CHAPTER II
LITERATURE REVIEW

Neisseria gonorrhoeae

Biology

The genus *Neisseria* belongs to the family *Neisseriaceae,* which consists of the "true Neisseria" and three additional species. The "true Neisseria" colonizing humans are *N. gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea, N. polysaccharea, N. subflava, N. sicca, N. mucosa, N. flavescens* and *N. elongate*. *N gonorrhoeae,* which causes gonorrhea, and *N. meningitidis,* which causes meningitis or septicemia, both are the main human pathogens among the *Neisseriaceae*. They are genetically and morphologically similar. However, molecular, cellular and biochemical differences exist, which probably reflect the differences in the diseases that the two species cause. The other *Neisseria* species are often part of the normal flora in humans, but can cause opportunistic infections. *N. meningitidis* frequently colonizes the human mucosa without any symptoms or signs of disease. *N gonorrhoeae,* however, although it may cause no symptoms, is still considered a pathogen, and if it is found, should always be followed by antibiotic treatment (3, 21).

N. gonorrhoeae is a Gram-negative coccus, often seen in the microscope as diplococci shaped like coffee beans. The bacteria are aerobic organism, requiring pyruvate or lactate as an energy source, and do not grow without glucose. The amino acid cysteine is essential, and some strains also require other amino acids, pyrimidines or purines and are adversely affected by drying and fatty acids. The optimum growth temperature is 35°C to 37°C, with poor survival of the organism at cooler temperatures. A humid atmosphere supplemented with carbon dioxide (CO_2) is either required or enhances growth of *N. gonorrhoeae* (21). Although the fastidious nature of this organism makes recovery from clinical specimens difficult, it is nevertheless easy for the organism to be sexually transmitted from person to

person. The structure of *N. gonorrhoeae* is typical of gram-negative bacteria, with thin peptidoglycan layer sandwiched between the inner cytoplasmic membrane and the outer membrane. The outer surface is not covered with a true carbohydrate capsule, as is found in *N. meningitidis*. The cell surface of *N. gonorrhoea* however, has a capsule-like negative charge. Fresh clinical isolates have pili, which extend from the cytoplasmic membrane through the outer membrane. The pili are composed of repeating protein subunits (pilins), whose expression is controlled by the *pil* gene complex. Pili expression is associated with virulence, because the pili mediate attachment to non-ciliated epithelial cells as well as provide resistance to killing by neutrophils. Other prominent families of proteins are present in the outer membrane. The Por proteins (formerly protein I) are porin proteins that form pores or channels in the outer membrane. Two classes of Por protein (PorA and PorB) and the antigenic variation in Por proteins have been exploited for the serotype classification of *N. gonorrhoeae.* Opa proteins (opacity proteins or protein II) are a family of membrane proteins that mediate binding to epithelial cells. Multiple alleles of these proteins can be expressed by an individual isolate. Bacteria expressing the Opa proteins appear opaque and facilitate bacterial adherence to each other and eukaryotic cells. The third group of proteins in the outer membrane is the highly conserved Rmp proteins (protein III). These proteins stimulate antibodies which block serum bactericidal activity against *N. gonorrhoeae.* Another major antigen in the cell wall is lipo-oligosaccharide (LOS). This antigen is composed of lipid A and a core oligosaccharide, similar to gram-negative lipopolysaccharide (LPS), and possesses endotoxin activity. Other important gonococcal proteins are an immunoglobulin (Ig) A protease, which degrades secretory IgA, and β-lactamase, which degrades penicillin (22). *N. gonorrhoeae* has a small to medium sized genome of approximately 2.15 million base pairs. Plasmid content varies from strain to strain, and plasmids may also be absent. A cryptic plasmid is present in a majority of strains, but the plasmids can also be partly or fully integrated in the chromosome. Plasmids are known to carry antibiotic resistance genes (21).

The gonococcus, in the majority of cases, gains entry into the body by way of the genital tract. The type of mucosa found in a particular part of the genitourinary tract basically determines the susceptibility of that tissue to gonococcal infection (23).

Life cycle and pathogenesis

Neisseria gonorrhoeae occurs only within the human host. Apparently, the bacteria have developed resistance to the human antibacterial defense mechanism. The infection by gonococcus and its outcome depend to a large extent on the bacterial cell envelope constituents such as pili, outer membrane proteins Por, Opa, Rmp, lipo-oligosaccharide, peptidoglycan and also extracellular products: the IgA protease, therefore, probably helps the gonococcus to withstand mucosal immunity to some extent (24).

After entering the host, the gonococcus must manage to deal with the hostile mucosal environment, and in order to prevent elimination a rapid association with the epithelial cell-layer is necessary. Several factors influence the manner in which gonococci mediate their pathogenicity and virulence. Attachment of gonococci to mucosal cells is mediated in part by pili, which have two roles. (i) Proteins at the end of pili are necessary for attachment to membrane proteins on the surface of epithelial cells and also contribute to resistance by preventing ingestion and killing by neutrophils. The microbes are able to attach to sperm, and this capability may allow invasion into the upper urethra. The importance of pili is demonstrated by the fact that strains unable to express pili also lose their virulence. (ii) Pili are involved in exchange of genetic material through natural transformation because *N. gonorrhoeae* expressing pili readily take up DNA and incorporate it into their chromosome. This may explain their ability to rapidly develop drug resistance to the antibiotics that have been used against them. The Opa proteins influence colony morphology of the gonococcus via Opa-LOS interactions that occur between adjacent bacteria; therefore, the interaction of Opa with a target host cell may be modulated by specific LOS moieties expressed by a gonococcus,

(25) and is thus important for adhesion to hosts. Opa help in binding to different cell receptors on different types of cells in the body, including to macrophages. This last binding mechanism helps *N. gonorrhoeae* bypasses the classic phagocytic route into macrophages and prevents microbe destruction, allowing bacteria to survive and grow inside the phagocyte (26, 27). The bacteria enter the epithelial cells by a process called parasite directed endocytosis. During endocytosis the membrane of the mucosal cell retracts and pinches off a membrane-bound vacuole that contains the bacteria. The vacuole is transported to the base of the cell, where the bacteria are released by exocytosis into the subepithelial tissue. The *N. gonorrhoeae* are not destroyed within the endocytic vacuole (27, 28).

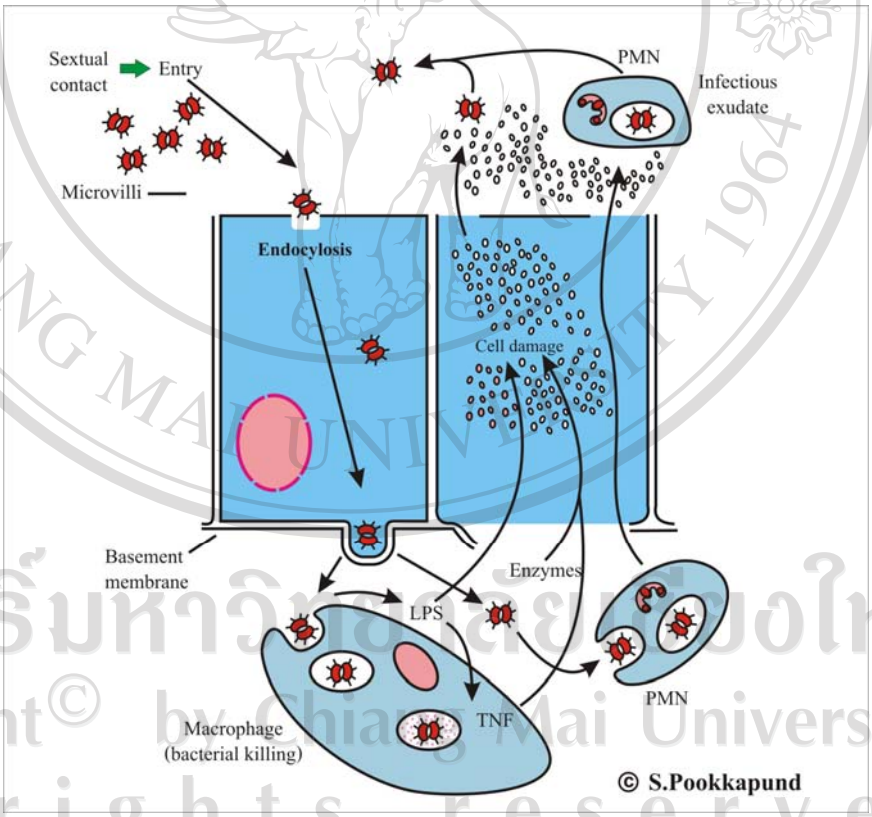

Figure 1 Illustration of *N. gonorrhoeae* life cycle and pathogenesis

Porin is the major outer-membrane protein (OMP) of *N. gonorrhoeae* and is closely related to the PorB OMP of *N. meningitidis.* The protein is

encoded by a single copy gene, *por,* which has 2 alleles, PIA and PIB (29). There are major differences in the protein and carbohydrate surface antigens of meningococci and gonococci; the meningococcus expresses two major porins, PorA and PorB, while the gonococcus is known to express only one, Por. The porins are diverse both within and among *Neisseria* species, particularly in those parts of the proteins predicted to form surface-exposed loops, indicating strong selection for variation in these proteins. Relationships among the Neisserial porins are not consistent with species divisions. For example, the meningococcal PorB proteins are more closely related to the gonococcal Por proteins than to the meningococcal PorA protein. Genes encoding one of two mutually exclusive porin families occur at both the gonococcal por and meningococcal porB loci. The most parsimonious explanation of these relationships is that porin genes have been exchanged among *Neisseria* species. The presence of a PorA gene in the genome of the gonococcus has been reported, which was an unexpected finding, as no gonococcal strain expressing a PorA protein has been described. Inactivating mutations in the promoter region and frameshift mutations in the coding region demonstrate that it is a pseudogene, which is almost identical in six unrelated gonococcal samples, comprising four isolates and two clinical specimens from patients with gonococcal infection. These findings are consistent with expression of the gonococcal porA gene being lost during the divergence of gonococci and meningococci (30-32).

The Por protein resembles a hydrophilic pore, allowing the passage of small nutrients and waste products across the outer membrane. With regard to the infection process, it has multiple functions. During infection, *N. gonorrhoeae* encounters the bactericidal action of PMNs, porin selectively interferes with the phosphatidylcholine dependent phospholipase C (PC-PLC) signaling machinery of PMNs, thereby inhibiting degranulation or phagosomal maturation while the NADPH-oxidase activity is not affected (33). Proin protein is thought to be the invasin that mediates penetration of a host cell. Each *N. gonorrhoeae* strain expresses only one type of Por; however, there are

several variations of Por that partly account for different antigenic types of the bacterium.

The pathogenic *Neisseria* possess within their outer membranes LOS molecules, which lack the repeating O-antigen sugar that comprises the polysaccharide side chain of LPS. This spontaneous conversion of oligosaccharide determinants can change the manner in which the gonococcus associates with host tissues and, hence, can potentially alter the course of gonococcal disease. LOS oligosaccharide side chains terminate in epitopes that mimic sugar moieties of mammalian glyco-sphingolipids. This form of molecular mimicry not only provides the bacterium with a method of immune avoidance but also allows the bacterium to use host-derived molecules that normally associate with the mimicked structure (25).

