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Pooling of Urine Specimens for Diagnosis of Asymptomatic Chlamydia Trachomatis Infection by PCR in a Population of Low Frequency, a Cost-Saving Technique for Epidemiological and Screening Programs.

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ABSTRACT

Objective: To describe the frequency of Chlamydia trachomatis in local community visiting a tertiary care hospital and to estimate the cost saving achieved as a result of pooling strategy.
Study Design: Cross-sectional study.
Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from Jan 2022 to Jun 2022.
Methodology: A pool of three urine samples was created after individual manual DNA extraction of each sample and tested by RT PCR. Any pool signaling positive was identified and all samples in that pool were retested individually to determine the positive sample. A total of 66 asymptomatic young people including males and females were tested.
Results: The frequency of Chlamydia trachomatis was found to be 7.57%. About 22 pools were created resulting in a 48.0% savings in costs.
Conclusion: The Pooling strategy adopted with the objective of saving test costs resulted in getting timely and reliable results in a resource limited setting. It also provided with the means to keep Sexually Transmitted Infections detection programs going on at various healthcare levels and in screening a larger number of populations.

Keywords: Chlamydia Trachomatis, Pooling, PCR, Urine.

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INTRODUCTION

Sexually transmitted infections (STIs) have always posed a risk to the reproductive health of sexually active individuals especially the young population. In regions like Pakistan, STIs remains largely underdiagnosed due to the paucity of testing facilities as well as certain religious and social stigmas associated with the diagnosis of sexually transmitted diseases and its aftermath.[1]

Among the ten leading infectious diseases that require notification of the Centre for Disease Control and Prevention (CDC) in the United States (US), five of them are transmitted via sexual route. These five infections include genital chlamydial infection, gonorrhea, Human Immunodeficiency Virus infection, syphilis, and hepatitis B.[2] Risk factors associated with various sexually transmitted infections including chlamydia trachomatis include young age of onset of intercourse, non-usage of barrier methods, multiple sexual partners, and gender (females as compared to males).[3]

Most Chlamydia infections are asymptomatic, especially in women.[4] If this infection is left undiagnosed, it can lead to various urological and gynecological conditions such as urethritis, cervicitis, salpingitis, and PID. It can even lead to ectopic pregnancies and secondary infertility.[5,6] Infants born to mothers with active chlamydia trachomatis infection can suffer from bacterial conjunctivitis and pneumonia.[7,8] The asymptomatic nature poses a challenge in the early detection of this disease. However, timely diagnosis can lead to effective treatment as well as a reduction in transmission rates.

The development of molecular techniques has dramatically transformed the diagnosis of sexually transmitted infections. Non-culturable bacteria such as Chlamydia trachomatis are more easily detected by means of Nucleic Acid Amplification Tests.[9,10] The urine sample remains one of the most easily self-collected samples amongst male and female patients both as compared to giving endocervical or urethral swabs making the basis of our study to detect this organism in urine samples.

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METHODOLOGY

This study was performed at the Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi from Jan 2022 to Jun 2022 after taking approval from Institutional Review Board (IRB), vide reference number FC-MIC19-5/READ-IRB/23/17923. After a thorough literature search, we calculated a sample size of 66 via the WHO calculator, keeping the margin of error at 5%, a confidence level at 95%, and a prevalence at 4%.[8] Sampling was done using a non-probability consecutive sampling technique.

Inclusion Criteria: Asymptomatic patinets of either gender visiting gynecology or urology OPD of Combined Military Hospital, Rawalpindi, were included.

Exclusion Crietia: None.

Of the 66 patients sampled, 42 were female and 24 were male. These patients were visiting gynecology or urology OPD of Combined Military Hospital, Rawalpindi. Informed consent was taken and information from the patients was recorded on the patient datasheet designed for the study.

After approval for participation, all patients were asked to submit a fresh-catch urine sample. Patients were instructed to give sample after at least gap of 1 hour or more from last voiding.

