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Detection of *Chlamydia trachomatis and Neisseria gonorrhoeae* in Genitourinary Specimens in Iraq Women by Real Time PCR assay.

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ABSTRACT

The *Chlamydia trachomatis(CT), Neisseria gonorrhoeae* (*N.gonorrhoeae*) are the most common Sexually transmitted diseases *(STD)* among women in the developing countries represents a public health problem of growing importance among the high risk group. The study aims to evaluate more rapid and accurate STD diagnosis by molecular technology using Real time PCR*(RT-PCR)* (*Chlamydia trachomatis*/*Neisseria gonorrhoeae*) CT/Ng test kit for diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoea* in women with and without symptoms and comparing the test with routine works. A hospital based cross sectional study was conducted from February ,2017 to December,2017 from different hospitals and Special Clinic at Baghdad governorate was included in this study.. *One hundred study participants who fulfill the criteria were included.* high vaginal swabs were taken from females at different ages (15-55 years) representing patients group complaining of abnormal vaginal discharge with or without other symptoms, questionnaire was applied *Different laboratory methods and techniques were applied to identify the possible microorganisms.* The results were analyzed using descriptive, chi-square as appropriate. In RT-PCR the rate of infection was 30(30%) Neisseria gonorrhoea and 22(22%) Chlamydia trachomatis , Highest rate of infection in women with vaginal infection [Chlamydia trachomatis/Neisseria gonorrhoeae] were among age groups (15-20), (21-25) and (30-35), the lowest rate was among age group (40-55) age group . 18(28.6%) of patients with Chlamydia trachomatis were symptomatic, and only 4(10.8%)were asymptomatic, in Neisseria gonorrhoeae shows17(27%) were complaining from symptomatic, and only 13(35.1%)were asymptomatic**.** The commonest genital tract infections among women were Chlamydia trachomatis/Neisseria gonorrhoeae. Molecular methods are considered the gold standard for diagnosis . Presence of clinical symptoms helps and lab diagnosis of genital tract infection.

Keywords: *C. trachomatis; N. gonorrhoeae;* Molecular methods.

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INTRODUCTION

Sexually transmitted diseases with increasing number of new infections are a global burden Chlamydia trachomatis and Neisseria gonorrhoeae are the most prevalent bacterial sexually transmitted infections (STI) in the world [1] Sexual risk behaviors such as 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]. The course of these infections is unpredictable and diverse . Most infections in women are asymptomatic and frequently remain unrecognized, which increases the risk for reproductive tract complications [3]CT is an obligate intracellular bacteria and the causative agent of genital chlamydial infection [4]

Chlamydia trachomatis (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[5,6]

Neisseria gonorrhoeae (Ng)is an obligate human pathogen and is the etiological agent of gonorrhoeae which is the second most prevalent bacterial STI[7,8]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.[9,10]As estimated by the World Health Organization, 350-500 million cases are annually identified as harboring Neisseria gonorrhoeae (N.gonorrhoeae), Chlamydiatrachomatis (C.trachomatis) which all classified as sexually transmitted infections (STIs) [11,12] .More than 1 million people are infected by an STI every day, with the largest proportion in the region of south and south-east Asia. Totally, over 30 bacterial, viral and parasitic pathogens are causative agents of STIs [13,14]. The last published report of World Health Organization estimated that globally 3.0 million adults are infected with C.trachomatis, 1.0 million with N.gonorrhoeae, [15.16].

Laboratory diagnosis of these infections is done by antigen detection for *C. trachomatis* and gram staining , culture and antigen detection 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 [17,18]. 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 [19].

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 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*[20]. 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 [21]. The RT-PCR CT/NG Test is *in vitro* test for the detection of N. gonorrhoeae and *C. trachomatis* DNA in endocervical and urethral swab specimens from symptomatic or asymptomatic females [22]. 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 [23].The general objective of the study is to evaluate the performance of molecular diagnosis as a detection method for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urogenital specimen in women at high risk of STIs.

MATERIALS AND METHODS

This study was conducted from February ,2017 to December,2017 from different hospitals and Special Clinic at Baghdad governorate was included in this study, all women aged 15-55 yr. Exclusion criteria included administration of systemic or topical antibiotics within one month prior to sampling. After examination of each volunteer by a gynecologist, demographic data, history and clinical signs/symptoms were recorded in a questionnaire. A total of one hunderns endocervical specimens were included in the study. 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. Five endocervical/vaginal swab samples were collected from each volunteer under aseptic conditions and used for smear preparation, culture, and DNA extraction.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.

Detection of N.gonorrhoeae infection by use modified Thayer-Martin agar (mTM) medium was prepared from Blood Agar Base No.2 (Oxoid, UK) supplemented with 5% sheep blood and Vancomycin, Colistin, Nystatin, and Trimethoprim (VCNT) antibiotics (Sigma, St. Louis, MO, USA). Swabs were cultured on modified TM agar and plates were kept on candle jar and incubated at 37oC for 48 hr.