Gonococcal lipooligosaccharide (LOS) has toxic effects (endotoxin), for instance on epithelial cells. LOS as part of outer membrane blebs are constantly released by gonococci and this might facilitate the invasion of epithelial cells. During infection, bacterial lipooligosaccharide (LOS) and peptidoglycan are released by autolysis of cells. Both bacterial polysaccharides activate the host alternative complement pathway, while LOS also stimulates the production of tumor necrosis factor (TNF) that causes cell damage. Neutrophils are immediately attracted to the site and phagocytize the bacteria (24, 28). Gonococcal LOS produces mucosal damage, which then causes the release of enzymes, such as proteases and phospholipases, which are important in pathogenesis and are also involved in the resistance of *N. gonorrhoeae* to the bactericidal activity of normal human serum. Specific LOS oligosaccharide types are associated with serum-resistant phenotypes of *N. gonorrhoeae.*

Experimental infection of men has shown that the chemokine interleukin-8 (IL-8) and cytokines IL-6 and tumor necrosis factor alpha (TNF-α) are prevalent within the urethral lumen with progressive gonococcal disease. Thus, LOS elicits TNF- α, IL-1β, IL-6, and IL-8 secretion from primary urethral epithelial cells. Release of cytokines and chemokines from the urethral epithelium may potentially initiate the inflammatory response associated with gonococcal urethritis by triggering Neutrophil influx (25).

Strains of *N. gonorrhoeae* produce two distinct extracellular IgA1 proteases which cleave the heavy chain of the human immunoglobulin at different points within the hinge region. Split products of IgA1 have been found in the genital secretions of women with gonorrhea, suggesting that bacterial IgA1 protease is present and active during genital infection. It is thought that the Fab fragments of IgA1 may bind to the bacterial cell surface and block the Fc-mediated functions of other immunoglobulins (28).

Disseminated bloodstream infection with gonococci (DGI) often occurs in the absence of local genital inflammation and is caused predominantly by gonococci that resist killing by nonimmune normal human serum (NHS) independent of sialylation. Complement component C3, which is synthesized in the endometrial glandular epithelium, is present in functional amounts at the cervix and binds to gonococci in vivo. Serum-sensitize gonococci can abrogate killing by NHS by adding sialic acid to their LOS, which later permits direct binding of the complement regulatory protein factor H to sialylated LOS. Strains of *N. gonorrhoeae* that cause disseminated infections are usually resistant to complement and the serum bactericidal reaction. This accounts for their ability to persist in the bacteremia (34).

Virulence and Competence (21)

N. gonorrhoeae adhere to and invade human mucosal epithelia. In order to escape the human immune system, *N. gonorrhoeae* utilizes phase variation and antigenic variation. In phase variation the control of expression is on or off and in antigenic variation mutations lead to protein changes in the cell wall. The cell wall of *N. gonorrhoeae* is highly homologous to that of other Gram-negative bacteria. The outer membrane contains pili, opacity (opa) proteins, porins and lipooligosaccharides (LOS), all of which are involved in virulence.

The correlations between virulence and clinical strains, as well as loss of virulence due to in vitro passages, have been established as has a correlation

between colony morphology and virulence. Piliated *N. gonorrhoeae* are competent and naturally transformable, which means the bacterium is able to take up external genetic components. Sparling found that naturally occurring, virulent clones of *N. gonorrhoeae* had greater competence than laboratory strains, and reported transformation rates of up to 1 percent.

Type IV pili are long hair-like filaments on the surface of *N. gonorrhoeae.* They are involved in adherence, motility and host cell responses. Type IV pili from adhesive strains trigger mobilization of cytosolic free calcium in epithelial cells, which, in turn, is a signal known to control many intracellular processes. Following the initial pilus-mediated interaction, the bacteria adhere more tightly, invade epithelial cells, and disseminate into the bloodstream (35). Pili undergo phase variation and may be expressed or not. Type IV pili are composed of several components; the outer layer of the pilus is hypervariable and the core is highly conserved. Expression of pili is highly related to degree of competence.

The Opa proteins are expressed in about eleven *opa* loci. The name "opa" originates from the former name opacity proteins. Phase and antigenic variation in the *opa* genes changes the color and opacity of the gonococcal colony. Opa proteins or fimbrial adhesins are involved in uptake into host cells and mediate firm attachment of gonococci to epithelial cells (36).

The major outer membrane protein in *N. gonorrhoeae,* Porin B1, is important in pathogenesis and is involved in uptake of gonococci in epithelial cells. Its function is mainly to transport ions and small nutrients between the bacteria and the environment. When the gonococcal membrane is in intimate contact with the host cell membrane, the Por protein is transferred to the host cell, resulting in alterations in ionic permeability of the host cell plasma membrane (36). Unlike Opa proteins and type IV pili, PorB does not undergo phase variation, which means that PorB is always expressed during infection and under laboratory conditions.

Neisseria produce LOS, potent endotoxins that activate the immune response and cause host tissue damage. LOS in *Neisseria* equals lipopolysaccharides (LPS) in other Gram-negative bacteria. Endotoxin activity

of LOS thought to be responsible for most of the symptoms of gonorrhea. The oligosaccharide chains of LOS are similar in sequence and linkage with the oligosaccharides expressed on the surface of human cells. The terminal oligosaccharides of LOS molecules undergo rapid phase variation, which results in alterations in the expression of various LOS isoforms (37). Thus, termination of this gonococcus infection by the immune response is quite difficult.

Other virulence factors of *N. gonorrhoeae* are important, such as Reduction–modifiable protein (Rmp or Protein III), which associates with Por in the formation of pores at the cell surface so as to protect other surface antigens (Por-LOS complex) from bactericidal antibodies. Transferrin-binding proteins (Tbp1, Tbp2) and Lactoferrin-binding protein (Lpb) mediate acquisition of iron directly from transferrin and lactoferrin, which is significant for colonization of mucosal surfaces and to establish an infection. Despite the relative abundance of iron in the host, in serum and interstitial fluid iron is associated with transferrin and lactoferrin. As a result, gonococci must possess mechanisms for utilizing the iron associated with the host iron binding proteins as well as other potential in vivo iron sources, such as heme and hemoglobin (38).

Extracellular products of *N. gonorrhoeae* such as IgA 1 protease, which inactivates human secretory immunoglobulin by cleavage on the C-terminal side of specific residues in the hinge region, are examples of other virulence factors (39).

Epidemiology

Gonorrhea is one of the most common sexually transmitted disease. WHO estimated 62 million new case gonorrhoea infection in 1999 world wide. The estimated incidences have been substantially higher in developing nations than in developed nations, and the global number of new gonorrhoea cases among men and women 15 to 49 years of age was estimated at 62.35 million (40). The highest prevalence was in South and Southeast Asia (27.2 million) where 2.85 % of

the over 15 year old population is infected (1). In the United States, from 1975 through 1997, the national gonorrhea rate declined by 74% following implementation of the national gonorrhea control program in the mid-1970s. Gonorrhea rates subsequently appeared to plateau for several years. However, in 2006, rates increased slightly from 2004, with 358,366 cases of gonorrhea reported in the United States, which was 120.9 cases per 100,000 population, the first increase rate in gonorrhea since 1999 and an increase of 5.5% over 2005. Gonorrhea rates increased in 2006 for the second consecutive year (40, 41). The highest reported rates of gonorrhea were in women 15 to 19 years of age (715.6 per 100,000 population) and in men 20 to 24 years of age (589.7 per 100,000 population) and rates of gonococcal infection have increased among men who report having sex with other men. In a surveillance project of six sexually transmitted disease clinics in five U.S. cities, positivity of urethral gonorrhea was 21% for those who were HIV positive and 12% for those who were HIV negative (43). The prevalence rate of the disease varies greatly among countries in the developed and developing world, the highest being in south and Southeast Asia, followed by sub-Saharan Africa and Latin America, where it continues to be a major public health problem. The Regional STD Teaching, Training and Research Centre at New Delhi showed an increasing trend in prevalence from 14% to 19.4% between 1990 and 2001. Overall, the rate varied between 4.2% and 16.2% in the other STD clinics of the country. In Bangladesh, the incidence is around 35.5 %. The success of the HIV Control Program in Thailand has led to decline in gonorrhea by more than 80% (44).

In Thailand, the office of sexually transmitted disease, department of disease control, Ministry of Public Health has reported annual STDs data from 1967 to 1987 showing that the rate of *N. gonorrhoeae* infection significantly increased from 36,467 cases to 232,586 cases per 100,000 population. After the Thai government began an HIV-control program in 1989(45), the infection rate decreased to 62,457 cases per 100,000 population in 1992, and continued to decrease, with 30,000 and 10,509 cases per 100,000 population in 1994 and 1996 respectively. In 1998 the rate of gonococcus infection was 5,849 cases

per 100,000 population and the rate appeared to plateau for few years until 2002. Since then, the reported rate has slightly increased due to increased disregard for protection during sexual intercourse (46).

Risk factor and Transmission

Gonorrhea may be transmitted during sexual intercourse. As a result, all sexually active populations are at risk, and the level of risk rises with the number of sexual partners. Multiple episodes of sexual intercourse increase the risk of gonorrhea and STD infection, probably by increasing the number of contacts with an infected individual, and rates of infection also vary by the type of intercourse. For example, vaginal or rectal penetration is more likely to lead to STD infection than oral sex. Another STD infection may make gonorrhea infection easier (47).

Risk factors commonly demonstrated for gonorrhea include young age, low socioeconomic status, early onset of sexual activity, single marital status, a history of prior gonococcal infection, and same-sex activity among men. *N. gonorrhoeae* can be passed through intercourse, anal sex, and cunnilingus or even from an infected mother to a newborn infant during birth. The transmission is by contact of fluids from mucous membranes of infected individuals. Most frequently, the bacteria infect the man's urethra, the tube that carries urine and semen through the penis, and the woman's cervix, the canal into the uterus (48).

During sexual contact, the risk of transmission of *N. gonorrhoeae* from an infected woman to the urethra of her partner is approximately 20% per episode of vaginal intercourse and rises to 60-80% after 4 or more exposures. In contrast, the risk of male-to-female transmission is approximately 50-70% per contact. Transmission through penile-rectal contact is fairly efficient (22, 49).

Clinical Manifestations

The name 'gonorrhoea' derives from the Greek words *gonos* (seed) and *rhoia* (flow) and described a condition in which semen flowed from the male organ without erection.

N. gonorrhoeae is exclusively a human pathogen. Infection is generally limited to superficial mucosal surfaces lined with columnar epithelium. The areas most frequently involved are the urethra, cervix, rectum, pharynx, and conjunctiva. In the male, genital infection is primarily restricted to the urethra. The commonest clinical presentation of the disease is acute urethritis, dysuria and urethral discharge that may range from a scanty, clear or cloudy fluid to one that is copious and purulent. Symptoms may develop after a 2 to 5 days of incubation, after unprotected vaginal or anal sexual intercourse. Inflammation of the urethral tissues results in the characteristic redness, swelling, irritation, itching, burning urination, voiding powerless and dripping urine as well. Dysuria and a purulent penile discharge make most sufferers seek treatment rapidly. The causal organism may invade the prostate, resulting in prostatitis, or extend to the testicles resulting in orchitis. A few men have relatively minor symptoms which may disappear rapidly. Truly asymptomatic infection is rare in the active male. However, up to 5% may carry the organism as a major reservoir without apparent distress or increased risk for developing complications. Rectal and pharyngeal infection is less often symptomatic and may be discovered only after tracing contacts.

