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Prevalence of *Neisseria gonorrhoeae* infection in symptomatic and asymptomatic women attending obstetrics and gynecology outpatient department at Lahore general hospital Lahore, Pakistan

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Abstract
Gonorrhoeae or gonococcal infection is worldwide sexually transmitted bacterial problem caused by *Neisseria gonorrhoeae*. The current study has been conducted to develop rapid and reliable diagnostics techniques for identification of gonorrhoeae and its incidence in Lahore region of Punjab, Pakistan. Total of 200 endo-cervical discharge samples were collected from symptomatic and asymptomatic patients reported with the history of gynecological problems attending OPD of Lahore general hospital, Lahore Pakistan. Each of the labeled sample was streaked on chocolate agar/Thayer-Martin Medium and incubated for 24-72 hours in the presence of 5% CO_2. Grayish-white, opaque, rounded 1-2mm colonies showed gram negative diplococcic in microscopy and revealed positive oxidase and catalase reaction. Results of PCR based analysis on screened colonies showed that 12% of the isolates developed 260bp amplicon size products against ORF1 type specific primer in gel electrophoresis system. Prevalence of *N. gonorrhoeae* in symptomatic patients of age group ≥30 and ≤30 is 20% and 11% respectively as compared to the asymptomatic patients of age group of ≥30 and ≤30 is 7% and 10% respectively. Similarly, prevalence of married and un-married symptomatic patients was 6% and 36% to that of asymptomatic patients (8% and 3%). The prevalence of *N. gonorrhoeae* in symptomatic patients of PID and cervicitus is 37.5% and 6.25% and the prevalence of *N. gonorrhoeae* in asymptomatic patients of PID and cervicitus is 5% and 6% respectively. Culture sensitivity results showed that all isolates were sensitive to Penicillin, Tetracycline, Spectinomycin, Ciprofloxacin and Ceftriaxone.

Keywords: *N. gonorrhoeae*, asymptomatic, symptomatic, thayer-martin medium, polymerase chain reaction

1. Introduction
Gonrorrhoea is a sexually transmitted bacterial infection caused by *Neisseria gonorrhoeae* with high prevalence in developing countries. In females it is generally asymptomatic or characterized by vaginal discharge and pain [1]. In different communities the prevalence of disease varies from 0.3% to 0.22% [2]. It is of great significance to reduce the rate by limiting the transmission from partner to partner and it can be achieved by estimation of prevalence along with the risk factors and other conditions that provide fertile grounds for manipulation of *N. gonorrhoeae*. Worldwide attempts are ongoing to treat, prevent and cure the gonorrhoea because of its drastic effects on fertility and being considered as one of the highlighted causes of infertility.

In 2001, the World Health Organization (WHO) estimated that more than 62 million new gonorrhoeae cases occur each year worldwide [3]. The number of new cases is now estimated to be over 82 million each year [4]. Although realistic goals for the reduction and elimination of *N. gonorrhoeae* infections have been established by many countries since the 1990s, following a worldwide reduction in prevalence, the incidence of gonorrhoeae is now rising again [5]. Non treated gonorrhoeael infection can transform into more complicated diseases affecting female reproductive organ, joints and hearts. Moreover, it can also increase the risk of sexually transmitted infection like human immunodeficiency virus and Chlamydia infection. Women have more chances to get infected with gonorrhoeae than male with single act of vaginal intercourse with an infected partner.

The disease remains the second most frequent bacterial sexually transmitted infection in the United States [6].

It is a major public health concern globally that requires immediate international public health resources and attention. While the most of symptomatic infected women were remained untreated because they did not seek care and 50% of asymptomatic infected women were undetected and untreated. Thus, the control of this disease will require strategies such as screening and mass treatment, in addition to improved clinical services for patients with symptoms [7].

The approach to diagnosis and management is likely to differ from country to country depending on available resources. Most developing countries rely on the syndromic approach to manage STIs in pregnancy. This approach, however, is notoriously poor in identifying infections particularly with *N. gonorrhoea* and *C. trachomatis* and will miss a high proportion of infection because a significant proportion of infections are asymptomatic. Risk assessment approaches have been used to reduce the proportion of women with cervicitis who are over treated using the syndromic management approach but this approach still lacks sensitivity and specificity. There is an urgent need for affordable, rapid, point of care screening tests for STI screening in resource constrained antenatal care settings [8].

