CHAPTER IV
RESULTS

1. Preparation of recombinant plasmid containing *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) DNA from the transformed *E. coli*

After extraction and purification, the recombinant plasmid containing *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) DNA was tested again by PCR. As expected, DNA fragments of 152 and 108 bps were obtained from *N. Gonorrhoeae* and *C. trachomatis* DNA respectively. The results were shown in figure 7.

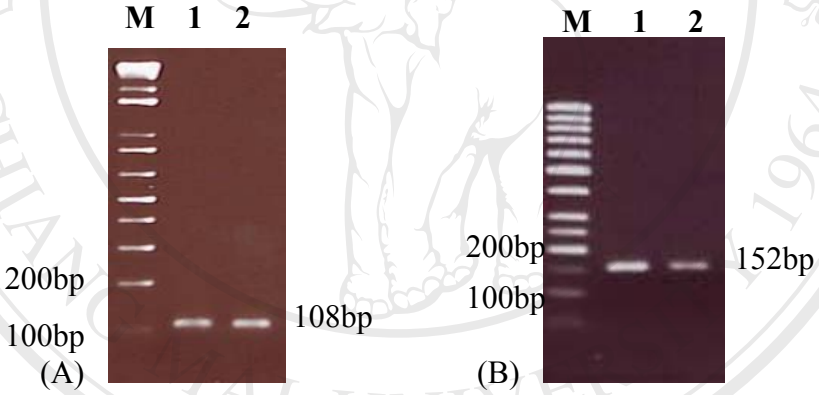

Figure 7. (A) The amplification of the plasmid *C. trachomatis* (pCHL1) DNA fragments from colonies of transformed *E. coli* by PCR. Lane M shows the DNA marker, lane 1 and 2 show the 108 bp PCR product of the plasmid DNA from different colonies. (B) The amplification of the plasmid *N. gonorrhoeae* (pJD1) DNA fragments from colonies of transformed *E. coli* by PCR. Lane M shows the DNA marker, lane 1 and 2 show the 152 bp PCR product of the plasmid DNA from different colonies.

The plasmid DNA was then quantified by using the spectrophotometry. The quality of DNA was determined by the ratio between the O.D. at 260 and 280. The

O.D. ratio of 1.8 or more demonstrates the high quality of DNA preparation. The performance of DNA from each preparation was shown in table 4.

Table 4. Showed the results of plasmid DNA preparation

Plasmid DNA	O.D.260	O.D.280	O.D.260 / O.D.280 ratio	Dilution factor	DNA µg/ml
N. gonorrhoeae	0.736	0.358	2.05	100	3,680
C. trachomatis	0.611	0.280	2.18	100	3,053

2. Sensitivity of In-house Multiplex Single-tube nested PCR (M-SN PCR)

Two sets of 10-fold serially dilutions of recombinant plasmid DNA containing *C. trachomatis* (pCHL1) and *N. gonorrhoeae* (pJD1) DNA from concentration 100 ng/µl to 1 fg/µl were used for determination the sensitivity of the assays. One was diluted in distilled water and the other was diluted with extract from pooled negative cervical swabs. Ten microliters of each dilution was amplified by M-SN PCR and detected by using agarose gel electrophoresis. The results were shown in figure 8-11. For *N. gonorrhoeae*, when DNA was diluted in water the sensitivity of the assay was at 10 pg/µl (figure 8), while diluted in the negative swabs extract, the sensitivity was lower at 100 pg/µl (figure 9). Sensitivity of M-SN PCR for detection of *C. trachomatis* DNA was at 100 fg/µl and 10 pg/µl when diluted in water and negative swabs extract respectively (figure 10-11). Although, the sensitivity of the assay was lower when both *N. gonorrhoeae* and *C. trachomatis* DNA were diluted in the negative swabs extract than in water but it represents the true sensitivity when tested with clinical samples.