In females with vaginal infection, the primary site of infection is the cervix, because the bacteria infect the endocervical columnar epithelial cells. The organism cannot infect the squamous epithelial cells that line the vagina of post-pubescent women. Thus, the pre-pubescent vaginal epithelium, which has not been keratinized under the influence of estrogen, may be infected. Gonorrhea in young girls may present as vulvovaginitis. Mucosal infections are usually characterized by a purulent discharge and only 50 percent may have symptoms of cervicovaginal discharge, dysuria, lower abdominal pain and abnormal or intermenstrual bleeding. Most seek attention because of their

partner's symptoms, as part of contact tracing or screening of high-risk individuals. Symptoms of uncomplicated endocervical infection often resemble those of other conditions such as cystitis, vaginitis or cervicitis which early symptoms often not consciously such as tenderness, purulent secretions more often, genital itching, burning sensation, abdominal pain and low back pain under. These atypical symptoms so that patients often do not attendance treatment have thus become a major source of infection. However, asymptomatic carriage in women is more common, especially in the endocervical canal. At menstruation or after instrumentation, particularly termination of pregnancy, gonococci may ascend to the fallopian tubes and give rise to acute salpingitis, tubo-ovarian abscesses which may be followed by pelvic inflammatory disease (PID) in 10% to 20% of women with a high probability of sterility if inadequately treated. Peritoneal spread occasionally occurs and may produce a perihepatic inflammation. Disseminated infection is seen more commonly in women and may present as painful joints, fever and hemorrhagic skin lesions on the extremities; this occurs in 1 % to 3 % of infected women and in a much lower percentage of infected men. The site of infection also determines whether carriage occurs, with rectal and pharyngeal infections more commonly asymptomatic than genital infections. Other diseases associated with *N. gonorrhoeae* are perihepatitis, purulent conjunctivitis particularly in newborns infected during vaginal delivery (ophthalmia neonatorum) or ocular gonococcal infections in adults who become inoculated with infected genital secretions, orogenital/anogenital gonorrhea in women and homosexual men (22, 50-52).

Proctitis or rectal infection by gonorrhea is defined as inflammation of the rectal mucosa extending up to 15 cm. into the sigmoid colon. Rectal gonorrhoea is common and asymptomatic in a high proportion of people; patients may have a sexual history taken to establish whether anorectal intercourse has occurred. The most common symptom of proctitis is a frequent or continuous urge to have a bowel movement, anorectal pain or discomfort, anal discharge, which may be purulent, mucoid or bloodstained, tenesmus, urgency of defecation, rectal bleeding and constipation. Proctocolitis presents

with the symptoms of proctitis and diarrhea, abdominal pain, and bloating. Systemic symptoms such as fever may also occur (53, 54).

Between 30% and 50% of infants exposed to gonococci will develop ophthalmia in the absence of treatment. Gonococcal ophthalmia can cause severe conjunctivitis (eyelid redness, purulent secretions) and lead to corneal scarring, abscess, eye perforation, and permanent blindness (52).

Laboratory Diagnosis

N. gonorrhoeae may be diagnosed presumptively by direct visualization on a Gram stain smear of secretions from a compatible clinical site, by growth in culture or detection by antigens, or by a non-amplified DNA probe or nucleic acid amplification tests (NAATs).

Specimen collection and transport

Since *N. gonorrhoeae* can cause infection at a variety of body sites, the collection of appropriate specimens for culture and diagnosis is dependent on the sex and sexual practices of the patient and on the clinical presentation. In all cases, specimens from genital sites (male urethra, female endocervix) should be collected. If the patient has a history of orogenital or anogenital sexual contacts, appropriate oro-pharyngeal or anal canal specimens are also collected. Specimens should be collected with Dacron or rayon swabs. Some lots of calcium alginate may be toxic to certain gonococcal strains. Cotton swabs may also be used. Some brands of cotton contain fatty acids that may be inhibitory for gonococci; therefore, calcium alginate and cotton swabs should be used only if the specimen is inoculated directly onto growth media or transported in non-nutritive media containing charcoal to adsorb or neutralize inhibitory materials. Although maximal recovery of gonococci is obtained when specimens are plated directly onto growth medium after collection, this technique might not always be possible or practical, particularly in busy clinics or hospital emergency departments. For these situations, Stuart's or Amie's buffered semisolid transport medium can be used for the transport of swab specimens for *N. gonorrhoeae.* However, there is a decrease in the

numbers of viable organisms with time. With these systems, long delays in transport and exposure to extremes in temperature (e.g., refrigeration) may compromise successful recovery of the organism. Transport of specimens on culture media presents certain advantages and several systems for this purpose are commercially available. Varieties of enriched selective media for culture of *N. gonorrhoeae* are available and include modified Thayer-Martin (MTM) medium and Martin-Lewis (ML) medium. These media allow selective recovery of *N. gonorrhoeae* from body sites harboring a large endogenous bacterial flora (51).

Microscopy

Gram stain is a rapid tool and has comparable sensitivity to bacterial culture for symptomatic urethral gonorrhea in men. This method is very sensitive (greater than 90%) and specific (98%) in detecting gonococcal infection in men with purulent urethritis. However, its sensitivity in detecting infection in asymptomatic men is only 60% or less. The test is also relatively insensitive in detecting gonococcal cervicitis in both symptomatic and asymptomatic women, although a positive result is considered reliable when an experienced microscopist sees the gram-negative diplococci within polymorphonuclear leukocytes. Thus, the Gram stain can be reliably used to diagnose infections in men with purulent urethritis, but all negative results in women and asymptomatic men must be confirmed by culture or another tests.

Gram stain is also useful for the early diagnosis of purulent arthritis but is insensitive for the detection of *N. gonorrhoeae* in patients with skin lesions, anorectal infections, or pharyngitis. Commensal *Neisseria* species in the oropharynx and morphologically similar bacteria in the gastrointestinal tract can be confused with *N. gonorrhoeae* (22).

Isolation and identification through culture

N. gonorrhoeae can be readily isolated from genital specimens if care is taken in collecting and processing the specimens. Because other commensal organisms normally colonize mucosal surfaces, all genital, rectal, and pharyngeal specimens must be inoculated onto both selective media (e.g., modified Thayer-Martin medium) and nonselective media (e.g., chocolate

blood agar) in 5% CO_2 at 37°C over night. Selective media suppress the growth of contaminating organisms. A nonselective medium should also be used, however, because some gonococcal strains are inhibited by the vancomycin present in most selective media. The organisms are also inhibited by the fatty acids and trace metals present in the peptone hydrolysates and agar in other common laboratory media (e.g., blood agar, nutrient agar). The gonococci die rapidly if specimens are allowed to dry. Therefore, drying as well as cold temperatures should be avoided through direct inoculation of the specimen onto prewarmed media at the time of collection.

The endocervix must be properly exposed to ensure that an adequate specimen is collected. Although the endocervix is the most common site of infection in women, the rectal specimen may be the only one positive for gonococci in women who have asymptomatic infections as well as in homosexual and bisexual men. Blood culture results are generally positive for gonococci only during the first week of the infection in patients with disseminated disease. In addition, special handling of blood specimens is required to ensure the adequate recovery of gonococci because supplements present in the blood culture media can be toxic to *Neisseria.* Culture results of specimens from infected joints are positive for the organism if the specimens are collected at the time the arthritis develops, but skin specimen cultures are generally unrewarding. *N. gonorrhoeae* is identified preliminarily on the basis of the isolation of oxidase-positive, growth on chocolate blood agar or on media that are selective for pathogenic *Neisseria* species. Definitive identification is guided by the detection of acid produced oxidatively from glucose but not from other sugars (22). Superoxol is another helpful test for the rapid presumptive identification of *N. gonorrhoeae.* Superoxol is 30% hydrogen peroxide (not the 3% solution routinely used for the catalase test). *N. gonorrhoeae* strains produce immediate, brisk bubbling when some of the colony material is emulsified with the reagent on a glass slide. *N meningitidis* and *N. lactamica,* the other species that grow on selective media, produce weak, delayed bubbling (51).

Immunological methods for culture confirmation of *N. gonorrhoeae*

The Fluorescent monoclonal Antibody test, which is used to identify *N. gonorrhoeae* by fluorescent antibody (FA) techniques, is fast and can identify both living and nonviable organisms. The FA procedure uses monoclonal antibodies that recognize epitopes on Porin protein, the principal outer membrane protein of *N. gonorrhoeae*. The commercial monoclonal FA test *(N. gonorrhoeae* Culture Confirmation Test, Syva Co, Palo Alto, CA) is performed by preparing a light suspension of the organism in 50 μL of water on an FA slide, allowing the suspension to dry, heat fixing the specimen, overlaying the smear with the FA reagent, and incubating the smear for 15 minutes. The smear is rinsed, air dried, mounted with a coverslip, and examined with a fluorescence microscope. Gonococci appear as apple-green fluorescent diplococci. The advantages of the Syva FA test include its rapidity, the ability to test colonies directly from primary cultures, and the small amount of growth required for test performance. However, the Syva FA test is not intended for direct detection and identification of organisms on smears from patient specimens (51).

Coagglutination test.

These tests make use of the ability of Protein A on *Staphylococcus aureus* cells to bind immunoglobulin G molecules by their Fc region. Binding of anti-gonococcal antibody to killed *S. aureus* cells and subsequent mixture with a suspension of gonococci causes visible agglutination of the suspension. Coagglutination tests for the identification of *N. gonorrhoeae* include the Phadebact GC OMNI (Karo-Bio Diagnostics AB, Huddinge, Sweden), the GonoGen I test (New Horizons Diagnostics, Columbia, MD),and the Meritec GC test (Meridian Diagnostics, Cincinnati, OH).

Molecular methods (55, 56)

Accurate and rapid diagnosis is important to implement adequate disease treatment and to prevent the spread of infectious disease. An infectious disease diagnostic assay should provide rapid, highly sensitive, and specific results. An insensitive assay can result in misdiagnosis of a truly infected

patient, leading to lack of treatment and spread of disease. A nonspecific assay can result in false diagnosis of disease in a healthy patient. Absence of reliable diagnostic methods resulting in erroneous diagnosis may have serious consequences, both to the individual patient and to public health in general. Recent developments in molecular diagnostic methods have made possible more sensitive, specific tests, which do not depend on the presence of viable microorganisms and which allow the use of several non-invasive sample collection methods and rapid diagnostic tests for infectious disease compared to traditional microbiology techniques. Evolution of diagnostic methods for detection of bacterial infections such as

a). Nucleic acid tests (NATs) methodologies

Non-amplified NATs utilize nucleic acid probes that are specific for a unique nucleic acid sequence, called the target sequence, present in the target organism. Probes are usually labeled with a fluorescent or chemiluminescent marker. The sample is treated to release nucleic acids from the target organism. If present, the labeled DNA probe specifically combines with the target sequence to form a stable probe-target sequence hybrid. The hybrid is separated or discriminated from non-hybridized probes, and the signal emitted by label in the hybrid is measured. NATs use various ways to increase the analytical sensitivity of the tests for direct detection of microorganisms in clinical specimens. Hybridization assays such as the Gen-Probe PACE II (Gen-Probe, San Diego, CA) target ribosomal RNA (rRNA), which exists in thousands of copies in most microorganisms; this greatly increases the sensitivity of the assay as there is so much more target molecule available to form hybrids. The rRNA targeting technology led to the first DNA probe tests widely used in the clinical laboratory for both culture confirmation, as well as direct detection. Digene Hybrid Capture II assays (Digene Corp., Beltsville, MD) increases the sensitivity of DNA probe assays by amplifying the signal molecules. By using antibodies that bind to RNA-DNA hybrids to detect hybrid formation, each antibody carries an enzyme label that is used to generate a colored product. Each enzyme molecule makes multiple colored product molecules for each hybrid molecule to which it binds. Multiple signal

molecules are generated from one hybrid molecule. These are known as "signal-amplification" methods. Signal-amplification methods are usually significantly less sensitive and specific than an improved assay method known as target amplification.

b). Nucleic acid amplification tests (NAATs) methodologies

Target-amplified nucleic acid amplification tests (NAATs) increase the assay sensitivity by producing millions of copies of the target sequence in a test tube, similar to the way culture increases detection sensitivity by producing more copies of the target DNA or RNA inside the bacteria as they multiply. Target amplification, however, is much faster than cell culture; it can be used to detect bacteria that are difficult or impossible to grow in the laboratory. The replicated sequences, or "amplicons," are usually identified using labeled DNA probes. Several target-amplification methods are currently in widespread use for the identification of gonococcal infections.