After collection, samples were sent to the microbiology department of Armed Forced institute of pathology and stored at 2°C–8°C awaiting analysis by PCR for Chlamydia trachomatis. Each sample was vortexed thoroughly. Extraction was carried out using BacterialXpress extraction kit. 200µl of nucleic acid extraction reagent was added to a nuclease free microfuge tube along with 50µl of sample and 10µl of internal standard, the whole mixture was vortexed for 10 seconds. The tube was then left at room temperature for 5 minutes. 250µl of isopropyl alcohol was added to each tube, it was again vortexed for 10 seconds and centrifuged at 16,000g for 10 minutes at room temperature. Supernatant was discarded, pellet was washed by adding 400µl of 70% ethanol, vortexed for 10 seconds and centrifuged at 6000g for 10 minutes at room temperature. Supernatant was removed and air-dryed. The pellet was resuspended in 50µl of nuclease free water.

PCR was carried out using Geneproof kit for chlamydia trachomatis RT PCR. 10µl of extracted DNA was supposed to be added along with 20µl of mastermix to each smart cycler tube. Following pooling strategy of 3 samples per pool, 3µl of extracted DNA was taken from 3 different samples in one smart cycler tube and 20µl of mastermix was added.[11] For positive control, 10µl of positive control was added along with 20µl of mastermix. For negative control, 10µl of nuclease free water was added along with 20µl of mastermix. Smart cycler tubes were placed in the RT PCR machine and program was started.

The resulting values were qualitative in terms of either positive or negative for Chlamydia trachomatis. Any pool signaling positive was identified and all 3 samples of that pool were tested individually to find out the positive sample. 10µl of extracted DNA was added to smart cycler tube along with 20µl of mastermix. RT PCR was carried out again and positive samples were determined individually.

RESULTS

A total of Sixty-six urine specimen were included. Mean age of the patients was 31.15±8.17 years range from 21.00 to 70.00 years. 41(62.1%) individuals were male and 25(37.9%) were females. The female Mean Age was 31.61±9.50 ranging from 22.00-70.00 years and the mean male age was 30.40±5.42 ranging from 21.00-40.00 years. Majority patients were reported among males and females in age group 21-35 years as shown in Table-I.

Table-I: Demographic Characteristics of the Patients (n=66)

Age groups	Females n(%)	Males n (%)	Total
21–35 Years	32(78.0%)	20(80.0%)	52(78.8%)
36–50 Years	7(17.1%)	5(20.0%)	12(18.2%)
50–65 Years	1(2.4%)	0(0%)	1(1.5%)
>65 Years	1(2.4%)	0(0%)	1(1.5%)
Total	41(100.0%)	25(100.0%)	66(100.0%)

Out of 66 urine specimens, 05(7.6%) tested positive for Chlamydia trachomatis and 61(92.4%) gave negative results. When the urine specimens were pooled by 3, 22 pools were created and tested by RT PCR. 04 pools were PCR positive (Table-II).

Table-II: Results of RT PCR Conducted on Urine Samples (n=66)

	Total	Pool
Urine Specimen	66 (100%)	22
Positive samples	05 (7.57%)	04
Negative samples	61 (92.43%)	18

For the confirmation of results and finding individual positive sample, all samples in the positive pools were tested individually. 1 urine sample from 3 different pools and 2 urine samples from a 4th pool tested positive. Rest of the samples from these 4 pools

tested negative. Instead of performing 66 PCRs, we performed a total of 34 PCRs including the individual samples tested (Table-III). Keeping in mind reagent and kit costs, the total cost saving achieved was approximately 48.0% compared to individual testing.

Table-III: Test & Cost Saving Achieved as a Result of Pooling Strategy

Total samples (n)	**66**
PCRs conducted (on pools)	22
PCRs conducted on positive pools (4 pools with 3 samples each)	12
Totals PCRs with pooling	34
Total PCRs without pooling	66
No. of tests saved	32

DISCUSSION

In 2020, WHO estimated 374 million new infections with 1 of 4 sexually transmitted infections. About 129 million cases of Chlamydia trachomatis were reported. Other STIs including gonorrhea had 82 million cases, syphilis had 7.1 million and trichomoniasis had 156 million cases globally.[12] In 2021, a total of 1,644,416 cases of *Chlamydia trachomatis* infection were reported to the CDC, making it the most common notifiable sexually transmitted infection for that year. This case count corresponds to a rate of 495.5 cases per 100,000 population, an increase of 3.9% compared with the rate in 2020.[13]

The asymptomatic nature of chlamydia infection also accounts to it being most common yet remaining undiagnosed.[4] Chlamydia can lead to various urological and reproductive complications in females as well as males.[14] Owing to these morbidities associated with this organism, it is vital that it is timely diagnosed and treated so that these long term effects may be avoided.[15]