Currently, the data regarding isolation and prevalence of the disease is scanty and the treatment for such suspected cases is being carried on basis of tentative diagnosis. Moreover, most of the gonorrhoeael cases are prevailing in females are asymptomatic but risk factors like urinary tract infection and hormonal changes during pregnancy might get it active along with further complications. Furthermore, disease diagnosis is still a major problem in the control of gonorrhoeael infection. The agent has been identified on the basis of "Gram staining" and microscopic features which could not be considered as reliable method in the presence of variety of commercial available microbial biochemical kits and advanced molecular diagnosis. Therefore, the current study has undertaken to diagnose the agent using different universal and type specific primers in polymerase chain reaction for incidence of gonorrhoeael infection in symptomatic and asymptomatic pregnant woman along with anti-biogram assessment to treat the infected patient in best possible way.

2. Material and Methods

2.1 Study Design and Protocol

2.1.1 Sample Size

The sample size was calculated by using the formula of Kish & Lisle states that [9]

$$n = z2\ p\ (1-p) / d2$$

Where, z = Score for 95% confidence interval = 1.96

p = Prevalence to estimate the proportion of *N. gonorrhoeae* in pregnant women

d = Sampling error that could be tolerate= 5%.
1-p= Probability

Present study was conducted in co-ordination with Gynecology and obstetrics department of Lahore general hospital (LGH) located on Ferozpur road Lahore, Pakistan. The study population comprised of all married, non-married, pregnant and non-pregnant woman attending the antenatal outdoor patient clinic in hospital during study period. The 200 endo-cervical discharge swabs samples were collected from the gynecological patients. Swabs carrying samples were immediately be placed into Stuart transport medium (Fig. 7).

2.2 Isolation and Growth of Bacteria

Each of such as chocolate agar, specified grams of thayer martin agar was weighed using digital balance (Shimadzu Scientific Instruments, Japan) and suspended in 1000 ml glass flask containing distilled water according to manufacturing instruction (Oxoid - England). Let the flask to dissolve medium completely with the help of digital shaker followed by sterilization at 121 °C for 15 minutes at 15lb pressure. Each of the labelled samples were streaked on the surface of chocolate agar media and incubated at 37 °C for 48 hours. Suspected freshly grown Neisseria colonies were then shifted aseptically with sterile platinum loop to thayer martin agar and incubated at 37 °C for 24 hrs. (Fig. 8).

2.3 Gram Staining

Every pure culture grown on the surface of selective medium showing standard characteristics colony were selected for Gram Staining. Smear was established by placing a loop full of culture on microscope glass slide re-suspended with drop of distilled water in circular pattern. The smear was fixed by passing 3-4 times on flame followed by heat air drying. Straining was performed according to the method described by Bergey (Fig. 9) [10].

2.4 Biochemical Assay

Biochemical test were performed on the pure culture of isolates often getting significant growth on thayer martin medium. A loop full of pure culture was mixed with the specific reagent followed by prescribed incubation time. Biochemical tests such as catalase, Oxidase and DNase (DNA hydrolysis test) were performed according to method described by King [11].

2.5 Polymerase Chain Reaction (PCR)

All the positive samples were subjected for DNA extraction by using QAIamp DNA Mini kit (QIAGEN-Germany) followed by PCR amplification of the desired genes *orfl,* (260bp) *23SrRNA* (200bp) and nspA (270bp) of *N. gonorrhoeae*. For the PCR reaction, 2 µl of 10X PCR buffer, 1µl of forward and reverse primer, 2µl of 80mM $MgCl_2$, 2 µl of DNTPs mixture and 1 µl of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) were added to the reaction mixture. Reaction volume was obtained by adding 9µl of Nuclease-free Water and 2 µl of extracted DNA sample. The PCR tubes were incubated in thermal cycler (Applied Biosystems) with an initial denaturation of 94 °C for 2 minutes. Followed by 40 cycles of denaturation at 94 °C for 30 secs, annealing at 51 °C for 30 secs and extension at 72 °C for 1 minute. Final extension was conducted at 72 °C for 10 minutes Fig. (10, 11).