There are four main commercial *N. gonorrhoeae* NAAT assays, the Roche Cobas Amplicor (Roche Molecular Systems, Branchburg, NJ), the Gen-Probe APTIMA Combo 2 (AC2; Gen-Probe), the Becton Dickinson ProbeTec assay (Becton Dickinson, Sparks, MD) and the Abbott Ligase Chain Reaction (LCx) (Abbott Laboratories). These assays target sequences present in genomic DNA or in plasmids that are usually, but not always, present in the organisms.

Treatment

The CDC's treatment guidelines for uncomplicated gonococcal infections are Cefixime (Suprax) 400mg orally, Ceftriaxone (Rocephin) 125 mg intramuscularly, Ciprofloxacin (Cipro) 500 mg orally, Levofloxacin (Levaquin) 250 mg orally and Ofloxacin (Floxin) 400 mg orally. All medications are administered as a single dose. Nevertheless, Quinolone-resistant *N. gonorrhoeae* (QRNG) continues to spread, making the treatment of gonorrhea with quinolones such as ciprofloxacin inadvisable in many areas

and populations. Resistance to ciprofloxacin usually indicates resistance to other quinolones as well. Quinolone-resistant *N. gonorrhoeae* is common in parts of Europe, the Middle East, Asia, and the Pacific; in the United States, QRNG is becoming increasingly common. Previously, the CDC had advised that quinolones must not be used in California and Hawaii because of the high prevalence of QRNG in these areas. Thus, fluoroquinolones should not be used in geographic areas of high resistance or in men who have sex with men (57).

Patients infected with *N. gonorrhoeae* frequently are co-infected with *C. trachomatis*; this finding has led to the recommendation that patients treated for gonococcal infection also be treated routinely with a regimen that is effective against uncomplicated genital *C. trachomatis* infection. Because the majority of gonococci in the United States are susceptible to doxycycline and azithromycin, routine co-treatment might also hinder the development of antimicrobial-resistant *N. gonorrhoeae* (20).

Chlamydia trachomatis

Biology (14, 22, 58, 59)

Chlamydia trachomatis is one member of genus *Chlamydia* in the Chlamydiaceae family. The genus *Chlamydia* is a well defined group of prokaryotic organisms, characterized as small Gram negative cocci which contribute to prevalent sexually transmitted, ocular and respiratory tract infectious diseases in humans. The organisms live an obligate intracellular parasitic existence, with two distinct forms that share a common group antigen. The two distinct forms of the organism are termed the elementary body (EB) and the initial or reticulate body (RB). The elementary body is the smaller particle, of around 300 nm in size. This is the extracellular transport form. The bacteria do not replicate in the EB form even though EB is highly infectious and characteristically stains bluish-red with Giesma stain. The cell wall is a rigid trilaminar structure; analogous to the cell wall of Gram-negative

bacteria and much like a spore, the EB is resistant to many harsh environmental factors. The reticulate body is between 800 - 1200 nm in size. The RB is the metabolically active, replicating chlamydial form in the cytoplasm of the host cell and stains bluish with Giemsa stain. Chlamydiae are highly adapted to their intracellular life. They may well have derived from other bacteria which led an increasingly intracellular existence, overcoming the efforts of the host cell phagocytic systems to destroy them. Certainly the almost total absence of biochemical mechanisms for producing energy would alone restrict them to an intracellular existence. Other important structural components of Chlamydiaceae are a genus-specific LPS or lipopolysaccharide that can be detected in a complement fixation (CF) test and species- and strain-specific outer membrane proteins.

The genus Chlamydia contains 3 species that infect humans, including *C. pneumoniae*, *C. psittaci*, and *C. trachomatis* which cause several distinct clinical syndromes in humans including trachoma, urogenital infections, conjunctivitis and lymphogranuloma venereum (LGV). Moreover, *C. trachomatis* has been subdivided into two biovars, trachoma and LGV (lymphogranuloma venereum). The biovars have been farther divided into 19 serotypes on the basis of antigenic differences in the major outer membrane protein (MOMP). The LGV biovar consists of 4 serovars (L1, L2, L2a, and L3); the remaining 15 serovars (A, B, Ba, C, D, Da, E, F, G, Ga, H, I, la, J, and K) are in the trachoma biovar. Meanwhile, *C. pneumoniae* and *C. psittaci* are etiological agents mostly of respiratory infections.

There is an enormous amount of information available on the cellular biology of *C. trachomatis*. The first sequenced chlamydial genome consists of a 1,042,519-base pair chromosome (58.7% A+T) and 5 to 10 copies of a well conserved plasmid, pCT, of approximately 7,493-base pairs (available at GenBank under accession number AE001273). Analysis of the genome sequence shows that *C. trachomatis* encodes only 894 proteins. The genome contains genes that are homologous with those encoding virulence factors in other bacteria. The chlamydial plasmid, in which the sequence is highly conserved among different isolates of *C. trachomatis,* has great practical

importance as it can be used as a target for DNA-based diagnosis of *C. trachomatis* infection. This is because there are approximately 7-10 copies of the plasmid present per chlamydial particle. Use of a multi-copy gene is a built-in amplification factor enhancing the possibility of detecting an individual particle (60-63).

Counterparts of enzymes characterized in other bacteria have been identified in *C. trachomatis*; these account for the minimal requirements for DNA replication, repair, transcription and translation (60).

The identification of genes in chlamydiae encoding a type three secretion (tts) system provides a newly understood mechanism by which chlamydiae might interact with the host cell. The tts system functions as a kind of molecular syringe, enabling gram-negative bacteria to inject virulence-related proteins into the cytoplasm of host cells. The tts system differs from all other bacterial secretion mechanisms in that it requires and is triggered by the intimate contact of the bacterium with the host cell membrane (64).

A conserved chlamydial protease, proteasome-like activity factor (Cpaf), is secreted into the host cell cytoplasm, where it interferes with the assembly and surface expression of human leukocyte antigen molecules and inhibits apoptosis. Also, a family of polymorphic membrane proteins (Pmps) is thought to function as adhesins, and a large number of related proteins (Inc proteins) are located in the inclusion membrane and probably regulate traffic between the inclusion and chlamydial cells (62).

Strains of *C. trachomatis* are antigenically variable and can be classified into serovars. The predominant antigen that defines individual serovars is the major outer membrane protein (MOMP), the main surface antigen of *C. trachomatis*. The single-copy gene *omp1* encodes for MOMP and consists of five regions of conserved sequence that alternate with four variable regions. The *omp1* variable domains can be used for genotyping isolates due to their extensive genetic variation. There is a high degree of concordance between the serological classification and *omp1*-based genotypes, with 19 currently identified genotypes (65).

Chlamydial development cycle

C. trachomatis replicate by means of a unique growth cycle that occurs within susceptible host cells. The cycle is initiated by the attachment of the infectious EBs near the base of microvilli, followed by active endocytosis by the host cell via the receptor into the tight endocytic vesicles. After they are internalized, the bacteria remain within cytoplasmic phagosomes, where the replicative cycle proceeds. The fusion of cellular lysosomes with the EB-containing phagosome and subsequent intracellular killing is inhibited. Phagolysosomal fusion is prevented if the outer membrane is intact. If the outer membrane is damaged or the bacteria are inactivated by heat or coated with antibodies, phagolysosomal fusion occurs with subsequent bacterial killing. Within 6 to 8 hours after entering the cell, the EBs reform into the metabolically active RBs, which are able to synthesize their own DNA, RNA, and protein but which lack the necessary metabolic pathways to produce their own high-energy phosphate compounds. The RBs are replicate by binary fission and are tightly associated with the inclusion membrane throughout their development, possibly using pores in the membrane to access directly the cytoplasm for nutrients such as host-derived ATP. The phagosome with accumulated RBs, called an inclusion, can be readily detected by histologic stains. Approximately 18 to 24 hours after infection, the RBs begin reorganizing into the smaller EBs, and between 48 and 72 hours, the cell ruptures and then releases the infective EBs (66-68).

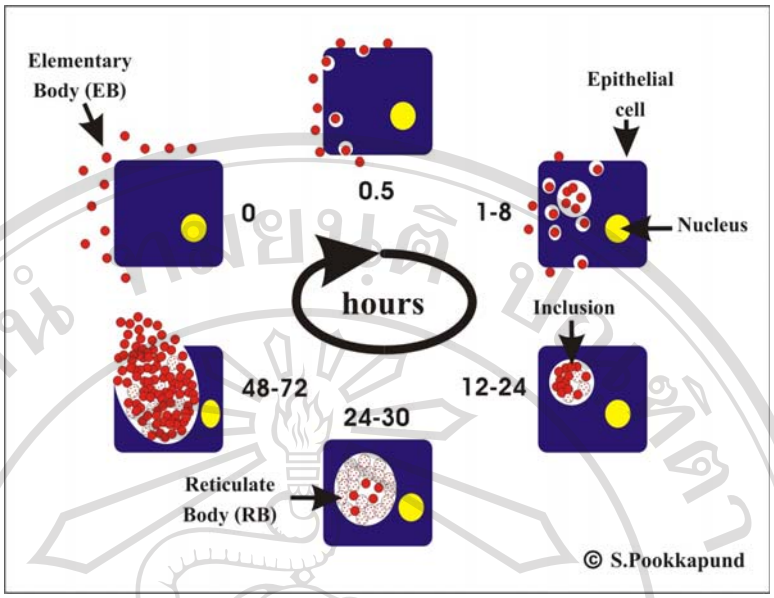

Figure 2 Illustration of the life cycle pathogenesis of *C. trachomatis*

Epidemiology

Prevalence and incidence (67, 69-71).

C. trachomatis is the most common sexually transmitted pathogen of humans, with an estimated 89 million new cases occurring world wide each year. In 2004, 50 states and the District of Columbia in the USA reported 929,462 cases of *C. trachomatis* infection to the CDC. This translates into a case rate of 319.6/100,000 population, which is a 5.9% increase compared to 2003. The reported numbers were more than two and half time that of gonorrhea (330,132 in 2004). The reported numbers were more than twice that of gonorrhea (335,104, 116.2/100,000 populations). Rates of chlamydial infections in women are over three times higher than rates in men (485/100,000 populations for women versus 147.1/100,000 populations for men); this most likely reflects more intense screening in women and sex partners of women infected with *C. trachomatis* not being tested. Indeed, rates in men have increased 47.7% from 2000 to 2004. The highest rates are in women between the ages of 15-19 followed by women 20-24.This is thought

to under represent the true incidence of *Chlamydia*, which is estimated at 3-4 million cases per year.

In Europe there are an estimated 10 million new cases of chlamydial genital tract infection per year with the prevalence of *C. trachomatis* infection in unscreened asymptomatic women ranging from 1.7 - 17% depending upon the setting, context and country.

In Thailand, the office of sexually transmitted disease, department of disease control, Ministry of Public Health report for 1967 to 2004 estimated the rate of Non-Gonococcal Urethritis (NGU), with approximately 40% of cases being due to infection with *C. trachomatis* (72). The rate of NGU in Thai population increased from 11,859 to 90,671 per 100,000 population in the years 1967 to 1987. After the Thai government implemented its HIV-control program in 1989 (45), the rate of NGU decreased to 39,126 in 1992 and continued to decrease from 1994, with 24,758 per 100,000 to 4,373 per 100,000 in 2003. However the incidence of NGU was increased slightly in 2004, with a rate of 5,044 per 100,000 (46).

