Sexually transmitted infections (STIs) are a major global cause of acute illness, causing long-term disabilities with severe physical and psychological consequences for millions of people. The World Health Organization (WHO) estimates that 340 million new cases of STIs have occurred worldwide since 1999. The largest numbers of new infections have occurred in the regions of South and Southeast Asia (151 million), with most of the STIs reported from this area being either trichomoniasis (76.5 million), chlamydia (43 million), gonorrhoea (27 million) or syphilis (4 million) (1). According to WHO, Neisseria gonorrhoeae, which causes gonorrhoea, and Chlamydia trachomatis, which usually causes non-gonococcal urethritis (NGU), are the most important public health problems. In Thailand, where an HIV prevention program has been vigorously carried out since 1984, STI prevalence decreased continuously from 1989 to 2001 (2). However, several STIs, especially gonorrhoea and NGU, have shown slight increases in prevalence from 2004 to 2008 in the North region (3).

Neisseria gonorrhoeae and Chlamydia trachomatis infections have similar signs and symptoms, although asymptomatic forms of the disease may be more common. In women, C. trachomatis and N. gonorrhoeae are major causes of cervicitis, endometritis, pelvic inflammatory disease with tubal factor infertility, and ectopic pregnancy. Without treatment, an infection may lead to urethritis in men and severe urethritis plus pelvic inflammation in women. Moreover, these pathogens can also cause oropharyngeal infections and conjunctivitis, which may lead to blindness, especially in newborns (4, 5).

The increased prevalences of C. trachomatis and N. gonorrhoeae infections worldwide suggests that prevention programs are necessary in order to control the corresponding illnesses. In an effort to prevent the spread of these diseases, increased attention is being focused on early diagnosis with high sensitivity and specificity in populations at risk, on development of more effective treatments, and on sex education (6).
Over the past 15 years the diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections have been done mainly via methods such as cell culture, enzyme immunoassays (EIA), direct fluorescent antibody (DFA) staining for *C. trachomatis*; for *N. gonorrhoeae*, cell cultures, Gram smears, and biochemical tests are used (7). To diagnose *C. trachomatis*, a cell culture is the preferred method when the diagnosis is problematic or when there is a case of suspected sexual assault or abuse. The main advantages of a cell culture are high specificity and the ability to preserve the organisms for additional studies, such as antibiotic susceptibility testing. Briefly, in cell cultures a specimen is collected in an appropriate transport medium and is inoculated onto a confluent monolayer of McCoy cells. After 48–72 h of growth, intra-cytoplasmic inclusions will have developed in infected cells. The inclusions are detected by staining with fluorescein-conjugated monoclonal antibodies (mAbs) that are specific for the MOMP of *C. trachomatis* or other Chlamydia-specific proteins. However, the cell culture method has disadvantages, such as the requirements for collection, storage, and transport, availability of results, time consumption and labor required. Thus cell cultures have generally been replaced by antigen or deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) detection methods since the 1990s.

At present, many laboratories also use antigen detection methods such as enzyme immunoassays (EIA). *C. trachomatis* EIA tests detect chlamydial lipopolysaccaride (LPS) using monoclonal or polyclonal antibodies. Manufacturers such as MicroTrac or Chlamydiazyme have developed blocking Abs that verify *C. trachomatis*-positive EIA results, since there is the potential for false-positives. The main advantages of EIA are the objective interpretation of the results, rapidity, and ease of use. At the same time, direct immunofluorescence assay (DFA) is an alternative method for detection of *C. trachomatis*. Briefly, a prepared specimen is placed directly on a slide. Fluorescein-conjugated Abs that react with the Chlamydial surface are added, and the stained slide is visualized by fluorescence microscopy. Depending on the commercial product used, the antigen detected by this method is either the LPS or major outer membrane protein (MOMP) component. This method
requires a skilled microscopist in order to distinguish between fluorescing chlamydial particles and non-specific fluorescence. DFA is mostly used as confirmatory test in routine laboratory testing. The disadvantages of EIA are that the anti-LPS antibodies can cross-react with *C. psittaci* and *C. pneumoniae*, as well as non-chlamydial bacterial species, whereas DFA with a *C. trachomatis*-specific anti-MOMP monoclonal antibody is considered highly specific (8,9).

