
E1 350L, (E11132) 5'-TRYRKGYYYTAAAACGAAAGT-3';
E1 547R, (E11263) 5'-TTCCACTTCAGWAYWGCCATA-3'

(S = G or C; R = A or G, Y = T or C, W = T or A, M = A or C; K = G or T; D = T, G, or A.)

Employing our system for the HPV E1 region, there are two options: (1) using a single primer pair, which is quite sufficient when working from biopsy material, or (2) using the one-tube nested PCR with four primers from the start in the same reaction. The nested model is more sensitive because of the two amplification steps and the small size of the final DNA fragment (180 bp), and therefore, suited for analysis from limited DNA copy numbers. It also eliminates the crosscontamination problem, which occurs when transferring PCR products from the first to the second amplification reaction. The general single PCR amplification system is normally sufficiently sensitive, and we have recently been able to use only a single primer pair also when analyzing HPV from archival cervical smears.

3.2.1. One-Tube Nested PCR

Add the reagents in the following order; buffer, detergent, dNTP, water, primers, *Taq* polymerase, oil if applicable, and last the template. This also applies to the single primer pair PCR system.

	1X
10X *Taq* polymerase buffer (15 m*M*)	10 µL
10 µ*M* outer primer 1 (E1 301L)	2 5 µL
10 µ*M* outer primer 2 (E1 847R)	2.5 µL
10 µ*M* inner primer 3 (E1 350L)	5 µL
10 µ*M* inner primer 4 (E1 547R)	5 µL
10% Nonidet P40/Tween-20	10 µL
25 m*M* dNTP (200 µ*M* of each nucleotide)	0 8 µL
AmpliTaq (5 U/µL)	0 45 µL
Template	2–10 µL
Sterile dH_2O	54–62 µL
Reaction volume	100 µL

Add oil on top (~40 µL) if applicable.

Put the samples in the thermocycler, and run a total of 45 cycles. The first 15 cycles are 1 min at 94°C, 1 min at 45°C, and 1.5 min at 72°C. Then lower the annealing temperature to 30°C, and run an additional 30 cycles with the other parameters unchanged. When using the Perkin-Elmer 9600 DNA Thermal Cycler, decrease the times to 40 s for each step in the cycle (*see* **Notes 2** and **3**).

3.2.2. Single Primer Pair PCR

This protocol could also be used with the L1 MY09/11 primer pair (50 pmol of each primer) by replacing the E1 primers by MY09/MY11.

	1X
10X *Taq* polymerase buffer (15 m*M*)	10 µL
10 µ*M* primer 1 (E1 301L) (MY09)	5 µL
10 µ*M* primer 2 (E1 547R) (MY11)	5 µL
10 mg/mL BSA	1 24 µL
10% Nonidet P40/Tween-20	10 µL
25 m*M* dNTP (200 µ*M* of each nucleotide)	0 8 µL
AmpliTaq (5 U/µL)	0 45 µL
Template	2–10 µL
Sterile dH_2O	58–66 µL
Reaction volume	100 µL

Add oil on top (~40 µL) if required depending on the type of thermal cycler used

Put the samples in the thermocycler, run a total of 45 cycles with 1 min at 94°C, 1 min at 45°C, and 1.5 min at 72°C. When using the Perkin-Elmer 9600 DNA Thermal Cycler, decrease the times to 40 s for each step in the cycle (*see* **Notes 2** and **3**).

3.2.3. Detection of PCR Products

1. Prepare a 4% agarose gel (4 g agarose, 100 mL TEB).
2. Stain the gel with 10 µL ethidium bromide from stock solution.
3. Mix 10 µL of the PCR amplification with 1 µL of loading buffer.
4. Load 10 µL.
5. Run the gel at 80 V for 20 min.
6. Visualize the DNA band using a transilluminator.
7. Note positive and negative samples (*see* **Note 4**).

3.3. Dot-Blot Typing

For the E1, we have designed an HPV typing system based on 19 different type-specific probes with similar hybridization kinetics to match the specific genital HPV types *(26)*.

Oligonucleotide probe	Sequence	T_m, °C
HPV6B E1B	B5'-TAAACTTACAAGACAG-3'	42
HPV11 E1B	B5'-TAAACTTACAACACAG-3'	42
HPV16 E1B	B5'-ATGTATAGAAAAACAAAGT-3'	46
HPV18 E1B	B5'-TTTAAATAGTGGGCAGA-3'	46
HPV30 E1B	B5'-TACAGACGTGGCCG-3'	46
HPV31 E1B	B5'-ATGCATAGAAAATAACAG-3'	46
HPV33 E1B	B5'-TAATAAAAATAAAGAATGCA-3'	46
HPV34 E1B	B5'-GACGATAAGGGACAC-3'	46
HPV35 E1B	B5'-TTGCATTGAAAATAAAAATA-3'	46
HPV39 E1B	B5'-TTCATTAAATGTAAGCAG-3'	46
HPV40 E1B	B5'-ACGGCTTGGCGGC-3'	46
HPV42 E1B	B5'-ACGCTATGTCGGGG-3'	46
HPV45 E1B	B5'-ATTAAATAGTGGGCACA-3'	46
HPV51 E1B	B5'-CAAACGAGTCACAAGT-3'	46
HPV52 E1B	B5'-GTGTAAATACAGAGTGT-3'	46
HPV53 E1B	B5'-GATACAGAAGTGCCG-3'	46
HPV56 E1B	B5'-TTTATCAGACCTACAAG-3'	46
HPV57 E1B	B5'-CTCGCTAAACAGAAAG-3'	46
HPV58 E1B	B5'-ATATAAAAATAAAGAATGC-3'	46

Oligonucleotide probes employed with the MY09/MY11 primers *(42,43)*.

Oligonucleotide probe		Sequence
HPV 6/11	(MY12)	5'-CATCCGTAACTACATCTTCCA-3'
	(MY13)	5'-TCTGTGTCTAAATCTGCTACA-3'
	(MY125)	5'-ACAATGAATCCYTCTGTTTTGG-3'

Oligonucleotide probe		Sequence
HPV 16	(MY95)	5'-GATATGGCAGCACATAATGAC-3'
	(MY133)	5'-GTAACATCCCAGGCAATTG-3'
HPV 18	(MY130)	5'-GGGCAATATGATGCTACCAAT-3'
	(WD74)	5'-GGATGCTGCACCGGCTGA-3'
HPV 26	(MY186)	5'-GCTGACAGGTAGTAGCAGAGTT-3'
	(MY187)	5'-GCCATAACATCTGTTGTAAGTG-3'
HPV 31	(MY92)	5'-CCAAAAGCCYAAGGAAGATC-3'
	(MY143)	5'-TTGCAAACAGTGATACTACATT-3'
HPV 32		5'-GCCATACGATGTCAAGCTAAG-3'
HPV 33	(MY16)	5'-CACACAAGTAACTAGTGACAG-3'
	(MY64)	5'-TCCTTTGGAGGTACTGTTTTT-3'
HPV 34		5'-CCACAAGTACAACTGCACCA-3'
HPV 35	(MY115)	5'-CTGCTGTGTCTTCTAGTGACAG-3'
	(MY117)	5'-ATCATCTTTAGGTTTTGGTGC-3'
HPV 39	(MY89)	5'-TAGAGTCTTCCATACCTTCTAC-3'
	(MY90)	5'-AGACACTTACAGATACCTACAG-3'
HPV 40	(MY176)	5'-CCCAAGGTACGGGAGGATCC-3'
HPV 42	(MY34)	5'-GGCTAAGGTAACAACGCC-3'
	(MY121)	5'-CACTGCAACATCTGGTGAT-3'
HPV 45	(MY69)	5'-ATACTACACCTCCAGAAAAGC-3'
	(MY129)	5'-GCACAGGATTTTGTGTAGAG-3'
HPV 51	(MY87)	5'-TATTAGCACTGCCACTGCTG-3'
	(MY88)	5'-CCCAACATTTACTCCAAGTAAC-3'
HPV 52	(MY81)	5'-CACTTCTACTGCTATAACTTGT-3'
	(MY82)	5'-ACACACCACCTAAAGGAAAGG-3'
HPV 53	(MY102)	5'-TTCTACCTTACTGGAAGACTGG-3'
	(MY182)	5'-GCAACCACACAGTCTATGTC-3'
HPV 54	(MY160)	5'-CAGCATCCACGCAGGATAG-3'
	(MY161)	5'-GAATAATGCCCCTGCAAAG-3'
HPV 55	(MY151)	5'-GTGCTGCTACAACTCAGTCT-3'
	(MY171)	5'-CCCTGAAAAGGCAAAGCAG-3'
HPV 56	(MY197)	5'-GCACAGCTATAACATGTCAACG-3'
	(MY199)	5'-CAGTTAAGTAAATATGATGCACG-3'
HPV 57	(MY154)	5'-AATGTCTCTTTGTGTGCCAC-3'
	(MY156)	5'-GGATCAGTAGGGGTCTTAGG-3'
HPV 58	(MY94)	5'-AGCACCCCCTAAAGAAAAGGA-3'
	(MY179)	5'-GACATTATGCACTGAAGTAACTAAG-3'
HPV 59	(MY123)	5'-GCCAGTTAAACAGGACCC-3'
	(MY162)	5'-CCTAATGWATACACACCTACCAG-3'
HPV 61		5'-CCATTTGTACTGCTACATCCCC-3'
		5'-TAAAGCCACGAGCTTTAGGG-3'