Use of NAAT in detection of infections caused by bacteria which are otherwise non-culturable or not very easily isolated has quiet revolutionized the diagnostic approaches towards these diseases.[15] Most of these tests require simple sample collection procedures which may include self-collected specimens, minimal sample transport requirements as well as effortless sample storage. Other testing techniques such as Indirect immunofluorescence, ELISA, Giemsa staining etc have also been employed by some institutes in diagnosing CT infection.[16]

In our study, we tested asymptomatic individuals reporting in gynecology and urology OPDs in order to estimate the general prevalence in our population. Keeping cost effectiveness and low economic resources of our setup, we ran Chlamydia trachomatis PCR on pooled samples making one pool containing three urine samples each.[17] Various studies have demonstrated pooling of urine samples for chlamydia trachomatis with multiple number of samples in one pool ranging from 2 samples to a maximum of 10 samples in one pool. Most studies have regarded a pool containing 2-3 samples each giving most accurate results.[11,17] Cost saving achieved as a result of pooling samples together has been used by many different setups in developed as well as developing countries.[18] This strategy has been known to save cost as well as easing mass public screening for STIs resulting public health benefits.[19]

When estimating cost saving, we estimated the pooling and non-pooling costs, based on reagents, lab equipment and lab technician time. We determined that our pooling strategy resulted in approximately 48% decrease in costs. The sensitivity of pooling is expected to be unaffected as long as the number of samples per pool is kept limited. This is extremely valuable in resource limited settings where there´s an increasing need to establish STI screening programs on mass level while keeping budget constrains in mind in a developing country with finite health resources.[20] However, as seen with the few positive cases of patients with barely any reportable clinical symptom, the need for devising and implementing mass screening programs is multiplied many folds.[21] Regular testing should be performed on sexually active individuals as well as at-risk communities regardless of their presence or absence of clinical findings.[22]

We did not find many studies utilizing pooling strategy as a cost-effective measure in our region. Studies conducted in Pakistan have reported a Chlamydia trachomatis prevalence of 12.25%. Another study has reported a prevalence of 4%.[8,16] However there is paucity of recent literature and studies available on this subject in our population. As mentioned in local as well regional studies and demonstrated by our study´s outcome as well, genital chlamydial infections are not very prevalent in our local population.

Although our study was carried out on fresh-catch urine samples, many studies have been carried out on other extragenital samples including throat swabs, semen etc.[19,23] These studies demonstrate that including extragenital samples along with genital

samples can result in a higher detection rate especially in asymptomatic individuals.[19] Since including more samples from various areas of the body can lead to an increased number of tests, pooling strategy can be employed in these situations as well to keep test costs limited. It is of interest to know the infected anatomical site as it can direct the course of treatment. For example, in the case of rectal chlamydia infections, doxycycline is the preferred treatment instead of azithromycin.[24] As an added advantage, knowledge of the infected site will also provide detailed epidemiological data.

As our test population consists of the general population without any specific risk of contracting STIs, this data may be extrapolated to a broader population. However, we would recommend more extensive sampling as well as including groups more exposed to the risk of STIs such as sex workers and people with multiple sexual contacts history. We limited our study on diagnosing genital Chlamydia trachomatis infections only, however, other STIs should also be kept in mind as in many cases multiple STIs can be encountered at the same time in a single patient.[1]

Many behavioral traits as well as case histories can indicate the patient to be possibly affected with an STI and while the patient may test negative for one of them, testing them by means of a panel of tests may catch a diagnosis which otherwise could have been missed.[23] Studies have demonstrated screening patients with panels of different STIs with a single diagnostic test.[25] Pooling strategy has also been employed by some of these studies resulting in cutting test costs while detecting multiple diseases.[4]

CONCLUSION

In conclusion, this is a unique study in its kind to explore pooling strategy in our setup as a technique to lower test costs without compromising on the quality of results. By reducing costs, more patient samples can be tested, resulting in public health benefits such as higher STI detection rates, interruption of transmission and prevention of long-term complications which otherwise are not possible in areas where resources are limited and tests costs are a big concern.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

NNC & IAM: Data acquisition, data analysis, critical review, approval of the final version to be published.

UK & AIM: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

SG & SHN: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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