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Development of Duplex *Polymerase Chain Reaction*in-house assay to detect of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*from genital discharge in Patient with Sexual Transmitted Infections

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Abstract

The incidence of sexually transmitted infections (STIs) with discharge in Indonesia to high-risk groups provide dominant pattern of Chlamydia trachomatis(CT) and Neisseriagonorrheae(NG) infection. Pathogenesis change of CT and NGinfection suggeststhat routine syndromic approach diagnostic is no longer accurate. To develop detection system of CTand NG using duplex polymerase chain reaction (PCR) assay to genital discharge in patient with STIs. Three phases: firstly was PCR assay optimalization to annealing time and temperature, primer concentration, centrifugation time and elution volume, secondly, specificity test and thirdly duplex PCR assay application to clinical specimen. Duplex PCR assay optimalization gave results as follow: annealing temperature and time 54°C and 60 seconds, CTand NG primer concentration were 0,7µM and0,5µM, centrifugation time 10 minutes, and elution volume 60 µl. Detection limit of duplex PCR to CTand NG 0.927 and 1.19 pg / PCR reaction, respectively. Duplex PCR to detect NG has ensitivity, specificity, positive predictive value and negative predictive value were 100%, 61.9%, 20%, and 100% in endocervical specimens, respectively and 75%, 40%, 50%, and 66.67%, in male urethral specimens respectively. Duplex PCR to detect CT was compared with chlamydial antigen detection test were show positive results higher both in endocervical and male urethral specimens (10:3 and 1:0). Development of detection system of CT and NG using duplex PCR assay is applicable to endocervical and male urethral swab specimens.

Keywords : sexual transmitted infections (STIs), genital discharge, Duplex PCR in-house assay,*Chlamydia trachomatis*, *Neisseria gonorrhoeae*

Abstrak

Insiden infeksi menular seksual (IMS) dengan duh genital di Indonesia pada kelompok berisiko tinggi memberikan pola dominan infeksi *Chlamydia trachomatis* (CT) dan *Neisseriagonorrheae* (NG). Perubahan pathogenesis CT dan NG menyebabkan diagnosis rutin menggunakan pendekatan sindrom tidak lagi akurat.
Untuk mengembangkan system deteksi CT dan NG menggunakan *polymerase chain reaction* (PCR) dupleks untuk duh genital pada pasien dengan infeksi menularseksual. Terdapat tiga langkah penelitian yang dilakukan. Pertama adalah optimalisasi PCR terhadap waktu dan suhu *annealing*, konsentrasi primer, waktu sentrifugasi dan volume elusi. Kedua, uji spesifisitas dan ketiga aplikasi PCR dupleks untuk specimen klinis. Optimalisasi PCR dupleks: suhu *annealing* 54 ° C, waktu *annealing* 60 detik, konsentrasi primer CT baik *reverse* maupun *forward* 0,7µM dan NG baik *reverse* maupun*forward* 0, 5µM, waktu sentrifugasi adalah 10 menit dan volume elusi 60 ml. Batas deteksi PCR duplex untuk CTadalah 0,927 pg / reaksi PCR, dan NG adalah 1,19 pg/ reaksi PCR. PCR dupleks untuk mendeteksi NG memiliki sensitivitas, spesifisitas, nilai prediksi positif dan nilai prediksi negative berturut-turut adalah100%, 61,9%, 20%, dan 100% pada spesimenen do serviks dan 75%, 40%, 50%, dan 66,67% pada specimen uretra laki-laki. PCR dupleks untuk mendeteksi CT dibandingkan dengan tes deteksi antigen klamidia yang menunjukkan hasil positif yang lebih tinggi baik dalam specimen endoserviks dan laki-laki uretra (10: 3 dan 1: 0). Pengembangan system deteksi CT dan NG menggunakan PCR dupleks dapat diaplikasikan pada specimen swab uretra laki-laki dan swab endoserviks.

Kata kunci: Infeksi Menular Seksual (IMS), duh genital, PCR dupleks*in-house*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*

Background

Sexual transmitted infections (STIs) is infection with marked morbidity and mortality. Each day there are 1 million STI cases had treated globally, with higher cases left untreated. With half number of cases occur in Asia, with Indonesia ranked as second highest of STI's prevalence.[1]

It is estimated 92 millions people suffered from chlamydial infection and 82 millions infected by *Neisseria gonorrhoeae* (WHO, 2001). STI surveillance in 10 cities of Indonesia in 2003 and 2005 showed prevalence of gonococcal infection 12-44%, chlamydial infection 35-56%, siphylis 6-22%.[1,2] The prevalence of STI in Indonesia suggested that gonococcal and chlamydial infection are dominant.