Oligonucleotide probe		Sequence
HPV 62		5'-TGCAGCAGAATACACGGCT-3'
		5'-CACTATTTCGAGTCTCGGGC-3'
HPV 64		5'-CCTAAGGCAGTCAGAAGAGATGT-3'
HPV 67		5'-CTGAGGAAAAATCAGAGGCTAC-3'
		5'-ATCCCCTCCAACAGCAAAG-3'
HPV 66	(MY83)	5'-ATTAATGCAGCTAAAAGCACATT-3'
	(MY178)	5'-CATGTCAGAGGGAACAGCC-3'
HPV 68	(MY191)	5'-CATACCGCTATCTGCAATCAG-3'
	(MY194)	5'-CTACTACTGAATCAGCTGTACC-3'
HPV 69		5'-CAATCTGCATCTGCCACTT-3'
		5'-GCCTTACCTTGCCTCCTACT-3'
HPV 70		5'-ATTGTCTGCCTGCACCGAA-3'
		5'-AAGCTTGGTGGACACGTATA-3'
HPV 72		5'-TCGTGAGTATCTTCGCCAC-3'
		5'-CCTCCTCCTAAAGAAGATCCAT-3'

(Y = T or C; W = T or A.)

The hybridization protocol is as follows (for the E1 probes) (*see* **Notes 5** and **6**):

1. Each membrane include 5–10 different controls (consisting of amplified products from HPV plasmid or cell lines with integrated HPV DNA) and about 85–90 patient samples.
2. Denature 5 µL PCR product in 100 µL denaturing solution.
3. Incubate for 20 min at room temperature
4. Cut the membrane into size, 8 × 12 cm
5. Pre-wet the membrane in 2 × SSC
6. Put the membrane on a piece of filter paper to remove excess buffer.
7. Put the membrane on top of the sealing gasket.
8. Mount the apparatus
9. Apply the DNA samples.
10. Turn the vacuum on and drain the wells
11. Remove the filter from the dot-blot apparatus
12. Put the membrane on a piece of filter paper to remove excess buffer.
13. Remove excess PCR product by immersing the membrane in 400 mL hot water (90°C) with ~3 mL 10% SDS for 10–15 min.
14. For hybridization, use a suitable plastic container with lid.
15. Add 10 mL of preheated hybridization buffer
16. Add 0.5 µL Streptavidin–POD conjugate and 4 µL probe (4 pmol/10 mL).
17. Incubate at 42°C for 30 min.
18. Discard the buffer solution.
19. Wash with stringency buffer
20. Wash in 1X PBS

21 Mix 1:1 of the two ECL detection solutions (0.125 mL/cm^2 membrane)
22 Incubate exactly 1 min at room temperature in the dark.
23 Put the membrane on a sheet of filter paper to remove the excess fluid
24. Wrap the filter in a cling-film sheet.
25 Fasten the membranes in a cassette
26 Apply the film, and expose for 1 min.
27. Strip the filter as in **step 13**, and apply a new probe
28 The filter can be stored by keeping it in cling-film at 4°C (*see* **Notes 5** and **6**).

For the MY probes, we refer for hybridization and washing conditions to the original descriptions *(42,43)*.

3.4. Discussion

The number of methods described for HPV detection and diagnosis is impressive. In general, methods based on detection of antibodies or native HPV DNA or RNA are analytically less sensitive and sometimes also less specific than PCR-based methods. Also, antibodies have been detected by serological methods in cleared DNA infection on average 19.9 mo after a seroconversion *(14)*. This delayed signal may be informative in some studies, but presents a problem if HPV serology is used for screening cervical disorders. Initially, PCR-based detection of HPV was perceived as being too prone to contamination, but this problem has subsequently been shown to be overestimated when using appropriate routines for contamination control. As a consequence, PCR-based methods have become wide-spread, in particular, for the analysis of the limited DNA present in routine cervical smears.

The PCR-based methods described are all characterized by high sensitivity in detecting a small initial copy number of HPV, but none appears to be ideal for large-scale studies or routine analyses because of:

1 Many manual steps
2 Amplification and typing being performed as two independent procedures
3. The nested PCR systems being too sensitive to contamination

The method described in detail in this chapter for the E1 method was designed to overcome some of the problems inherent in earlier methods. Thus, the PCR assay was designed to have the sensitivity of nested PCR, but without the most contamination-prone step in the procedure, i.e., the transfer of product from the first to the second amplification reaction, by performing both reactions in the same tube without opening. The subsequent typing procedure, using hybridization with a series of oligonucleotides to membranes with PCR products, is admittedly better suited for a research setting than a routine operation. A somewhat more doable method would be to perform the typing in a microtiter plate, with the different oligonucleotide probes attached to individual wells.

This would make the method more suited for complete typing of a few samples at a time. However, an ideal method for HPV detection should include both amplification and typing of HPV in a single assay. A number of technologies for such homogeneous assays have recently been described, one of the most promising being the 5'-exonuclease assay, which can be performed using fluorophores (the TAQMAN assay) ***(44,45)***. In this assay, the typing is performed simultaneously with the amplification, through the release of fluorophores from the probes included in the PCR, and the results are available immediately after the PCR without further laboratory steps. The TAQMAN assay also eliminates the problem of PCR products from previous or neighboring reactions contaminating the unamplified reaction, since the system is closed and no products are released into the laboratory. Preliminary results of using the TAQMAN assay with the E1 amplification system indicate that a combination of one-tube nested PCR and TAQMAN detection will be a sensitive and robust method for HPV detection (Josefsson et al., unpublished). Such an assay would fulfill many of the criteria for an ideal detection system for infectious agents.

4. Notes

1. To minimize contamination, we perform all DNA extractions in a dedicated room. Change gloves frequently during the handling of materials While using the humidity chamber, do not put the cervical smears too near to each other
2. To minimize the risk of contamination, we have a dedicated room with areas for clean reagents (without DNA) and others for adding DNA template to the PCR reactions The PCR amplifications are done in a second room where all the thermal cyclers are kept When using nested systems that require opening the tubes inbetween the two reactions, a third facility is employed. However, when using the nested primer system of Ylitalo et al. ***(26)***, we do not have to open the tubes to start the second round.
3. Purified BSA is useful in the PCR to bind *Taq* polymerase inhibitors present in the extracted material. For experimental use, a concentration of 2.5 μg/μL has been found to be appropriate
4. For detection of PCR products, NuSieve GTG agarose is used. This agarose has a high resolution of nucleic acid fragments <1000 bp and can detect fragments as small as 8 bp. Owing to its low viscosity, the agarose can be used at high concentrations (2–4%) DNA fragments are detected by using a low concentration of the fluorescent, intercalated dye, ethidium bromide. As little as 1–10 ng of DNA can be detected by direct examination of the gel under UV light ***(46)***.
5. Preheat the membrane after applying PCR products, prior to hybridization in order to wash off excess PCR product. To obtain repeatable results, it is important to prewarm the hybridization and washing solutions to the different temperatures Always include HPV controls in order to evaluate the stringency of the hybridization. The crosshybridization between controls are eliminated through

adjustment of the stringency If no signal is obtained from the appropriate control, the stringency of the wash was too high. The stringency can then be lowered either by increasing the salt concentration by a factor of two or by lowering the washing temperature If crosshybridization between different HPV controls occurs, dilute the SSPE twofold. The use of a 1X PBS-D wash gives a higher signal-to-noise ratio. Samples that remain positive after the elimination of all crosshybridizations between the controls are considered HPV-positive Normally, the intensity of the hybridization signal is correlated with the amount of PCR product detected on the agarose gel.

6. Handle the blots with care, since physical damage gives a higher background and causes difficulties when reprobing. ECL is a chemiluminescent detection system that uses the luminol as a substrate for the enzyme horseradish peroxidase (HRP). This detection solution can be used several times for about 1 h

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12

Quantitation of HIV-1 RNA in Dried Plasma Spots (DPS)

A Field Approach to Therapeutic Monitoring

Sharon A. Cassol, Francisco Diaz-Mitoma, and D. William Cameron

1. Introduction

The ability to measure accurately viral RNA in the plasma *(1–3)* and intracellular *(4–7)* compartments of HIV-1-infected persons has led to a dramatic improvement in our understanding of the natural history of HIV-1/AIDS. A number of recent studies have convincingly demonstrated that high levels of viral replication occur at all stages of disease *(8–10)*, and that changes in viral RNA load are predictive of disease outcome *(11,12)* and response to therapy *(13,14)*. These findings, combined with the introduction of potent new antivirals *(15,16)*, have stimulated a growing interest in viral load monitoring, both as a function of disease status, and as a predictor of disease progression and therapeutic efficacy.