For conventional *N. gonorrhoeae* diagnosis, detection of intracellular diplococci, direct microscopy of Gram- or methylene blue-stained smears of urethral specimens from men or women is still being used in STD clinics, even though endocervical samples have much lower sensitivity. This method is rapid, inexpensive and quite specific. In addition, identification can be done via cell culture via growth of *N. gonorrhoeae* on selective media and testing for Gram-negative staining cells, followed by biochemical tests. However, these methods are not sufficient for a conclusive identification of *N. gonorrhoeae*, for which further tests need to be performed (10). Different EIAs for antigen detection have also been developed for diagnosis of *N. gonorrhoeae*. These are rapid, easy to perform, do not require viable organisms, and may be sensitive and specific for detection of bacterial infection in male urethral specimens and first-void urine. However, the assays are less sensitive to endocervical specimens or to samples from asymptomatic infections. Rectal and pharyngeal specimens are not suitable for antibiotic susceptibility testing (8).

Although cell culture, DFA, and EIA have been useful in detecting chlamydia and gonorrhea infections, the tests still suffer from excessive labor intensity, complicated logistics, or suboptimal sensitivity. Newer diagnostic methods employing molecular genetic technology have the advantages of rapidity, automated technology, reasonable expense, and potential for improved sensitivity without significant loss of specificity. Nucleic Acid Techniques (NAT) are based on either the direct detection of organism-specific nucleic acid sequences with nucleic acid probes (nucleic acid hybridization). The true sensitivity of this test will be overestimated when
compared to the existing reference standard if the reference standard’s sensitivity is less than 100% and the sensitivity of the test being evaluated is less than or equal to that of the reference standard, as is the case with EIA and DFA (9).

Additionally, infection by *C. trachomatis* is often associated with *N. gonorrhoeae*; thus it is advisable to test simultaneously for both infections (10).

Detection of *C. trachomatis* and *N. gonorrhoeae* can be done via nucleic acid probes such as chemiluminescent-labeled DNA segments that are complementary to specific sequences of *C. trachomatis* or *N. gonorrhoeae* DNA/RNA. The most thoroughly evaluated of these assays is the Gen-Probe PACE-2 (PACE 2 CT, PACE 2 GC, or PACE 2C) (Gen-probe, San Diego, CA). The DNA probes offer the same advantages as EIA in that they are inexpensive and simply performed, with a total processing time of 2–3 hours. Large batches of samples can be processed easily. The sensitivity is at least comparable to that of EIA and superior to that of cell cultures. The specificity of PACE-2 can be set to virtually 100%. Unlike the EIA, where a confirmatory test is required for all positive specimens, PACE-2 requires a positive confirmatory assay supplementation only for borderline positive results (9). Other commercial test kits for *C. trachomatis* and *N. gonorrhoeae* detection are HC2® CT (GC) ID (Digene Corporation Gaithersburg, MD), APTIMA CT® Assay and PACE® 2 CT (GC) Probe Competition Assay Ct(GC)-confirmation test (Gen-Probe, Inc. San Diego, CA) (11).

The most recently introduced assays are commercial nucleic acid amplification tests (NAATs). NAATs offer several advantages over cell culture and other methods for detection of *C. trachomatis* and *N. gonorrhoeae* in clinical specimens. These advantages include increased sensitivity, high throughput, no requirement of viable organisms, and use of urine as an alternative to more difficult to obtain specimens (12). NAATs are designed to amplify nucleic acid sequences that are specific for the organism being detected. Commercial tests differ in their amplification methods and their target nucleic acid sequences. For *C. trachomatis*, The Roche Amplicor® test
(manufactured by Roche Diagnostics Corporation, Basel, Switzerland) uses polymerase chain reaction (PCR); the Abbott LCx® (Abbott Laboratories, Abbott Park, Illinois) test uses ligase chain reaction (LCR); and the Becton Dickinson BDProbeTec™ ET (Becton, Dickinson and Company, Franklin Lakes, New Jersey) test uses strand displacement amplification (SDA) to amplify *C. trachomatis* DNA sequences in the cryptic plasmid that is found in >99% of strains of *C. trachomatis*. The Gen-Probe APTIMA™ (Gen-Probe, Incorporated, San Diego, California) assay for *C. trachomatis* uses transcription-mediated amplification (TMA) to detect a specific 23S ribosomal RNA target. For *N. gonorrhoeae* detection, the target of the Roche Amplicor test is a 201 base pair sequence within the cytosine methyltransferase gene M:Ngo P11. The Abbott LCx test detects a 48 base-pair sequence in the Opa genes, ≤11 copies of which occur per cell, whereas the BDProbeTec ET detects DNA sequence within the multicopy pilin gene-inverting protein homologue. The Gen-Probe APTIMA Combo 2 version of TMA detects the 16S ribosomal RNA (rRNA). The majority of commercial NAATs have been cleared to detect *C. trachomatis* and *N. gonorrhoeae* in endocervical swabs from women, urethral swabs from men, and urine from both men and women. In addition, other specimens (e.g., those from the vagina and eye) have been used with satisfactory results, although these applications have not been cleared. Testing of rectal and oropharyngeal specimens with NAATs has had limited evaluation and is not recommended. However, the primers employed by certain NAATs for *N. gonorrhoeae* might cross-react with nongonococcal *Neisseria* species (4).