Table 1: Sequences of Oligonucleotide primers *N. gonorrhoeae* Gene Primers Amplicon size Sequences

Gene	Primers	Amplicon size	Sequences
Orfl	Forward Primer Reverse Primer	260bp	CAA CTA TTC CCG ATT GCG GTT ATA CAG CTT CGC CTG
23SrRNA *nspA*	Forward Primer Reverse Primer	200bp	CGT TCA TCG GCG TAG GGT AA CAC TTC TCG GTG TTA AGA AA
	Forward Primer Reverse Primer	270bp	GCC GCG TAT CTT GAG GCA TT TGA AGC CTT TGG CAG AAC CT

2.6 Culture Sensitivity Test

Antimicrobial susceptibility test (AST) was performed for the suspected isolates using Kirby-Bauer's disk diffusion method described by Bauer [12]. All procedures were done as recommended by Clinical and Laboratory Standard Institute (CLSI, 2015) [13]. Individual colonies were suspended in normal saline to 0.5 McFarland and using sterile swabs the suspensions were inoculated on Muller Hinton agar for 18-24hr. Antibiotics tested for susceptibility were Ceftriaxone (30µg), Ciprofloxacin (30µg), Penicillin (10U), Tetracycline (30mcg) and Spectinomycine (100 µg) (Fig. 13).

3. Results

The samples collected from married, non-married, pregnant and non-pregnant gynecological patients were categorized into Symptomatic and asymptomatic patients. On the basis of colony characteristics, microscopy and biochemical analysis of each isolate revealed that out of 200 vaginal samples, 60 samples were positive for *N. gonorrhoeae*. PCR amplification of extracted DNA of each positive isolate showed percentage presence of orfi gene 25 (41.66%) as compare to nspA 0 (0%) and 23SrRNA 0 (0%) which did not show amplification for either of the samples (Fig. 12).

Biochemical analysis of 120 symptomatic patients in age group of ≥30 showed high prevalence of *N. gonorrhoeae* 23 (46%) followed by the age group ≤30 20 (29%). Whereas, PCR based molecular analysis confirmed highest prevalence of orfi 10 (20%) in age group of ≥30 as compare to the age group ≤30 8 (11%). Significant bacterial count 56 was recorded in vaginal sample of ≥30 & ≤30 year in asymptomatic age group. Out of these positive samples 8 (27%) and 9 (17%) were confirmed as *N. gonorrhoeae* respectively. However, 2 (7%) and 5 (10%) *N. gonorrhoeae* genotype orfi were detected positive through PCR (Fig. 1, 2).

On the basis of marital status, out of 120 samples 80 married and 40 unmarried symptomatic patients were showed significant prevalence of N. gonorrhoeae 20 (25%) and 20 (50) in biochemical analysis respectively. Whereas, the prevalence of virulence gene orfi of N. gonorrhoeae in married and unmarried patients were 5 (6%) and 15 (36%). Moreover, vaginal samples of married and unmarried women suspected for asymptomatic showed bacterial count 40 (80%), 25 (83%) for biochemical analysis. The percentage prevalence of N. gonorrhoeae is 11 (22%) and 9 (30%) for married and unmarried respectively. PCR based identification of N. gonorrhoeae gene orfi gene was positive for 4 (8%) and 1 (3%) samples in asymptomatic married and unmarried patients (Fig. 3, 4).

In symptomatic patients, biochemical analysis revealed that significant bacterial count 35 (87%) and 60 (75%) were observed in PID and cervicitus. The positive samples showed high prevalence of *N. gonorrhoeae* in PID 18 (45%) followed by cervicitus 18 (22%). However, 15 (37.5%) and 5 (6.25%) were positive for PID and cervicitus virulence gene orfi through PCR. Furthermore, 80 samples of asymptomatic PID and cervicitus patients was recorded for significant bacterial count having percentage positivity of N. gonorrhoeae 6 (30%) and 11 (18%) in biochemical analysis. Asymptomatic women with cervicitus showed highest prevalence the frequency of orfi gene 4 (6%) as compare to PID 1 (5%) (Fig. 5, 6).

The PCR confirmed *N. gonorrhoeae* showed varying susceptibility patterns to 8 antimicrobial drugs. The results revealed that the isolate was highly sensitive to Penicillin, Tetracycline, Spectinomycine, and Ceftriaxone. Whereas, isolates of *N. gonorrhoeae* was highly resistant to Ciprofloxacin (33%) (Fig. 13).