DNA extraction was carried out on the vaginal/endocervical samples by using AccuPrep® Genomic DNA Extraction Kit according to manufacturer's instruction (Bioneer, South Korea). A real-time PCR assay targeting the porA pseudogene was performed as described by Whiley (12). Briefly, LightCycler® 8-Tube strips were used, each well of 20 µL reaction mixture contained: 10 µl of 2x FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Germany), 2 µl of primer pairs mix (0.5 µM each) NIS.F 5'-CGGTTTCCGTGCGTTACGA-3', and NIS.R 5'-CTGGTTTCATCTGATTACTTTCCA-3', 5 µL of DNA extract, and up to 20 µL sterile PCR-grade water. PCR amplification was performed on the LightCycler® system (Roche Diagnostics GmbH, Germany) with the following program: initial denaturation at 95oC for 10 min followed by 45 cycles of denaturation at 95oC for 10 sec, annealing at 55oC for 10 sec, and extension at 72oC for 20 sec

Statistical analysis Statistics Analysis

Statistical analysis was performed by using SPSS computing program for the analysis of the results [24].

Ethics approval and consent to participate

Research Ethical approval was obtained from the research ethics committee of Baghdad University, college of medicine, and permission letters was obtained from the hospital management committee. Before commencement of data collection the purpose of the study was explained to the participants and all of them provided written informed consent. Ng, CT screening was performed free of charge, and those found to have infection were managed by physicians.

RESULTS

All vaginal sawbs and endocervical samples (n=100) were investigated by Gram stain culture , ***and PCR*** to detect the presence of ***Ng*** by using specific primer set which revealed that 20/100(20%) 25/100(32%),30/100(30%)of samples were ***Ng*** positive respectively and use gram stain and PCR to detected **CT** which revealed that 18/100(18%) ,22/100(22%) respectively (Table 1) figure (1).

Table 1: Various techniques for detection of Ng and CT in study patients group.

Methods	Positive cases (%)
Neisseria gonorrhoeae	
Gram stain	**20(20%)**
Culture	**25(25%)**
PCR	**30(30%)**
Chlamydia trachomatis	
Gram stain	**18(18%)**
PCR	**22(22%)**

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The age of the study population ranged from 15-55 years, Women aged 50-55years had higher prevalence of infection than other age classes .

The study revealed that women with Illiterate education have the highest Infection while low percentage of infection was seen in higher educational level .Based on family income, moderate socioeconomic status had the highest rate in chlymiadia infection but in Neisseria infection low socioeconomic status had the highest rate infection.

In rural residence the infection rate was higher prevalence than infection rate in urban . In parity, The infection percentage increased in Multipara status.

Presence of clinical symptoms helps in the diagnosis of infection. . 40 (40%) had malodorous discharge, 25 (25%) had itching, 10 (10%) had dysuria and 20 (20%) had low abdominal pain respectively. Women with genital ulcer represent the highest percent of cases Followed by Vulrular itching ,Pain in lower abdomen , Profuse discharge ,dysuria respectively (table 2)

Table 2: Variable factors of sexually transmitted infections according to patients with questionnaire in the present study

Education levels			
Illiterate	**30(30%)**	**11(36.7%)**	**8(26.7%)**
Variable factors	No. of test cases (n =100) %	Positive cases NG N=30	Positive cases CT N=22
G1: 15-20	**27**	**9(33.3%)**	**7(26%)**
G2: 21-25	**20**	**8(40%)**	**6(30%)**
G3: 30-35	**17**	**6(35.3%)**	**4(23.5%)**
G4: 36-40	**15**	**4(26.7%)**	**3(20%)**
G5:41-45	**19**	**3(15.8%)**	**2(10.5%)**
G6:50-55	**2**	**0**	**0**
Primary	**28(28%)**	**9(32.1%)**	**7(25%)**
Secondary	**25(25%)**	**6(24%)**	**4(16%)**
Higher	**17(17%)**	**4(23.5%)**	**3(17.6%)**
Socioeconomic classes			
Low	**35(35%)**	**10(28.6%)**	**8(22.9%)**
Moderate	**40(40%)**	**15(37.5%)**	**7(17.5%)**
High	**25(25%)**	**5(20%)**	**5(20%)**
Location			

