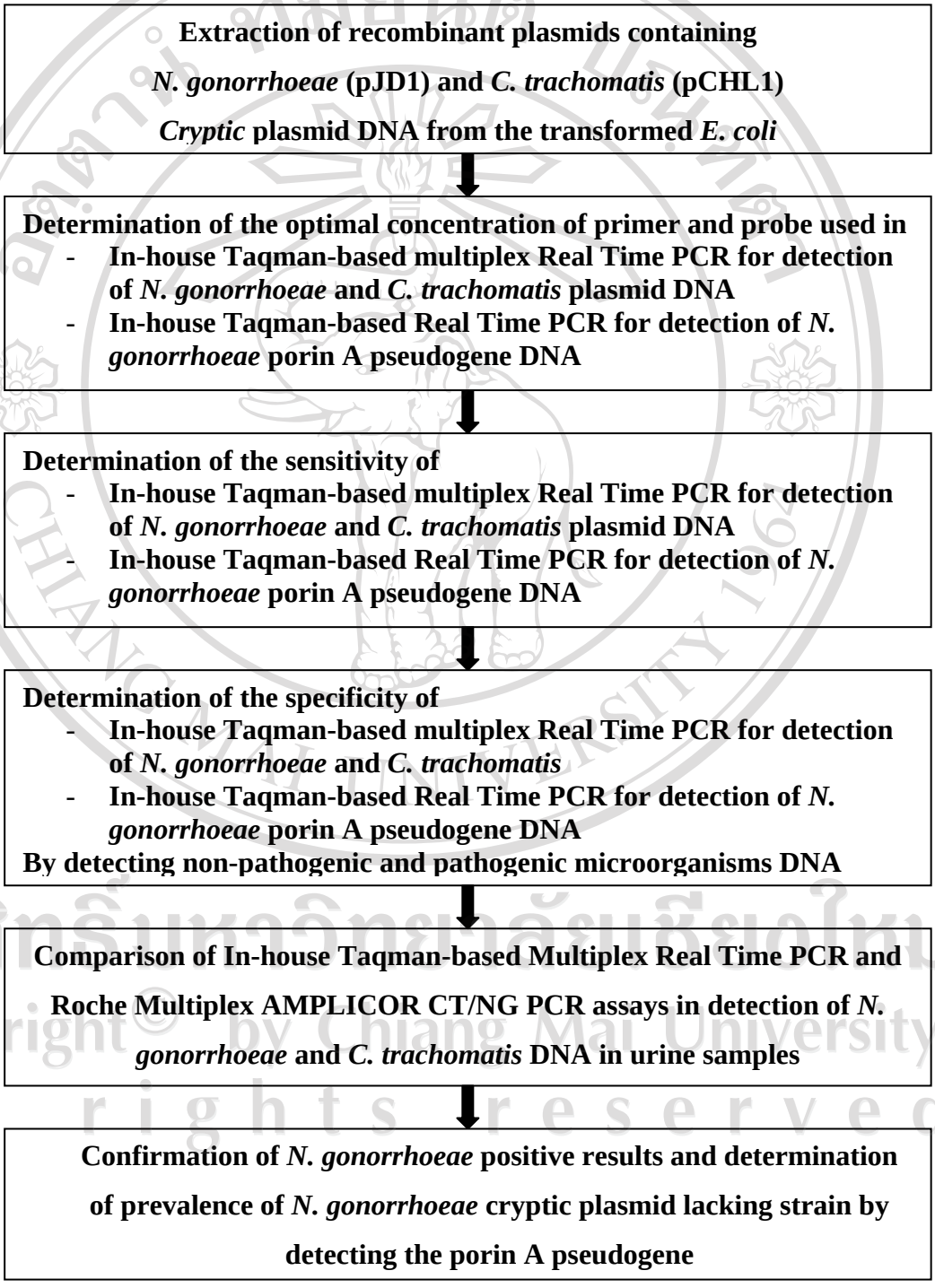
CHAPTER III

RESEARCH DESIGN, MATERIALS AND METHODS

1. Research design



2. Materials and methods

2.1. Extraction of recombinant plasmids containing *N. gonorrhoeae* (pJD1) and *C. trachomatis* (pCHL1) *cryptic* plasmid DNA from transformed *E. coli*