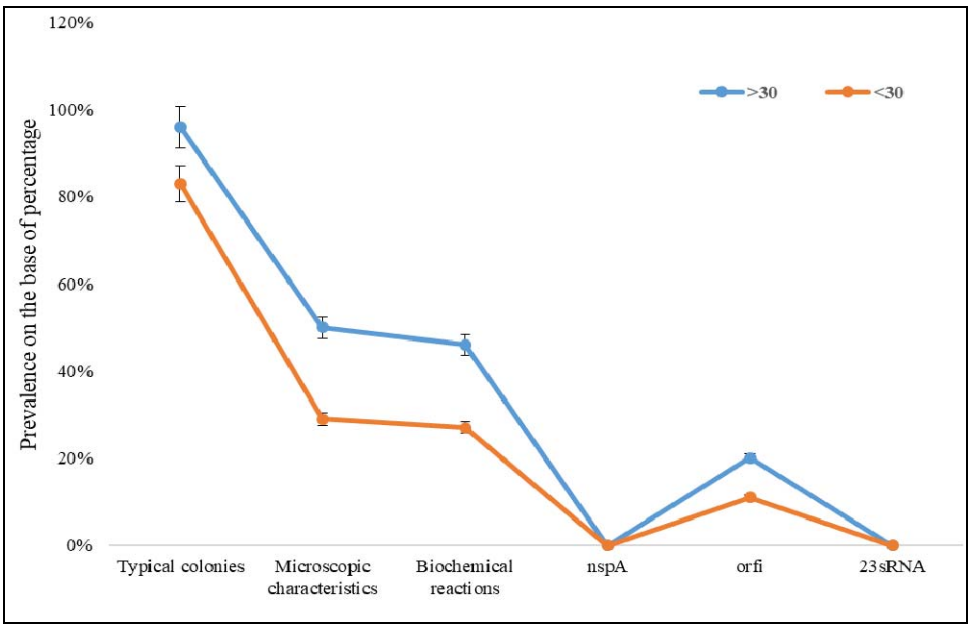

Fig 1: Age based prevalence in symptomatic patients

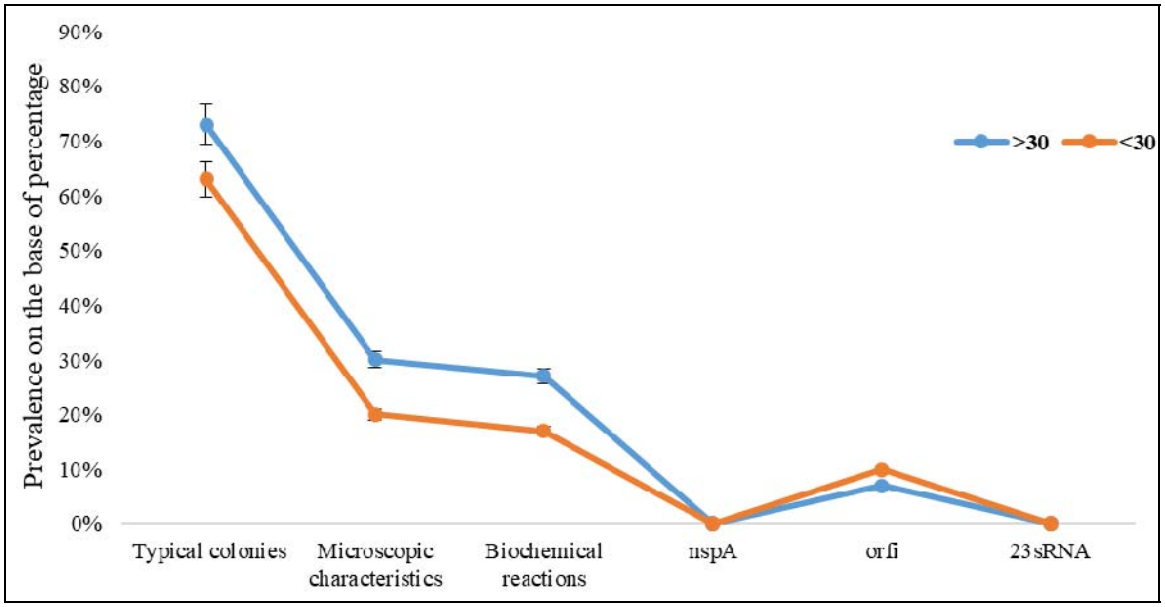

Fig 2: Age based prevalence in asymptomatic patients

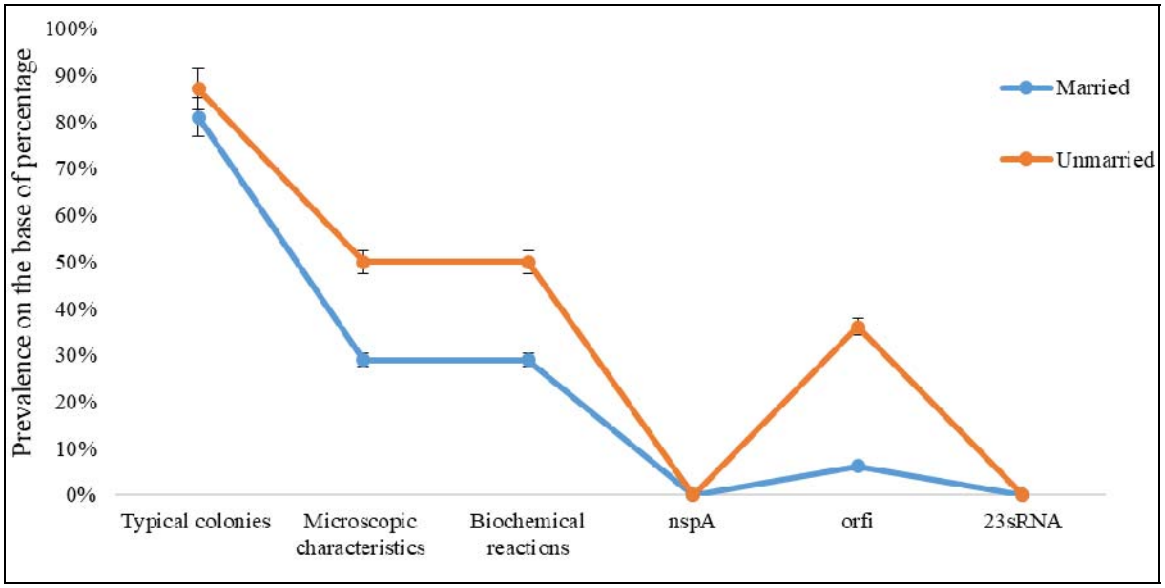

Fig 3: Marital status based prevalence of asymptomatic patients

