RESEARCH ARTICLE

Cytological and molecular screening of *Chlamydia trachomatis* in infertile women attending a maternity teaching hospital in Gezira State, Sudan: a cross-sectional study [version 1; peer review: awaiting peer review]

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Abstract

**Background:** *Chlamydia trachomatis* (CT) is a sexually transmitted pathogen that threatens reproductive health worldwide. This study aims to screen CT urogenital infection using cytology and molecular methods in women suffering infertility.

**Methods:** In total, 415 women suffering infertility, attending Wad Madani Maternity Hospital were included in this study and then classified into two groups: primary infertile women and secondary infertile women. Both urine (n= 415) and vaginal swab samples (n= 130) were collected and tested using Giemsa stain and Polymerase Chain Reaction (PCR) for detection of CT.

**Results:** CT was detected in 33.7% (140/415) of urine samples and 73.1% (95/130) of vaginal swab samples using Giemsa stain, compared with 44.6% (185/415) and 84.6% (110/130) using PCR, respectively. In the primary infertile group (n= 265), chlamydia was detected in 35.8% (95/265) of urine and 75% (60/80) of swab samples by Giemsa stain compared with 50.9% (135/265) and 75% (60/80) of the samples by PCR. In the secondary infertile group (n= 150), chlamydia was detected in 30% (45/150) of urine and 70% (35/50) of swab samples by Giemsa stain compared with 33.3% (50/150) and 100% (50/50) of the samples by PCR. The associated risk factors were age, lower abdominal pain, and urethritis (p< 0.05). The sensitivity, specificity, positive predictive value, and negative predictive value of Giemsa stain in detecting chlamydia compared to PCR were 86.4%, 100%, 100%, and 83.6%, respectively.

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Any reports and responses or comments on the article can be found at the end of the article.
Conclusions: Giemsa stain can be used as a screening test for detection of urogenital chlamydia in urine and vaginal samples in places where PCR is difficult to be performed.

Keywords
Chlamydia trachomatis, Infertility, Cytology, Polymerase Chain Reaction, Screening test.

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Introduction

Chlamydia is the most common bacterial sexually transmitted infection (STIs), with more than 100 million new cases per year, and is caused by an intracellular Gram-negative bacterium named *Chlamydia trachomatis* (CT)\(^1\-^3\). CT infections of the lower female genital tract are frequently asymptomatic. However, if these infections do not resolve or persist untreated, the organisms can ascend to the upper genital tract, potentially causing salpingitis and functional damage to the fallopian tubes\(^4\). The World Health Organization (WHO) estimated that in 2012 there were 357.4 million new global cases of STIs, including CT (130.9 million cases) and *Neisseria gonorrhoeae* (78.3 million cases)\(^5\).

Improvements in screening tests hold promises for increasing screening rates and preventing consequences of silent untreated infections. Until recently, asymptomatic infection and the lack of a simple and sensitive screening test has been a barrier to the accurate detection of CT infection. The prevailing concept was that chlamydia tests were conducted using cervical swabs for women and urethral swabs for men, but, due to the greater sensitivity and specificity of molecular tests, urine for both sexes can be used as a less invasive sampling technique\(^6\). Due to the asymptomatic nature of many chlamydia infections, screening is recognized as the most effective approach for reducing the sequel of this disease. Thus, the development of alternative, low-cost, easy-to-use, point-of-care methods for the detection and simultaneous screening of CT infections in clinical settings in a useful time frame remains a critical strategy for improving reproductive genital tract health worldwide\(^1\).

Sudan is one of the largest countries in Africa and most of its population live in rural areas. This country faces manifold problems including; low socioeconomic status, poor transportation and education, and health problems. One of these health problems is infertility. In Gezira State, Sudan, CT infections are neglected infections and there is no local preventive screening program or exhaustive information about the prevalence of these infections. In addition, to the best of our knowledge, no previous studies have been carried out to determine the size of the problem. This prompted the following study to be performed, which focusses on urogenital CT infections among women suffering infertility, which may be indicator for higher risk for more serious sequela of sexually transmitted diseases.

Methods

**Study design and setting**

A cross-sectional hospital-based study was conducted from May 2017 to May 2018, to screen CT in urine and high vaginal swab samples from infertile women attending Wad Medani Maternity Hospital, Medani, Sudan.