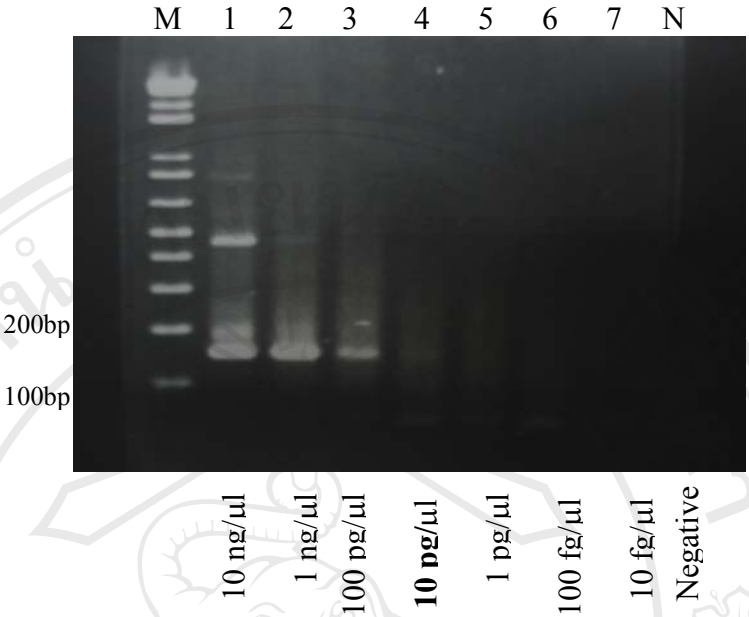

Figure 8 Illustration the sensitivity determination of in-house M-SN PCR in detection of *N. gonorrhoeae* DNA diluted in distilled water

Lane M; 1Kb plus DNA ladder,

Lane 1-7; serial dilution of *N. gonorrhoeae* DNA

Lane N; negative control using distilled water

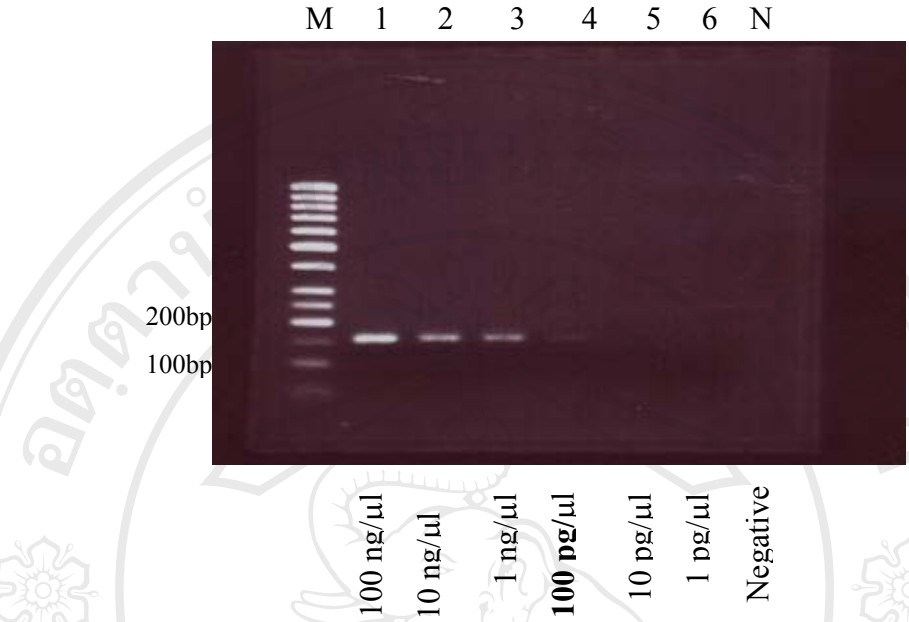

Figure 9. Illustration the sensitivity determination of in-house M-SN PCR in detection of *N. gonorrhoeae* DNA diluted in pooled negative cervical swabs extract

Lane M; 1Kb DNA ladder Plus (Gibco BRL, USA)

Lane 1-7; serial dilution of *N. gonorrhoeae* DNA

Lane N; negative control using distilled water

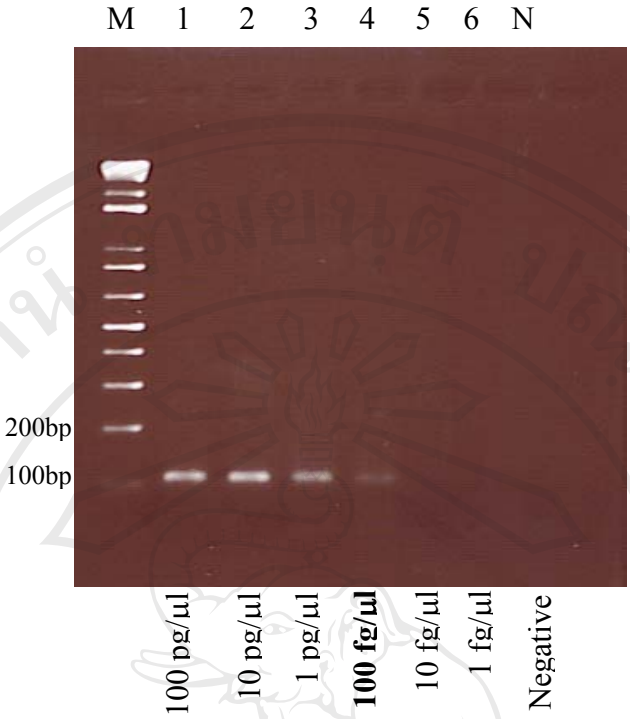

Figure.10 Illustration the sensitivity determination of in-house M-SN PCR in detection of *C. trachomatis* DNA diluted in water

Lane M; 1Kb DNA ladder Plus (Gibco BRL, USA)

Lane 1-6; serial dilution of *N. gonorrhoeae* DNA

Lane N; negative control using distilled water

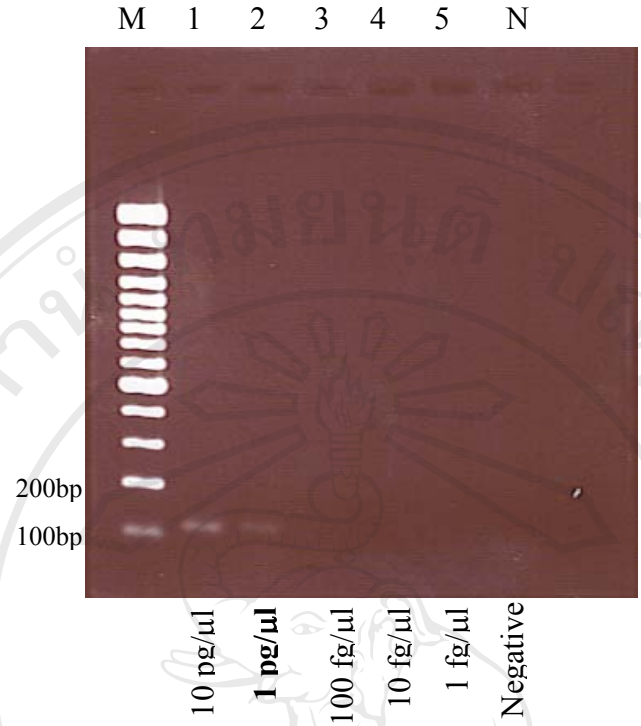

Figure 11 Illustration the sensitivity determination of in-house M-SN PCR in detection of *C. trachomatis* DNA diluted in pooled negative cervical swabs extract

Lane M; 1Kb DNA ladder Plus (Gibco BRL, USA)

Lane 1-5; serial dilution of *C. trachomatis* DNA

Lane N; negative control using distilled water

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

To evaluate the diagnostic efficacy of our in-house M-SN PCR, 278 first void urine samples were randomly collected from STD high-risk male age between 18-25 years old and tested for *C. trachomatis* and *N. gonorrhoeae* infection by using M-SN PCR and Roche Multiplex AMPLICOR CT/NG PCR as gold standard method. The two assays were blindly performed in two separated laboratories and technicians. After all samples had been done, the results were analyzed by comparing the positive and negative results obtained from the two assays. Samples with concordant results were determined as true positive. However, samples with discordant results were confirmed by the third assay which different in assay principle or primer specificity. In this study, we used the SN-PCR with primers specific to the chromosomal DNA; MOMP gene for *C. trachomatis* and methyltransferase gene for *N. gonorrhoeae*. Any positive or negative results that had been confirmed positive or negative respectively were determined as true positive or negative results.

Among 208 samples, 17 samples positive for *N. gonorrhoeae* DNA by Roche Multiplex AMPLICOR CT/NG PCR but only 16 samples positive by M-SN PCR. One sample that had been missed by our assay was later confirmed positive by SN PCR with primers specific to chromosomal DNA and was determined as a true positive. However, 191 samples negative for *N. gonorrhoeae* by Roche Multiplex AMPLICOR CT/NG PCR were all negative by M-SN PCR. They were all determined as true negative results. The results were shown in table 5.

Comparing with Roche Multiplex AMPLICOR CT/NG PCR, the M-SN PCR had sensitivity at 94.12% whereas the specificity was at 100%. The positive and negative predictive values were 100 and 99.48% respectively.

Table 5. Comparison between In-house M-SN PCR and Roche Multiplex AMPLICOR CT/NG PCR in detection of *N. gonorrhoeae* from urine samples

Assays		Roche Multiplex AMPLICOR CT/NG PCR		Total
		Positive	Negative	
In-house Multiplex Single-tube nested PCR (M-SN PCR)	Positive	16 (a)	0 (b)	16
	Negative	1 (c)	191 (d)	192
Total		17	191	208

Notes:

Sensitivity = (a / a+c) x 100 =(16 / 17) x 100 = 94.12 %

Specificity = (d / b+d) x 100 =(191 / 191) x 100 = 100 %

Positive predictive value = (a / a+b) x 100 =(16 / 16) x 100 = 100 %

Negative predictive value = (d / c+d) x 100 =(191 / 192) x 100 = 99.48 %

Concordant = 99.52% (207 of 208)

Discordant = 0.48% (1 of 208)

For *C. trachomatis* detection, 278 FVU samples were analyzed comparing with Roche Multiplex AMPLICOR CT/NG PCR. As shown in the table 6, *C. trachomatis* was detected in 66 from 278 samples by Roche Multiplex AMPLICOR CT/NG PCR while the M-SN PCR detected only 64 samples. After confirmation, the two discordant samples were the confirmed positive. However, all 212 samples negative by Roche Multiplex AMPLICOR CT/NG PCR were negative by M-SN PCR. When analyzed, the sensitivity of M-SN PCR was at 96.97% while the specificity was 100%. The positive and negative predictive values were 100% and 99.07% respectively.

Table 6. Comparison between In-house M-SN PCR and Roche Multiplex AMPLICOR CT/NG PCR in detection of *C. trachomatis* from urine samples.

Assays		Roche Multiplex AMPLICOR CT/NG PCR		Total
		Positive	Negative	
In-house Multiplex Single-tube nested PCR (M-SN PCR)	Positive	64 (a)	0 (b)	64
	Negative	2 (c)	212 (d)	214
Total		66	212	278

Notes:

Sensitivity = (a / a+c) x 100 = (64 / 66) x 100 = 96.97%

Specificity = (d / b+d) x 100 = (212 / 212) x 100 = 100 %

Positive predictive value = (a / a+b) x 100 = (64 / 64) x 100 = 100 %

Negative predictive value = (d / c+d) x 100 = (212 / 214) x 100 = 99.07 %

Concordant = 99.28% (276 of 278)

Discordant = 0.72% (2 of 278)

4. Detection of the *C. trachomatis* and *N. gonorrhoeae* in clinical samples by using In-house M-SN PCR.

For standard protocol, urethral and endocervical swabs are used for laboratory diagnosis of STI in men and women respectively. However, collection of these specimens is usually invasive and should be done by experience personnel like physicians or nurses. In this study, we compared the efficiency of non-invasive collected samples (urine or self-collected vaginal dry swab) with the invasive standard samples (urethral or endocervical swabs) in detection of *C. trachomatis* and *N. gonorrhoeae***.** Urethral swabs and FVU were collected from 195 men, endocervical and self-collected vaginal dry swabs were collected from 53 women. All samples were tested for presence of *C. trachomatis* and *N. gonorrhoeae* DNA by using M-SN PCR. Amplification of ß–globin gene was used as an internal control for quality of sample collection, sample preparation and amplification steps. After amplification,

any samples showing no ß–globin amplification DNA fragment were excluded from further analysis as unqualified samples. Among 195 urethral swabs and urine samples, 23 urethral swabs and 18 urine samples were excluded according to this criterion, only 154 sample pairs where both of them are qualify were used for analysis. The same criteria was also applied for using with endocervical and self-collected vaginal dry swabs, among 53 samples, 13 were excluded (2 for endocervical swabs and 11 for self-collected vaginal dry swabs), only 40 sample pairs were subjected for analysis later. The results of M- SN PCR in detection of *C. trachomatis* and *N. gonorrhoeae* were shown in figure 12.

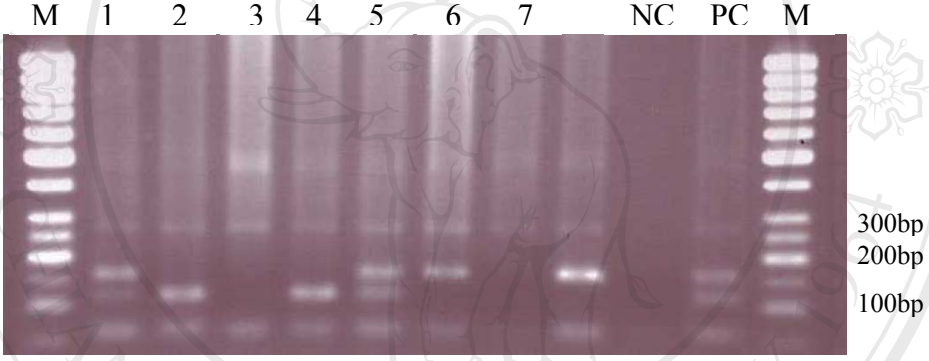

Figure 12. Agarose electrophoresis of *C. trachomatis* and *N. gonorrhoeae* detection by In-house M-SN PCR from genitourinary specimens

M = DNA marker (50bp Ladder)

Lane 1, 5 = Positive for *C. trachomatis* (108bp) and *N. gonorrhoeae* (152bp) with beta-globin (273bp)

Lane 2, 4 = Positive for *C. trachomatis* (108bp) with beta-globin (273bp)

Lane 3, 7 = Negative with beta-globin (273bp)

Lane 6, 8 = Positive for *N. gonorrhoeae* (152bp) with beta-globin (273bp)

NC = Negative control

PC = Positive control

***C. trachomatis* detection** In men, 144 of 154 (93.5%) urethral swab and urine pair samples had concordant positive and negative results, while only 10 (6.5%) sample pairs showed the discordant results (4 were positive by swabs but negative by

urine, 6 were negative by swabs but positive by urine). The results were shown in table 5. To see whether those men were infected with *C. trachomatis*, all discordant positive samples were confirmed for the true positivity by using other PCR with primers specific to MOMP gene. After confirmation, all discordant positive samples were confirmed positive with MOMP gene. Any subjects whose samples (either urethral swab or urine) were positive for *C. trachomatis* by two different assays (PCR with cryptic plasmid and PCR with chromosomal MOMP gene specificity) were determined as infected persons.