In clinical setting, gonococcal and chlamydial infection had similar sign and symptom, in addition of asymptomatic women (70-80%) and men (up to 50%) create a big reservoir of transmission.[3,4,5] Ministry of Health produce the STIs manual of treatment 2010 with syndromic approach for clinical diagnosis. The manual stated that syndrome of gonococcal and chlamydial infection is treated as coinfection, which lead to higher chance of overdiagnosis and overtreatment. The microbial diagnostic is important, considering that the chance of asymptomatic cases produced by both organisms or one of them, serious complication and antimicrobial therapy.[3]

Syndromic approach for clinical diagnosis is unreliable, while separated microbial diagnosis for both infections required higher volume of specimens. The character of specimens is differ in women and men will affected diagnostic test's accuracy. A molecular assay as diagnostic test had been widely used. CDC (2002) recommend molecular assay technique is nucleic acid amplification test (NAAT) to detect *C. trachomatis* and *N. gonorrhoeae* simultaneously.[6] The comercial NAAT for detection of *C. trachomatis*and *N. gonorrhoeae* is available with high price. The in-house NAAT for detection of *C. trachomatis* (CT) and *N. gonorrhoeae* (NG) with comparable sensitivity and specificity is need to develop.

Methods

This study is a diagnostic test study with a cross sectional study design, non-experimental. The study was conducted at the Laboratory of Clinical Microbiology, Faculty of Medicine University of Indonesia (FMUI) Jakarta, within periode of August 2011 - March 2012.Ethical committe board of FMUI has endorsed the study. The study was carried out in three phases: the first phase of optimization of PCR conditions, phase II specificity test and phase III application on clinical specimens (Table 1).

Table 1. Phases of study

Phases	Activities	Details
I	Optimization of PCR condition	Optimization of pre-PCR:
		-Sentrifugation gradient -Final volume of elusion
		Optimization of PCR:
		-Annealing temperature -Annealing time -Primer concentration
II	Spesificity test	
III	Application on Clinical specimens	

Specimen taken is genital discharge, women and men aged 18 years or more, that comes to sexual transmission infection clinic Department of Dermatology Ciptomangunkusumo hospital with chief complaint of genital discharge and willing to participate in the study by signing the informed consent form. Women who got pregnant or menstruating are not included in the study. Each subject was taken genital swab 3 times[7,8] : swab I direct smear and culture, swab II Chlamydia antigen detection test, and swab III duplex PCR test. Swab I and III using a Dacron swab , whereas the second swab using default swab Chlamydia antigen kit (Figure 1). Sampel size is determine using diagnostic test sample size: [9] $\mathbf{=Z\alpha^2PQ/d^2}$

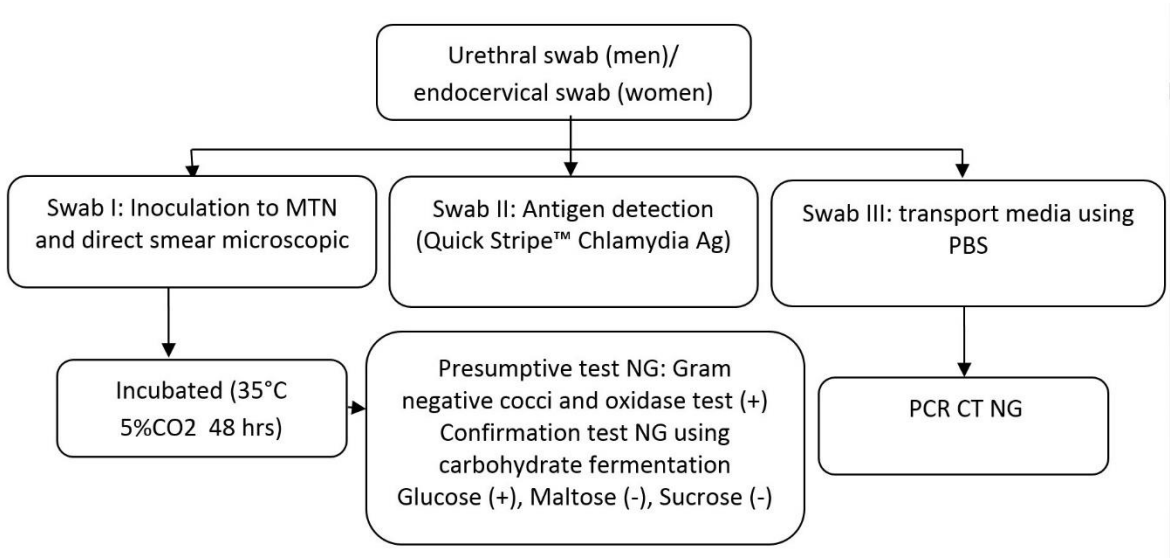

Figure 1. Specimen collection flow. PBS= Phosphat buffered saline; MTM: modified Thayer Martin; PCR=polymerase chain reaction; CT=Chlamydia trachomatis; NG=Neisseria gonorrhoeae

Coinfection prevalence in women 33%[1] and men 12% [10] prevalence of coinfection with a significance level of 95%, the absolute precision of 10%, the number of 85 female subjects and 41 male subjects.

Primer used for NG is the forward primer 5'-ACTGCGTTCTGAACTGGG TG and reverse primer 5'-GGCGGT CAATTTCACGCG that amplify 281-bp fragment of the 16S rRNA target gene and primers used for CT primer 5'-CTAGGCG TTTGTACTCCGTCA forward and reverse primer 5 '-TCCTCAGGAGTTTA TGCACT which amplify a 200-bp fragment of *orf8* gene.[11]

Optimization of PCR conditions using a DNA extraction kit Mini Blood® from Qiagen® and follow the manufacturer's instructions. Comparative test for the detection of NG is culture, while the detection of CT is the qualitative antigen detection QuickStripe™ Chlamydia Ag (Savyon Diagnostic).[12] Culture of NG using a modified Thayer Martin (MTM) in 10% CO_2 at a temperature of 35°C for 48-72 hours and was confirmed using a serial sugar test in Cystic Tripticase Agar.The fermentation of glucose (+), maltose (-) and sucrose (-) was presumptive diagnosis of NG.[7,13]Qualitative antigen detection test use the swabcomercial kit and follow the manufacturer's instructions. Direct specimen examination using Gram stain performs on glass slide using a Dacron swab applicator. The slides are then dried at room temperature. Gram staining is performed is maximum up to 4 hours after specimen collection. The gram stain procedure as follows: The slides were dried by passing it over and over 5 cm above the Bunsen flame, then dripped one ose of sterile NaCl 0.9% above the glass slide. Smeared specimen using swab, dry air, and then flooded the entire specimen using a Gram stain Aapproximately 1-2 minutes, rinse using running water. Then flooded the specimen using Gram stain B for 1 minute, rinse using running water. Again flood the specimen using Gram stain C for ½ minute, rinse under running water. The last flood the specimen with Gram stain D for 1 minute, rinse using running water. Let the slide dry at a temperature of room before microscopic reading. Results reported are the result of inspection microscopes with magnification X1000 (high power field with oil immersion) with a minimum of 10 visual field examination.

Specificitytest is tested to following organisms: Candida spp, Human Herpes Simplex virus, Mycobacterium tuberculosis, Klebsiellaoxytoca, Klebsiella pneumoniae, Acinetobacter baumannii, Acinetobacter anitratus, Salmonella typhii, Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, human Cytomegalovirus, Gardnerella vaginalis, Lactobacillus acidophilus, Enterococcus spp., and Streptococcus spp.

Results

Optimization of annealing temperatu re.

Annealing temperature optimization is done using a range of 50-60 ° C with duplex PCR conditions and using bacterial control of *C. trachomatis* and *N. gonorrhoeae.* Optimal annealing temperatures were obtained for the detection of *C. trachomatis* and *N. gonorrhoeae* is 53.9 °

C. Figure 2 shows the temperature gradient annealing *C. trachomatis*.

Optimization of annealing time and primer concentration. In comparison annealing time of 30 and 60 seconds there were variations in the thickness of the band corresponds to*C. trachomatis* and *N. gonorrhoeae*. Optimal thickness appears on annealing time of 60 seconds (Figure 3). Optimal primer concentration was found in the Mx1 band (figure 3; B) which

concentration of *C. trachomatis* primer with each forward and reverse (CTF / R) 0,7µM and primer concentrations of *N. gonorrhoeae* with each forward and reverse (NGF / R) 0,5µM.

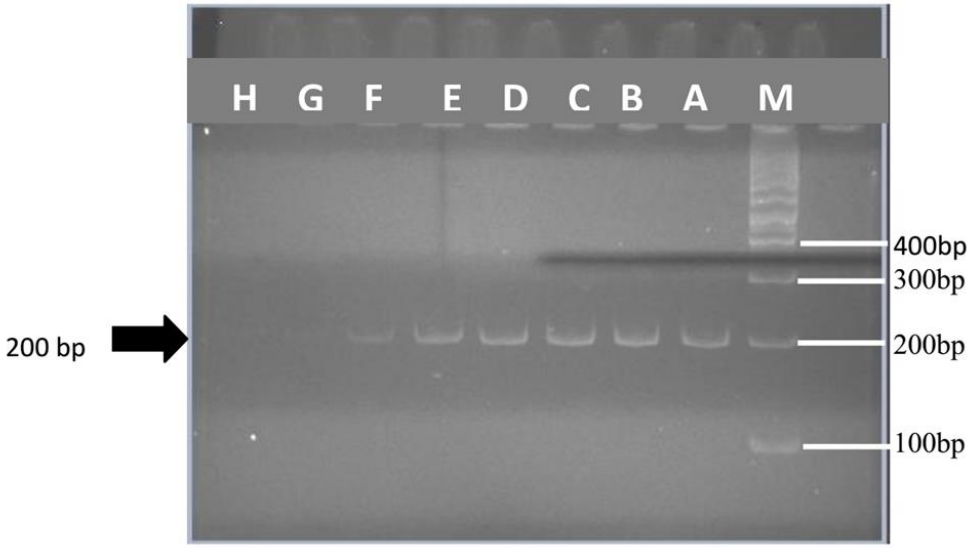

Figure 2. Bands of PCR amplification of *Chlamydia trachomatis* and *N. gonorrhoeae* with annealing temperature gradient of 50-60°C. M=marker 100 bp, 5 µl; A= 50,0°C; B= 50,8°C; C=52,1°C; D=53,9°C; E=56,4°C; F=58,3°C; G=59,5°C; H=60°C.

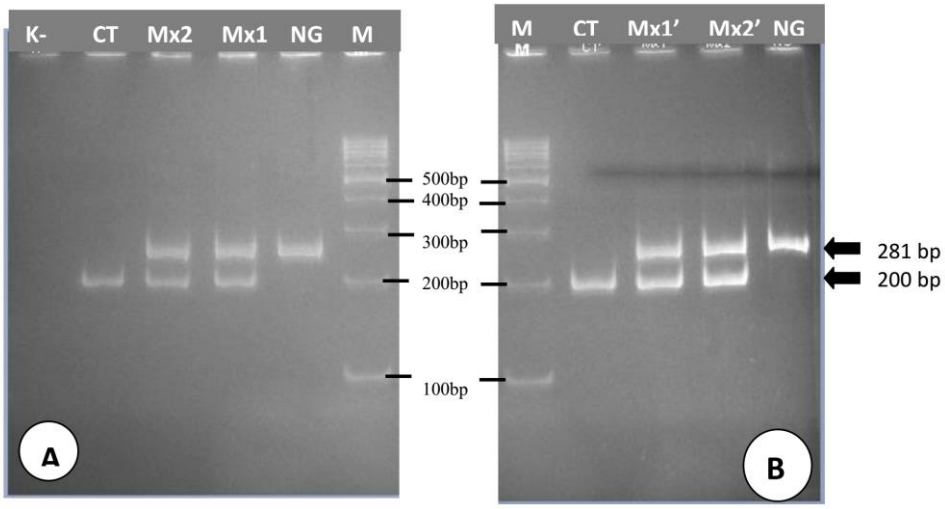

Figure 3. The result of amplification of *C. trachomatis* and *N. gonorrhoeae* with *annealing* time of 30 second (A) dan 60 second (B). M=marker 100 bp; Mx1=CTF/R 0,7µM, NGF/R 0,5µM; Mx2= CTF/R 0,6µM, NGF/R 0,5µM; CT=positive control of *C. trachomatis*; NG=positive control of *N. gonorrhoeae*

Optimization centrifugation.

Optimization centrifugation in this study conducted at a speed of 12,000 rpm between 5-90 minutes. In this study showed that the speed of 12,000 rpm and duration of 10 minutes is the optimal centrifugation gradient.

Optimization of the final elution volume.

Elution volume is the final volume of buffer AE that is used to maintain the stability of the extraction product after DNA extraction process that will be used as template DNA for PCR reaction. Elution volume carried between 40µl - 80 mL. Optimal elution volume is 60 mL.

Duplex PCR detection threshold.

PCR detection threshold based on the concentration of DNA duplex print pattern for *C. trachomatis* and *N. gonorrhoeae* diluted consecutive glow 0.927 pg / PCR reaction and 1.19 pg / PCR reaction. PCR detection threshold using DNA duplex print pattern controls the extraction of the bacterial suspension in 0.09% NaCl with serial dilution for *N. gonorrhoeae* is 1.5 x 10^2 CFU / ml or 12.5 CFU / PCR reaction.

Specificity test. To determine the specificity of the primers used, the assay was loaded against other microorganisms. Overall other microorganisms seen no band formation. Due to the limited stock of microorganism control in CML and difficultiesto obtain these bacteria that act as vaginal flora *Gardnerella vaginalis*, *Lactobacillus acidophilus*, *Enterococcus spp*., and *Streptococcus spp*. Those bacterias were tested using the method of GenBank BLAST (www.ncbi.nlm.gov) and showed no similarity.

Sequencing analysis of the two primer pairs.

Primers CTF / R and NGF / R is sequenced in orther to analyze whether the primary is a specific primers for intended organisms. The result showed that the primers CTF / R and NGF / R used a primer pair that is specific for *C. trachomatis* and *N. gonorrhoeae* based on GenBank reference (Figures 4 and 5).

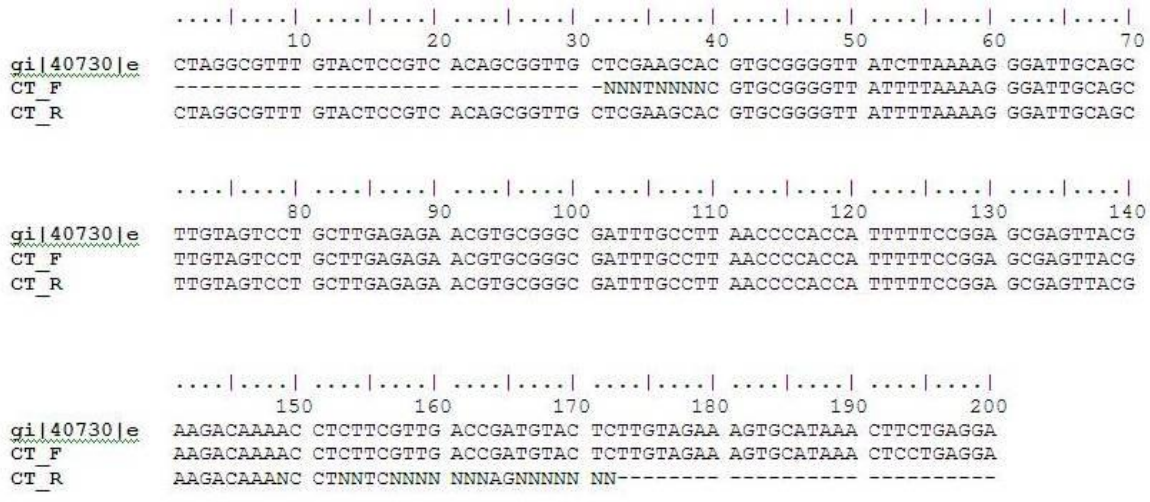

Figure 4. Primer CTR/F *alignment* to bacterial genbank reference

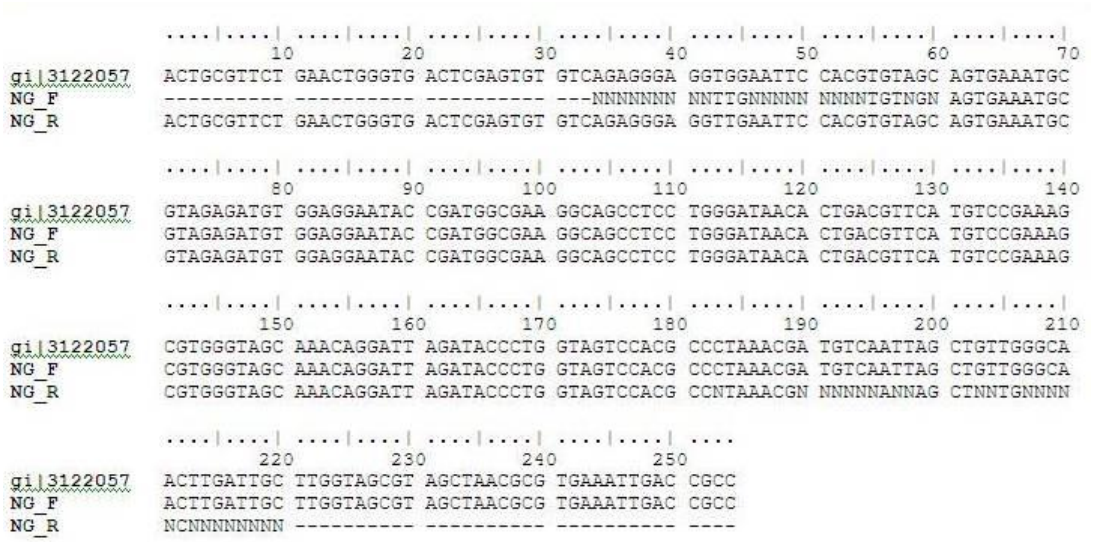

Figure 5. Primer NGR/F *alignment* to bacterial genbank reference

Characteristics of the subjects.

The adjustment the number of subject was made due to: budget limitation and it has reach the end of study period. A sum of 26 subjects women who signed informed-consent, include 23 subjects who met the inclusion criteria. Three samples did not meet the inclusion criteria due to existence of menstrual blood (2 subjects) and unspecified chlamydial antigen detection result. A total of 18 male subjects met inclusion criteria (Figure 6). Overall age range 18-54 years with a median age of 25 years.

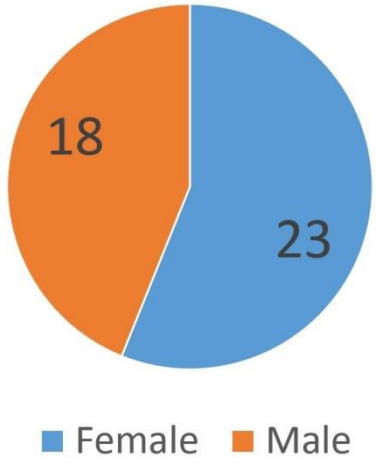

Figure 6. Sex Distribution of Research Subject

Statistical analysis of leukocytes count groups among various diagnostic modes.

The number of leukocytes as the proof of presence of infection is analysedamong diagnostic modes. The threshold of male

urethral discharge according to syndromic approach flowchart 2 (MOH, 2010): male urethral discharge with a microscope examination of the ≤ 5 / hpf and> 5 / hpf.[3] Syndromic approach on endocervical duh use threshold of ≤ 10 / hpf and > 10 / hpf.[2,3] Table 2 shows the statistical analysis resume the leukocyte count groups.

Table 2. Statistical analysis of leukocytes groups and other diagnostic modes of *C. trachomatis* and *N. gonorrhoeae.* PPV=positive predictive value; NPV=negative predictive value; f=female; m=male

Statistical analysis of leukocytes groups and:	Chlamydial antigen detection test		Duplex PCR assay in detecting *C. trachomatis*		Gram-negative intracelluler diplococci		Culture of *N. gonorrhoeae*		Duplex PCR assay in detecting *N. gonorrhoeae*	
Sex	f	m	f	M	f	m	f	m	f	m
Sensitivity (%)	66.7	0	40	0	33.3	0	0	0	40	8,3
Specificity (%)	40	100	23	82.4	35	72.7	33.3	70	23	66.7
PPV (%)	14.3	0	28.6	0	7	0	0	0	28.6	33.3
NPV (%)	88.9	100	33.3	93.3	77.8	53.3	77.8	46.7	33.3	26.7

Comparison test results.

Comparison test used in this study is the culture of gonorrhea and chlamydia antigen detection test. Table 3 shows that chlamydia antigen detection test results have a low positive result.

Table 3. Comparison test result. NG=*Neisseria gonorrhoeae;* f=female; m=male

	Positive		Negative		Total
	f	M	f	m	
Culture NG	2	8	21	10	41
Chlamydial antigen detection test	3	0	20	18	41

Duplex PCR applications in clinical specimens.

Duplex PCR test results of *C. trachomatis* and *N. gonorrhoeae*against utrethral and endocervical discharge produce the appropriate bands for *C. trachomatis* as many as 11 samples (10 women and 1 man) and the appropriate bands for *N. gonorrhoeae* as many as 22 samples (10 women and 12 men). Appropriate bands to both bacteria as much as 4 samples (Table 4).

dddd

Table 4. Duplex PCR assay result

	Positive		Negative		Total
	f	m	f	m	
C. trachomatis	10	1	13	17	41
N. gonorrhoeae	10	12	13	6	41
Co-infection	4	0	19	18	41

Statistical analysis duplex PCR assay in the detection of *N. gonorrhoeae*.

Diagnostic test analysis of Duplex PCR assay can be applied to the detection of *N. gonorrhoeae* compare to gold standard of Ddd *N. gonorrhoeae* culture. While the detection of *C. trachomatis* diagnostic test analysis can not be performed compare to chlamydial antigen detection test (Table 5).

Table 5. Statistic analysis duplex PCR assay in detection of *N. gonorrhoeae.* PPV=positive predictive value; NPV=negative predictive value

	Sensitivity (%)	Specificity (%)	PPV	NPV
Endocervical swab	100	61.9	20	100
Male urethral swab	75	40	50	66.7

Application of syndromic approach.

Application of syndromic approach in this research is in the flowchart 1 (MOH 2010): male urethral discharge and flowcharts 9: vaginal discharge. Table 6 shows that 100% of subjects positive for the chlamydial and gonorrhoeal infection (100% coinfection). Duplex PCR assay for the detection of *C. trachomatis* detection results in general show a more superior than antigen detection test (Table 6).

Table 6. Resume of comparison tests. CT=*Chlamydia trachomatis*; NG=*Neisseria gonorrhoeae*; f=female; m=male

		Syndromic approach		Comparison tests		Duplex PCR assay	
		f	m	f	m	f	m
CT	Pos	23	18	3	0	10	1
	Neg	0	0	20	18	13	17
Total		23	18	23	18	23	18
NG	Pos	23	18	2	8	10	12
	Neg	0	0	21	10	13	6

Coinfection.

Co-infection of both pathogens is an occurrence that possible in STI with genital discharge. In this study, the findings of co-infection in various diagnostic modes as follows (Table 7).

Table 7. Finding of co-infection cases to various diagnostic modes

	Syndromic approach		Comparison tests		Duplex PCR assay	
	Co-infection	Non co-infection	Co-infection	Non co-infection	Co-infection	Non co-infection
Endocervical swab	23	0	0	23	4	19
Male urethral swab	18	0	0	18	0	18

Discussion

Sequencing of two pairs of primers.

The result of 16SrRNA primer sequencing showed the amount of amplification of *N. gonorrhoeae* 254 *bp* lower than the amount of amplification of the reference amount of 281 bp. Examination using the sequencing analysis software Bioedit® twice and showed consistent results.

Significance of the number of leukocytes.

Table 2 said in general that the majority sensitivity value is below 80%. Significance value of specificity is more than 80% is between the number of leukocytes of urethral swab and chlamydia antigen detection test (100%), also between the number of urethral swab and duplex PCR in the detection of *C. trachomatis* (82.4%). The chance for applying the benefits of leukocyte count in the population can be seen in a negative predictive value (> 80%) seen in the statistical analysis of the number of leukocytes endocervical swab with chlamydia antigen detection test (88.9%), the number of leukocyte of male urethral swab and detection of chlamydia antigen (100%), the number of leukocytes of male urethral swab and duplex PCR detection of *C. trachomatis* (93.3%). Consistently large groups of the number of leukocytes that both men and women showed a low positive predictive value, or in other words, no direct evidence of inflammatory tendencies both bacterial infection.

Leukocytes number of swab specimens is important in pre-screening method to decide wetherpresence of the infection, especially in women with no or minimum of risk factors of cervicitis. Asbill et al (2000) in the study to detect *C. trachomatis* and *N. gonorrhoeae* that colonize the cervix in pregnancy provides a definition of leukocytes <10 / hpf. The results are 100% of Gram stainwith leukocytes value of <10 / hpf had a 100% negative predictive value, which means that the Gram staining leukocytes <10 / hpf as a good predictor for the absence of infection of *C. trachomatis* and *N. gonorrhoeae*.[14]In this study the benefits of the examination the number of leukocytes demonstrate low sensitivity (<80%), but showed an opportunity to exclude the disease (negative predictive value> 80%) especially male urethral swabs that the number of leukocytes 5 or less is a good predictor for the absence of chlamydial infection (chlamydia antigen detection test = 100%, duplex PCR = 93.3%), but a low positive predictive value (chlamydia antigen detection test = 0%, the duplex PCR = 0%). This analysis indicates that the number of leukocytes more than five is

not a predictor for the presence of infection chlamydia. In the endocervical specimen, the number of leukocytes of 10 or less is a good predictor for the absence of chlamydia (Chlamydia antigen detection test = 88.9%) but the number of leukocytes more than 10 nor as a predictor of chlamydial infection (Positive Predictive Value = 14.3%).

The syndromic approach.

In the book Manual for Management of Sexually Transmitted Infections (MOH, 2010) for the treatment of gonorrhea and chlamydia infections have been prepared flow chart 'treatment of STIs using syndromic approach'. The purpose of making the flow chart of this treatment is to facilitate the management of STIs in areas with limited resources, as well as cut the chain of transmission of STIs, improving service coverage and reducing the rate of increase of STI cases HIV.[3]

In this manual all specimens (women and men) showed significant overall coinfection cases do not distinguish between infections chlamydia and gonorrhea. Treatment of STIs using syndromic approach indicates overdiagnosis that leads to overtreatment.

According Mayaud (2004) there are advantages and disadvantages in syndromic approach management of STIs. These advantages is the treatment of STIs problem-oriented, has high sensitivity in detecting infection in symptomatic patients and did not miss a mixed infection, the treatment given at the beginning of the visit, provide opportunities of education and counseling of patients, avoiding the laboratory tests that are expensive, allows the selection of patients who require referral to a specialist, and can be implemented in primary health care. Its shortcomings are overdiagnosis that leads to overtreatment, require (re)training staff, the possibility of the denial from the medical community, and not be able to detect asymptomatic population.[15]

The co-infection cases.

The risk factors that contributes to co-infection cases are: multiple sex-partner and certain sexual orientation.[3]Co-infection is a diagnostic and treatment issues that are important in the prevention of STIs. The discovery of the case of co-infection mostly found throughduplex PCR assay and mostly from the endocervical specimen. Co-infection cases was not found in a comparison test examination, while 23 cases of endocervical specimen is detected through syndromic approach and 4 cases through dupelx PCR assay (Table 7). In patogenesis picture this coinfection can be described by Vonck et al (2011) well, using a mouse model.Vonck et al can show that chlamydia infections were made at lower genital tract of female mice can enhance the colonization gonore.[16]The interesting thing about the case of co-infection is all subject to sex woman. Pathophysiological process of infection of *C. trachomatis* and *N. gonorrhoeae* have different mechanisms, especially in terms of virulence factors and their interaction with genital mucosa in the early stages. Woman's vulnerability to co-infection is higher than men. Factors that affect these conditions include: factors anatomically female genital, histological endocervical conditions (area transitional epithelial cells and cuboid), the use of progesterone increases the susceptibility to infection CT, the use of estradiol are protective against infection CT and hormonal administration of estradiol in women can make gonorrhea infection becomes prolonged.[16,17,18,19]Further more three samples showed results in gram-negative intracellulardiplococci were negative, and one sample is positive, suggesting the possibility of duplex PCR assay is more sensitive than conventional examination.

Duplex PCR assay in STIs with genital discharge management.

Studies regarding the use of PCR in-house in dual detection of of these two organisms has revealed more superior than single test for each organism alone[6,8,11] and cheaper than comercial one (with average cost of 400.000 IDR compared to 850.000 IDR). With turn around time less than 2 hours may lead more rapid decision in individual therapy and epidemiology concern.

Some of the advantages of duplex PCR detection technique used in this study were 1) Needs specimen volume slightly, and 2) the value of better sensitivity. Based on these advantages, the position of the duplex PCR in-house assay in line with the STI services spending genital discharge may be addressed to:

1. The patient had a history of genital discharge STIs previously and return with the same complaint (diagnosis and therapy evaluation)
2. Patients who have genital discharge volume is low
3. Patients with genital discharge but the results ofGram stain of fresh specimen examination are not specific
4. Patients who are at risk, but no symptoms (asymptomatic)
5. Patients who are at risk and have the immunocompromissed status
6. Search the risk of infertility, ectopic pregnancy, pelvic inflammatory disease and other complications presumably caused by *C. trachomatis* or *N. gonorrhoeae*

Limitations of the study.

Limitations of this study lies on: 1) not include other types of urogenital specimens (urine and vaginal secretions), 2) All research subjects is limited to symptomatic subjects and the subjects are identified as having a high risk, 3) a comparison test of *C. trachomatis*PCR not the gold standard, and 4) the number of samples according calculations can not be met due to limited research costs and time.

Conclusion

Duplex PCR assay that has developed can be used on endocervicalswab and male urethral discharge.

Conflict of interest

There is no conflict of interest stated

Acknowledgment

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Reference

1. Ministry of Health Republic of Indonesia (MOH), **Rencana Aksi Pengendalian Infeksi Menular Seksual- Infeksi Saluran Reproduksi Sebagai Strategi Nasional 2008-2012**. 2007
2. WHO. **Global prevalence and incidence of selected curable Sexually Transmitted Infections: overview and estimates**. Revised edition, November 2001.
3. Ministry of Health Republic of Indonesia (MOH). **Pedoman Penatalaksanaan Infeksi Menular Seksual**. Jakarta. 2010
4. James Chin (Editor). Kandun IN (translator coordinator). **Manual Pemberantasan Penyakit Menular. Ditjen PP dan PL.** 2005. Jakarta available at http://www.pppl.depkes.go.id/catalogcdc/Wc707b89d46213.htmAccessed 20 Juli 2010
5. Black CM. **Current methods of laboratory diagnosis of Chlamydia trachomatis infections.**Clin. Microbiol. Rev. 1997; 10(1):160-184
6. Centers for Disease Control and Prevention. 2002. **Screening tests to detect Chlamydia trachomatis and Neisseria gonorrhoeae infections**—2002. Morb. Mortal. Wkly. Rep. **51:**1–38.
7. Goering RV, HM Dockrell, D Wakelin, M Zuckerman, PL Chiodini, IM Roitt, and C Mims. **Mims' Medical Microbiology**. 4th edition. 2008. Mosby Elsevier.
8. Tshibaka LM, M Alery, F Bernier, E van Dyck, CM Lowndes, A Guedou et al. **Diagnostic Performance of the Roche Amplicor PCR in Detecting Neisseria gonorroeae in Genitourinary Specimens from Female Sex Workers in Cotonou, Benin**. J ClinMicrob 2000; 38: 4076-4079
9. Mudiyono B, Moeslichan S, Sastroasmoro S, Budian I, Purwanto HS. **Perkiraan Besar Sampel**. Dalam: Sastroasmoro S, Ismael S, editor. **Dasar-Dasar Metodologi Penelitian Klinis**. Edisi 2. Jakarta: SagungSeto. 2002. Pp. 257-86

10. DE Mustikawati, G Morineau, Nurhayati, Y Irmaningrum, P Riono, S Priohutomo and R Magnani. **Sexual risk taking, sexually transmitted infections and HIV prevalence among four ''high-risk''occupational groups of Indonesian men**. Sex Transm Infect 2009;85:391–396.
11. Lee SR, JM Chung and YG Kim. **Rapid One Step Detection of Pathogenic Bacteria in Urine with Sexually Transmitted Disease (STD) and Prostatitis Patient by Multiplex PCR Assay (mPCR)**. J Microbiol. 2007; 45 (5): 453-459
12. Insert package. **QuickStripe® Chlamydia Ag. Savyon®Diagnostics Ltd.** Habosem St. Ashdod 77610 Israel
13. Jawetz, Melnick & Adelberg's **Medical Microbiology**, 24th edition. 2007. Lange / McGraw Hill
14. Asbill KK, Higgins RV, Bahrani-Mostafavi Z, Vachris JC, Kotrotsios SH, Elliot MC et al. **Detection of Neisseria gonorrhoeae and Chlamydia trachomatis colonization of the gravid cervix** . Am J ObstetGynecol 2000;183:340-6.
15. P Mayaud, D Mabey. **Approaches to the control of sexually transmitted infections in developing countries: old problems and modern challenges**. Sex Transm Infect 2004;80:174–182
16. RA Vonck, T Darville, CM O'Connell, and AE Jerse.**Chlamydial Infection Increases Gonococcal Colonization in a Novel Murine Coinfection Model.** Infect Immun 2011:79(4): 1566–1577
17. Edwards JL and MA Apicella. **The Molecular Mechanisms Used by Neisseria gonorrhoeae To Initiate Infection Differ between Men and Women.** Clin. Microbiol. Rev. 2004, 17(4):965
18. C Kaushic, F Zhou, AD Murdin and CRWira. **Effects of Estradiol and Progesterone on Susceptibility and Early Immune Responses to Chlamydia trachomatisInfection in the Female Reproductive Tract**. Infect. Immun. 2000, 68(7):4207
19. Juey-Shin L. Lin, S. Patrick Donegan, Timothy C. Heeren, Marilyn Greenberg, Elizabeth E. Flaherty, Ruth Haivanis, Xiao-Hong Su, Deborah Dean, Wilbert J. Newhall, Joan S. Knapp, Samuel K. Sarafian, Roselyn J. Ri**c**e, Stephen A. Morse, and Peter A. Ri**c**e. **Transmission of Chlamydia trachomatis and Neisseria gonorrhoeae among Menwith Urethritis and Their Female Sex Partners. The Journal of Infectious Disease,** Dec 1998; 178: 1707 – 1712