Risk Factors (14, 53, 73-75)

Risk factors independently associated with chlamydial infection include having vaginal sex, being a female less than 25 years old, multiple sex partners, high frequency of sexual intercourse, use of non-barrier or oral contraceptives, cervical douching, being African-American, and low socioeconomic status. Chlamydial infection during pregnancy may be transmitted to the infant during delivery. An infant born to a mother with active infection has a risk of acquiring the infection.

In recent years, there have been numerous reports of increasing risk taking during sexual behavior, particularly of unprotected anal intercourse. It has been established that unprotected anal intercourse is the highest risk activity associated with sexual transmission of HIV and that the presence of a concomitant STI greatly increases this risk.

Transmission (14, 70)

Chlamydial infection is highly transmissible, with 65-70% of exposed sex partners concurrently infected. Transmission rates are probably similar between genders. The exact rates are unknown, but can be as high as 68% from men to women. . Chlamydia infection is transmitted in two ways, from one person to another by close personal contact such as through sexual intercourse or from mother to child during the passage of the child through the birth canal.

Since chlamydia can be transmitted by oral or anal sex, men who have sex with men are also at risk for chlamydial infection.

Clinical Manifestations

Non-Gonococcal Urethritis (NGU)

Urethritis, or inflammation of the urethra, is a multifactorial condition which is primarily sexually acquired. The diagnosis of urethritis is confirmed by demonstrating an excess of polymorphonuclear leucocytes (PMNLs) in the anterior urethra. Urethritis is described as either gonococcal, when *N. gonorrhoeae* is detected or non-gonococcal (NGU) when it is not. Urethritis are often due to infection with *C. trachomatis,* which causes approximately 40% of cases.

C. trachomatis is the commonest cause of NGU, accounting for 30-50% of cases. Between 20-30% of men with NGU have no organism detected. Asymptomatic urethritis, without an observable discharge, may have a different etiology from symptomatic urethritis, with *C. trachomatis* being detected less frequently. Burning during or after urination is the primary symptom. There may also be a discharge visible at the tip of the penis, especially early in the morning. Symptoms usually appear within 3-21 days after exposure and may last only a few hours or days. Even if the symptoms go away spontaneously, the infection will still be present and treatment is needed (76).

Trachoma

In hyperendemic areas, trachoma is caused by serotypes A, B, Ba and C of *C. trachomatis*. Transmission is from eye to eye, often via vectors such as flies, and the disease is always worse in poor countries. Trachoma begins in

childhood, and young children contribute most to the reservoir of chlamydial infection. The clinical features of trachoma are acute or subacute conjunctivitis with diffuse hyperaemia, oedema and conjunctival infiltration, followed by the appearance of lymphoid follicles initially on both the lower and upper tarsal conjunctivae and, in the chronic stage, trachoma follicles are readily visible, and corneal infiltration and pannus increase to a maximum. Cicatrisation occurs to a variable extent, and if involvement of the upper lid is severe this may lead to entropion and corneal ulceration. The inflammatory process may leave a permanently damaged eye (58).

Urogenital infection

Urogenital infections with *C. trachomatis* in women have a clinical course varying from asymptomatic to various complications. *C. trachomatis* infections leading to pelvic inflammatory disease are associated with late ectopic pregnancy and tubal infertility. In men, urogenital *C. trachomatis* infection causes urethritis. Ascending infections (epididymitis) are rarely seen (77). Clinical manifestations are often found in females who experience vaginal discharge, dysuria and post-coital or intermenstrual bleeding. Less frequent manifestations include urethral syndrome (dysuria and pyuria), bartholinitis, perihepatitis and proctitis.

Lower genital tract infection in men

Chlamydial genital tract infections are reproductive tract infections. Symptomatic chlamydial urethritis present with dysuria, have a whitish, grey or sometimes clear urethral discharge. This discharge is usually most prominent in the morning. The incubation period for chlamydial urethritis is approximately 7 - 14 days (78). Additionally, *C. trachomatis* is often the cause of post gonococcal urethritis, which occurs when gonococcal infection is treated with an antibiotic regime that does not simultaneously eradicate any co-incident chlamydial infection; multiple infections with agents of urethritis is common (79).

Upper genital tract infection in men

Half of all men experience symptoms of prostatitis at some time in their lives, usually when they are 50 years old or more. The symptoms of prostatitis (inflammation of the prostate) include back pain, discomfort when

passing urine or at ejaculation, and sometimes an ache in the region immediately behind the penis. The association of prostatitis with raised semenal white blood cells suggests that infection may be caused by bacteria such as chlamydiae (80).

Epididymitis

Epididymitis presents as pain in the affected testicle. Swelling and tenderness begin in the lower pole of the epididymis but as the epididymis is lined with a single cell layer of susceptible columnar epithelium, frequently involves the whole of the epididymis and adjacent testicle. Marked swelling may make the two testes difficult to distinguish**.** When severe, the condition may be accompanied by abdominal pain, malaise and fever**.** Epididymitis is usually a complication of urinary tract infection with coliforms arising from prostatic enlargement or urogenital surgery leading to urinary obstruction (81).

Chlamydial epididymitis cannot be differentiated with certainty from the other causes of epididymitis on clinical grounds alone, and chlamydial epididymitis is milder but persists longer than epididymitis from other causes, and is not always preceded by symptoms of urethritis**.** Moreover, epididymitis is important because fertility may be impaired as a result of inflammation and obstruction of the small diameter sperm collecting tubes and vasa, particularly where both testes are affected (82).

***Chlamydia* and male infertility**

Male infertility is cause by the association of *C. trachomatis* with chronic epididymitis and epididymal granuloma formation. Under these circumstances it is reasonable to suppose that chlamydial infection will lead to blockage of the epididymis and thus to infertility due to azoospermia**.** There is clinical and pathologic evidence linking these pathogens to urethritis, linking urethritis to epididymo-orchitis, and finally linking epididymo-orchitis to infertility. Chlamydial infection of the upper genital tract of men leads to the presence both white blood cells and of anti-chlamydial antibodies in semen. It has been suggested that the presence of antibodies to *C. trachomatis* is correlated with the presence of white blood cells in semen and with the development of an autoimmune response to spermatozoa (79).

Reproductive tract infections in women

Women are less likely to be able to prevent STD exposure than men. Female anatomy makes women more susceptible to STDs. *Chlamydia* is more efficiently transferred from males to females than the other way around. In women, the abdominal cavity and its organs can be infected by STD agents via ascending infection through the uterus and fallopian tubes. Susceptibility varies with the menstrual cycle and women are more likely than men to be asymptomatically infected. Women with lower genital tract infection often are infected with *C. trachomatis,* which is one of the organisms thought to be associated with the urethral syndrome called lower tract urinary infection (84).

Cervicitis

The prime target of chlamydial infection in the lower genital tract of women is the columnar epithelial cells lining the endocervical canal. Chlamydial cervicitis is caused by *C. trachomatis* organisms of serovars D to K. Serovar E is particularly common (85).Cervicitis is inflammation of the cervix and the signs of cervicitis include: pain in passing urine, frequency, soreness, and a cervical discharge. The cervix itself may be swollen, reddened and may bleed easily. Occasionally, lymphoid follicles may be observed on the cervix (86).

Pelvic Inflammatory Disease (PID)

PID is infection of the uterus, fallopian tubes, and adjacent pelvic structures. It is caused by ascending infection from the lower genital tract of bacteria associated either with sexually transmitted disease such as *C. trachomatis*. Symptoms of PID vary from none to severe. When PID is caused by chlamydial infection, the clinical spectrum may range from virtually asymptomatic endometritis to severe salpingitis, pyosalpinx, tubo-ovarian abscess, pelvic peritonitis, and perihepatitis. Clinical signs include; low grade fever, exudate from the cervix, sensitivity of the cervix to movement and abdominal tenderness due to locally enlarged lymph nodes. A woman may experience mild symptoms or no symptoms at all, while serious damage is being done to her reproductive organs. Because of vague symptoms, PID goes unrecognized by women and their health care providers about two thirds of the

time. Women who have symptoms of PID most commonly have lower abdominal pain, fever, unusual vaginal discharge that may have a foul odor, painful intercourse and urination, irregular menstrual bleeding, and pain in the right upper abdomen (rare). PID can cause permanent damage to the female reproductive organs. Infection-causing bacteria can silently invade the fallopian tubes, causing normal tissue to turn into scar tissue. This scar tissue blocks or interrupts the normal movement of eggs into the uterus. If the fallopian tubes are totally blocked by scar tissue, sperm cannot fertilize an egg, and the woman becomes infertile. Infertility also can occur if the fallopian tubes are partially blocked or even slightly damaged. About one in eight women with PID becomes infertile, and if a woman has multiple episodes of PID, her chances of becoming infertile increase. In addition, a partially blocked or slightly damaged fallopian tube may cause a fertilized egg to remain in the fallopian tube. If this fertilized egg begins to grow in the tube as if it were in the uterus, it is called an ectopic pregnancy. As it grows, an ectopic pregnancy can rupture the fallopian tube causing severe pain, internal bleeding, and even death (87).

Perihepatitis (Fitz-Hugh-Curtis Syndrome)

This rare syndrome is characterized by right upper quadrant pain, nausea, vomiting, fever, and normal transaminases. About 70% of perihepatitis cases are associated with chlamydia. PID may not be clinically evident. Although thought to be caused by direct spread of the organism from the infected fallopian tubes to the capsule of the liver, hematogenous and lymphatic spread may also occur. Inflammation of the liver capsule may lead to intra-abdominal adhesions (88).

Chlamydial infections in pregnancy

Prematurity is one of the leading causes of perinatal mortality. Uterine contractions may be induced by cytokines, proteolytic enzymes or prostaglandins released or induced by microorganisms. Asymptomatic bacteriuria, cervicitis and vaginosis are strongly associated with preterm delivery, but the role of *C. trachomatis* is less clear. However, a substantial

number of studies suggest that maternal *C. trachomatis* infection in pregnancy is associated with premature delivery.

Anorectal infection (proctitis)

Proctitis, or inflammation of the rectum, has several infectious and non-infectious causes, the infectious pathogens typically being sexually acquired. Proctitis is seen almost exclusively in homosexual men engaging in receptive anal intercourse; however, women also are susceptible. Rectal infections are generally asymptomatic but may cause symptoms characteristic of proctitis. Proctitis is manifested by rectal discharge, bleeding, tenesmus, and pain during defecation (53).

Reactive arthritis (Reiter's syndrome)

Reactive arthritis is an aseptic inflammatory polyarthritis that usually follows nongonococcal urethritis or infectious dysentery, especially by *C. trachomatis*. Chronic inflammation is probably stimulated by microbial infection of the joint synovial fibroblasts, in which intracellular persistence of viable chlamydiae is the probable trigger for the initiation and maintenance of the inflammatory process. Thus chlamydial-induced cytokine release from synovial fibroblasts might contribute to alterations in the synovial membrane that promote the development of joint inflammation (89). There appears to be some geographic variation in the major organisms associated with reactive arthritis (90). The main symptoms of reactive arthritis are pain and swelling of fairly sudden onset in the knees, ankles or toes. The fingers, wrists, elbows and sacroiliac joints (base of spine) may also be affected. The signs of scaly skin rash on the palms, soles of the feet, or tip of the penis, and urethritis may present as well. Acute and chronic reactive arthritis are regarded as being diseases of respectively less or more than six months duration. The interval between symptoms and arthritis is from a minimum of 7 days to a maximum of 4 weeks.

