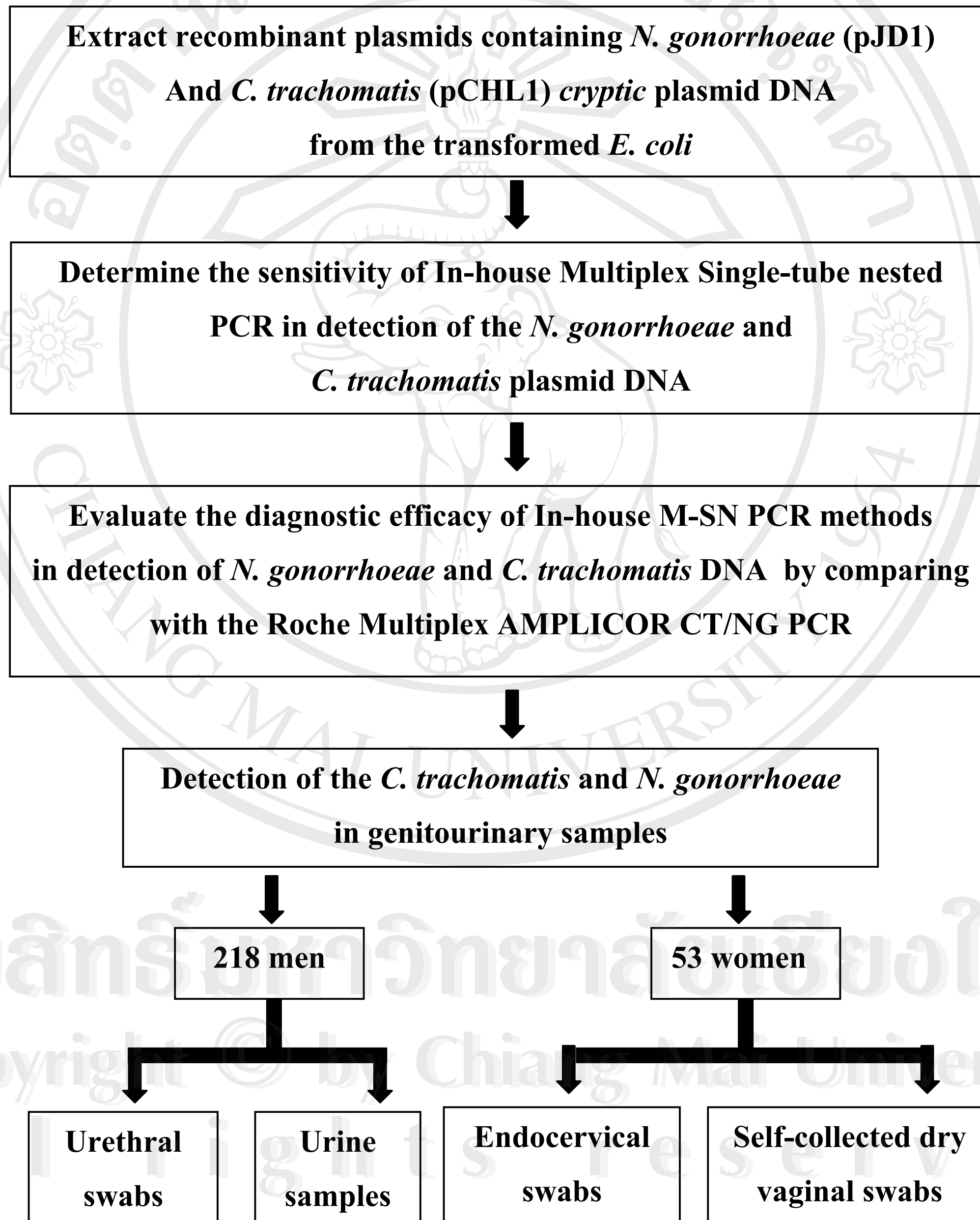
CHAPTER III

RESEARCH DESIGN, MATERAILS AND METHODS

1. Research design



ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2. Materials and methods

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

Recombinant plasmids containing *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) *cryptic* plasmid DNA have previously been preformed and were kindly provided by Dr. Pranee Leechanachai and Miss Tanawan Samleerat at Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. These plasmids were used to determine the sensitivity of the assay and as a positive control DNA. The plasmid DNA was extracted from transformed *E. coli* by minipreparation using alkaline lysis with SDS (Samdrook et al., 1987). In Brief, a single bacterial colony was picked up and cultured in 2 ml of LB medium containing 100 µg/ml of ampicillin in a loosely capped 15 ml tube, and incubated at 37 °C for 18-24 hours with vigorous shaking. After incubation, the culture was transferred into a sterile 2.0 ml polystyrene tube and centrifuge at 12,000 x g for 30 seconds at 4 °C. The supernatant was removed and the bacterial pellet suspended in 100 µl of ice-cold Solution I (glucose solution) and vortex quickly. Then, 200 µl of Solution II (NaOH and SDS solution) were added and the contents mixed by inverting the tube and incubating on ice for 5 min. One hundred and fifty microliters of ice-cold Solution III (Ammonium acetate solution) were added and mixed by gently vortexing, and the mixture was then incubated on ice for 5 min. After centrifugation at 12,000 x g for 5 min at 4 °C, the supernatant was collected and the DNA was precipitated with 2 volumes of 95% ethanol before the same centrifugation process was repeated. The supernatant was removed by gentle aspiration. To ensure that impurities were removed, a 0.5 volume of 7.5 M ammonium acetate was added, stored at -70 °C for 10 min, and centrifuged at 12,000 x g for 5 min at 4 °C, supernatant was collected. Two volumes of 95% ethanol were added again to precipitate the DNA. The DNA pellet was rinsed with 1 ml of 70% ethanol, centrifuged as above and allowed the pellet air dry for 30 minnutes. Finally, the DNA was dissolved in 100 µl of distilled water and stored at -20 °C until further examination.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

The concentration of the plasmid DNA was determined by using the spectrophotometry method. The DNA was diluted in distilled water at an appropriate dilution and the optical density (O.D.) was then measured for nucleic acid and protein at a wavelength 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:

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 plasmid DNA preparation could be estimated by the ratio between O.D. 260 and 280. Pure preparation of DNA has the O.D. 260/O.D.280 values of 1.8 or more. If the value had been lower, 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 mathematical formulas as follows:

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

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

1 mol = 6×10^{23} molecules (copies)

2.2. In-house Multiplex Single-tube nested PCR (M-SN PCR)

In-house Multiplex Single-tube nested PCR (M-SN PCR) was designed and optimized by Miss Tanawan Samleerat and Dr. Pranee Leechanachai at Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Two set of amplification primers specific to *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) *cryptic* plasmid DNA shown

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

in figure 6 were used to amplify *C. trachomatis* and *N. gonorrhoeae* DNA and β-globin gene was used as a target for internal control of the assay. Any discordant results observed between assays or specimens were confirmed by using Single-tube nested PCR (SN PCR) with primer specific to chromosomal DNA; methyltransferase gene for *N. gonorrhoeae* or MOMP gene for *C. trachomatis*