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Urban	**65(65%)**	**21(32.3%)**	**15(23.1%)**
Rural	**35(35%)**	**9(25.7%)**	**7(20%)**
Parity			
Nullipara	**32(32%)**	**10(31.3%)**	**5(15.6.%)**
Unipara	**40(40%)**	**11(27.5%)**	**7(17.5%)**
Multipara	**28(28%)**	9(32.1%)	10(35.7%)
Symptoms and signs			
Vulrular itching	**25(25%)**	**9(36%)**	**5(20%)**
Pain in lower abdomen	**20(20%)**	**7(35%)**	**7(35%)**
Vaginal discharge	**40(40%)**	**10(25%)**	**6(15%)**
Dysuria	**10(10%)**	**2(20%)**	**2(20%)**
Genital ulcer	**2(2%)**	**1(50%)**	**1(50%)**
Swelling in groin	**3(3%)**	**1(33.3%)**	**1(33.3%)**

Table (3) shows that 17(27%) of patients with *NG infection* were symptomatic, and 13(35.1%)were asymptomatic, in CT shows 18(28.6%) were complaining from symptomatic, and only 4(10.8%)were asymptomatic, On application of Chi square test, highly significant difference between types vaginal infection in relation to vaginal symptoms was found.

Table 3: Types of vaginal infection according to vaginal symptoms

Vaginal symptoms Total =100	Positive no NG(%)	Positive no CT(%)
Symptomatic N=63	**17(27%)**	**18(28.6%)**
Asymptomatic N=37	**13(35.1%)**	**4(10.8%)**
P value	**P=0.390**	**P=0.038**

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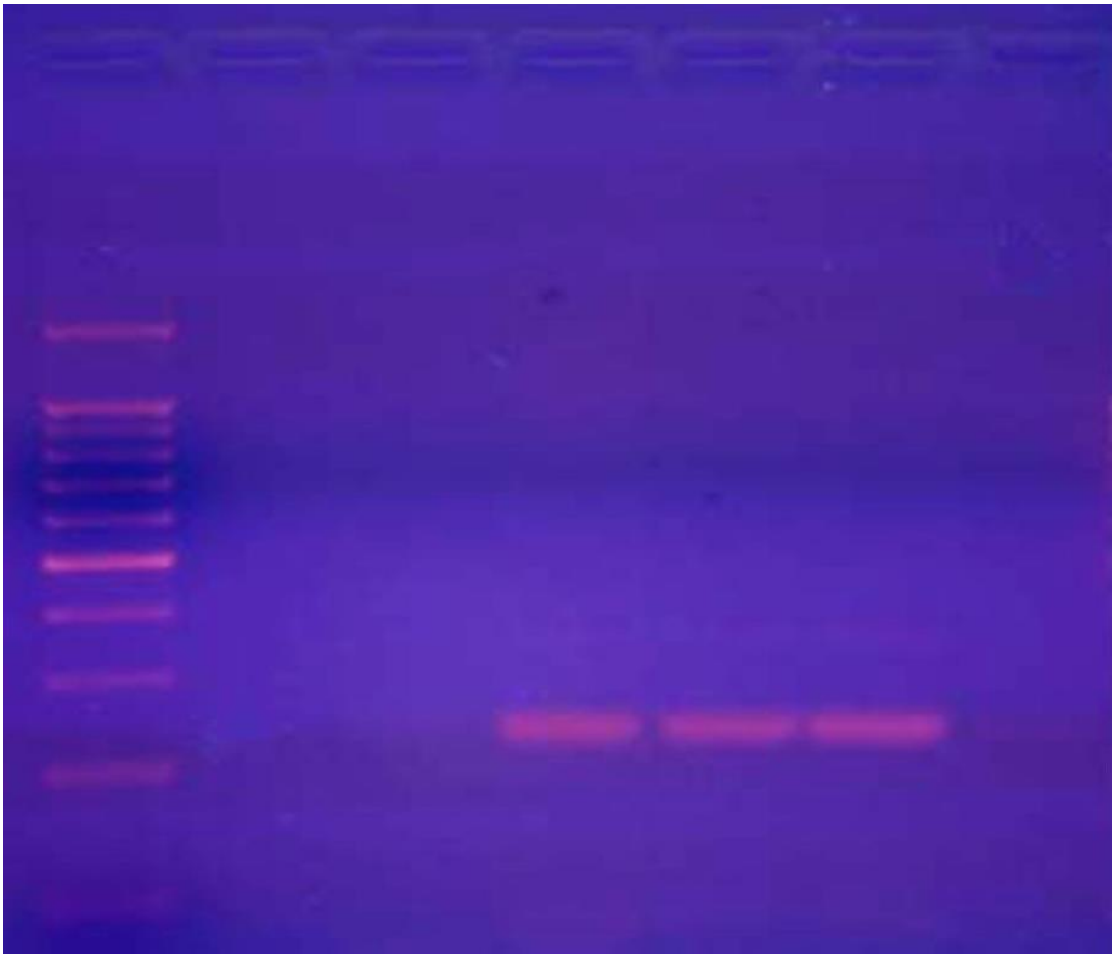

Figure 1: Agarose electrophoresis of total DNA extraction

DISCUSSION

Sexually transmitted pathogens regard as the most serious causative agent for vaginal infection. However, vaginal infection are possible increased prevalent in reproductive ages due to biological factors such as age, hormonal changes, cervical ectopic (25).