NAATs have been shown to be more sensitive than non-amplified tests for the detection of *C. trachomatis* and *N. gonorrhoeae* in asymptomatic patients. The Centers for Disease Control and Prevention (CDC) recommends using NAAT for *C. trachomatis* and *N. gonorrhoeae* screening of female endocervical/urethral swabs, as well as male and urine samples (13,14). Although, NAATs technology provides highly sensitivity and specificity, however, NAATs do have some limitations, such as the expense of the commercial test kits. In theory, this problem can be circumvented by the use...
of multiplex NAAT assays, whereby reagents for the detection of both *C. trachomatis* and *N. gonorrhoeae* are incorporated into the one reaction mix. Therefore, a single multiplex reaction would in theory be able to simultaneously detect and distinguish the presence of both organisms. Potentially, a multiplex assay detecting three targets could decrease assay costs and hands-on time by two-thirds. However, the use of multiplex assays may create further problems due to the inclusion of multiple primers and probes in one reaction mix, which could decrease the ability to detect individual targets through either non-specific interaction between the primers and probes or by competition between the amplification of the different NAAT reactions (15).

The polymerase chain reaction (PCR) which is a method that allows logarithmic amplification of short DNA sequences within a longer double stranded DNA molecule by using a pair of primers (16). PCR is the most sensitive method but still has some disadvantages, such as post PCR processing and beneath specificity. Thus, conventional PCR is replaced by Real Time PCR, in which the progress of a PCR reaction can be monitored in real time and which has greater sensitivity, and for which post PCR processing is not required (17).

With reference to all of the above methods, genitourinary tract infections due to *C. trachomatis* and *N. gonorrhoeae* are a major cause of morbidity in sexually active individuals and also continue to rise among homosexual and/or heterosexual males (MSM) and young worker populations of both sexes (18,19). Untreated, *C. trachomatis* and *N. gonorrhoeae* infections can lead to serious complications. Even though, Azithromycin 1 g orally in a single dose or Doxycycline 100 mg orally twice a day for 7 days for *C. trachomatis* and Ofloxacin 400 mg orally in a single dose or Levofloxacin 250 mg orally in a single dose for *N. gonorrhoeae* are efficient treatments, prevention and early diagnosis is probably the best way to reduce the prevalence of infection (20).

The last 10 years have seen major improvements in the ability to detect STIs, first with the advent of newer technologies such as DNA hybridization
and nucleic acid amplification, and second via PCR, including Real Time PCR. In the meantime, evaluations of several commercial tests are now available. Nucleic acid amplification tests have generally been more sensitive than traditional tests for the detection of *C. trachomatis*, but the methods have not been extensively evaluated for *N. gonorrhoeae*. Although multiplex PCR has been applied to the detection of *C. trachomatis* and *N. gonorrhoeae* in clinical specimens, this approach to diagnosis using the Real time PCR multiplex method has only recently been applied to the detection of bacterial STIs (7).

Standard laboratory diagnosis of these pathogens is based mainly on direct microscopy or isolation and identification methods of the viable organisms. Although they are relatively inexpensive and highly specific, such methods are logistically complicated and limited in sensitivity. Rapid, easily performed, and accurate diagnostic tests are urgently need to identify individuals who harbor these organisms. Accurate identification of individuals at risk for chlamydial or gonococcal infection is highly desirable, as it would facilitate early recognition and treatment of these infections (3).

In this study, we describe a rapid and inexpensive In-house Taqman-based multiplex Real Time polymerase chain reaction for simultaneous detection of *N. gonorrhoeae* and *C. trachomatis* in a single reaction.
Objectives

1. To develop In-house Taqman-based multiplex Real Time polymerase chain reaction for detection of *N. gonorrhoeae* and *C. trachomatis* cryptic plasmid DNA.

2. To compare the performance of In-house Taqman-based multiplex Real Time polymerase chain reaction for detection of *N. gonorrhoeae* and *C. trachomatis* cryptic plasmid DNA method in urine samples with the Roche multiplex AMPLICOR CT/NG PCR test.

3. To assess the extent of lack of the cryptic plasmid in one *N. gonorrhoeae* strain.