Conjunctivitis

Inclusion conjunctivitis in adults usually presents a history of several weeks or months of a red, irritable eye with a sticky discharge. Often the symptoms are in one eye only. Chronic follicular conjunctivitis is usually

unilateral, with sub-acute onset. Symptoms include foreign-body sensation, tearing, mucoid discharge, redness, photophobia, and swelling of lids. Incubation is usually 1 - 3 weeks. Palpebral conjunctiva may show follicles, predominantly in upper and lower fornices but also around the caruncle and on the plica. Cornea are usually not affected, but occasionally these is a fine punctate keratitis which resolves spontaneously. Chlamydial inclusion conjunctivitis is caused by genital tract serovars D to K of C. trachomatis. Many cases will be found to have a concomitant chlamydial genital tract infection, usually asymptomatic (91).

Lymphogranuloma veneeum

Lymphogranuloma venereum (LGV) is an uncommon form of sexually transmitted disease caused by serovars L1, L2 or L3 of *Chlamydia trachomatis*. Clinically the LGV biovar is remarkable for its tropism for lymphoid cells and its ability to cause systemic disease. LGV is commonest in tropical areas of the world. However, its distribution is world wide (92). The disease usually presents as painful inguinal adenopathy up to 6 weeks after exposure to infection. The clinical course of LGV is classically considered as occurring in three stages. In the Primary stage, after an incubation period ranging as wide as 3 to 30 days, a lesion reportedly appears on the glans of the penis or, in women, on the vaginal wall, labia or, occasionally, the cervix. Rarely lesions may occur at extra-genital sites such as the anus, fingers or tongue. The initial lesion is usually transient, barely perceptible and painless, occurring as a papule, a shallow ulcer or erosion. In the secondary stage, occurs from one to several weeks after infection. Most patients attend clinics because of the marked lymphadenopathy which is usually unilateral, involving the retro-peritoneal lymph nodes in women or the inguinal lymph nodes in men. Both the inguinal and femoral lymph nodes may be involved giving rise and other lymph nodes may become involved by lymphatic drainage of infection from the infected node, giving rise in some cases to a whole chain of swollen nodes. The infected nodes become abscesses which eventually suppurate and may give rise to draining fistulae. Lastly in the tertiary stage, progressive spread of the disease leads to increasing and devastating tissue

destruction and LGV proctitis initially occurs, followed by rectal damage, strictures and, in women, the formation of recto-vaginal fistulae. There may be substantial urethral destruction, in which epithelial tissue is destroyed and replaced with granulation tissue and infiltrating plasma cells as well (93, 94).

Neonatal conjunctivitis and neonatal pneumonia

Often known by its Latin name of *Ophthalmia neonatorum,* conjunctivitis of the eyes of newborns may be caused by bacterial infection. Usually the infection is derived from the mother's genital tract at birth, in which case the causative organism is the genital serovars D to K of *C. trachomatis*. The observation of chlamydial neonatal conjunctivitis that developed three days after a child was born by Caesarian section with intact amniotic membranes suggests the possibility also of a rare trans-placental or trans-membrane route of infection (95). The orbit of the eye is usually quite swollen, and there is a mucoid discharge which is less purulent than that usually seen with overt gonococcal ophthalmia. The infection is particularly common in pre-term babies, who are often born to women at particular risk of *C. trachomatis* infection. It does not respond well to treatment with chloramphenicol eye ointment. In neonatal conjunctivitis, the nasopharynx is also commonly infected with *C. trachomatis*, presumably via drainage from the oto-lachrymal duct, so it is important to treat the infants with systemic rather than topical antibiotics. As the causative organism is a sexually transmitted infection, it is vital to ensure that the mother and her sexual partner are also treated (96). If left untreated, approximately 10 - 20% of infants will develop neonatal pneumonia**.** Occasionally neonatal *C. trachomatis* pneumonia may be sufficiently severe for the infant to require ventilation**.** The infants develop a chronic, staccato, "machine gun-like" cough and are febrile with minimal malaise but show marked chest X-ray changes, and frequently have a history of failure to thrive. On physical examination the infants tend to have diffuse riles in the chest and are congested and tachypnoeic. Laboratory examination usually reveals hyper-gammaglobulinaemia and eosinophilia. Significant limitations of expiratory airflow have been reported, as well as signs of abnormally elevated volumes of trapped air (97).

Laboratory diagnosis

Chlamydial infections are frequently asymptomatic. Confirmation of chlamydial infection usually depends on taking an appropriate clinical sample from the patient followed by the direct detection of the organism using a suitable laboratory-based diagnostic test. The demonstration of chlamydial antibodies in an individual is rarely diagnostic. For many years, the optimum method of confirming the presence of chlamydial infection was the growth of the infecting organism in cell culture and the demonstration of characteristic chlamydial inclusions. However, this method necessitated the availability of good transport and cold-storage facilities in order to maintain the viability of the organism prior to inoculation. Moreover growth and isolation of the organisms in cell culture was relatively tedious and difficult to control. It is now widely recognized that cell culture techniques were, at best, only 60 to 80% sensitive (98). A key advance in the laboratory diagnosis of chlamydial infections has been the development of non-viability dependent tests that place less demand on specimen transport. The first of these tests were the chlamydial antigen detection tests, which relied either on the direct detection of chlamydial elementary bodies in clinical material using fluorochrome-labelled, chlamydial specific, monoclonal antibodies or the capture and detection of chlamydial antigen in an extract of clinical material using enzyme immunoassay based procedures. However, they are gradually being superseded by newer methods based on the detection of chlamydial nucleic acids, either by direct hybridization or by nucleic acid amplification. The latter uses a variety of amplification reactions, including the polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA) or transcription mediated amplification (TMA). Nucleic acid-based methods generally offer superior sensitivity and specificity to the antigen detection tests, but at greater cost and a greater requirement for trained staff. However, depending on the prevalence of infection in the test population, costs may be reduced by combining different specimens.

Sample collection

In females, any extraneous pus or discharge should first be removed from the endocervix for Gram stain and culture using a conventional swab or gauze. A fine endocervical swab is then introduced into the endocervical canal, rotated gently for 15 to 30 seconds, then removed for laboratory testing. Wooden-tipped swabs and swabs made of calcium alginate should be avoided, as some cotton swabs are toxic to *Chlamydia;* Dacron or rayon material is preferred. In males, urethral and nasopharyngeal specimens may be collected with a fine swab on a flexible wire. The swab should be inserted 3 to 5 cm into the urethra rotated well before removed. Urine, semen, and purulent urethral discharge are not considered adequate specimens for culture but urine is the most non-invasive sample used for NAATs.

The precise swab and transport material used will depend on the test being performed; the manufacturer's instructions should be followed. An adequate specimen would be expected to contain susceptible infected cells. Even with a sensitive nucleic acid amplification-based method like the Abbott LCR, the rate of specimen positives was much lower if infected cells were absent and inadequate specimens reduce the sensitivity of other NAATs methods as well (99).

Classical Diagnosis

a). Cell culture

Up until the early 1980s, the main method of confirming a diagnosis of chlamydial infection, was the inoculation of clinical material into animals, embryonated hens eggs or (usually) tissue culture cells and the demonstration of characteristic chlamydial inclusions which could be seen in cell cultures, either by staining with iodine, by staining with Giemsa stain, fluorochrome-labelled poly- or mono-clonal antibody or by enzyme immunohistochemistry. The usual tissue culture cells were HeLa 229, L434 mouse fibroblasts or McCoy cells in the case of *C. trachomatis*. After three days it was usually necessary to homogenize the cells and do at least one

'blind passage' in order to ensure one had not missed a low-level infection. Fields of monolayer cells also had to be searched for telltale inclusions. Even now cell culture is one reference standard against which new diagnostic tests may be compared. The disadvantages were many; for example, the inoculum had to be kept viable until it could grow in cells; this usually meant good transport arrangements and maintenance. The tissue culture service was expensive to maintain and there were many variables that were difficult to quantify, including the tissue culture water, the particular clone of cells in use; the quality of the fetal calf serum; the absence of adventitious mycoplasma infection; the quality and sterility of the laboratory ware, etc. All of this has been replaced by the demanding, but less variable protocols associated with modern, non viability-dependent diagnostic products with much better sensitivity than was routinely achieved by cell culture (100).

b). Antigen Detection Methods

Use of polyclonal antisera dates from the 1960s. This enabled the production of highly reproducible antisera of defined specificity, and lead to the elucidation of the relationship between the newly discovered MOMP and the serotypes of *C. trachomatis*. The first product was the Syva Microtrak® based on a fluorescein-conjugated and species specific monoclonal antibody to *C. trachomatis*'s major outer membrane protein, which was used for the direct detection of chlamydial elementary bodies in clinical specimens. Syva Microtrak® is dependent upon proper interpretation by a skilled microscopist; the test has a sensitivity of 70%-75% and a reported specificity of 95-99%. Direct immunofluorescence with monoclonal antibodies was the start of the present day trend towards the use of non viability-dependent methods for the laboratory diagnosis of *C. trachomatis* infection. Other companies brought out genus specific monoclonal antibodies, directed against chlamydial lipopolysaccharides which, while they gave less clear staining than those directed against MOMP, also required careful, painstaking microscopy. Throughput for screening purposes was limited, with few people able to tolerate the eyestrain of looking at fluorescing 'pin pricks' for hours on end. What was needed was a way to harness the antigen detection capability of

antibodies to the new technique of enzyme immunoassay (EIA). Enter the Abbott Chlamydiazyme® test (101).

c). Antigen detection enzyme immunoassays

The first enzyme immunoassay (EIA) for the detection of chlamydial antigen in clinical specimens was the Abbott Chlamydiazyme® which appeared around the same time as the Syva Microtrak® direct immunofluorescence test. Unlike the Syva test, the Chlamydiazyme was based on an adsorbed polyclonal antiserum, rather than a monoclonal antibody. The test which could be completed in four hours, had sensitivity against male and female genital tract specimens of around 60 to 90%, depending on the sample and the reference technique (102). Specificity was problematic, however. Lack of specificity was associated with the finding that the enzyme immunoassay antibody reacted with strains of *Acinetobacter calcoaceticus*, *Escherichia coli*, *Gardnerella vaginalis*, *Neisseria gonorrhoeae* and group B streptococci (103). Subsequently three further chlamydial antigen detection EIAs were introduced to the market, the Syva Microtrak EIA, the Pathfinder® EIA (Sanofi/Kallestad) and the Boots-Celltech IDEIA®. The latter was a radically different immunoassay, being based on the improved specificity of monoclonal antibody to the chlamydia-specific lipopolysaccharide epitope plus an ingenious, redox-linked, signal amplification system. Many laboratories still use chlamydial antigen detection immunoassays because of their lower cost and ease of use. Research interest in this methodology, however, waned with the introduction of superior methods based on nucleic acid hybridization or amplification (104).