Two permissions were granted to carry out this study; firstly from the Faculty of Medical Laboratory Sciences, University of Gezira, and secondly from the Ministry of Health, Gezira State. Written informed consent was obtained from all study subjects after they had been informed about the study objectives (for participants under the age of 18 years, consent was obtained from the husbands as per Sudanese policy).

**Participants and selection criteria**

In total 415 Sudanese, married women, suffering infertility were enrolled during the routine clinic of Wad Madani Maternity Hospital. The women were classified into two groups as follow; primary infertile women (n= 265), those failed to become pregnant for at least one year after marriage; and secondary infertile women (n= 150), those failed to become pregnant after a history of abortion and/or successful pregnancy/ies before. Women suffering vaginal bleeding were excluded from this study.

The study sample size (415) was calculated according to the following equation: 

\[
(n = \frac{z^2 \cdot p \cdot (1 - p)}{d^2})
\]

where \(n\) stands for number, \(z\) equal 1.96 at 95% level of confidence, \(p\) for estimated proportion of the population, and \(d\) for margin of error 0.05.

**Data collection**

A questionnaire (Extended data\(^7\) was designed and filled out before clinical sample collection in order to obtain participant’s demographic characteristics and relevant clinical symptoms.

Socioeconomic status was classified according to the participant’s income, or husband’s monthly income if the participant was not in employment as follows: high status, >30000 SDG per month; moderate, 30000–8000 SDG; low, <8000 SDG.

Education level was classed according to the last degree achieved by the participant as follows: high for undergraduate and post graduate degrees certificate; medium for secondary school degree certificate; and low for primary and pre-school level.

**Sampling**

Participants were instructed on the proper self-collection of the first-void urine (i.e., the initial 10–30 mL of voided urine) using a sterile plastic wide neck leak-proof cup and transported to the laboratory within two hours. Also, high vaginal swabs were taken using a Dacron swab (COPAN DIAGNOSTICS INC.) by a trained medical officer.

**Cytological technique.** The urine sample was centrifuged to obtain a pellet to make a smear slide. Another smear was performed from the swab sample by rolling the swab gently on a glass slide. All smears were fixed using alcohol and then stained using weak solution of Giemsa stain for a longer staining time. The presence of intracellular inclusions of chlamydia was tested microscopically.

**Molecular technique.** The DNA was extracted from urine and high vaginal swab samples using the manual chloroform phenol method, as described by Mohammed\(^1\). In brief, 500 µl of the sample was added to 500 µl of guanidine chloride, 5 µl proteinase K, and 150 µl ammonium acetate. The mixture was incubated overnight at 37°C, then boiled and cooled at room temperature. One milliliter (1 mL) of pre-chilled chloroform was added, vortexed, and centrifuged for 5 minutes at 3000 rpm. The upper layer was transferred to another tube, and 3 ml of cold absolute ethanol was added and kept at -20°C for 2 hrs. Then the tube was centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded, the tube dried, the pellet was re-suspended with...
70% ethanol, and centrifuged at 3000 rpm for 15 minutes. The supernatant was poured off again, the tube was dried and 200 µl of de-ionized water was used to re-suspend the last pellet. Finally, the tube was vortexed gently and stored at -20°C. The DNA purity was measured by spectrophotometer.

The desired conserved region of cryptic plasmid published DNA sequences for CT species was amplified by PCR using CT forward primer (5’-TagTAACTgCCACTTCATCA-3’) and CT reverse primer (5’-TTCCCCTTgTAATTCgTTgC-3’), to produce 201 bp length end product. For the PCR setups; 25 ml PCR reaction mixture containing 5 µl of extracted DNA, 0.5 µl of each primer, 5 µl of PCR premix (containing 10 Mm Tris, 50 Mm KCl, 0.01% gelatin, 200 µM deoxynucleoside triphosphate, and 2.5 mM MgCl₂), and 14 µl of sterile deionized water (Ready Master mix; MaximeTM PCR pre Mix Kit (i-Taq for 20 ul rxn), iNtRON). The first cycle was optimized to 5 min denaturation at 94°C, followed by 35 cycles each of 30s at 94°C, 45s at 55°C and 30s at 72°C with a final extension for 5 minutes at 72°C. PCR was run on GeneAmp PCR System 9700 version 3.1. DNA fragments were visualized in 1.5% agarose gels under UV light using gel documentation system (Ingenus, USA).