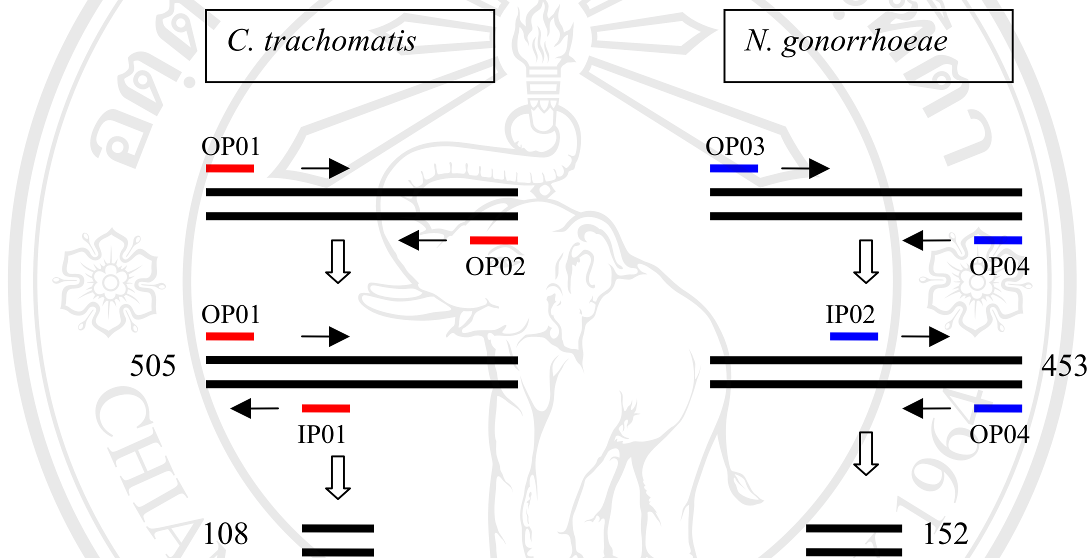


Figure 6. Illustration and location of primers specific to *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) *cryptic* plasmid DNA using in In-house Multiplex Single-tube nested PCR (M-SN PCR) for detection of *C. trachomatis* and *N. gonorrhoeae* DNA in genitourinary specimens

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

For detection of *C. trachomatis,* OP01 and OP02, which amplify a 505-bp-fragment, were used as outer primers for the first round amplification and OP01 and IP01 were used as inner primers amplify inner DNA product of 108-bp.. For N. *gonorrhoeae,* OP03 and OP04, which amplify a 453-bp-fragment, were used as the first primer pair. Inner primers for second PCR were OP04 and IP02, which amplify a 152-bp-fragment. The PCR reaction was performed with all primers including primers specific to β-globin gene in a 50 μl reaction volume. The M-SN PCR was performed as follow: 1.25 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 10x PCR

buffer (Promega, USA; 500 mm KCl, 100 mm Tris-HCl pH 9.2, 1% Triton X-100), 25 mM of $MgCl_2$ and 5 units of *Taq* DNA Polymerase. 10 μl of extracted samples was used as a target DNA. The amplification was performed in thermal cycle (GeneAmp PCR system 2700, Applied Biosystems, USA). The PCR program was set up as the following; the first PCR commenced with 45 seconds at 94 °C, 45 seconds at 68 °C, and 60 seconds at 72 °C for 5 cycles and subsequently followed by the second PCR at 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 45 seconds for 50 cycles, then at 72°C for 7 minutes. and finally hold at 4 °C. The PCR products were analyzed by agarose gel electrophoresis. Ten micro liters of the amplification products were electrophoresed on 2 % agarose gel in 0.5x TAE (Tris-Acetate-EDTA, pH 8.0) buffer at 100 volts for 40 minutes. The DNA fragments were stained with 2 μg/ml of ethidium bromide in water for 15 minutes then destained with distilled water for 10 minutes and visualized under ultraviolet light using an ultraviolet transilluminator. The DNA fragment size was determined by using the standard DNA size marker, 1 kb Plus DNA Ladder (GibcoBRL,USA).