Molecular diagnostic methods

a). Direct hybridization probe tests

All nucleic acid-based chlamydial diagnostic assays depend on hybridization. In direct hybridisation probe tests, the signal is generated without any intermediary target amplification. The Gen-Probe PACE 2 test was first introduced in 1988. Its sensitivity is founded on the fact that it is

targeted against a high copy number of chlamydial rRNAs, which effectively serves as a natural pre-amplification. As rRNA is mostly single stranded, no thermal denaturation for strand separation is necessary prior to hybridization. The test is completed in approximately two hours. The test is popular because of its relative simplicity to perform and its scalability using conventional laboratory equipment. Subsequently a probe competition assay was introduced to help resolve any border line results, or to verify positive results. Early results indicated that the Gen-Probe PACE 2 was highly specific, with sensitivity roughly equivalent to the IDEIA® III antigen detection test and better than the Chlamydiazyme® test (105). The PACE 2 test has the advantage of being relatively simple to perform but has reduced sensitivity compared to the leading nucleic acid amplification based techniques (106).

b). Nucleic acid amplification based tests

The development of chlamydial tests based on nucleic acid amplification technology (NAAT) has been considered the most important advance for the detection of chlamydial infections since cell culture (98). These tests amplify the target nucleic acid, DNA or RNA or probe after it has annealed to target nucleic acid. Such tests are generally more sensitive than liquid or solid phase hybridization tests which do not embody an amplification process and are considerably more sensitive than culture or antigen detection methods (107). The major targets for amplification--based tests against *C. trachomatis* are generally multiple-copy gene products, such as the cryptic chlamydial plasmid or ribosomal RNA. Starting with a multiple copy gene offers a clear starting advantage with respect to sensitivity. Initial nucleic acid amplification tests were based on the detection of chlamydial DNA in clinical samples. The well known Polymerase Chain Reaction (PCR), the Ligase Chain Reaction (LCR), Strand Displacement Amplification (SDA) and Transcription Mediated Amplification (TMA) are the methods use for *C. trachomatis* infections by NAATs. The main advantage of the nucleic acid amplification-based diagnosis for Chlamydiae is that such methods combine unsurpassed sensitivity with high specificity (108). Nevertheless, Nucleic acid

amplification tests tend to be more expensive (but more effective) than other laboratory methods of testing for chlamydial infection.

c). Polymerase Chain Reaction

The Roche AMPLICOR® PCR for *C. trachomatis* was the first commercial nucleic acid amplification test to be introduced for the diagnosis of chlamydial infection. Subsequently a multiplex assay became available that permitted simultaneous PCR amplification of *C. trachomatis* (CT) and *N. gonorrhoeae* (NG) target DNA as well as internal control DNA. Currently the test has two main formulations; the Roche AMPLICOR CT/NG MWP (multi well plate) PCR and the fully automated Roche COBAS® AMPLICOR PCR. Urine, swabs etc. are treated with detergent to release the target chlamydial DNA, which is directed against the multi-copy cryptic chlamydial plasmid. The test is not isothermal but involves rapid thermal cycling in a specially programmed thermal cycler. Theoretically, this is a disadvantage of PCR compared to isothermal processes because of the time required for thermal cycling and equilibration. The sensitivity, specificity and predictive values for the Roche AMPLICOR were 93.4 and 96.7 respectively (108).

d). Ligase Chain Reaction

The Abbott LCx® (Abbott Laboratories, Illinois, USA), based on a ligase chain reaction, was the second nucleic acid amplification-based method to be introduced for the diagnosis of chlamydial genital tract infections. It is based upon the cryptic chlamydial plasmid. Four oligonucleotide probes, working in pairs, hybridize to complementary single strand DNA. Polymerase fills in the gap of a few nucleotides between a given probe pair. Once the gap is filled, ligase joins the pair of probes so that an amplification product complementary to the original target sequence has been created. This product then serves as a target for subsequent rounds of amplification in a thermal cycler. Discordant results (PCR or LCR positive, but culture negative) can be confirmed by using a sequence including the other DNA amplification test, direct fluorescent antibody testing or DNA amplification test to detect the gene encoding chlamydial major outer membrane protein. Very few false-positive results are usually found, indicating that the specificity levels of PCR,

LCR, and cell culture are very high. The sensitivity of cell culture was estimated at between 60 and 65%, giving a true sensitivity of PCR and LCR of between 90 and 97% (109). The excellent sensitivity of the LCx® assay makes it suitable for screening studies in large populations, with urine the usual sample (110). There is a need for 'second generation', simpler, higher throughput, more user-friendly systems which incorporate real-time detection of amplified product within a sealed system, thereby minimizing opportunity for contamination of other samples by amplicon.

e). Strand Displacement Amplification

The Becton Dickinson ProbeTecET® is a commercial attempt to achieve this. In essence, the system uses isothermal strand displacement (SDA) technology and the target gene is chlamydial cryptic plasmid. In practical terms the performance characteristics of ProbeTecET® seem to be similar to the other leading nucleic acid amplification based technologies (111). The BD ProbeTecET® results for *C. trachomatis* was 100% sensitive and 100% specific relative to the LCx. In male urines the sensitivity of the ProbeTecET® assay was 95.5%, specificity 100% (112).

f). Transcription Mediated Amplification

In 1997 two studies compared the performance of the Gen-Probe AMP-CT, either against the Abbott LCx® LCR, the Roche COBAS AMPLICOR® PCR and cell culture. The sensitivities of LCx, AMPLICOR and AMP-CT against cell culture were 79, 86, and 78%, respectively. After discrepant analysis using an in-house PCR, the sensitivities of LCx, COBAS AMPLICOR, and AMP-CT were 84, 93, and 85%, respectively. Specificity exceeded 99% for all three of these amplification assays while sensitivity for all three methods was consistently lower for urine from females compared to males (113). Amplification assays are potentially susceptible to inhibitors in urine, which can lead to false negative results. In an interesting and relevant study, the effect of inhibitors in the urine of pregnant or non pregnant women on various amplification-based tests was assessed (114). For the second generation TMA nucleic acid amplification test Gen-Probe APTIMA-Combo 2, a relatively new FDA-approved test, overall sensitivity and specificity for

C. trachomatis in all specimens was approximately 95.8 and 98.2% respectively. In comparison to the sensitivity and specificity of the Gen-Probe APTIMA® Combo 2 test, the Abbott LCx® and the Roche AMPLICOR® CT showed that Combo 2 was more sensitive than LCx or AMPLICOR for *C. trachomatis* in men and women, but the specificities of LCx and AMPLICOR were slightly higher than that of the APTIMA 2 (115).

Treatment

Treating infected patients prevents transmission to sex partners. In addition, treating pregnant women usually prevents transmission of *C. trachomatis* to infants during birth. Treatment of sex partners helps to prevent re-infection of the index patient and infection of other partners.

Co-infection with *C. trachomatis* frequently occurs among patients who have gonococcal infection; therefore, presumptive treatment of such patients for chlamydia is appropriate. The following recommended treatment regimens and alternative regimens cure infection and usually relieve symptoms. Azithromycin 1 g orally in a single dose or Doxycycline 100 mg orally twice a day for 7 days are the recommend regimens (20).

Polymerase Chain Reaction (PCR) Amplification

PCR is based on the recognition by the primer (a short piece of DNA) of a sequence on template strand, (a larger single stranded fragment of DNA). When the primer recognizes the template and anneals to the recognition sequence, the 3'-end of the primer is used by DNA polymerase to synthesize a new DNA strand (elongation). When the temperature is raised, the new DNA strand will melt away (denature) from the template, and the template is once again open for annealing of a new primer when the temperature is decreased. By adding a second primer which recognizes the template strand complementary to the first template, the elongation can proceed in the direction of the first primer. New production of template strands take place in

every temperature cycle. In this way the DNA sequence between the two primer sequences is amplified exponentially, yielding high concentrations of double-stranded DNA of the same length. There are three major steps in a PCR (figure 3), which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time: (i) Denaturation at around 94°C, during the denaturation, the reaction solution containing DNA molecules, polymerases, primers and nucleotides is heated, causing the double helical strand to open into single stranded DNA, and all enzymatic reactions stop; (ii) lowering the temperature to 55°C causes the primers to bind to the DNA, a process known as hybridization or annealing. The resulting bonds are stable only if the primer and DNA segment are complementary. The polymerases then begin to attach additional complementary nucleotides at these sites, thus strengthening the bonding between the primers and the DNA; (iii) extension: the temperature is again increased, this time to 72°C. This is the ideal working temperature for the polymerases used, which add further nucleotides to the developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments that are not fully complementary are broken. Generally, 30 to 50 thermal cycles result in detectable amounts of the target sequence originally present in less than 100 copies.

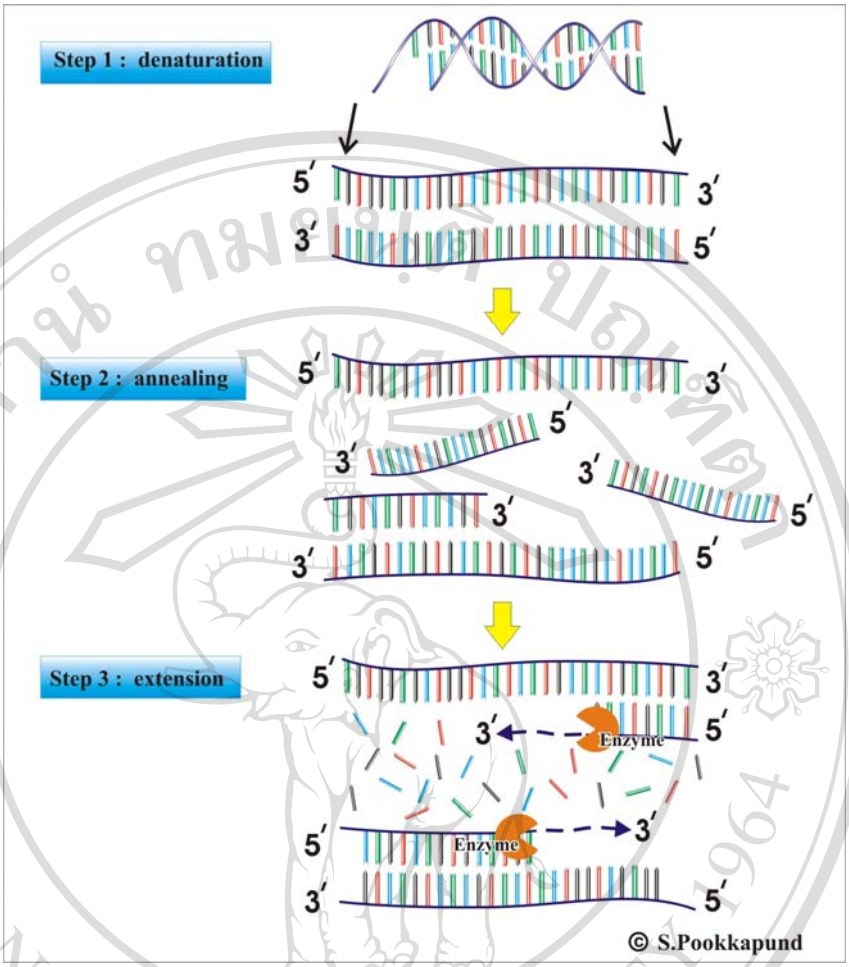

Figure 3 Illustration of the Polymerase Chain Reaction procedure. There are three steps in the PCR. With alternating temperatures the PCR technique denatures target strands, anneals primers and amplifies the strand by replicating the coordinate strand from the primed complexes, creating double stranded nucleic acid products called amplicons. The cycle is repeated 35 to 45 times. The amplicon can be detected either by separation on an agarose gel under the influence of an electric current or by an enzyme immunoassay color detection method or by using oligonucleotide complementary probe.

The PCR process is exquisitely sensitive. While most biochemical analyses, including nucleic acid detection with radioisotopes, require the input of significant amounts of biological material, the PCR process requires very little (116).

Multiplex PCR

To overcome the inherent disadvantage of cost and to improve the diagnostic capacity of the test, multiplex PCR, a variant of the test in which more than one target sequence is amplified using more than one pair of primers, has been developed. Multiplex PCRs to detect viral, bacterial, and/or other infectious agents in one reaction tube have been described. In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility (117). Primers used in multiplex reactions must be designed carefully in order to have similar annealing temperatures and to lack complementarity in order to avoid dimerization (118).