The most commonly used quantification methods measure HIV-1 virion RNA levels in plasma, either by the reverse transcriptase polymerase chain reaction (RT-PCR) *(17–19)* or the branched DNA (bDNA) signal amplification assay *(20,21)*. As currently formatted, both assays are performed on fresh plasma processed within 4–6 h of collection, or on cryopreserved plasma that has been separated from whole blood and frozen immediately after collection at –20°C or colder. If in-house quantification is not available, the frozen plasma is then shipped on dry ice to a designated reference laboratory for analysis. These specialized handling requirements render the technology unsuitable for use in developing countries, where dry ice is unavailable, and facilities and resources are limited. Simple and improved methods that eliminate the need

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for cryopreservation, and extensive on-site processing and analysis would be highly advantageous.

The collection of blood specimens on filter paper blotters (Guthrie cards) provides an innovative and powerful approach for the PCR-based analysis of HIV-1 *(22,23,25–29)*. Using filter paper sampling, large numbers of difficult field specimens can be systematically collected, dried, and shipped without cryopreservation *(29)* or biosafety hazard *(30)*. Since first introduced for HIV-1 genetic testing in 1991 *(22)*, dried whole-blood specimens, in combination with DNA PCR and sequencing, have been used to screen rapidly for the presence of virus in newborns *(23–25,31)*, monitor the emergence of drug-resistant mutations *(26)*, characterize the genotype of transmitted virus *(26)*, and track the spread of HIV-1 subtypes in Asia *(27)*.

This chapter will describe the extension of filter paper technology to the quantification of HIV-1 RNA in dried plasma. After the plasma has saturated the filter, it is simply air-dried, placed in a high-quality bond envelope *(30)*, and shipped at ambient temperature to a suitable reference or research laboratory for RNA extraction and analysis using modifications of a commercially available RT-PCR kit. When evaluated under a variety of different environmental conditions and across the spectrum of HIV-1 disease, and the results of dried plasma testing were biologically equivalent to those obtained using more conventional HIV-1 RNA quantification methods (**Fig. 1**). It is anticipated that dried plasma spots (DPS) will prove particularly valuable for monitoring therapeutic efficacy among isolated and hard-to-reach populations and to assess viral replication kinetics in patients infected with different HIV-1 subtypes.

2. Materials

1. Blood collection tubes with EDTA anticoagulant (*see* **Note 4**)
2. Blood collection paper (Schleicher & Schuell, Keene, NH) (*see* **Note 7**).
3. AMPLICOR HIV Monitor Kit (Roche Molecular Systems, Somerville, NJ).
4. PE 9600 Thermocycler (Perkin-Elmer, Foster City, CA).
5. Microfuge.
6. Multichannel pipetter
7. Microwell plate washer and reader with computer.
8. Thermomixer
9. Vortex mixer
10. Micropipets, adjustable volumes 1–1000 µL.

3. Methods

3.1. Preparation of Dried Plasma Spots (DPS)

Manipulation of infected blood specimens should be performed under strict biosafety precautions (*see* **Note 1**) *(32)*, and in an environment that is free of

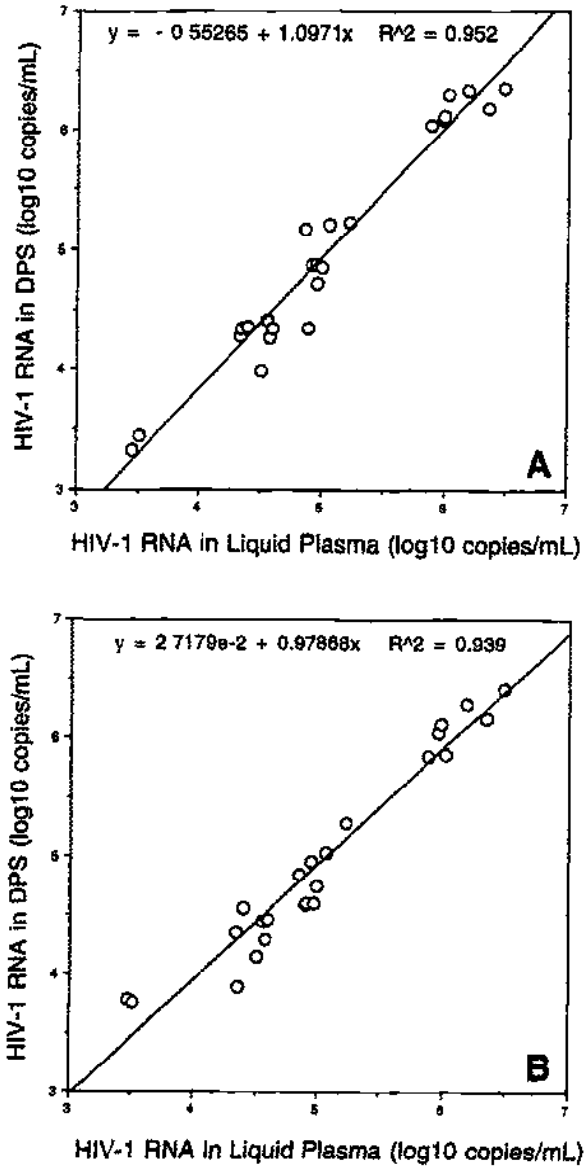

Fig. 1. Regression analysis of viral RNA measurement in paired DPS and fresh frozen plasma. DPS were **(A)** refrigerated or **(B)** stored at room temperature (20°C) for 7 to 16 days. (Figure taken from **ref.** *38*).

contaminating PCR amplicons and cloned viral sequences (*see* **Note 2**) *(33)*. A variety of different techniques are acceptable for the preparation of plasma, provided that there is no substantial inhibition of subsequent PCR amplifica-

tion reactions and that the same procedure is used throughout an entire study (*see* **Note 3**). The following protocol is routinely used in our laboratory.

1. Collect whole blood by venipuncture in Vacutainer tubes containing EDTA as the anticoagulant (*see* **Note 4**).
2. Transport tube to the virology laboratory at room temperature.
3. Centrifuge tube at 1200*g* for 20 min at room temperature to separate plasma from whole blood. For reliable results, plasma separation should be completed within 6 h of blood collection (*see* **Notes 3** and **5**).
4. Remove the separated plasma, and transfer to a new sterile tube
5. Using a micropipetter and sterile plugged tips, apply 50-µL aliquots of the clarified plasma to individual circles of a standard newborn screening blotter (Schleicher & Schuell, #903) (*see* **Notes 6** and **7**).
6. Air-dry for at least 3 h in a biological safety cabinet, when possible
7. If shipping is required, place each labeled filter in an individual bond envelope containing desiccant (*see* **Note 8**).
8. Seal envelope and enclose in a second, outer bond envelope, and ship to a reference laboratory by courier at ambient temperatures (*see* **Note 9**)
9. At the reference laboratory, the DPS can be analyzed immediately, or they can be stored frozen at –70°C and used for "batch testing" and retrospective analysis (*see* **Note 10**).

3.2. Reagent Preparation

Although different in-house methods exist for the extraction, reverse transcription, and amplification of HIV-1 RNA, we have opted to use a commercially available, quality-controlled kit, the Roche HIV-1 Monitor assay (*see* **Notes 11** and **12**). The use of a commercial method maximizes reproducibility and renders the technology suitable for large-scale efficacy testing in clinical trials. At the time of analysis (in the research or reference laboratory):

1. Prepare a working lysis reagent by adding a known amount (25 µL) of Quantitation Standard RNA (QS RNA) to one bottle of lysis reagent (AMPLICOR HIV-1 Monitor Kit, Roche) (*see* **Note 13**) The QS RNA acts as an internal standard to monitor the efficiency of sample preparation, reverse transcription, and amplification. The pink dye confirms the presence QS RNA in the extraction reagent.
2. After determining the number of quantifications required, prepare the appropriate amount of PCR reaction mix For 12 quantifications, mix 100 µL of manganese solution with one tube of master mix (AMPLICOR HIV-1 Monitor Kit). Dispense 50 µL of the working master mix into PCR reaction tubes in a MicroAmp™ tray. Seal the tray in a plastic bag and transport to the specimen preparation area.

3.3. RNA Extraction

1. Excise each circle of dried plasma (approx 1.0 cm^2) using clean, acid-depurinated scissors, cut in three pieces, and place in a 1.5-mL screw-cap microfuge tube

Use a different pair of scissors for each patient and each control specimen (*see* **Notes 14** and **15**)

2. Reconstitute the dried plasma by adding 200 µL of sterile, diethylpyrocarbonate-treated water to each tube.
3. Dispense 600 µL of working lysis reagent (containing QS RNA) into each tube, cap, and incubate for 15 min at 65°C with continuous shaking on an Eppendorf Thermomixer set at 1000 rpm to extract the RNA (*see* **Note 16**).
4. Following incubation, transfer 750 µL of the supernatant to a new tube.
5. Add 750 µL of 100% isopropanol, recap, and vortex briefly (3–5 s) to precipitate the RNA
6. Centrifuge at ~16,000*g* for 15 min at room temperature.
7. Aspirate the supernatant, taking great care not to disturb the RNA pellet on the outer shoulder of the tube Use a fine-tipped, sterile transfer pipet or a pipeting device with disposable tips. It is important to maintain a constant negative pressure during removal of the liquid.
8. Wash the pellet with 1 0 mL of 70% ethanol. Recap and vortex (3–5 s).
9. Recentrifuge at ~16,000*g* for 5 min at room temperature.
10. Again, carefully aspirate the supernatant without disturbing the pellet.
11. Add 100 µL of specimen diluent (AMPLICOR HIV-1 Monitor Kit), recap, and vortex vigorously for 10 s to resuspend the extracted RNA (*see* **Note 17**).