In the present study, Women in the age groups (21-25) (26-30)and (30-35)years had the highest prevalence of infection compared with older age category in a study of women according to the pH level, vaginal discharges were varied with age, use of contraceptives, menstrual cycle and with the estrogen level, douching used. Sexually transmitted pathogens (STPs) regard as the most serious causative agent for vaginal infection. However, while vaginal infection is possible increased prevalent in reproductive ages due to biological factors such as age, hormonal changes, cervical ectopic [26,27].

The study revealed that women with primary education have the highest number and percentage (36.7%) while low percentage of infection was seen in higher educational level (23.5%) .In residence, was higher infection rate in urban than infection rate in rural for infection of (CT and Ng) (probably because of the difference between the city and countryside in lifestyle and vaginal infection mainly affecting people living in poor or disadvantaged communities [28,29]. In parity, the infection percent of two microorganism increase in multipara status followed by unipara status, and finally the nullipara status .It appeared that infection with is strongly correlated with reactivation of some agents, abortion, through vaginal delivery and vaginal hygiene practices (such as douching) and type of contraceptive this result agreement with (30) .

In the current study, the majority of symptoms were genital ulcer, vulvar itching, pain in lower abdomen , swelling in groin than the other symptoms occur with less frequency. Symptoms alone are not sufficient to make reliable diagnosis of CT and NG pathogens . No association between the occurrences of

strong vaginal discharge and the two infections was found, as well as no positive correlation for dysuria with infection , corroborating other studies.[31]The disparity in the prevalence of vaginal infection could be attributed to many reasons; environmental and socio-economic factors, accurate take of sample form patient by gynecologist, diagnostic method, number of tested samples, type of samples and cultural factors. [32,33]

Vaginal culture is one of the most difficult cultures to be evaluated in a clinical microbiology practice. The necessity of some expensive and complicated processes for diagnosis of some specific agents, age related variability of normal vaginal flora, and failure to make a diagnosis caused by the temporary presence of some pathogens in normal flora can be listed among the probable causes of that problem (34).In vitro culture is still the reference method for the diagnosis of gonorrhea. [35]. Detection of gonococcal infection in females as defined by culture is significantly underdiagnosed. Reasons for false-negative *N. gonorrhoeae* culture could include prior antimicrobial therapy, loss of viability of the organisms during transport, low concentrations of the organisms, or sampling error.[36]

The present results showed the highly sensitive as determined by q PCR assay for presence of Neisseria gonorrhea and C. trachomatis among effected women. Similar detection rates of Neisseria gonorrhea *and C. trachomatis* in effected women by PCR have been reported in previous studies from developing countries [37].Ahmed by a specific PCR for the *Chlamydia* plasmid (KL1 and KL2) genes detection revealed that *Chlamydia trachomatis* of endocervical swabs from women Iraqi [38].El Qouqa *et al.* [39] detected C. trachomatis in 20.2 % of from infertile Palestine women infertility by plasmid-based PCR. Also C. trachomatis infection detection in 200 Indian infertile women found in 13.5 % ,11.5 % .6.5 % by real time-PCR,cryptic plasmid , EIA Respectively [40,41]. In infertile Iranian women was found 13.7 %frequency of C. trachomatis infection by PCR [42]. In a study from Iran, by using a multiplex PCR, N. gonorrhoeae was detected in 31 (46.0%), C. trachomatis in 15 (22.4%), and mixed two infections in 7 (10.4%) urine samples from patients with urethritis [43]. For C.Tand Ng infection, PCR assay is more sensitive than culture [44,45]. It has been shown that culture and immunoassay have sensitivity of as low for CT, Ng [46,47].

RT-PCR is more accurate to detect the pathogens . Molecular methods are considered the gold standard for diagnosis ,given the excellent sensitivities and specificities in diagnosis. Presence of clinical symptoms ,lab diagnosis of infection supported by cultivation were used for diagnosis of genital tract infections.

CONCLUSIONS

Present multiplex PCR assay showed same specificity and equivalent or higher sensitivity compared to uniplex PCR and other routine assays. Multiple infections are difficult to detect, because a few clinics and laboratories perform diagnoses to N. gonorrhoeae and C. trachomatis some centers may not look for a second STI after one is detected. The multiplex PCR offers a sensitive and more rapid method for the detection of multiple pathogens involved in STIs in a single specimen. Additional studies performed with larger numbers of women, symptomatic and asymptomatic and other specimens such as urine will be required to determine the true performance of this multiplex PCR for detection of C. trachomatis and N. gonorrhoeae simultaneously.

Competing interests

The authors declare that they have no competing interests.

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