Table 2. Oligonucleotide primer sequences specific to *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) *cryptic* plasmid DNA used in M-SN PCR and the fragment size of amplified products

Primers	Sequence	Target	Product size (bp)
OP-01	5′CCTGCTTGAGAGAACGTGCGGGCGA 3′	pCHL1 (CT)	-
OP-02	5′CTGCCAAGACTTCTTGGACCGAAGG 3′	pCHL1 (CT)	505
IP-01	5′GCACTTTCTACAAGAGTACATCGG 3′	pCHL1 (CT)	108
OP-03	5′ GCCCAAGTGCGTTAAGGCTTTCATC 3′	pJD1 (GC)	453
OP-04	5′ GCCGCCAGCATAGAGCAACAAACG 3′	pJD1 (GC)	-
IP-02	5′ CACAGATACGGCTTGAATACTGC 3′	pJD1 (GC)	152

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Table 3. In-house Multiplex Single-tube nested PCR for detection of *C. trachomatis* and *N. gonorrhoeae* master mix

Reagent	1 Reaction (µl)
Distilled water	13
10X buffer	5
25 mM MgCl2	5
1.25 mM dNTPs	8
20 pmol/µl OP-01	1
20 pmol/µl OP-04	1
18 pmol/µl IP-01	1
18 pmol/µl IP-02	1
2 pmol/µl OP-02	1
2 pmol/µl OP-03	1
4 pmol/µl ß-BGL-1	1
4 pmol/µl ß-BGL-2	1
5U *Taq* DNA polymerase	1
Extracted DNA	10
Total	**50**

2.3. Determination of the sensitivity of In-house Multiplex Single-tube nested PCR in detection of the *C. trachomatis* and *N. gonorrhoeae* plasmid DNA

To determine the sensitivity or lower detection limit of the assay, purified *C. trachomatis* and *N. gonorrhoeae* plasmid DNA were 10-fold serially diluted in water and in the extract of pooled *C. trachomatis* and *N. gonorrhoeae* negative cervical samples from 100 ng/µl to 1 fg/µl. The smallest concentration of target DNA that can be detected was determined as the lower limit of the assay.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.4. Evaluation the diagnostic efficacy of In-house M-SN PCR methods in detection of *C. trachomatis* and *N. gonorrhoeae* DNA in urine samples by comparing with the Roche Multiplex AMPLICOR CT/NG PCR

Approximately 20 ml of first void urine (FVU) samples were collected from 278 men aged 18-25 years old. Ten milliliters of sample was pipetted into two sterile 15-ml screw cap plastic tubes, one was used for M-SN PCR and the other was sent for Roche Multiplex AMPLICOR CT/NG PCR as gold standard method. Blind experiment was designed for this study. Roche Multiplex AMPLICOR CT/NG PCR was performed by researchers at the Research Institute for Health Science, Chiang Mai University while the M-SN PCR 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 both laboratories. Samples that positive or negative by both assays were accounted as the true positive or the true negative samples. The discordant results observed between assays were confirmed by using PCR with primer specific to chromosomal DNA; methyltransferase gene for *N. gonorrhoeae* or MOMP gene for *C. trachomatis*. Samples that were positive by either assay and by confirmatory PCR were considered as “true positive”, in reverse samples that were negative by either assay and by confirmatory PCR were considered as “true negative” results.

2.5. Detection of the *C. trachomatis* and *N. gonorrhoeae* in different types of genitourinary samples

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Genitourinary Samples Urethral swabs and first void urine, cervical and self-collected vaginal swabs were randomly obtained from 218 mem and 53 women attending at the Sexually Transmitted Infection Center 10 Chiang Mai from November 2004 through December 2005 for their routine check up or health problems. Gram staining and cultivation of *N. gonorrhoeae* were routine service at the center and the rest of samples were sent to confirm by using M-SNPCR. Urethral swabs were collected by physician or nurse by inserting a narrow-shafted Dacron-

tipped swab 2 to 3 cm into the urethra, turn the swab around 2 to 3 times and then placed in transport screw-cap sterile tube containing 2 ml sterile normal saline solution (0.9% NaCl). Approximately 20 ml of first-voided urine (FVU) was collected in a sterile 50-ml screw-cap plastic cup without preservatives. For women, pelvic examination was routinely performed by physician or nurse and endocervical swab was obtained and placed in transport screw-cap sterile tube containing 2 ml sterile normal saline solution Afterwards women were instructed how to take the self-collected vaginal swab; put the tip of the cotton swab stick about 2 cm inside vagina. Turn the swab a round once and count to ten whilst leaving the cotton swab stick in the vagina, then air dried at room temperature. The self-collected dry vaginal swab was pack into sealed plastic bag. All specimens were transported at 4°C to the laboratory on the same day as collection.