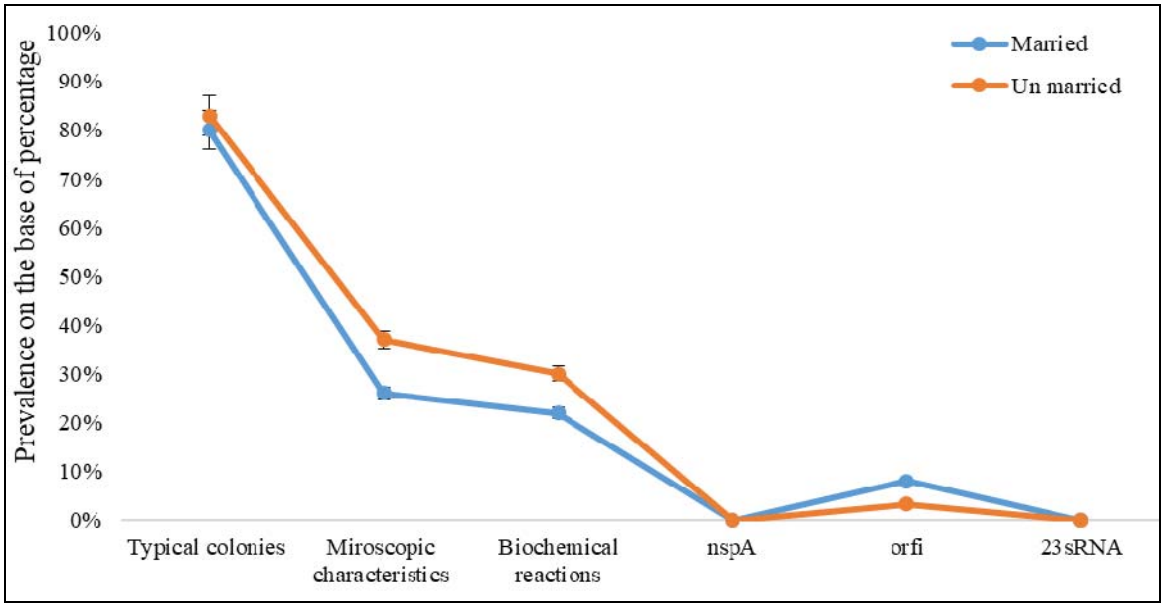

Fig 4: Marital status based prevalence of asymptomatic patients

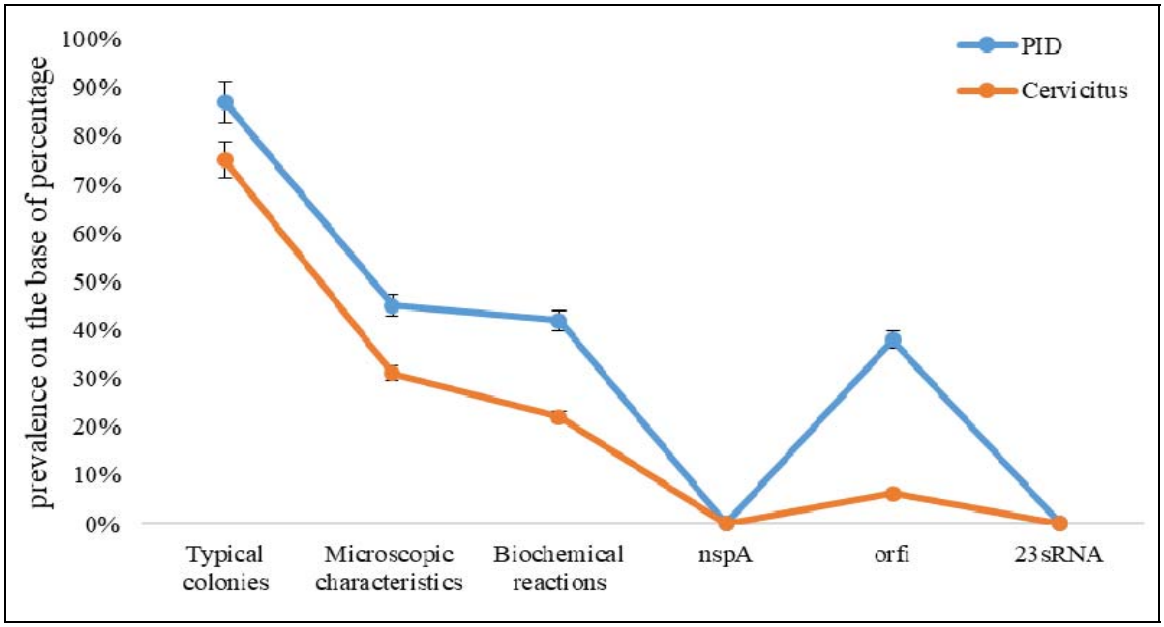

Fig 5: STI based prevalence of symptomatic patients

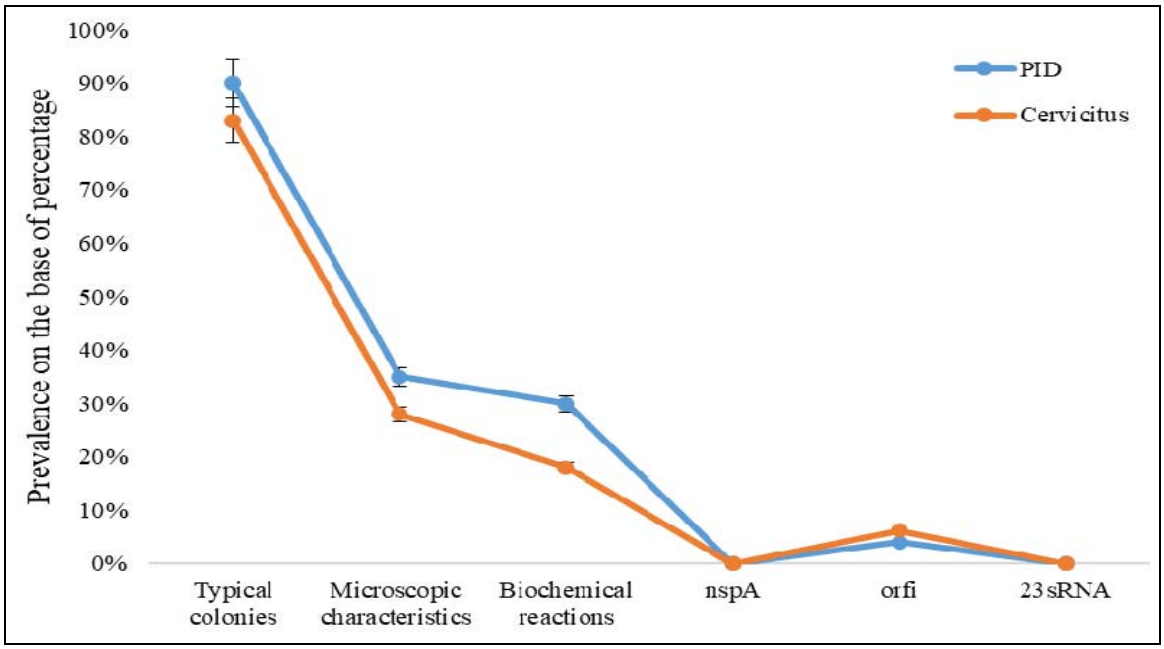

Fig 6: STI based prevalence of asymptomatic patients