3.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Pipet 50 µL of each patient RNA and control specimen into the appropriate PCR tube containing 50 µL of working master mix (*see* **Subheading 3.2., step 2**). Cap the tubes, record specimen positions, and proceed with amplification and detection, exactly as specified in the Amplicor Monitor package insert (**steps 1–14** *below*).

1. Place tubes in a Perkin-Elmer 9600 thermocycler and amplify as follows:

Hold	2 min at 50°C
Hold	30 min at 60°C
4 Cycles	10 s at 95°C; 10 s at 55°C; 10 s at 72°C
26 Cycles	10 s at 90°C; 10 s at 60°C; 10 s at 72°C
Hold	15 min at 72°C

2. Remove the tray during the final 72°C hold program, and using a multichannel pipetter, add 100 µL of monitor denaturation solution. Mix and analyze immediately, or store denatured amplicons at 2–8°C for up to 1 wk.
3. At the time of analysis, remove an Amplicor Monitor microwell plate from the refrigerator, allow the plate to warm to room temperature, and add 100 µL of hybridization buffer to each microwell. The individual microwells have been precoated with HIV-1-specific (rows A–F), and QS-specific (rows G and H) oligonucleotide probes
4. Add 25 µL of each denaturated amplicon to a separate well in row A and make serial fivefold dilutions in the HIV-1 wells in rows B–F. Pipet each dilution up

and down 10 times to mix. After the final dilution in row F, mix as before and discard 25 µL of the mixture.

5 Add 25 µL of each denatured amplicon to the corresponding QS well in row G Make one fivefold dilution into row H, mix, and discard 25 µL of the mixture

6 Cover the plate, and hybridize for 1 h at 37°C

7 Wash the plate five time with working wash solution in an automated plate washer

8 Add 100 µL of avidin–horseradish peroxidase conjugate to each well, cover, and incubate for 15 min at 37°C.

9 Wash the plate as described in **step 7**, and add 100 µL of substrate solution Allow color development to proceed for 10 min in the dark at room temperature

10 Add 100 µL of stop reagent to each well, and within 10 min, measure the optical density at 450 nm

11 Choose the HIV-1 well with the lowest OD value in the range of 0.200–2.000 OD units. Subtract background (0.070 OD), and multiply by the dilution factor associated with that well

12 Choose the QS well with the lowest OD value in the range 0 300–2.000 OD units Subtract background (0 070 OD), and multiply by the dilution factor associated with that well.

13. Calculate HIV-1 RNA copies per mL of plasma according the following formula

$$\text{HIV-1 RNA copies/mL plasma} = (\text{Total HIV-1 OD/Total QS OD}) \times \text{input QS copies/PCR reaction} \times 40 \quad (1)$$

14. If the QS OD value falls within the expected range (0.300–2.000), but the HIV-1 OD value lies below 0.200, report the result as "No HIV-1 RNA detected, <200 copies/mL plasma."

3.5. Discussion and Future Directions

Few studies have specifically addressed issues of specimen processing, transport, and analysis in isolated and developing country regions. Given that the developing world bears a disproportionate share of the global HIV-1/AIDS burden *(34,35)* and that clinical trials are planned for many of these country regions, there is an urgent need for innovative technologies that can be widely applied to the quantification of HIV-1 RNA in field specimens.

The filter paper method described in this chapter offers substantial promise for large-scale therapeutic monitoring in developing, as well as developed countries, particularly in the pediatric setting where only minute amounts of samples are available. Based on the ease, economy, and safety of transporting filter papers, combined with unexpected and marked stability of HIV-1 RNA in dried specimens, and the biological equivalency of dried and cryopreserved plasma specimens, it is anticipated that the DPS method will continue to be refined and evaluated as a practical tool for monitoring

the effects of drugs, vaccines, and other interventions on viral burden. In particular, it will be important to simplify the procedure further to eliminate the need for an initial, on-site, low-speed centrifugation to separate the plasma. Elimination of this step would broaden the applicability of the method and render it suitable for use in geographic regions that lack electricity.

4. Notes

1. Universal precautions are to be used whenever handling blood or other potentially infectious specimens. Essential precautions include the availability of adequate hand-washing facilities and appropriate work practice protocols, as well as the proper use of warning labels and protective equipment, such as gloves and lab coats, the routine decontamination of work areas, and the proper disposal of waste material.
2. To reduce the risk of false-positive results and inaccurate quantification, we routinely use in-house and commercial assays that incorporate uracil-*N*-glycosylase (UNG) for the prevention of PCR product carryover contamination Although this approach is highly effective in controlling against small amounts of contamination, it is not a substitute for the extreme care that is required of all PCR-based technologies. Other universal precautions to guard against carryover of PCR amplicons and cloned sequences include performing sample processing, pre-PCR, amplification, and detection in separate laboratories or biological safety cabinets, using designated pipetters and plugged tips, cleaning work areas with bleach after each assay, using disposable gloves that are changed frequently, and aliquoting reagents in small volumes suitable for a single assay.
3. To achieve accurate and reproducible quantification, it is important that cells and platelets be effectively removed from the plasma preparation and that the same specimen processing procedure be used for all comparative studies *(36)*. If cells are not removed from the preparation, HIV-1 DNA will be amplified in addition to viral RNA Platelets, if present, may confound quantification by binding small amounts of circulating virus To remove these components, the Roche Monitor assay recommends a single centrifugation at 800–1600*g* for 20 min or longer. Other methods have used a two-step protocol involving a low-speed centrifugation at 400–800*g* for 20 min, followed by removal of the plasma and recentrifugation for an additional 20 min at 800*g*. Despite these differences, it is reassuring that several commercially developed assays are giving comparable HIV-1 RNA quantification results. In the near future, however, it is likely that methods will become increasingly standardized and that consistent guidelines will be developed to minimize interassay and interlaboratory variability. This is especially important for RT-PCR, where small differences in HIV-1 target RNA, when exponentially amplified, can lead to substantial variations in PCR product yield.

4 Although heparin, acid-citrate-dextrose (ACD), and citrate cell preparation tubes (CCPT) have been successfully used for collection and quantification of HIV-1 RNA in plasma, EDTA is now emerging as the preferred anticoagulant. Heparinized samples require a rather tedious extraction with silica to purify the RNA and prevent heparin inhibition of PCR. Citrate, which is used in solution, dilutes the plasma by an unknown amount (~15%) and leads to variable results
5. Early studies recommended that the plasma be processed within 2–3 h of collection to preserve the integrity of the viral RNA. However, with increased experience and, as shown in this chapter, it is becomingly apparent that the HIV-1 virion RNA in plasma is more stable than previously appreciated, presumably owing to the protective presence of the viral coat. As a result, the 2–3 h processing time has now been extended to 6 h
6 All steps in the collection, isolation, and extraction procedure, as well as handling of the filter papers, should be performed using aseptic techniques to prevent degradation of the RNA by nucleases. This includes the use of sterile tubes, pipet tips, and reagents, as well as the wearing of disposable gloves
7. We have routinely used Schleicher & Schuell #903 paper, originally designed for neonatal metabolic screening, as an absorbent matrix and noninfectious transport system *(22,23,25–29)* This collection device consists of a blotter containing five 1-cm^2 circles for specimen collection and sturdy paper overlay that covers the absorbent blotter and dried sample. A 50-µL aliquot of plasma saturates the circle and may extend slightly beyond In future studies, it may be possible to use blotters that have been preimpregnated with 2 *M* guanidine thiocyanate *(37)* or other preservatives to reduce the risk of microbial contamination.
8 The plasma spots should be thoroughly dried at room temperature before covering them with the attached paper overlay and sealing them with high-quality, sturdy envelopes, preferably ones that are air-permeable and water-resistant. In regions of excessive humidity, it may be prudent to air-dry in the presence of desiccant The plastic seal-a-bags used in some early studies are to be avoided, since they release undesirable chemicals and cause heat build-up, leading to specimen degradation
9 When double-packaged using two extra-strong bond envelopes, as recommended by the Centers for Disease Control in Atlanta *(30)*, filter specimens can be safely shipped and transported by mail or courier
10. Real-time analysis may be required to determine eligibility for a clinical trial, or to alter therapy, whereas batch-testing of serially collected samples may be more appropriate for evaluating therapeutic efficacy in large-scale, blinded trials
11 HIV-1 RNA quantification is still technically challenging, requiring skilled technologists, stringent quality controls, and standardized reagents and protocols At present, quantification assays are best performed in a controlled setting using commercial technology Three assays that show significant promise in this regard are the bDNA assay from Chiron *(20,21)*, the AMPLICOR RT-PCR assay from Roche *(17–19)*, and the NASBA amplification system developed by Organon Teknika. Although each assay has its strengths and limitations, the results appear to be closely related. RT-PCR was selected for this study, since it is currently the

most sensitive assay, is readily available, and requires the least amount of specimen, although a small-volume assay with improved sensitivity is currently under development at Chiron. A disadvantage of RT-PCR is that it requires a high level of technical skill to avoid crosscontamination of amplification reactions. In addition, since RT-PCR is based on the exponential amplification of HIV-1 RNA target sequences, small changes in sample processing can alter the input RNA copy number and lead to significant variation in the final PCR signal With respect to PCR, there has also been some concern that a single set of primers may not recognize all HIV-1 subtypes equally. This would lead to nonuniform, differential amplification across HIV-1 subtypes. Strict quality control and judicious selection of primers are needed to avoid these potential pitfalls.

