CHAPTER V
DISCUSSION AND CONCLUSION

For many decades, the standard methods for detection of *N. gonorrhoeae* and *C. trachomatis* in patient specimens were culture (liquid and/or solid media) and staining methods, such as Gram stain for *N. gonorrhoeae* and tissue culture for *C. trachomatis*. Less commonly, enzyme immunoassays (EIAs) and direct fluorescent antibody (DFA) staining methods were used to detect bacterial antigens using specific antibodies. Clinical diagnostic methods were revolutionized by the development of nucleic acid tests (NATs), which use molecular biological techniques to detect microorganismal nucleic acids by hybridization with a labelledDNA probe specific to a DNA sequence of bacteria. NATs that detected organisms directly in the specimens without the need for cell culture provided shorter times to obtain results and were generally more accurate than direct immunological methods. Subsequently, a crucial technological improvement to direct NATs was made by adding a target amplification step, which amplified the target sequence prior to probe hybridization. Since then, improvements have been made in NAATs in several amplification methods, such as Stand Displacement Amplification (SDA), Transcription Mediated Amplification (TMA), Ligase Chain Reaction (LCR) and Polymerase Chain Reaction (PCR) (55).

PCR is the most sensitive assay compared to previously available diagnostic tests such as culture, antigen detection, or nucleic acid hybridization and has a powerful potential for screening and diagnosis of various STIs, especially genital chlamydial and gonorrhea infections. It can provide higher sensitivity by at least 20- 30% when compared to the traditional method. Moreover, with PCR, a non-invasive sample collection method such as urine is possible. Comparing using urine and urethral swab, Sugunendran H reported that using urine was more sensitive than the urethral swab in detection of *C. trachomatis* infection in males (134). Thus first-catch urine samples (FCU) from men or women, which is a key

advantage of NAATs, may be the sample of choice for detection of gonorrhea and chlamydia infection. In addition, there is the possibility of screening males and females in other than traditional venues (4, 126, 133). The faster the detection of bacterial infection, the treatment of infection can commence, which can prevent spreading STIs that increase the likelihood of HIV type 1 infection (135-137).

STIs screening programs in many countries have demonstrated the prevalence of co-infection of both organisms at rates of 20 % in men and 21% in women, and have regularly found *C. trachomatis* infection after gonorrhoeae treatment (138, 139). Mariani SM. et al suggested simultaneous treatment of both gonorrhea and chlamydia infection. However, multi-treatment of these microorganisms might lead to inappropriate treatment and/or more expensive treatment (10). The appropriate treatment needs to be directed to the cause of infection using methods specific to those pathogens.

Nowadays, there are many commercial assays available for *C. trachomatis* and *N. gonorrhoeae* detection such as Ligase Chain Reaction (Abbott LCx), Stand Displacement Assay (BDProbeTecET) and COBAS AMPLICOR ™ CT/NG multiplex Polymerase Chain Reaction assay (Roche Diagnostics). They provide a useful platform for STI evaluation and have been evaluated by several research groups. (125). The sensitivity of PCR ranges from 95-100 % with standard specimens; urethral swab and endocervical swab may be used. However, with the alternative non-invasive samples; urine or vaginal swabs, the sensitivity is lower (70-90%). The performance of the PCR techniques used for diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections may vary among different settings, format of assays, and types of clinical specimen (15). However, when comparing conventional PCR to real time PCR, there are many advantages of real time PCR, such as non-post PCR procedure which reduces the vertical and horizontal contamination and is available for massive screening, during which the results can be obtained within 4-5 hours. Moreover, real time PCR reaction detects the reaction with probe

hybridization at the amplifying step, which yields more reliable results than conventional PCR that detects PCR products at the end of the reaction.

However, the major drawback of commercial test kits has been the cost for the assays, and some lack specificity such as the COBAS AMPLICOR test for *N. gonorrhoeae* (COBAS AMPLICOR CT/NG; Roche Diagnostics), which produces false-positive results with certain nonpathogenic *Neisseria* species (*N. subflava* and *N. cinerea*) and lactobacilli, and thus a subsequent confirmation test is necessary (128, 140).