Fig 7: Collection of Vaginal Samples from Symptomatic and Asymptomatic Patients

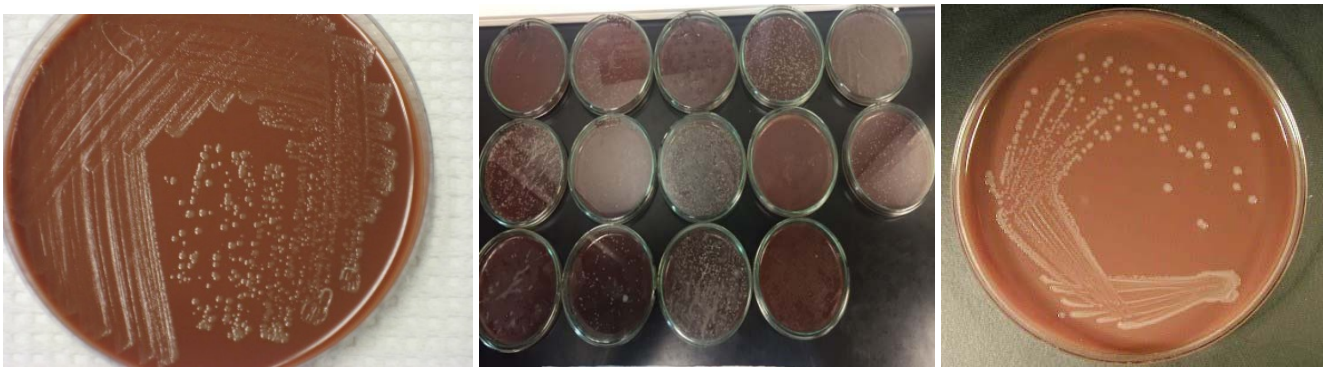

Fig 8: Isolation and Growth of *N. Gonorrhoeae* on Selective Media

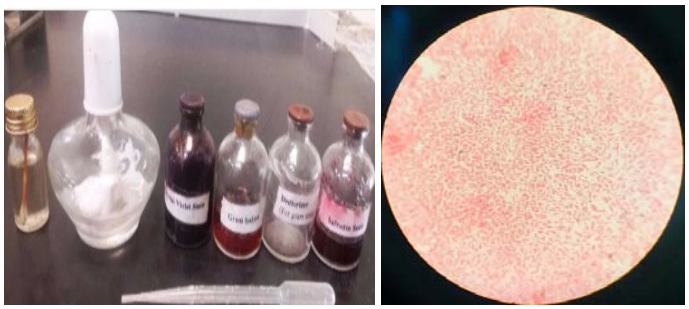

Fig 9: Gram Staining and Microscopy