When analyzed, *C. trachomatis* infection was observed in 37 of 154 (24.0%) men. Using standard urethral swabs could detect *C. trachomatis* infection in 31 of 37 (83.78%) infected men while using of urine samples detected more infected cases, 33 of 37 (89.19%), than the standard swabs. The performance of samples (sensitivity, specificity, positive and negative predictive values) was shown in table 7.

In women, among 40 sample pairs (endocervical and self-collected vaginal dry swabs) concordant positive and negative results in detection of *C. trachomatis* were 38 (95%) while only 2 pairs of sample (endocervical swabs positive but vaginal swabs negative) were discordant. Again after confirmation, all 2 positive endocervical swabs were confirmed positive. The performance of endocervical and self-collected vaginal dry swabs in detection of *C. trachomatis* was shown in table 8.

Table 7. Comparison the performance of urethral swabs and urine samples in detection of *C. trachomatis* by using In-house M-SN PCR

Samples (n=154)	**Results of *C. trachomatis* detection by using M-SN PCR**				**Infection (n=37)**	**No infection (n=117)**	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**
Urethral swabs	+	+	-	-	31	123	83.8	100	100	95.1
Urine	+	-	+	-	33	121	89.2	100	100	96.7
No of specimens	27	4	6	117						

Note; Concordant = 93.50 % (144 of 154), Discordant = 6.50 % (10 of 154)

Table 8. Comparison the performance of endocervical swabs and self-collected dry vaginal swabs in detection of *C. trachomatis* by using M-SN PCR

Samples (n=40)	**Results of *C. trachomatis* detection by using M-SN PCR**				**Infection (n=18)**	**No infection (n=22)**	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**
Endocervical swabs	+	+	-	-	18	22	100	100	100	100
Self-collected dry - vaginal swabs	+	-	+	-	16	24	88.9	100	100	91.2
No of specimens	16	2	0	22						

Note; Concordant = 95.00 % (38 of 40), Discordant = 5.00 % (2 of 38)

***N. gonorrhoeae* detection** In men, 154 paired samples (urethral swab and urine) were tested for *N. gonorrhoeae* infection by M-SN PCR. As the results shown in table 7, the concordant positive and negative results were observed in 149 (96.75%) sample pairs while only 5 pairs were discrepant (2 were swab positive but urine negative and 3 were swab negative but urine positive). To indicate infection with *N. gonorrhoeae*, all discrepant positive samples were confirmed by PCR with primers specific to chromosomal methyltransferase gene. After confirmation, all samples were confirmed positive. Taken together, total *N. gonorrhoeae* infected men were observed in 129 of 154 (83.77%). Sensitivity of urethral swab in detection of *N. gonorrhoeae* infection was 97.67% (126 of 129 samples) while, urine has higher sensitivity, 98.45% (127 of 129 samples) than swab. Specificity, positive and negative predictive values were calculated and shown in table 9.

In women, 40 paired samples (endocervical and self-collected dry vaginal swab) were tested for *N. gonorrhoeae*. The positive and negative concordant samples were observed in 38 of 40 (95.0%), the discrepant (endocervical swab positive but vaginal sawb negative) was only in 2 of 40 (5.0%). After confirmation, they were again positive. So that *N. gonorrhoeae* infection was observed in 24 of 40 (60.0%) women. The sensitivity of endocervical swab for detection of *N. gonorrhoeae* infection in women was 100% (24 of 24 samples) while vaginal swab was 91.67% (22 of 24 samples). Specificity, positive and negative predictive values were calculated and shown in table 10.

Table 9. Comparison the performance of urethral swabs and urine samples in detection of *N. gonorrhoeae* by using M-SN PCR

Samples (n=154)	**Results of *N. gonorrhoeae* detection by using M-SN PCR**				**Infection (n=129)**	**No infection (n=25)**	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**
Urethral swabs	+	+	-	-	126	28	93.0	100	100	89.3
Urine	+	-	+	-	127	27	98.4	100	100	92.6
No of specimens	124	2	3	25						

Note; Concordant = 96.75 % (149 of 154), Discordant = 3.25 % (5 of 154)

Table 10. Comparison the performance of endocervical swabs and self-collected dry vaginal swabs in detection of *N. gonorrhoeae* by using M-SN PCR

Samples (n=40)	**Results of *N. gonorrhoeae* Detection by using M-SN PCR**				**Infection (n=24)**	**No infection (n=16)**	**Sensitivity %**	**Specificity %**	**PPV %**	**NPV %**
Endocervical swabs	+	+	+	-	24	16	100	100	100	100
Self-collected dry vaginal swabs	-	+	-	-	22	18	91.7	100	100	88.9
No of specimens	22	2	0	16						

Note; Concordant = 95.00 % (38 of 40), Discordant = 5.00 % (10 of 40)

Although both *C. trachomatis* and *N. gonorrhoeae* are the most commond STI reported worldwide. Co-infections between them are also common. In this study, we showed that *N. gonorrhoeae* infection was also commond and more prevalence than *C. trachomatis* in both men and women (table 11). In men, *N. gonorrhoeae* was detected in much higher rate (70.8%) than *C. trachomatis* (11.0%). In contrast, in women, the detection rate of both *C. trachomatis* and *N. gonorrhoeae* was not that much difference; 27.5.0% vs. 42.5.0%. Moreover, *C. trachomatis* infection was detected higher in women (27.5%) than in men (11.0%). We also demonstrated here that the co-infection between these two organisms was not rare among the study group. They were not significantly difference among men and women (13.0% vs. 17.5%, $p < 0.05$)

Table 11. Detection rate of the *C. trachomatis* and *N. gonorrhoeae* among the STI high risk group at the Sexually Transmitted Infection Center 10 Chiang Mai

Gender	Number of CT+ only (%)	Number of GC+ only (%)	Number of co-infection CT+ and GC+ (%)
Male (n=154)	17 (11.0)	109 (70.8)	20 (13.0)
Female (n= 40)	11 (27.5)	17 (42.5)	7 (17.5)

5. Cost analysis of in-house M-SN PCR for detection of *C. trachomatis* and *N. Gonorrhoeae*

The assays cost was analyzed based mainly on the assay procedure; DNA extraction, amplification and detection in each test. The cost of instruments and others service contact was not included. The labor cost which is highly varied among institution was also not included for calculation. Cost of all reagent and consumable equipments were based on price list on August 2006 and no discount pricing was used

for analysis. In this study, we compare the cost of our assay with Roche Multiplex AMPLICOR CT/NG PCR which has a biggest market shared in Thailand. The results in table 12 revealed that, including all consumable and reagents, cost of the in-house M-SN PCR assay has 8.6 times (188.24 vs. 1,615.40 baht) lower than Roche Multiplex AMPLICOR CT/NG PCR assay.

Table 12. Comparison of cost between in-house M-SN PCR and Roche Multiplex AMPLICOR CT/NG PCR for detection of *C. trachomatis* and *N. gonorrhoeae*

Cost category	Roche Multiplex AMPLICOR CT/NG PCR(baht)	in-house M-SN PCR (baht)
Costs of disposables		
Sample preparation		
polystyrene tube-2ml	0.80 x 1=0.80	0.80 x 2=1.60
aerosol barrier 1000-µl tips	3.65 x 3=10.95	3.65 x 6=21.90
Amplification		
aerosol barrier 200-µl tips	3.65x 1=3.65	3.65 x 13=47.45
PCR reaction tube	-	2.00 x 1=2.00
Detection		
1 barrier 200-µl tips	-	3.65x 1=3.65
Costs of reagents		
Multiplex AMPLICOR CT/NG PCR kits	1,600	-
5U Taq Polymerase	-	50
1.25 mM dNTPs	-	50
Primers	-	1.64
Others reagents	-	10
Total cost	**1,615.40**	**188.24**