We reported here the establishment of In-house Taqman based multiplex real time PCR for simultaneously screening of *N. gonorrhoeae* (cppB cryptic plasmid) and *C. trachomatis* (cryptic plasmid) in urine specimens. In general, multiplex real time PCR has sensitivity as high as the real time PCR reaction but can reduce the cost of the test and give better performance. Moreover, with the multiplex format, our assay can detect both bacteria simultaneously within one reaction. This makes the assay more accurate, convenient, less time consuming. Multiplex real time is sensitive for both *N. gonorrhoeae* and *C. trachomatis* DNA detection at 5 fg per reaction. According to Mahony JB et al, the multiplex PCR method detecting *C. trachomatis* and *N. gonorrhoeae* in genitourinary specimens demonstrated a sensitivity of 10 fg of total DNA for both bacteria. When compared to other in- house real time PCR, Whiley DM. et al. reported that their in-house real time PCR had a sensitivity for detection of *N. gonorrhoeae* at 5 colonies (141). Our method provides sensitivity for detection *N. gonorrhoeae* comparable to those from previous reports. For *C. trachomatis* detection, Jaton K. et al. reported the sensitivity of their novel real time PCR for detection *C. trachomatis* in first void urine at lower than 10 copies per reaction (142) which is also comparable to the assay developed in this study.

In addition, the results from specificity testing indicate that our assay is highly specific only to *N. gonorrhoeae* and *C. trachomatis* and

can be applied to use with other clinical sample methods, including throat swabs .

Unfortunately, Hjelmevoll S. et al. reported that cppB cryptic plasmid, the target of our real time PCR assay, was absent in some *N. gonorrhoeae* strains. As the result, confirmation test was needed. In this study, we performed the real time PCR specific to *N. gonorrhoeae* porin A pseudogene to confirm the negative results and determined the plasmid lacking strain *N. gonorrhoeae* as well (30, 31, 56, 127, 143). We reported 2.66% prevalence of plasmid lacking strain *N. gonorrhoeae* in these subjects. However this number needs to be confirmed with a larger sample size.

Furthermore, the performance of the multiplex real time PCR *N. gonorrhoeae* and *C. trachomatis* assay was compared with a commercial test kit; Roche AMPLICOR CT/NG assay from urine samples.. The 9 samples positive only from our assay were later confirmed positive with porin A pseudogene detection, indicating the high sensitivity of the real-time PCR assay. For *C. trachomatis*, 4 samples positive by our assay were also confirmed positive by conventional PCR detecting the *C. trachomatis* MOMP gene. This discordant result might be due to higher sensitivity of the real time PCR than COBAS AMPLICOR. Moreover, our test had eliminated the cross reaction found in COBAS AMPLICOR, for instance cross reactivity with certain nonpathogenic *Neisseria* species (*N. cinerea* and *N. subflava*)(128, 146-148).

Regarding the nature of the urine sample, as epithelial cells may not be presence in all samples, the use of β-globin gene as an internal control is not possible. To confirm the negative result, human β-globin gene extracted from PBMC was added to the negative samples and detected by conventional PCR. From 191 samples, 3 samples gave a negative result, which may due to the presence of inhibitors in the samples. Although incorporation of internal control DNA into the assay system is more convenient, this might reduce the sensitivity. However, the suitable internal control system should be designed and tested further.

Even though there are several commercial test kits available, the cost of the test may be limiting. Our In-house Taqman-based multiplex Real Time PCR could efficiently be applied for clinical usage, according to its high sensitivity and specificity. Nowadays there is a need to develop better and more easily available techniques for diagnosing STDs at reasonable expense. The estimated cost of multiplex Real Time PCR method was less than 500 Baht or 4 times cheaper than the commercial test kits.

In-house Taqman-base multiplex real time PCR is easy to perform, as the amplification can simultaneously detect both *C. trachomatis* and *N. gonorrhoeae* within 4-5 hours turnaround time. The confirmation assay with porin A pseudogene enhances the specificity of the screening assay with plasmid DNA. Taken together with using non-invasive urine sampling, this assay is convenient and suitable for epidemiological studies in screening large number of samples.