Specimen processing

Urine samples Ten milliliters of FVU samples were centrifuged at 3000g for 20 minutes. The supernatant was discarded, and the pellet was lysis with 200 µl of lysis buffer containing 100 µg/ml of proteinase K.

Swab samples For urethral and endocervical swabs, after votexing and discarded the swabs, 2.0 ml of samples were centrifuged at 10,000 x g for 10 minutes at room temperature. The supernatant was removed and pellet was lysis with 200 µl of lysis buffer containing 100 µg/ml of proteinase K. For dry vaginal swabs, before extraction swabs were soaked in 2 ml sterile normal saline solution for overnight, after vortexing for 30 seconds they were discarded. The sample solutions obtained from this step were further processed as described above.

After adding lysis buffer containing proteinase K, all samples were incubated in water bath at 56 °C for 60 minutes and proteinase K was inactivated immediately by incubating at 95°C for 10 minutes. A 0.5 volume of 7.5 M ammonium acetate was added and mixed well then centrifuge at 10,000 x g for 10 minutes at room temperature, the supernatant was collected and the DNA was precipitated with 2 volumes of 95% ethanol and stored at -70 °C for 10 minutes or -20 °C for 2 hours. After centrifugation at 10,000 x g for 10 minutes at 4°C, the supernatant was removed by gentle aspiration. The DNA pellet was rinsed with 1 ml of 70% ethanol by centrifuged at 10,000 x g for 5 minutes at 4°C, removed the supernatant by gentle

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

aspiration and the pellet was allowed to air dry for 30 min. Finally, the DNA was dissolved in 50 µl of distilled water and stored at -20 °C until further examination.

2.6. Comparative cost analysis

For comparative analysis, costs of reagents and disposables was analyzed followed the steps of the assays; the in-house M-SN PCR as described above and the Roche Multiplex AMPLICOR CT/NG PCR, as described in the package inserts. Costs for disposables were based on price lists on August 2006 from the vendors in Thailand. No discounted pricing was used.

Costs of disposables for the Roche Multiplex AMPLICOR CT/NG PCR assays were determined for each of the three workflow steps. Costs of sample preparation disposables were calculated for the following; 1 polystyrene tube-2ml (800 baht/1000) and 3 aerosol barrier 1000-µl tips (350 baht/96). And costs of disposables for amplification and detection were calculated for the following; 1 aerosol barrier 200-µl tip (350 baht/96).

Costs of disposables for the in-house M-SN PCR assay were determined for each of the three workflow steps. Costs of sample preparation disposables were calculated for the following; 2 polystyrene tubes-2.0ml (800 baht/1000) and 6 aerosol barrier 1000-µl tips (350 baht/96). Costs of disposables for amplification were calculated for the following; 13 aerosol barrier 200-µl tips (350 baht/96) and 1 PCR reaction tube (200 baht/100). And costs of disposables for detection were calculated for the following; 1 barrier 200-µl tip (350 baht/96).

Cost of reagent for the Roche Multiplex AMPLICOR CT/NG PCR assays is approximately 1,600 baht/test (consumable equipments not included). And cost of reagents or the in-house M-SN PCR assay were calculated for the following; 5U Taq Polymerase, 10X buffer, 25 mM $MgCl_2$, 1.25 mM mixed dNTPs, primers and others reagents (distilled water PCR grade, lysis buffer, 10 mg/ml proteinase K, 7.5 M ammonium acetate, ethanol, 2% agarose gel, 0.5X tris acetate buffer, loading buffer, 10 mg/ml ethidium bromide and 1 kb plus DNA ladder) is approximately 118.64 baht/test.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved