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Molecular Diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Women at High Risk of Sexually Transmitted Infections

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Abstract

Introduction: The genital Chlamydial and Gonococcal infections are the most common Sexually transmitted diseases (STD) among women in the developing countries and co-infection of HIV-1 with these infections represents a public health problem of growing importance among the high risk groups.

Objective: The study aims to evaluate more rapid and accurate STD diagnosis by molecular technology using Amplicor CT/NG (*Chlamydia trachomatis/Neisseria gonorrhoeae*) test kit for diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoea* in HIV positive and negative women with and without symptoms and comparing the test with conventional gram staining method.

Methods: Ninety four female sex workers who were HIV (Human Immunodeficiency Virus) positive and HIV negative were included in the ratio 1:1 and endocervical specimen from them were processed at National Public Health Laboratory, Teku, over a period of 6 months, from March to end of July, 2014. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were detected by Nucleic Acid Amplification test (NAATs) and gram staining using standard protocols.

Results: This study observes that among ninety four participants twenty five patients showed positive result by Amplicor test. The rate of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* test results in the clinical study among HIV positive and negative were 38.2% and 14.8%, respectively. The total illiterate and literate cases showed 65.9% and 34.04%, respectively. The measures of accuracy of Amplicor test showed sensitivity of 27.03% as compared to Gram staining to detect CT and NG from endocervical swab which was 9.46%. In this study, relationship of the STD was found statistically significant ($p<0.005$) with education, HIV status, symptoms and sex. Whereas, it was not significant ($p>0.005$) with age, case type, contraceptive method, *Chlamydia trachomatis* infection status and *Neisseria gonorrhoeae* infection status, respectively.

Conclusion: It can be concluded that nucleic acid amplification test as compared to gram staining maintains high sensitivity for diagnosing *C. trachomatis* and *N. gonorrhoeae* in a low prevalence population.

Keywords: *C. trachomatis; N. gonorrhoeae;* Sensitivity; HIV; Amplicor test

Introduction

Sexually transmitted diseases with increasing number of new infections are a global burden [1]. *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) continue to be important sexually transmissible infective agents worldwide. Sexual risk behaviors such as multiple partners, history of STDs, and lack of condom use have been reported as the crucial cause of these infections ranging from asymptomatic to life threatening [2].

CT is an obligate intracellular bacteria and the causative agent of genital chlamydial infection [3]. CT infection often transmitted via asymptomatic individuals in 70 to 75% of infected women during vaginal, oral, or anal sexual contact. Pelvic inflammatory disease, a serious complication of CT infection, is a major cause of infertility in women.

NG is an obligate human pathogen and is the etiological agent of gonorrhoeae which is the second most prevalent bacterial STI [4]. Similar to CT, 80% of women infected with NG are asymptomatic and the most common and serious complications of the infection are pelvic inflammatory disease, ectopic pregnancy, and infertility. Female Sex Workers (FSWs) are a high-risk population for STIs and human immunodeficiency virus and also considered to be an important reservoir of STIs. In most parts of Asia and Africa, 80 to 90% of the venereal infections, including gonorrhea and chlamydia, originate from FSWs, a substantial proportion of who have asymptomatic infections [5].

Laboratory diagnosis of these infections is done by cell culture or antigen detection for *C. trachomatis* and gram staining and culture for *N. gonorrhoeae* traditionally. However, due to the difficulty in maintaining the viability of organisms during transport and storage in the diverse settings better and advanced tests are required [6]. In addition, the tissue culture methods for *C. trachomatis* isolation are difficult to standardize, technically demanding and expensive. Due to the fastidious nature of the organisms or unavailability of sensitive culture methods, traditional methods of detecting these organisms have often been hampered. Thus, non-culture tests were developed by diagnostic test manufacturers [7].

Enzyme immunoassays, which detect specific chlamydial or gonococcal antigens, and direct fluorescent antibody tests for *C. trachomatis*, which use fluorescein-conjugated monoclonal antibodies that bind specifically to bacterial antigen in smears are the first non-

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culture tests for *C. trachomatis* and *N. gonorrhoeae*. But these tests failed to detect a substantial proportion of infections which is the primary drawback, especially for *C. trachomatis* [8]. This changed with the introduction of Nucleic Acid Amplification Tests that amplify and detect *C. trachomatis* or *N. gonorrhoeae* specific DNA (Deoxyribonucleic acid) or RNA (Ribonucleicacid) sequences in individuals with a low number of infectious units. NAATs are approximately 20%-35% more sensitive than the earlier non-culture tests that do not require viable organisms or difficult culture methods [9].

The AMPLICOR CT/NG Test is *in vitro* test for the detection of *N. gonorrhoeae* and *C. trachomatis* DNA in urine from symptomatic or asymptomatic males, in endocervical and urethral swab specimens from symptomatic or asymptomatic females [10]. This test is a multiplex assay that permits the simultaneous amplification of *C. trachomatis* target DNA; *N. gonorrhoeae* target DNA from the infected individual and for sensitivity and specificity are clearly the highest of any of the test platforms for the diagnosis of chlamydial and Gonococcal infections [11].

The general objective of the study is to evaluate the performance of molecular diagnosis as a sensitive detection method for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urogenital specimen in women at high risk of STIs.

Materials and Methods

The study was carried at National Public Health Laboratory, Teku from beginning of March to end of July, 2014. This is a comparative, prospective and cross-sectional study among street female sex workers with and without HIV. The mean age of the 94 participants was 26.94 years ± 8.6 SD ranging from age group of 14 years to 50 years. A total of ninety four endocervical specimens were included in the study. First patients are allowed to lie on their back with their feet in stirrups. The health care provider will insert an instrument called a speculum into the vagina. Specimen collection for both organisms is invasive requiring insertion of a swab 1-2 cm into the endocervical canal through the speculum followed by two or three rotations. The swabs after sample collection are sent to a laboratory, where it will be smeared on a slide using standard procedure. In case of *N. gonorrhoeae* the color, size and shape of the cells help identify the type of bacteria and in *C. trachomatis* columnar or cuboidal epithelial cells are detected. The Gram stained smear was evaluated for the number of PMN/HPF (Polymorphonuclear/High Power Field), presence of bacteria, yeast, red blood cells and clue cells.

In the case of Amplicor CT/NG test following processes

Sample processing: 1) Specimens are treated with a detergent solution to lyses cells. 2) A second detergent solution is then added to prepare the specimen for amplification.

Amplification: 1) The GeneAmp PCR (Polymerase Chain reaction) System (9700 thermal cyclers) was started before 30 min for warm up. 2)The tubes with 100 µL sample were assembled into the thermal cycler sample block. 3) The 5 programs linked together into a method was started and program was run for approximately 2 h. 4) The samples were removed from the thermal cycler anytime during the final hold program and final PCR product was obtained.

Denaturation of PCR product: 1) 10 µl of DN was placed in all PCR tube. 2) The tube was then incubated at room temperature for 10 min.

Enzyme linked immunosorbent assay (ELISA)

1) 100 µl CT/NG hybridization solution was added into each well. 2) Then 25 µl DN Amplicon was added to each well. 3) It was incubated at 37°C for 2-4 min. 4) After incubation, the tray was tapped 10 to 15 times to change colour from blue to yellow. 5) The well was covered with lid and again incubated for 1 h at 37°C. 6) The well was then washed into ELISA microplate washer with wash buffer (250-300 µl) for 5 times. 7) The micro plate was tapped for complete drying into the blotting paper. 8) Then 100 µL Horse Radish Peroxidase (HRP) was added to each well. 9) It was incubated at 37°C for 15 min. 10) Again, the well was washed into ELISA microplate washer with wash buffer (250-300 µl) for 5 times. 11) The microplate was tapped for complete drying into the blotting paper. 12) 100 µL Substrate solution was added into each well. 13) It was then incubated at room temperature in complete darkness for 10 min. 14) 100 µl of stop solution was then added. 15) Absorbance of the specimen was read at 450 nm within 1 h.

Data collection and statistical analysis

The study utilized a socio-epidemiological questionnaire to gather information on risk factors and related health history. The questionnaire was administered via verbal interviews by the researcher at the clinic. Information was gathered on age (self-reported), educational level, use of contraceptives and disease status. The sexual risk behaviors (self-reported), were determined by the results of the questions that pertain to age of first gestation, number of sex partners in the past 6 and 12 month period, usage of condoms, sexual partners 3 years older, history of sexually transmitted infections and history of sexual abuse. The sexual knowledge was determined by a series of questions that tested knowledge about sexually transmitted infections specifically Gonorrhea and Chlamydia. All the data obtained was statistically analyzed by using Statistical Package for Social Science (SPSS) version 16 software package and RX64 3.0.3 software package. Sensitivity of the test was compared using formula (Sensitivity=(a/a+c)*100%). A p-value of <0.05 was regarded as significant.

Limitations

1) There are many sexually transmitted infections that have devastating outcomes; this study was limited to only Chlamydia and Gonorrhea. 2) Limiting sampling parameters to persons seeking care at the clinic may not provide a random sample generalizable to the entire community.

Results

Out of 94 study cases of sexually active women that were tested for Neisseria and Chlamydia, 25 (26.6%) cases with highest positive cases was in the age group less than or equal to 20 years were found infected with the CT and NG infection when diagnosed. Among all, 49 (52.1%) participants used condoms as method of contraceptive. The overall rate of STIs in the clinical study among both forty seven HIV positive and HIV negative were 38.2% and 14.8%. CT and NG co-infection was found in 1 (1.06%) participants. For univariable analysis, the variable for STI risk was reclassified into Sex, Age, Education, NG infection status, CT infection status, HIV status, case type, contraceptive method and symptoms. In our study, relationship of the STI was found statistically significant ($p<0.005$) with education, HIV status, symptoms and sex. Whereas, it was not significant ($p>0.005$) with age, case type, contraceptive method CT infection status and NG infection.

Among 94 cases, positive cases in symptomatic group by amplicor test and gram staining were 20 and 7, respectively. Whereas 5 cases were shown positive by amplicor test and gram staining showed no positive cases in asymptomatic (Table 1). Out of total cases, most of the specimen showed the absorbance in the range 0 to 0.5 both for *N.*

Study group	Study Characteristics		
	Tests in relation to presenting symptoms of the cases		
	No. of cases	Tests with no. of positive cases	
		Amplicor test	Gram Staining
Symptomatic cases	74	20	7
Asymptomatic cases	20	5	0
Result by Amplicor test (Absorbance at 450 nm) Mean Absorbance			
Cases with *C. trachomatis*	0.313		
Cases with *N. gonorrhoeae*	0.506		
Comparison between the test positivity			
	Tests	**No. of positive tests**	**Percent of test positivity**
Cases with *C. trachomatis*	Amplicor	11	11.7
	Gram Staining	6	6.3
Cases with *N. gonorrhoeae*	Amplicor	14	14.8
	Gram Staining	1	1.06
Accuracy of tests			
Sensitivity			
Amplicor test	27.03		
Gram Staining	9.46		

Table 1: Clinical characteristic of patients.

gonorrhoeae and *C. trachomatis*. The mean absorbance of Amplicor test at 450 nm for *C. trachomatis* was 0.3137 and that for *N. gonorrhoea* was 0.5066 (Table 1). In both cases, the highest percent of test positivity was shown by Amplicor test, i.e., 11.7% for 14.8% for CT and NG, respectively as compared to gram staining. The total number of positive cases shown by Amplicor test was 11 in case of CT and 14 in case of NG. Whereas, 6 cases was shown positive by Gram staining for CT and 1 for NG (Table 1). The measures of accuracy of Amplicor test for detection of CT and NG from endocervical swabs comparing with gram staining showed sensitivity of 27.03%. The sensitivity of Gram staining to detect CT and NG from endocervical swabs was found 9.46% (Table 2).

Discussion

This study conducted in National Public Health Laboratory, Teku, provides new information about STIs of public health importance in high risk population, i.e., female street prostitutes with and without HIV attending STIs clinic. This study was designed to provide important data to help formulate modifications to the standards of care in the clinic to ensure effective protocols for diagnosis of STIs such as Gonorrhea and Chlamydia. It is important for clinicians to understand the risk factors that contribute to the transmission of these diseases. A total of 94 participants and two controls (i.e., one for NG and one for CG; only to check whether the results were correct) completed the core study. In the present study, the highest number of positive cases was in the age group less than or equal to 20 followed by 21 to 25 years. The youngest and oldest positive cases were in the age less than 20 and 50 years respectively. Dibua et al. showed that the age group 15-19 were the most infected with STI; followed by those within the age bracket 20-24 [12]. However, the infectivity was lower among those in the 40-44 (22%) and >45 (13%) age brackets. The study determines contraceptive status and among which 10 (10.6%) used no method of contraceptive, 49 (52.1%) used condoms only, 30 (31.9%) used both oral contraceptive pills and condoms and 5 (5.4%) used pills only respectively. Hossan reported that 66.2% of the participants stated they used other contraceptive means than condoms and 33.7% participates stated they used condoms sometimes [5]. Mayans et al. showed that

Variables	Female sex workers with respect to STI status		
	Female Sex Workers (n=94)		p-value
	N	%	
Sex			
Female	94	100	0.01
Male	-	-	
Age			
≤ 25	35	37.2	0.074
>25	59	62.8	
N. gonorrhoeae			
Yes	14	14.8	0.108
No	80	85.1	
C. trachomatis			
Yes	11	11.7	0.108
No	83	88.3	
HIV-1			
Yes	47	50	0.01
No	47	50	
Eucation			
Illiterate	62	65.9	0.002
Literate (Primary studies)	32	34.04	
Case type			
Relapse	10	10.6	
Chronic	12	12.7	0.17
Follow up	52	55.3	
New	20	21.2	
Contraceptive method			
No method	10	10.6	
Condom only	49	52.1	0.08
Pills only	5	5.4	
Both (Oral pills and condom)	30	31.9	
Symptoms			
Symptomatic	74	78.7	0.022
Asymptomatic	20	21.3	