Recombinant plasmids containing *N. gonorrhoeae* (pJD1) and *C. trachomatis* (pCHL1) *cryptic* plasmid DNA were kindly provided by Dr. Pranee Leechanachai and Miss Tanawan Samleerat at the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. These plasmids were used for optimization and determination of the sensitivity of the assay and as a positive control DNA. The recombinant plasmid DNA was extracted from transformed *E. coli* by minipreparation using alkaline lysis solution with SDS (Samdrook et al., 1987). Briefly, a single bacterial colony was cultured in 5 ml of Luria-Bertai medium containing 100 µg/ml of ampicillin in a loosely capped 15 ml tube and incubated at 37 °C for 18 hours with vigorous shaking. After incubation, the culture was transferred into a sterile 1.5 ml micro centrifuge tube and centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was removed and the bacterial pellet resuspended in 100 µl of ice-cold Solution I and gently mixed. Then, 200 µl of Solution II were added and the contents mixed by tapping gently, and the tube was incubated on ice for 10 minutes. One hundred and fifty microliters of ice-cold Solution III were added and mixed by gently vortexing, and the mixture was then incubated on ice for 10 min. After centrifugation at 12,000 x g for 10 minutes at 4 °C, the supernatant was collected and DNA was precipitated with equal volumes of phenol-chloroform. The mixture was vortexed and the same centrifugation process was repeated, then the supernatant was collected by gentle aspiration. Absolute ethanol in double volume was added and the tube gently tapped. After being stored for 60 minutes at –70°C the same centrifugation process was repeated. After being centrifuged as above, the supernatant was

removed and 1 milliliter of 70% ethanol was added, and the mixture was gently vortexed. After being centrifuged as above, the supernatant was removed by gentle pouring. The DNA pellet was allowed to air dry until almost 70% of the ethanol had evaporated. The DNA was dissolved in 100 µl of distilled water and stored at 4 °C for 2 hours. Finally, the DNA was stored at -20 °C for further examination.

The concentration of the plasmid DNA was determined by spectrophotometry. The DNA was diluted in distilled water at an appropriate dilution (10^{-2}-10^{-3}) and the optical density (O.D.) was then measured for nucleic acid and protein at wavelengths of 260 and 280 nm, respectively, using a UV spectrophotometer (Shimadz model 1101, Japan). The quantity of DNA was calculated by using the following equation (3):

Quantity of DNA (ng/µl) = $O.D._{260}$ x dilution factor x 1 $O.D._{260}$ unit of double- stranded DNA concentration.

= O.D. at 260 nm x dilution factor x 50

1 $O.D._{260}$ unit contains double-stranded DNA = 50 ng/µl

The purity of the plasmid DNA preparation could be estimated by the ratio between O.D. 260 and 280. A pure preparation of DNA has an O.D. 260/O.D.280 value of 1.8 or more. If the ratio had been lower than 1.8, the preparation would have contained some contaminants (*e.g.*, protein or phenol). The DNA concentration (ng/µl) was converted into a number of copies by using the copy number formula as follows (3):

$$\frac{6 \times 10^{23} \text{ (copies/mol)} \times \text{concentration (g/µl)}}{\text{MW (g/mol)}} = \text{amount (copies/µl)}$$

MW = (number of base pairs) x (660 daltons/base pairs)

1 mol = 6×10^{23} molecules (copies)

2.2. In-house Taqman-based Real Time PCR

2.2.1. In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* plasmid DNA

In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* was designed as a screening test by Miss Tanawan Samleerat and Asst. Prof. Dr. Pranee Leechanachai at the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. The location of primers specific to *N. gonorrhoeae* (pJD1) and *C. trachomatis* (pCHL1) *cryptic* plasmid DNA are shown in figure 7. These were synthesized by the Operon Biotechnologies Company (Operon Biotechnologies GmbH, Germany) and used to amplify *N. gonorrhoeae* and *C. trachomatis* plasmid DNA.

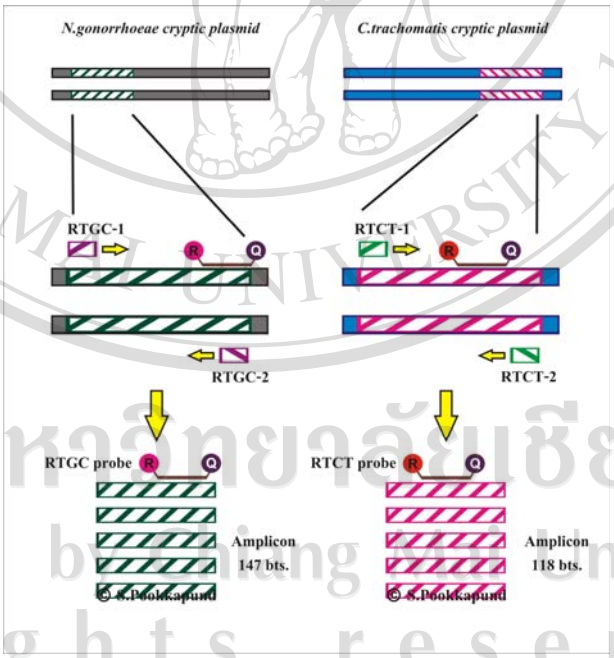


Figure 7. Illustration of the location of primers and probes used in the In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* plasmid DNA assay.

Two sets of primers, RTGC1, RTGC2 and RTCT1, RTCT2 that are specific to the cryptic plasmid of *N. gonorrhoeae* and *C. trachomatis* respectively, as well an RTGC probe, were mixed and used for the Multiplex PCR reaction, which was performed in 25 µl of reaction volume. The procedure for the In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* was performed by using 2x QuantiTect Multiplex PCR Master Mix, (QIAGEN, USA) comprising HotStarTaq® DNA Polymerase, QuantiTect Multiplex PCR Buffer, dNTP mix including dUTP, ROX (passive reference dye) and 11 mM $MgCl_2$. Five microliters of extracted sample were added and used as target DNA. The amplification was performed in a real time thermal cycler (Chromo 4™, BIO-RAD, USA). The PCR program was set up as follows; the first PCR was run for 10 minute at 95 °C to activate the enzyme, then 1 minute at 60 °C, 1 minute at 72 °C, followed by 30 seconds at 94 °C and 1 minute at 60 °C; and the last 2 steps were repeated for 44 cycles. The signal of the PCR reaction for each cycle was recorded during the annealing step at 60°C.

2.2.2. In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* porin A pseudogene DNA

In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* porin A pseudogene DNA was design by Hjelmevoll SO. *et al* (127). This method was used to confirm the detection of *N. gonorrhoeae* from the screening assay. The sequence and location of primers and probe specific to the *N. gonorrhoeae* Porin A pseudogene are shown in Figure 8 and Table 1.

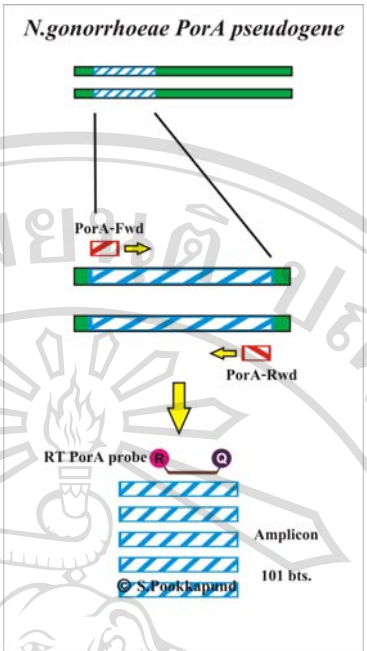


Figure 8 Illustration the location of primers and probe specific to *N. gonorrhoeae* porin A pseudogene used in In-house Taqman-based Real Time PCR for confirmation of the detection of *N. gonorrhoeae*.

For detection of *N. gonorrhoeae* Porin A pseudogene DNA, PorA-Fwd and PorA-Rwd, which amplify a 101 bp DNA fragment, were used as primers and RT PorA probe was use as a probe. The Real Time PCR reaction mixture was performed as described in 2.2.1.

Table 1 Sequence of oligonucleotide primers and probe used in In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* Porin A pseudogene (127).

Primers and probe	Sequence	Target	Product size
PorA-FWD PorA-RWD PorA-Probe	5′ CCAGAACTGGTTTCATCTGATT 3′ 5′ GTTTCAGCGGCAGCATTCA 3′ 5′ (6~FAM)CGTGAAAGTAGCAGGCG (BHQ1~dT)ATAGGCGGACTT 3′	PorA Pseudogene PorA Pseudogene PCR product	101 bp

2.3. Determination of the optimal concentration of primers and probes.

2.3.1. In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* plasmid DNA

To determine the optimal ratio of the concentrations of primers and probes used in this assay, several ratios of concentrations of primers and probe were used as follows: 400 nM/300 nM, 400 nM/200 nM, 200 nM/200 nM and 200 nM/100 nM and these were tested with purified *N. gonorrhoeae* and *C. trachomatis* plasmid DNA mixture at concentrations of 50, 5,000 and 500,000 copies per reaction. The lowest concentration ratio which yielded a similar cycle threshold compared to the others was the optimum concentration.

2.3.2. In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* Porin A pseudogene DNA

To determine the optimal ratio of the concentrations of primers and probes used in this assay, three sets of ratios of concentrations of primers and probe were used as follows:

Pattern 1: 800 nM/300 nM, 800 nM/250 nM, 800 nM/200 nM
Pattern 2; 400 nM/300 nM, 400 nM/250 nM, 400 nM/200 nM
Pattern 3: 200 nM/300 nM, 200 nM/250 nM, 200 nM/200 nM

The assay was performed in duplicate and used *N. gonorrhoeae* genomic DNA at a concentration of 500 pg per reaction as the target of the PCR amplification. The lowest concentration ratio which yielded a similar cycle threshold compared to the others was the optimum concentration.

2.4. Determination sensitivity of In-house Taqman-based Real Time PCR.

2.4.1. In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* plasmid DNA

To determine the sensitivity of the assay, a purified *N. gonorrhoeae* and *C. trachomatis* plasmid DNA mixture was 10-fold serially diluted in distilled RNase free water at concentrations from 1 pg/µl to 10 ag/µl and was used as a template for amplification. The assay was performed in duplicate 3 times. The lowest concentration of target DNA that could be detected in all assays (100 percent) was designated as the sensitivity of the assay.

2.4.2. In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* Porin A pseudogene DNA

To determine the sensitivity of the assay, purified *N. gonorrhoeae* genomic DNA was 10-fold serially diluted in distilled RNsae free water at concentrations from 100 pg/µl to 1 fg/µl and was used as a target for amplification. The assay was performed in duplicate 2 times. The minimal concentration of template DNA that could be detected in all assays (100 percent) was designated as the sensitivity of the method.

2.5. Determination of the specificity of In-house Taqman-based multiplex Real Time PCR for detection of *N. gonorrhoeae* and *C. trachomatis* and In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* porin A pseudogene.

To determine the specificity of the assays, two types of investigationwere performed, nucleotide alignment using computer software and PRC amplification of related pathogenic and non pathogenic

organisms. In the former, all primers and probed used in the In-house assays were tested by aligning with non-pathogenic and pathogenic organismal DNA sequences downloaded from http://www.ncbi.nlm.nih. gov/Genbank (Table 10). The study used the programs Primer Premier 5, AmplifX v. 1.37 and BioEdit available at http://www.premierbiosoft.com, http://www.versiontracker.com and http://www.mbio.ncsu. edu). Comparisons were made with *N. gonorrhoeae* (pJD1), *C. trachomatis* (pCHL1) cryptic plasmid and *N. gonorrhoeae* Porin A pseudogene DNA (available at http://www.ncbi.nlm.nih.gov/ Genbank/). For the latter experiment, 2-4 clinical or reference isolates of related non-pathogenic and pathogenic micro-organisms from the upper respiratory tract and urogenital tract (Table 2) were extracted for DNA by adding 100 ul of lysis buffer containing 100 µg/ml of proteinase K in a 1.5 ml micro-centrifuge tube. After vortexing, the sample was incubated at 56°C for 1 hour and the Proteinase K was inactivated immediately by incubating at 100°C for 10 minutes. After being centrifuged at 7280xg (Denville 260D Scientific Metuchen, USA) the supernatant was collected by gentle aspiration. DNA was stored at -20 °C and used for specificity testing by performing the real time PCR with all sets of primers and probes.

Table 2 List of non-pathogenic and pathogenic organisms from clinical isolates and standard strains used in specificity testing by real time PCR

Bacteria	Number of isolate	Habitat
Neisseria species		
1. *Neisseria lactamica*	2	Commensal of throat
2. *Neisseria subflava*	2	Commensal of throat
3. *Neisseria sicca*	2	Commensal of throat
4. *Neisseria cinera*	2	Commensal of throat
5. *Neisseria meningitidis*	4	Pathogen
6. Neisseria gonorrhoeae	10	Pathogen
Urogenital related organisms		
7. *Candida albicans*	4	Pathogen
8. *Escherichia coli*	4	Commensal, Pathogen
9. *Tricomonas vaginalis*	4	Pathogen
10. *Gardnerella vaginalis*	2	Pathogen
11. *Proteus mirabilis*	2	Pathogen
12. *Proteus vulgaris*	2	Pathogen
13. *Lactobacillus*	2	Normal flora
Respiratory related organisms		
14. *Staphylococcus epidermidis*	3	Commensal
15. *Staphlycoccus aureus*	4	Pathogen
16. *Streptococcus pneumoniae*	3	Pathogen
17. *Haemophilus influenzae*	4	Commensal, Pathogen
18. *Klebsiella pneumoniae*	3	Pathogen
19. *Pseudomonas aeruginosa*	3	Pathogen

2.6. Comparison between In-house Taqman-based multiplex Real Time PCR and Roche Multiplex AMPLICOR CT/NG PCR method in detection of *N. gonorrhoeae* and *C. trachomatis* urine samples.