12 Of particular note for filter paper methods is the potential of the NASBA assay. Although not extensively tested in the clinical setting, a major advantage of NASBA is its ability to amplify RNA selectively in the presence of whole-blood DNA. In the future, it will be important to determine whether NASBA can be applied to dried, whole-blood spots. The ability to use whole blood, rather than plasma spots would further simplify HIV-1 field studies by eliminating the need for on-site separation and isolation of plasma

13 To compensate for the smaller plasma volume of DPS specimens (50 µL instead of the 200 µL routinely used for fresh or cryopreserved plasma) and to maintain the same relative ratio of QS RNA to specimen RNA, the amount of QS RNA used to prepare the working lysis reagent has been reduced from 100 to 25 µL.

14. To depurinate and prevent crosscontamination between specimens, scissors are routinely washed in 0.25 *N* HCl for 10 min followed by a thorough rinsing in sterile water

15 In addition to clinical specimens, it is recommended that at least one negative and three positive controls be included in each DPS RT-PCR assay. Controls are prepared by applying measured aliquots (50 µL) of pooled plasma (from uninfected individuals, and infected individuals with known low-, intermediate, and high-HIV-1 RNA copy number) to replicate sets of no. 903 Schleicher & Schuell filter paper. After air-drying in a laminar flow hood, individual filters can be enveloped and stored at −70°C for use as reference standards.

16. DPS are heated at 65°C to ensure that the RNA is efficiently eluted from the filter and to eliminate any RNA secondary structure.

17 Again, the amount of specimen diluent has been reduced (from 400 to 100 µL) to compensate for the smaller volume of dried plasma specimens. When adjusted in this manner, the RNA input into the PCR reaction is identical to that used in conventional "fresh plasma" assays.

18. The Amplicor HIV Monitor test involves RNA extraction in guanidine thiocyanate, precipitation with isopropanol, RT-PCR amplification of a 142-bp HIV-1 *gag* sequence using biotinylated primers, and a single thermostable enzyme (rTth DNA polymerase) that has both RT and DNA polymerase activities. Serial dilutions of the biotinylated PCR product are hybridized to individual wells a microwell plate coated with HIV-specific and QS-specific oligonucleotide probes and quantified

in an avidin–horseradish peroxidase colorimetric reaction The input HIV-1 RNA copy number is then calculated from the known copy number of the QS RNA standard (Amplicor HIV Monitor package insert, Roche Molecular Systems)

19. Since preparation of this chapter, accurate quantification of HIV-1 RNA from dried plasma and dried blood spots (DBS) has been achieved using NASBA/Nuclisense technology *(38–40)*. As with the AMPLICOR method, there is strong correlation between viral RNA levels in liquid plasma, dried plasma, and dried whole blood. In patients with primary HIV-1 infection, the testing of DPS/DBS allows accurate measurement of viral RNA during the initial spike of viremia, and in the subsequent period of suppressed viral replication. In the pediatric setting, testing of dried filters is facilitating natural history and perinatal intervention studies in both developed and developing countries (*39,40*; manuscripts in preparation)

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13

Collection and Processing of Seminal Plasma for the Quantitation of HIV-1 RNA by NASBA and RT-PCR

Susan A. Fiscus and Myron S. Cohen

1. Introduction

Semen is the major vehicle for the sexual transmission of HIV-1. The ability to isolate infectious HIV from the semen and to quantitate viral burden in the form of cell-free or cell-associated HIV-1 RNA in semen are important for epidemiologic and public health aspects of the epidemic. Earlier studies used viral culture to detect HIV in semen. Cell associated culturable virus recovery rates ranged from 8 to 55% *(1–8)*. Much lower recovery rates (3–15%) were reported by these investigators for cell-free seminal plasma. In general, subjects with lower CD4 counts, higher seminal plasma viral load (>3.5–4 $\log_{10}$), and an AIDS diagnosis were more apt to have positive seminal cell HIV cultures

More recently, quantitative HIV RNA and DNA assays have been employed *(6,8–15)*. Overall these studies have demonstrated that 60–75% of men shed HIV RNA in the seminal plasma and that 65–80% have detectable HIV DNA in seminal cell pellets. Recent crosssectional studies using commercially available RNA kits have concluded that seminal plasma RNA levels are significantly correlated both with blood plasma RNA levels *(8,11)*, and the recovery of infectious virus from seminal cells *(8,9)*, but not with CD4 cell count *(8,9,11)*, stage of disease *(8,9)* or antiviral therapy *(8,9,11)*. In contrast to the crosssectional analyses, longitudinal studies have demonstrated that the amount of HIV RNA in seminal plasma increases with time in individuals who progress to AIDS *(16)*, and decreases with effective antiviral therapy *(11,16,17)*. Antibiotic treatment of pathogens causing urethritis, especially gonorrhea, can also reduce seminal plasma viral load *(18)*. In addition, since the viral burden in genital secretions may serve as a reservoir in patients who have had their virus

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seemingly eliminated from the peripheral blood, the quantitation of virus in semen has become a major focus of clinical trials. The following protocols can be used for conducting studies involving semen.

2. Materials

2.1. Collection of Semen

1. Gloves
2. Sterile specimen cups.
3. 15 mL conical centrifuge tubes.
4. 5 mL pipets.
5. 1.5 mL–2.0 mL sterile cryovials.
6. Sterile 100 mL brown or foil wrapped bottle.
7. Benzidine stock solution: Dissolve 125 mg benzidine (Sigma, St. Louis, MO) in 50 mL 96% ethanol. This may take as long as 24 h. Add 50 mL distilled water. Store in a brown bottle at room temperature. Label with a one year outdate

   Working benzidine solution - To 2 mL stock benzidine solution add 25 µL of 3% hydrogen peroxide. If kept in a brown bottle, this will last for several months at room temperature. However, since contamination with peroxidase can frequently occur, it is probably best to make this up fresh daily.
8. Centrifuge.
9. Microscope.
10. Pipet aid
11. 200 and 1000 µL Pipetmen.

2.2. HIV-1 RNA Quantitation Assay (Roche Monitor Assay)

1. DEPC distilled water.
2. Trizma Base.
3. Concentrated HCl
4. Guanidinium thiocyanate.
5. Triton-X 100.
6. Silicon dioxide (Sigma).
7. 70% Ethanol.
8. Acetone (reagent grade >99% pure).
9. EDTA.
10. 0.2 *M* EDTA, pH 8.0: add 9 g EDTA to 121 mL DEPC distilled water and check pH.
11. L2 Buffer (2 L):
    a. Dissolve 24.22 g Trizma base in 1600 mL DEPC distilled water.
    b. Adjust pH to 6.4 with concentrated HCl (approx 15.5 mL).
    c. Cool to room temperature.
    d. Check pH and readjust to pH 6.4.
    e. Add DEPC water to 2000 mL.
    f. Store at room temperature in the dark.

12. L2 Washing Buffer (approx 2 L):
    a. Dissolve 1200 g GuSCN in 1000 mL L2 buffer. Heating to 60–65°C with shaking facilitates this process.
    b. Store at room temperature in the dark.
13. L6 Lysis Buffer (approx 1 L):
    a. Dissolve 660 g GuSCN in 550 mL L2 buffer (NOT washing buffer). Heating to 60–65°C with shaking facilitates this process.
    b. Add 121 mL 0.2 mL EDTA, pH 8.0
    c Add 14.3 g Triton-X-100
    d. Mix and store at room temperature in the dark.
14. Silica Reagent:
    a Place 60 g silicon dioxide in a 500 mL glass cylinder.
    b. Add dH_2O to 500 mL and allow to stand overnight at room temperature
    c. Remove supernatant (approx 430 mL) by suction.
    d Add dH_2O to 500 mL and shake vigorously to resuspend silica
    e. Let stand 5 h at room temperature.
    f. Remove supernatant (approx 440 mL) by suction.
    g Adjust pH to 2 0 with approx 400 µL of concentrated HCl.
    h. Dispense in glass containers and autoclave for 15 min
    i Store at room temperature in the dark.
15. Roche Monitor kit
16 2 mL Sarstedt tubes.
17. Vortex mixer
18 Microcentrifuge (≥12,000*g*).
19. Transfer pipets with thin tips.
20 Dry heating block at 56°C
21. Water bath.
22 Aspiration device.
23. pH Meter.
24 Mixer
25. P1000 and P200 pipettors.
26. Aerosol barrier pipet tips.

2.3. In House HIV-1 RNA Quantitation Assay

1. Tri-reagent (Molecular Research Center, Cincinnati, OH, TR 118.500)
2 D-PBS (GIBCO-BRL, Gaithersburg, MD, # 14190-144).
3. Chloroform (Sigma, St. Louis, MO, #C 6038 or #C 2432).
4. Glycogen (Boehringer Mannheim, Indianapolis, IN, #901-393).
5 70% Ethanol.
6. Carrier tRNA, 7.5 Kb poly A (GIBCO-BRL, # 15621-014). Store at –80°C.
7 Isopropanol.
8 RNasin (Promega, Madison, WI # N2514).
9 DTT (Boehringer Mannheim, #100-032)
10. BSA, Acetylated (Sigma, # B2518).

11 DEPC deionized water (Quality Biologicals, Gaithersburg, MD, 50-125-1);
12. RNA Suspension Buffer.
    a. Add 25 µL RNasin, 40 U/µL, and 10 µL 0.1 *M* DTT to 965 µL sterile water
    b. Store at –20°C or –80°C.
13 Poly A RNA Carrier:
    a. Add 1 µL of 1 µg/mL 7.5 Kb poly A RNA to 1 mL DEPC deionized water
    b Store at –80°C.
14. BSA Solution.
    a. Add 12.5 µL of 20 mg/mL BSA to 50 mL of D-PBS
    b Store at 4°C.
15 2 µg/mL Glycogen Stock:
    a Add 0.1 mL of 20 µg/mL Glycogen to 0.9 mL DEPC deionized water.
    b. Store at –80°C
16 1 5–2 mL Sterile microfuge tubes.
17 Pipet tips.
18. Fine-tipped transfer pipets
19. Gloves.
20. Microcentrifuge.
21 Water bath or heat block
22 Pipetmen.

3. Methods

3.1. Collection and Processing of Semen

1 The subject should refrain from sexual activity for 48 h prior to donation.
2 The subject should wash his hands and penis and then use an antiseptic towelet to wipe the head of the penis including the opening If the subject is uncircumcised, the foreskin should be pulled back before cleaning the head and opening.
3 The subject should masturbate and collect the specimen in a sterile container The time that the specimen was produced should be noted on paper work accompanying the specimen. The container should be placed in a zip-lock bag and then in a brown paper bag and transported to the clinic and then to the lab rapidly, within 4 h if possible
4 Allow liquefaction of the semen to occur. This typically occurs within 30–60 min of specimen collection
5 Transfer the sample to a conical centrifuge tube using a pipet and measure and record the volume of semen
6. Count the cells using the Endtz test *(19,20)* to document inflammation
    a. Place 20 µL of liquefied semen in a microfuge tube, add 20 µL phosphate buffered saline, and 40 µL of working benzidine solution
    b Vortex briefly and allow to sit at room temperature for 5 min.
    c. Count brown staining (peroxidase positive) cells in the four large squares of a hemacytometer
    d. Calculate and record the number of peroxidase positive cells/ejaculate = Brown cells in all 4 squares × 10^4. Inflammation is considered present when there are ≥ 1 × 10^6 peroxidase positive cells/mL of semen.

7 Centrifuge 600–800*g* for 10 min.
8. Remove supernatant, aliquot into 0 5 mL aliquots, and freeze at –70°C

3.2. Seminal Plasma HIV-1 RNA Quantitation Protocol

There are probably as many versions of this as there are investigators working in this field. One thing is clear: there are factors in seminal plasma that inhibit the PCR reaction unless they are removed. The standard Roche RNA isolation method does not remove these inhibitors *(9–11)*. Options are to use Boom's silica bead extraction assay *(21)*, use Organon Teknika RNA assays that include the silica bead extraction procedure, or pellet the virus using ultracentrifugation. Here are some of the options. (*See* **Note 1**.)

3.2.1. Organon-Teknika

Use Organon Teknika's NASBA assay following the manufacturer's instructions except use the diluted calibrators to get increased sensitivity at the lower end of the dynamic range, or use Organon-Teknika's NucliSens assay following instructions found in the package insert. (*See* **Note 2**.)

3.2.2. Roche Monitor Assay

3.2.2.1. Procedure

1 Add.. µL of Roche QS (Lot # ...) to 12 mL of L6 Lysis Buffer. (The amount of QS will depend on the particular lot number.)
2 Mix well by vortexing for 5 s and tilting tube several times
3 Aliquot 900 µL into each labeled 2.0 mL Sarstedt tube
4. Resuspend silica solution by vigorous mixing.
5 Add 40 µL to each tube of L6 Lysis buffer.
6 Vortex each tube until silica pellet is resuspended.
7 Add 200 µL of seminal plasma or control (Roche Monitor kit) and vortex immediately until solution is homogeneous (5–10 s).
8. Incubate at room temperature for 10 min.
9 Vortex for 5 s.
10 Centrifuge for 15 s at 12,000*g*. Aspirate supernatant with fine tipped transfer pipet and discard.
11 Wash **step 1**
   a Add 1 mL of L2 Washing Buffer and vortex until pellet is completely resuspended.
   b. Centrifuge for 15 s at 12,000*g*.
   c. Aspirate supernatant.
12 Wash **step 2**: repeat **step 11**
13 Wash **step 3**:
   a Add 1.0 mL 70% ethanol and vortex until pellet is completely resuspended This may be somewhat difficult

b. Centrifuge for 15 s at 12,000*g*.
c. Aspirate supernatant.
14. Wash **step 4**: repeat **step 13**.
15. Wash **step 5**:
a. Add 1.0 mL acetone and vortex well.
b. Centrifuge for 15 s at 12,000*g*.
c. Aspirate supernatant.
16. Evaporate acetone by incubating open tubes at 56°C for 10–15 min. Pellet should be dry.
17. Add 400 µL sample diluent (Roche Monitor kit) to each tube.
18. Vortex until pellet is resuspended.
19. Incubate 10 min at 56°C.
20. Vortex 5 s.
21. Incubate at 56°C for 10–20 min.
22. Vortex 5 s.
23. Centrifuge at 12,000*g* for 2 min to pellet silica. The supernatant contains the RNA.
24. Amplify immediately or store froze at –20°C until ready to proceed with the HIV Monitor Assay. If samples are frozen prior to amplification, thaw them, vortex to resuspend the silica, heat to 56°C 10 min, and centrifuge at 12,000*g* for 2 min before adding 50 µL of the RNA containing supernatant to the Roche PCR tubes.

3.2.3. In House Assay (see **Note 3**)

1. Transfer 0.5 mL seminal plasma to microfuge tubes containing 1 mL of PBS with 5 µg/mL pg BSA. As little as 200 µL of specimen can be processed. Note volume when less than 0.5 mL is used.
2. Pellet HIV virions from the seminal plasma by centrifugation at ≥14,000*g* for 60 min at 4°C.
3. Remove the supernatant using sterile, fine-tipped transfer pipets without disturbing the viral pellet.
4. Add 0.8 mL Tri-reagent and vortex.
5. Allow the mixture to stand for 5 min at room temperature.
6. Add 0.16 mL chloroform and vortex.
7. Let stand at room temperature for 3 min.
8. Centrifuge at 12,000*g* for 15 min at 4°C.
9. Transfer the upper aqueous phase to a snap-cap microfuge tube containing 0.16 mL chloroform.
10. Centrifuge at 12,000*g* for 5 min.
11. Remove and transfer the upper aqueous phase to a fresh snap-capped microfuge tube.
12. Add 5 µL 1 ng/µL carrier tRNA (7.5 Kb Poly A) and 5 µL of 2 µg/mL glycogen.
13. Vortex for 15 s.
14. Add 450 µL or equal volume of isopropanol.
15. Vortex for 15 s.
16. Place at –20°C overnight (or at least for 60 min) or at –70°C for 30 min.

17. Centrifuge at 12,000*g* at 4°C for 15 min.
18. Decant supernatant, being careful not to disturb the viral pellet.
19. Wash the pellet with 0.5 mL ice cold 75% ethanol.
20. Centrifuge at 12,000*g* at 4°C for 5–10 min.
21. Decant the supernatant, being careful not to disturb the pellet.
22. Air dry the samples for 20–30 min at room temperature. Do not heat the samples.
23. Resuspend the pellet in 50 µL RNA suspension buffer.
24. Incubate at 42°C for 15 min.
25. Vortex.
26. Place in wet ice for 5 min.
27. Centrifuge briefly.
28. Store at –70°C until assayed.
29. Proceed with in-house RT-PCR assay. (*See* Chapters 8 and 12.)

4. Notes

1. Attempting to increase the sensitivity of the RNA assays by increasing the specimen volume may prove difficult with the Boom extraction procedure, though it should not be a problem with the ultracentrifugation method.
2. Invalid results are immediately flagged by the Organon-Teknika software and are obvious in the Roche method by careful examination of the ODs for each series of wells.
3. Quality control is more problematic with in-house assays.