Table 2: Variables distribution among female sex workers with respect to STI status.

out of 301 female prostitutes, only 40% uses condoms during sexual intercourse. In this study, the comparison of the infection among the study subjects in relation to their presenting symptoms shows 74 symptomatic cases and 20 asymptomatic cases with 27.02 and 25 percent of positive cases, respectively, which accounts for 26.59% of positive cases in total? Carre showed 47.2% and 41.1% of positive cases among symptomatic and asymptomatic population which is 45.3% in total population [13,14]. On comparison of the result of Amplicor and Gram staining among symptomatic cases, the percent of positive cases by amplicor test and gram staining were 21.27 and 7.44, respectively. Whereas, 5.31% positive result was obtained by Amplicor test and gram staining showed no positive cases in asymptomatic group. Out of 94 patients, the study includes 47 HIV positive and 47 HIV negative respectively with the ratio of 1:1. Among 47 HIV positive cases, 8 are CT positive and 10 NG positive which accounts for 38.2%. Similarly, 3 are CT positive and 4 are NG positive with 14.8% of positive cases among HIV negative cases. Sharma et al. stated that Vaginal infections including sexually transmitted infections (STIs) were found in 47 (57%) HIV positive women and 30 (34%) HIV negative women [15]. In the

study, the mean absorbance of result of CT infection by Amplicor tests at 450 nm was 0.3137, whereas, 0.50667 for NG at 450 nm. Livengood et al. showed that the mean absorbance at 450 nm of CT and NG are 3.851 and 2.797, respectively. Similarly, for *N. gonorrhoeae* the Amplicor test result showed positivity of 14.8%, higher than that compared to *C. trachomatis* [9]. The positivity of gram staining was 1.06%. In both cases, the highest percent of test positivity was shown by Amplicor test, i.e., 11.7% for CT and 14.8% for NG, respectively, as compared to gram staining. Dyck et al. showed that out of 396 specimens, the test results showed, 63 (15.9%) specimens were *N. gonorrhoeae* positive by Amplicor test. Out of 94 individuals, 73.4% showed no infection whereas 10.6% came with Chlamydial infection, 13.8 with gonorrhoea and 1.06% participants with the co-infection of both gonorrhoea and chlamydia [16]. Bromhead et al. have found that 42.2% of those infected with *N. gonorrhoeae* had a concurrent *C. trachomatis* infection [17]. In the study the sensitivity of Amplicor test to detect the infection from endocervical swabs was found 27.03%.The sensitivity of gram staining to detect the organism was found 9.46%. Schepetiuk et al. showed that the Roche PCR (95% sensitive) was more sensitive than the gram staining (75% sensitive) [18].

In overall, when variables were analyzed, the highest percentage of FSWs were from age group more than 25 (62.8%).The total illiterate cases showed 65.9% whereas literate cases showed 34.04%, respectively. The highest case type was those who had come for follow up (55.3%). In our study, relationship of the STI was found statistically significant ($p<0.005$) with education (p-value 0.002), HIV status (p-value 0.010), symptoms (0.022) and sex (p-value 0.010).Whereas, it was not significant with Age (p-value 0.074), case type (p-value 0.17), contraceptive method (p-value 0.008), CT infection status (0.100) and NG infection status (0.082), respectively.

Conclusion

Analyzing the findings of the present study, it can be concluded that Nucleic Acid Amplification Test exhibited equally high sensitivity and are thus well suited for screening of *C. trachomatis* and *N. gonorrhoea* in female swab specimens. Thus, the AMPLICOR CT/NG test makes it possible to screen for both pathogens by processing and amplifying a specimen simultaneously. This study observes that among ninety four participants twenty five patients showed positive result by Amplicor test. The rate of sexual infection was drastically higher in HIV positive as compared to HIV negative. The measures of accuracy of Amplicor test showed 21.27% sensitivity as compared to Gram staining to detect *C. trachomatis* and *N. gonorrhoeae*. The highest percentage, i.e., 52.1% of the patients used condoms as the contraceptive methods. Twenty Patients were positive among symptomatic cases which was higher than that of asymptomatic cases. In this study, relationship of the STI was found statistically significant ($p<0.005$) with education, HIV status, symptoms and sex. Among all patients only one case was found co-infected with both the infections.

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