Urine samples were kindly provided by Regional Medical Sciences Center, Chiang Rai.

Approximately 25 ml urine samples were collected from men and women at high risk for STDs in Chiang Rai province. The samples were kept in a sterile 50-ml screw-cap plastic cups without preservatives and were sent for detection of *N. gonorrhoeae* and *C. trachomatis* using the

Roche Multiplex AMPLICOR CT/NG PCR method at the Regional Medical Sciences Center. The leftover samples were sent to the Division of Clinical Microbiology, Faculty of Associated Medical Science, Chiang Mai University for further analysis. Ten milliliters of sample were pipetted into sterile 15-ml screw cap plastic tubes and processed for the testing.

Specimen processing

Urine samples were centrifuged at 1855xg (Sorvall, Germany) for 20 minutes. The supernatant was discarded and the pellet was divided into 2 tubes and stored at -20°C for later use. For the extraction, the pellet was lysed with 100 µl of lysis buffer containing 100 µg/ml of proteinase K. After adding lysis buffer, samples were incubated in a water bath at 56 °C for 60 minutes and proteinase K was inactivated immediately by incubating at 100°C for 10 minutes. After being centrifuged at 7280xg for 10 minutes, the supernatant was collected by gentle aspiration. DNA was stored at -20 °C for further use.

The Roche Multiplex AMPLICOR CT/NG PCR assay was performed by researchers at the Regional Medical Sciences Center, Chiang Rai, while the In-house Taqman-based multiplex Real Time PCR test was performed at the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. The results obtained from both methods were analyzed by persons who were not involved in either laboratory. Samples that were positive or negative by both assays were accounted as positive or negative results. All *N. gonorrhoeae* positive samples were confirmed by In-house Taqman-based Real Time PCR for detection of *N. gonorrhoeae* porin A pseudogene, while *C. trachomatis* positive samples were confirmed by conventional PCR for the MOMP gene. Negative results from the In-house Taqman-based multiplex Real Time PCR were confirmed with conventional PCR detection of the human β-globin gene. Positive results from the β-globin gene were considered as a “true negative” result, as there was DNA in the samples and no PCR inhibitor. Samples that were

negative by this test were considered as false negative and were not accounted counted in the analysis.

2.7. Confirmation assay for *N. gonorrhoeae* by using In-house Taqman-based Real Time PCR detecting Porin A pseudogene

In-house Taqman-based Real Time PCR specific to Porin A pseudogene of *N. gonorrhea* was performed as described in 2.2.2

2.8. Confirmation assay of *C. trachomatis* cryptic plasmid positive results by detecting Major Outer Membrane Protein gene using conventional PCR method.

All *C. trachomatis* positive results from the screening assay were confirmed by performing conventional PCR using the MOMP gene. Five microliters of *C. trachomatis* positive sample were added to the PCR master mix containing 10x PCR buffer, 25 mM Magnesium Chloride, 1.25 mM dNTP, Taq DNA polymerase enzyme, 20 pico mol of MOMP forward and reverse primers. The PCR program was set up as follows; the PCR cycle commenced with 45 seconds at 94 °C, followed by 45 seconds at 55 °C, and 45 seconds at 72 °C, and this was repeated for 30 cycles. Then the reaction was maintained at 72 °C for 7minutes, after which it was kept at 4 °C forfor further use.

2.9. β-globuin gene testing using conventional PCR method.

All negative results were confirmed by conventional PCR for the human β-globuin gene. Fifty picograms of human DNA extracted from the Buffy coat were added to the conventional PCR master mix containing 10x PCR buffer, 25 mM Magnesium Chloride, 1.25 mM dNTP, Taq DNA polymerase enzyme, 10 pico mol of forward and reverse primer and 5 μl of extracted DNA from negative samples. The PCR program was

set up as follows; the PCR cycle commenced with 45 seconds at 94 °C, and 45s second at 55 °C and 45 seconds at 72 °C, and this was repeated for 35 cycles. The reaction was maintained at 72 °C for 60 seconds and held at 4 °C. The amplified product was detected by agarose gel electrophoresis for 30 minutes, then stained with ethidium bromide. The DNA bands were visualized via a UV-transilluminator. The DNA size fragment was determined against standard DNA molecular size markers (GibcoBRL,USA).