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IV

Molecular Technology for STD Diagnosis and Research: The New Challenges

14

Molecular Techniques for HIV and STDs

Implications for Research and Disease Control in the New Millennium

Rosanna W. Peeling, David C.W. Mabey, and King K. Holmes

1. Introduction

1.1. Paradigm Shift

Molecular techniques have gradually shifted the paradigm in the laboratory diagnosis of sexually transmitted infections from biological to molecular amplification. It is now possible to combine the sensitivity and specificity of culture with all the convenience of nonculture tests, such as ambient specimen transport, automation, and fast turnaround time. Pathogens that cannot be cultivated in vitro, such as the human papillomavirus (HPV), can now be detected and typed to determine if they have oncogenic potential. These powerful tools have improved and will continue to have a significant impact on our ability to design strategies and programs for the control and prevention of sexually transmitted infections worldwide.

It is estimated that approximately 50% of sexually transmitted infections are asymptomatic. Asymptomatic infections form a reservoir of infection that persists in a community. Individuals with asymptomatic infections often have a lower microbial load, hence requiring more sensitive detection techniques than conventional nonculture tests *(1,2)*. Now nucleic-acid-based amplification techniques offer the increased sensitivity to allow us to screen for these infections, interrupt the chains of transmission within the community, and decrease or eliminate the reservoir of infection.

Traditionally, screening can only be carried out in individuals who access the health care system. Those at highest risk of acquiring or transmitting sexually

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Table 1
The Paradigm Shift: Molecular Techniques for Screening and Diagnosis of STDs

Advantages	Disadvantages
sensitivity	high cost
use of noninvasive specimens	false positive (contamination)
ease of specimen collection	false negative (inhibition)
tolerance to specimen transport conditions	cannot monitor antimicrobial
multiplex testing	susceptibility
pooling of specimens for testing	
quantitation	
fingerprinting	

transmitted infections often do not exhibit good health seeking behavior and seldom access health care. The improved sensitivity of molecular amplification methods has facilitated changes in the types of specimens that can be used for testing. Thus screening can now be carried out in nontraditional settings where at-risk individuals can be targeted, irrespective of their health-seeking behavior. Noninvasive specimens such as urine can be used for the screening and laboratory diagnosis of genital chlamydial or gonococcal infections *(3–5)*. Saliva has been used successfully for the detection of antibodies to HIV *(6)*.

The increased patient acceptability of noninvasive specimens, the ease of specimen collection, and the tolerance of DNA and RNA to various specimen-transport conditions further increase the flexibility in the design of screening strategies. HIV RNA can be detected in whole blood lysates or dried blood spots, which are easily transported for field studies *(7,8)*. *Neisseria gonorrhoeae* can be detected in air-dried slides using a single-tube PCR assay *(9)*. Self-obtained vaginal or vulval swabs have been shown to be comparable to specimens taken in clinic by care providers for the diagnosis of genital chlamydial infections *(10)*. Innovative STD control programs can now be designed for active screening in nontraditional settings and even from home.

However, in spite of these advantages, molecular testing cannot be easily adopted by most laboratories (**Table 1**). The first and foremost reason is the cost. At present, these tests cost much more than other nonculture tests such as antigen detection tests. There are, however, cost effective means of utilizing this technology. Targeting molecular screening at populations with high risk behaviors or core groups will not only result in significant reduction in the reservoir of infection in the community but offers an opportunity to carry out molecular epidemiology studies on the dynamics of disease transmission within

these groups. The test format of molecular tests allows the simultaneous amplification of several pathogens from a single specimen in a single reaction. Such multiplex testing can result in tremendous savings in costs and time, both for care providers and for the laboratory. STD diagnostic panels based on clinical syndromes have been developed. A duplex PCR test is commercially available for the detection of *N. gonorrhoeae* and *Chlamydia trachomatis* in urine or genital swabs from patients presenting with urethritis or cervicitis *(11,12)*. A multiplex PCR reaction for the simultaneous detection of *Haemophilus ducreyi, Treponema pallidum*, and Herpes simplex Types 1 and 2 from genital ulcers has been shown to be more sensitive than standard diagnostic methods *(13)*. For large population-based studies, pooling of urine specimens for PCR testing can substantially reduce laboratory costs *(14)*.

Apart from economic reasons, this paradigm shift requires careful consideration of a number of issues. First, antimicrobial susceptibility testing cannot be performed without growing the isolate in culture. *N. gonorrhoeae,* which has been shown to develop antimicrobial resistance readily, is relatively easy and inexpensive to culture. The use of molecular tests for such STD pathogens may only be warranted if specimen transport is difficult. Under these circumstances, trends in antimicrobial resistance for the region need to be monitored by performing culture and antimicrobial susceptibility testing on isolates from patients who are at high risk of infection and those with a history of repeated infections. It is conceivable in the near future that resistance markers could be incorporated as probes into molecular diagnostic kits for the detection of antimicrobial resistance.

Second, inherent in the exquisite sensitivity of molecular amplification techniques is the risk of specimen to specimen contamination as well as environmental contamination of specimens, causing false positive results. The risk of run to run contamination can be reduced with the use of enzymes such as uracil *N*-glycosylase or physical methods such as ultraviolet irradiation *(15)*. Nested single-tube PCR techniques, which increase assay sensitivity without the necessity of post-amplification manipulations before the second round of amplification, have been developed to overcome problems of contamination. The preformulated PCR reaction mix for the second round of amplification can be dried in a trehalose matrix and embedded onto the inside of the cap of the PCR reaction tube. Nested single-tube PCR assays have been developed for *Haemophilus ducreyi, N gonorrhoeae,* and HPV *(2,9,16)*.

Third, field studies have shown that false negative results may be caused by the presence of inhibitors in different types of specimens. It is known that PCR and LCR reactions can be inhibited by a variety of substances such as phosphate and nitrite ions, detergents, heparin, and heme from bloody specimens. The nature of these inhibitors and how the inhibitory reactions can be over-

come are still being investigated *(17)*. Internal controls to identify inhibitory specimens are now included in some amplification assays.

Finally, can these molecular techniques replace culture for the detection of STD pathogens in specimens for medicolegal purposes? The specificity of molecular techniques should be in excess of 99% compared to culture because of the high fidelity of polymerases and other enzymes used in molecular amplification essays, provided appropriate precautions are taken to avoid false positive results due to contamination during laboratory processing. The rate of mismatching of base-pairs for *Taq* polymerase is estimated to be between 1 in 9,000 to 1 in 14,000 *(18)*. Thus, molecular techniques should be acceptable for medicolegal specimens if performed properly and confirmed with another test of similar sensitivity and specificity. An advantage of molecular techniques is that detection assays can be adapted to carry out DNA fingerprinting on the specimens, which may be useful for medicolegal cases.

1.2. Recent Technical Developments

A majority of the molecular amplification technology to date utilizes thermostable enzymes to amplify DNA under thermocycling conditions. The amplified products are then detected by gel electrophoresis or various colorimetric or chemiluminescent methods in an ELISA format. A major improvement on these systems is the concept of real time PCR, which accumulates signals of amplified products with each cycle of amplification using chemical or fluorescent probes. It is now possible to achieve single-tube amplification with real-time built-in detection during amplification, using 5' nuclease technology and fluorescent probes. With this type of system, the assay turnaround time is cut by half, and specimen to specimen or environmental contamination is minimized by the elimination of any postamplification manipulation. Such tools have been developed for the detection of Hepatitis C virus in serum *(19)*. Real-time quantitative PCR assays are also being developed using dual labeled fluorogenic probes *(20,21)*. These rapid systems will soon have wider application outside the research laboratory.

Molecular tests using isothermal amplification technology such as the transcription-mediated amplification (TMA) assay or nucleic acid sequence based amplification (NASBA) assay are now available for the detection of *C. trachomatis* and HIV-1 RNA *(22–24)*. These assays utilize enzymes capable of 10^5-fold amplification in 15 min under isothermal conditions. As RNA rather than DNA is amplified, these assays can be adapted for the detection of active infection with replicating organisms or for antimicrobial susceptibility testing. Other isothermal amplification techniques are continuously being improved. A thermophilic strand displacement amplification (SDA) assay has been described which is capable of 10^{10}-fold amplification within 20 min at 60°C,

and with the promise of real time detection *(25,26)*. Isothermal amplification reactions use enzymatic denaturation of DNA rather than heating to high temperature, hence eliminating the need for expensive machinery to carry out thermocycling. Isothermic reactions are, however, more susceptible to nonspecific amplification than amplification reactions requiring thermocycling.

With rapid advances in miniaturization technology and the availability of room-temperature stable reagents, the next challenge will be to package amplification reactions and the visual detection of amplified products in a miniature cassette for on-site testing. The development of simple rapid test kits may be on the horizon. Hand-held PCR technology, which has been in development as a research tool for field work, may eventually be available for disease control. The pressure for development of rapid miniaturized molecular diagnostic kits may also come from increasing public sector access to disease control information through audiovisual and electronic media and a growing and an often profitable market for technologically sophisticated point-of-care or home testing.

The main advantage of point-of-care testing is that a physician or health provider's diagnosis can be confirmed during the patient's visit. Thus, appropriate treatment can be initiated or prescribed, and, where the disease is reportable, reporting and contact tracing can be initiated without delay. The expedited management will likely result in lowering the risks of further disease transmission or long term complications of disease for the patient. Thus, the development of tests that combine the convenience and advantages of point-of-care testing with the sensitivity and specificity of molecular tests are desirable.