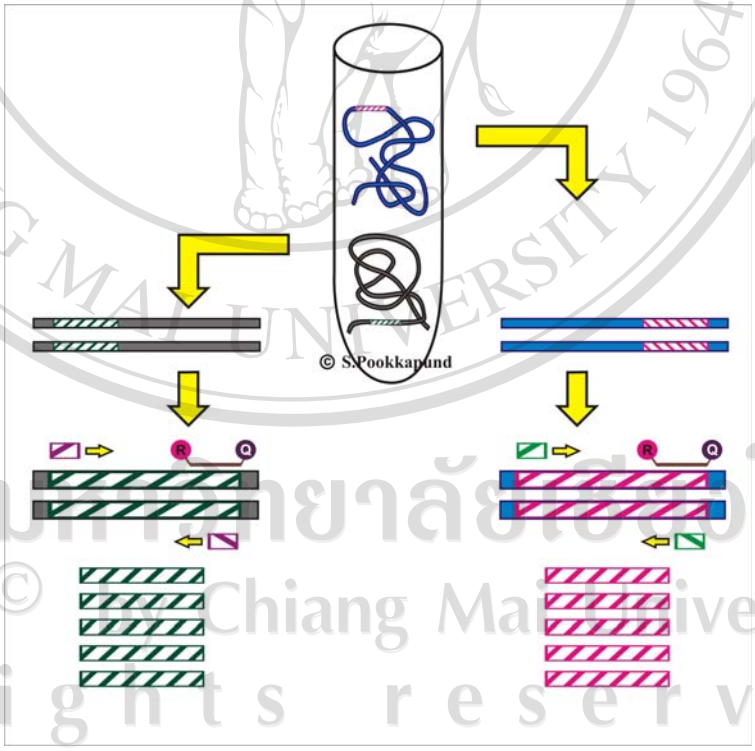

Figure 4. Diagram of multiplex PCR with probe detection systems. Two DNA templates with two different targets can be amplified in single tube simultaneously. The amplicon can be detected with the Taqman probe system.

Real Time PCR

Real-time PCR is a widely used method to detect and quantify even very low amounts of DNA or RNA of varying origins. This is called “real-time PCR” because it allows the user to actually view the increase in the amount of DNA as it is amplified during the early phases of the reaction. Real-time systems for PCR are improved by probe-based, rather than intercalator-based, PCR product detection. The principal drawback to intercalator-based detection of amplicon accumulation is that both specific and nonspecific products generate signal. An alternative method, the 5' nuclease assay, provides a real-time method for detecting only specific amplification products. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerization ensures that cleavage of the probe occurs only if the target sequence is being amplified (119).

Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction, which is very time consuming. Agarose gel results are obtained from the end point of the reaction based on size discrimination, which may not be very precise. The end point is variable from sample to sample. While gels may not be able to resolve this variability in yield, real-time PCR is sensitive enough to detect these changes. Agarose gel resolution is very poor, about 10 fold. Real-Time PCR can detect as little as a two-fold change and automate which no need of Post PCR processing as well.

As the PCR progresses, the samples begin to amplify in a very precise manner. Amplification occurs exponentially, that is a doubling of amplicon number occurs every cycle. This type of amplification occurs in the exponential phase because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon. However, as the reaction progresses, some of the reagents are being consumed

as a result of amplification. This depletion will occur at different rates for each replicate. The reactions start to slow down and the PCR product is no longer being doubled at each cycle. Eventually the reactions begin to slow down and stop altogether, or “plateau”. Each tube or reaction will plateau at a different point, due to the different reaction kinetics for each sample. The plateau phase is where traditional PCR takes its measurement. Therefore, it will be more precise to take measurements during the exponential phase, where the replicate samples are amplifying exponentially. By contrast, Real-Time chemistry provides fast, precise and accurate results that are designed to collect data as the reaction is proceeding, which is more accurate for DNA quantification and does not require laborious post PCR methods.

Detection of PCR products can be achieved using the sequence-specific probes, such as dual-labeled (TaqMan®) probes, LightCycler® hybridization (FRET) probes, or Molecular Beacons. For optimal results in real time PCR, time-consuming optimization of the reaction parameters is often necessary. In this research, we employed a TaqMan®-based probe which contains oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10^{o}C higher) that contain a fluorescent dye, usually on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of quencher (no FRET) and the reporter dye starts to fluorescence, which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye. One specific requirement for fluorogenic probes is that there must be no G at the 5' end. A 'G' adjacent to the reporter

dye quenches reporter fluorescence even after cleavage. Well-designed TaqMan probes require very little optimisation (120-122).

Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. An amplification plot is a plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline.

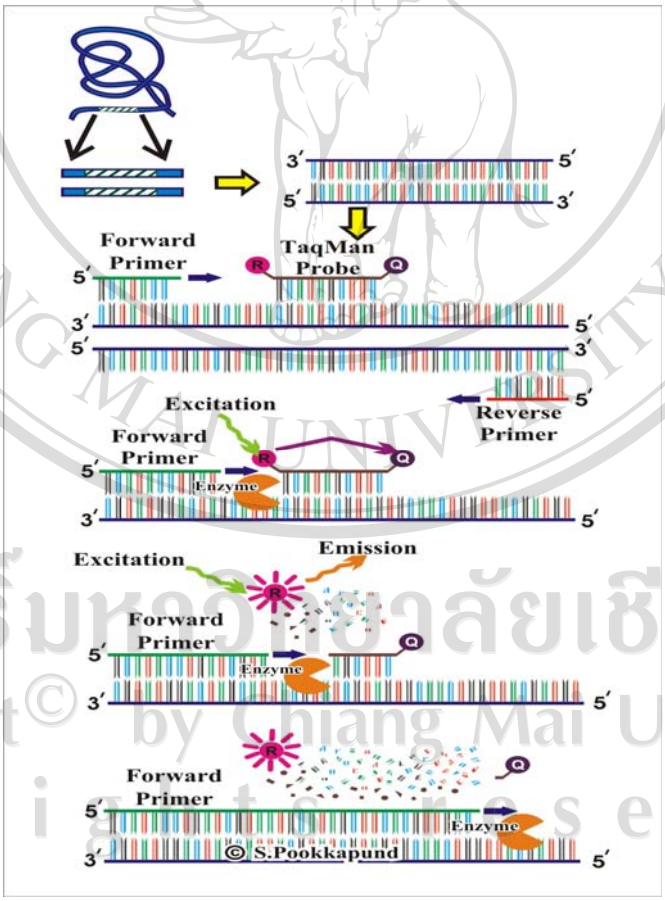

Figure 5. The principle of TaqMan probes in real-time PCR. Both the TaqMan probe and PCR primers anneal to the target sequence during the PCR

annealing step. The proximity of the fluorophore with the quencher results in efficient quenching of fluorescence from the fluorophore. *Taq* DNA polymerase extends the primer. When the enzyme reaches the TaqMan probe, its 5' to 3' exonuclease activity degrades the probe, resulting in physical separation of the fluorophore from the quencher. Increased fluorescence from the released fluorophore is proportional to the amount of accumulated PCR product.

Cycle threshold

The parameter Ct (cycle threshold) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. A plot of the log of initial target copy number for a set of standards versus CT is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number (123).

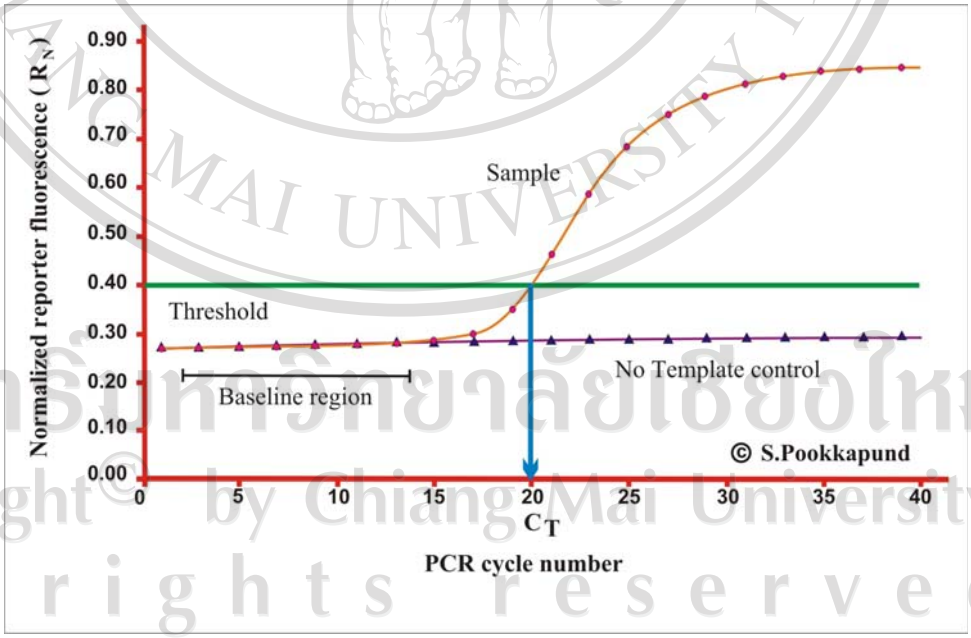

Figure 6 Shows a representative amplification plot and defines the terms used in the quantitation analysis. An amplification plot is the plot of fluorescence signal versus cycle number. This defines the baseline for the amplification

plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter Ct (cycle threshold) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Traditional laboratory diagnosis of these infections is done by culture for *N. gonorrhoeae* and cell culture or antigen detection for *C. trachomatis*. Recently, nucleic acid amplification tests (NAATs) have become widely used; these tests have shown a greater sensitivity and have improved the ability to detect *C. trachomatis* and *N. gonorrhoeae* infections. Several studies have shown that NAATs are more accurate than the former standard tests. Currently available commercial *C. trachomatis* and *N. gonorrhoeae* DNA amplification tests include PCR (Roche Molecular Systems, Branchburg, N.J.)(124). Because these organisms are regularly found together, in this study, we evaluated the In house Taqman-based multiplex real time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* that simultaneously detects both pathogens in a single amplified urine specimen. Noninvasive screening options, such as urine testing could eliminate some of the barriers to screening and detection for *C. trachomatis* and *N. gonorrhoeae* infections.

The COBAS AMPLICOR *C. trachomatis*/*N. gonorrhoeae* (CA CT/NG) multiplex PCR test (Roche Diagnostics, Mannheim, Germany) provides a useful platform for STI evaluation. An internal control greatly reduces false-negative results. The CA CT component has proven to be a useful diagnostic tool for the detection of *C. trachomatis* in cervical scraping and urine specimens. The CA NG component, however, lacks specificity, as some non-*N.gonorrhoeae Neisseria* species and even lactobacilli have been reported to give false-positive results. Therefore, confirmation by a second, more specific PCR assay is required (125). With reference to these data, we have developed In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* using cryptic plasmid B gene as target. Unfortunately, the cppB gene of *N. gonorrhoeae* is present in some commensal Neisseria strains and absent in some *N. gonorrhoeae* strains; this influenced us to develop a confirmation test for *N. gonorrhoeae,* an In-house Taqman-based Real Time

PCR for detection of *N.gonorrhoeae* porin A pseudogene DNA (126-127). Our methods employ Taqman-based real time PCR, which has high sensitivity similar to SDA, LCR or TMA but which uses only one pair of primers for each DNA target and only DNA polymerase enzyme in a single reaction. Detection using this method is in real time, with no need of a post PCR procedure or even of separation steps (128-131). For specificity, TaqMan probes are designed to anneal to an internal region of a PCR product, which is highly specific to target sequence and will be tested with bacterial species other than *C. trachomatis* and *N. gonorrhoeae* (132).