Fig 10: DNA Extraction and Polymerase Chain Reaction

Fig 11: Gel Electrophoresis analysis of PCR product

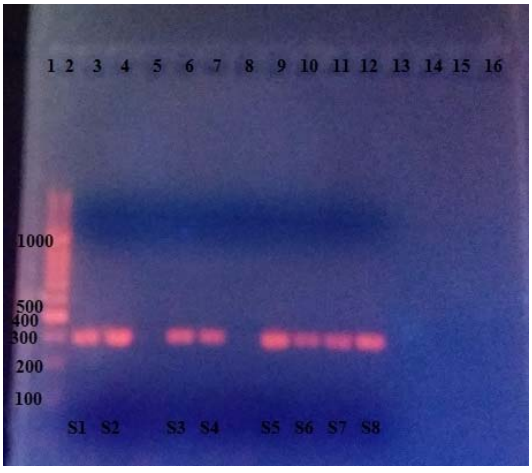

Fig 12: Electrophoresis Analysis of PCR Products

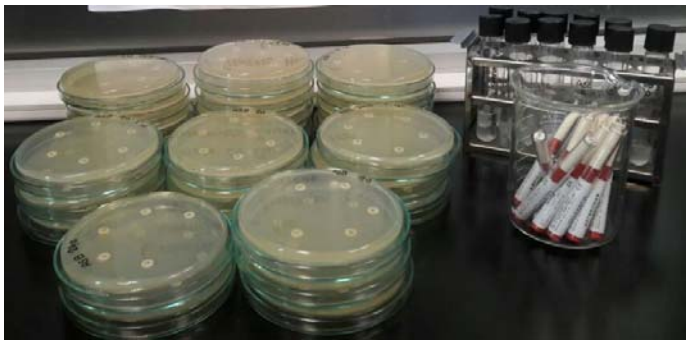

Fig 13: Kirby-Bauer's Disk Diffusion Method for Antimicrobial Susceptibility Test