A 2-min erythrocyte agglutination test and a peptide impregnated test strip to detect HIV antibody in serum have been shown to perform well compared to ELISA kits *(27,28)*. But false positive results were obtained on a rapid on-site EIA test for HIV antibody when the temperature in the clinic was 3°C above the recommended temperature of 20–25°C *(29)*. Given the psychosocial and medicolegal implications of a positive diagnosis for a sexually transmitted disease, and the wide variety of conditions under which these kits may be used, it is important that there is adequate built-in quality assurance and extensive evaluation of the performance of these test kits.

1.3. The Role of Molecular Techniques for STD Diagnosis and Research

Early and accurate laboratory diagnosis is the cornerstone of an effective STD control program. The role of molecular tests in STD control can be considered at several levels. At the personal level, molecular tests provide the most sensitive and specific diagnosis that leads to treatment of the infected individual and the prevention of adverse sequelae. At the community level, molecular tests improve our ability to screen for infection as a means of inter-

rupting the chain of transmission and decreasing the disease reservoir within a population. At a public health level, molecular techniques can provide the most accurate laboratory-based data for epidemiologic surveillance. Accurate data and trends are important for the formulation of effective public health policy. Molecular techniques for DNA fingerprinting and typing will also improve our ability to provide outbreak investigation support and to discern sexual networks for effective behavioral intervention.

Approximately 90% of sexually transmitted infections worldwide are in settings where resources for laboratory testing are limited or where social and economic factors hinder STD control efforts *(30)*. In these settings, syndromic management is often the only option available to the health care providers. Thus, the utilization and impact of molecular diagnostic and screening tests on STD control and research are different for developed and developing countries.

2. Implications for Developed Countries

2.1. Disease Control

Cost-effectiveness studies have shown that despite the higher cost of nucleic-acid-based amplification tests, the increased sensitivity of these tests makes them more cost-effective than antigen detection tests for some STD control programs *(31)*. Screening is especially cost-effective for bacterial STDs such as genital chlamydial infections, where curative therapy is available and the long-term reproductive sequelae are serious and costly *(32)*. Unless a simple, rapid, sensitive, and inexpensive test such as the RPR for syphilis is available, universal screening for most STDs is neither cost effective nor warranted. Risk assessment based on age, marital status, and sexual behavior is therefore useful in targeting testing toward individuals most at risk of acquiring STDs. Molecular tests can be used to give the most accurate assessment of risk factors or markers to be used as criteria for screening. These criteria for risk assessment should be validated and continuously monitored in target populations using laboratory-based studies.

2.1. Patient Management

Molecular techniques can be adapted not only to detect infection but also to quantitate microbial load. For patients infected with HIV, quantitation of viral load by PCR, NASBA, and other RNA detection assays provide an important means of monitoring treatment efficacy and indirectly for the emergence of drug resistance *(33,34)*. Viral load may also be important for hepatitis B infections, where treatment is costly and is not uniformly effective *(35)*. Quantitation of HIV RNA and herpes virus in genital tract secretions have improved our understanding of subclinical shedding and the effect of antiviral therapy on the shedding of viruses at mucosal sites *(36,37)*. The ability to quantitate accu-

rately viral load in genital secretions is important for epidemiologic and public health reasons as genital secretion is a major vehicle of transmission of sexually transmitted infections.

2.3. Research

Molecular techniques have provided a better understanding of the epidemiology and the pathogenesis of sexually transmitted infections. This is especially true of noncultivable pathogens. Molecular epidemiologic tools to fingerprint isolates have been used to determine geographic clusters in outbreak investigations as well as disease transmission dynamics through sexual networks *(38,39)*. The identification of drug resistant plasmids and tracking of spread of resistance worldwide are possible only with molecular tools. Molecular techniques have also provided a better understanding of the pathogenesis of sexually transmitted infections through defining the molecular basis of antigenic variation and generation of mutants to delineating structure–function relationships. Such knowledge serves as an important basis for rational vaccine design and development *(40,41)*. DNA vaccines are being developed for a number of sexually transmitted diseases. Experimental studies in animals have shown that DNA vaccines may be promising for HIV, hepatitis B virus, and chlamydial infections *(42–44)*.

3. Implications for Developing Countries

3.1. Disease Control

In most developing countries, laboratory facilities for the diagnosis of STDs are limited or inadequate *(45)*. The WHO therefore advocates syndromic management, in which patients presenting with the common STD syndromes (e.g., urethral or vaginal discharge, or genital ulcer) are treated for all the likely causes of that syndrome at their first visit. This approach has a number of advantages (**Table 2**), and when implemented at the primary health-care level in Tanzania was shown to reduce substantially the incidence of HIV infection by reducing the duration of symptomatic STDs (which facilitate the sexual transmission of HIV) *(46,47)*.

However, if it is to be effective, syndromic management needs to be based on laboratory studies of the prevailing etiologies of the common syndromes and on the local antimicrobial susceptibility patterns, which may be specific to the population as well as geographical location *(48)*. Molecular diagnostic techniques such as the multiplex PCR for *T. pallidum, H. ducreyi*, and *H. simplex* can be extremely useful in designing locally appropriate syndromic management algorithms for genital ulcers *(13)*.

Whereas the WHO algorithms for syndromic management of urethral discharge in males, and for genital ulcers, have been widely and successfully used

Table 2
Syndromic Management

Advantages	Disadvantages
expedited management: no risk of further transmission and less complications	failure to detect atypical/subclinical infection
rapid	difficulties with contact tracing
convenient	cost and risks of overtreatment
low cost. no laboratory tests needed	
assurance of treatment for laboratory false negative cases	

In many developing countries *(49)*, the algorithm for vaginal discharge has been more problematic. At its simplest level, this algorithm recommends that all women complaining of vaginal discharge should be treated for all the likely causes (*Trichomonas vaginalis, Candida albicans*, bacterial vaginosis, *N. gonorrhoeae*, and *C. trachomatis)*. This has been shown to lead to very considerable overtreatment for *N. gonorrhoeae* and *C. trachomatis*, which are usually present in less than 10% of women with this complaint *(50–52)*, leading not only to a waste of scarce resources, but also to the potential for social problems for women who are mistakenly informed that they have an STD and asked to refer their partners for treatment. If a speculum is available, the appearance of the discharge and simple bedside tests such as vaginal pH, the KOH "whiff" test, or the microscopic examination of a wet preparation can help to establish the diagnosis of candidiasis, BV, or *T. vaginalis* infection, but the problem of identifying gonococcal and chlamydial infections remains. Overtreatment can be reduced by including a "risk assessment" step, but even then the sensitivity and specificity of such an algorithm for the identification of women with these infections is unlikely to exceed 70% *(53,54,56)*.

This problem will only be solved by the development of simple, rapid, inexpensive, and accurate bedside tests for the detection of *N. gonorrhoeae* and *C. trachomatis*, which will no doubt be possible thanks to some of the technological advances described in this book. The Rockefeller Foundation have offered a prize of $1 million for the development of such a test *(30)*. Although the prize is yet unclaimed, this initiative should stimulate more research into making this a reality.

The other, and greater, advantage of such a test would be for screening asymptomatic individuals. A high prevalence of asymptomatic gonococcal and chlamydial infections has been documented among both men and women in developing countries *(47,52–54,56)*. Data from community based studies in sub-Saharan Africa suggest that, owing to a combination of asymptomatic

infection, lack of access to treatment, inappropriate treatment seeking behavior and suboptimal case management, <10% of infected individuals are likely to receive adequate treatment *(57)*. Improved case management alone is unlikely to lead to effective STD control in these circumstances. The leukocyte esterase urinary dip-stick test has been used to screen for asymptomatic gonococcal and chlamydial infection, but recent studies have found that this test is neither sensitive nor specific for genital chlamydial infections when a combination of molecular tests and culture was used as the gold standard *(58,59)*.

3.2. Research

Molecular techniques can be used by national reference laboratories in developing countries to carry out epidemiologic studies to determine changes in the pattern of disease, to identify those at risk, and, hence, to target those most in need of services. Pooling of specimens for large population-based studies and multiplex testing can offer cost savings in time and expense. It should also be possible to use these strategies in well-defined studies to measure the impact of control programs.

4. Summary

In the last decade, there has been rapid and exciting progress in the development of molecular techniques for the screening and diagnosis of sexually transmitted diseases including HIV. Research studies using molecular tools have allowed us to learn more about the epidemiology of STDs in developed and developing countries and may give us a rational basis for the development of vaccines as well as of behavior intervention strategies. The increased sensitivity of molecular amplification techniques and the tolerance of DNA and RNA to various transport conditions means that noninvasive specimens, either self-obtained or obtained outside physician's offices, can be used for testing. These tests have been shown to be cost-effective for bacterial STDs where curative therapies are available. The cost of these tests may also be reduced through multiplex testing based on clinical syndromes, pooling of specimens for testing, or targeting their use in high risk populations. The stage is now set for the use of these tools in the design of innovative programs that will make a significant impact on the control and prevention of sexually transmitted diseases and their sequelae worldwide.

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Index

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