Discussion

In the present study bacterial isolates recovered from married, non-married, pregnant and non-pregnant gynecological patients were confirmed for *N. gonorrhoeae* by using conventional microbiological techniques and polymerase chain reaction. Typical colonies were observed on selective media (Thayer martin), and enrichment media (Chocolate agar). These were further characterized on the basis of form, shape, color and microscopic features. Gram negative diplococci were recorded as the prominent isolates grown on selective media. These results were further analyzed by biochemical reaction (oxidase, catalase) and molecular characterization (PCR) using orfi, nspA and 23SrRNA genes.

Neisseria gonorrhoeae showed positive results for oxidase and catalase test with almost the same percentage distribution, supported the results obtained by Gram staining. This finding is highly corroborates to the observation of King who confirmed *Neisseria gonorrhoeae* by using same series of biochemical reaction [11]. Similarly, the results are also similar to Koumans by using the same conventional biochemical reaction [14].

PCR based analysis for genotype nspA, Orf1 and 23SrRNA revealed amplicon size of 260bp for orf1 whereas nspA and 23SrRNA did not show amplification for either of the sample. The results are partially in agreement with chaudhry who reported amplicon size of 260bp, 200bp and 270bp for Orf1, 23SrRNA and nspA respectively [15]. The difference in results may be due to the source of chemicals used in the study, optimization of PCR technique and difference in method for the extraction of nucleic acid.

The current results were analyzed for the prevalence of *N. gonorrhoeae* in different age groups. The results showed that the prevalence of *N. gonorrhoeae* in symptomatic woman 10(20%) are significantly higher than asymptomatic woman (27%). Our results are in line with Ahmed that reported the prevalence of *N. gonorrhoeae* in symptomatic patients is higher than asymptomatic woman [16]. Whereas, Bhatta reported the age

based prevalence of *N. gonorrhoeae*. According to him the disease is significantly higher in age group >20(77.9%) as compare to age group 17(3.7%) (Fig. 1, 2) [17].

The result of the current study showed that 6% of symptomatic married woman were declared as positive of *Neisseria gonorrhoeae* on the basis of microbiological evaluation supported by molecular characterization of orfi gene. Our results are partially in agreement with Molaei who reported that 4% of married gonorrhoeae suspected patients were confirmed through bacterial culture and serological test [18]. In the current study 3% of un married asymptomatic women were declared as positive of *Neisseria gonorrhoeae* by molecular characterization of orf1 gene. Our results are partially in line with Hailmariam who reported that 5.1% of gonorrhoeae suspected patients were confirmed through bacterial culture, gram staining and biochemical reaction [19]. In our study 5% prevalence of *Neisseria gonorrhoeae* in married asymptomatic woman were recorded. The results are not in line with the finding of Mayta who reported that 1.7% of gonorrhoeae suspected asymptomatic woman were confirmed through PCR analysis (Fig. 3, 4) [20].

4% of PID asymptomatic infected woman were declared as positive of *Neisseria* by molecular characteristics of Orf1 gene. Our results are partially in agreement with Elkayl who reported that 2% of gonorrhoeae suspected patients was confirmed through PCR analysis in Egyptian woman [21].

Our results are consistence with janpor that reported that 9% of gonorrhoeae suspected patients was confirmed through PCR based analysis. The present study revealed that 38% of PID infected symptomatic married woman were declared positive for *N. gonorrhoeae*. Kasanda revealed similar observation which supports the current findings. According to him 37% of PID infected woman were confirmed to have gonorrhoeae through gram staining and immunoassay rapid test [22]. Results of symptomatic cervicitus showed that 6% of symptomatic cervicitus infected women were declared as positive of *Neisseria* gonorrhoeae. The results are contradictory with the observation of Ujvec who reported that only 0.8% of cervicitus infected woman were declared as positive of *Neisseria gonorrhoeae* [23]. The difference in the data may be due to collection of sample method, processing method of sample, technique used for extraction of DNA, source of kits for either of the sample, difference in procedure run technique and recipie used for the amplification of DNA (Fig. 5, 6).

Results of bacterial antibiotic sensitivity are in agreement with finding of King where the result revealed that *Neisseria gonorrhoeae* is sensitive to Penicillin, Tetracycline, Spectinomycin, Ceftriaxone whereas Ciprofloxacin showed resistance (Fig. 13) [11].

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