**Statistical analysis**

Data were analyzed using Statistical Package for the Social Science (SPSS) software version 20.0 (IBM, USA). Descriptive statistics including frequencies, mean, range, and standard deviation were calculated for all demographic, clinical, laboratory and other parameters. Specificity, sensitivity, positive and negative predictive values (PPV and NPV, respectively) were also calculated. Chi square test was used to determine the significance and associations. P<0.05 was considered significant.

**Results**

A total of 415 women were tested for CT in urine and high vaginal swab samples, their age range between 17 and 52 years. The study subjects divided into 265 (63.9%) women suffering primary infertility and 150 (36.1%) women suffering secondary infertility. Their mean age was 32.47±7.80 and 31.27±8.02 years, respectively. The majority of the women were between 31–40 years (43.4%) and 21–30 years (46.7%), and were housewives, resided in an urban city, with varying educational levels, and with moderate socioeconomic status (Table 1).

A total of 415 urine samples were collected from participants, and, due to some traditional/social beliefs and the painful nature of the swab sampling, only 130 vaginal swab samples were obtained (Table 2). Using Giemsa stain, 33.7% and 73.1% were positive for CT, compared with 44.6% and 84.6% positive using PCR (Table 3). In the primary infertile group (n=265), chlamydia was detected in 35.8% (95/265) of urine samples and 75% (60/80) of swab samples using Giemsa stain, compared with 50.9% (135/265) and 75% (60/80) of the samples by PCR. In the secondary infertile group (n=150), chlamydia was detected in 30% (40/150) of urine samples and 70% (50/50) of swab samples by cytology, compared with 33.3% (50/150) of urine samples and 100% of swab samples (50/50) by PCR (Table 4). The risk factors associated with genital chlamydia infection were
age, lower abdominal pain (PID), and urethritis (p<0.05; Table 5).

The sensitivity, specificity, PPV, and NPV of cytology in detecting chlamydia compared to PCR were 86.4%, 100%, 100%, and 83.6%, respectively (Table 6).

Discussion

Chlamydia is the most prevalent sexually transmitted bacterial infection worldwide, screening of the causative pathogen is important, not only to identify symptomatic individuals for the management of the infection and preventing the more serious sequelae caused by the organism, but also to identify asymptomatic individuals who serve as reservoirs for the disease.

Age is shown to be a risk factor for chlamydia infection among the sexually active population. In total, 29 of 34 studies in women have shown a significant relationship between age

| Table 5. Risk factors associated with Chlamydia infection in study subjects. |
|-----------------------------|----------------|------------|----------------|----------------|----------------|
| Age group, years            | Positive  | Negative  | Total       |     | P Value      |
| 0–20                        | 15 (8.1)  | 15 (6.5)  | 30 (7.2)    | 0.000 |
| 21–30                       | 50 (27.0) | 110 (47.8)| 160 (38.6)  |      |
| 31–40                       | 90 (48.6) | 75 (32.6) | 165 (39.8)  |      |
| 41–50                       | 30 (16.2) | 30 (13.0) | 60 (14.5)   |      |
| Total                       | 185 (100) | 230 (100) | 415 (100)   |      |
| Lower abdominal pain        | Yes       | 150 (81.1)| 160 (69.6)  | 310 (74.7)| 0.007 |
| No                          | 35 (18.9) | 70 (30.4) | 105 (25.3)  |      |
| Total                       | 185 (100) | 230 (100) | 415 (100)   |      |
| Burning in urination        | Yes       | 115 (62.2)| 155 (67.4)  | 270 (65.1)| 0.301 |
| No                          | 70 (37.8) | 75 (32.6) | 145 (34.9)  |      |
| Total                       | 185 (100) | 230 (100) | 415 (100)   |      |
| Vaginal discharge           | Yes       | 135 (73.0)| 150 (65.2)  | 285 (68.7)| 0.110 |
| No                          | 50 (27.0) | 80 (34.8) | 130 (31.3)  |      |
| Total                       | 185 (100) | 230 (100) | 415 (100)   |      |
| Urethritis                  | Yes       | 115 (62.2)| 85 (37.0)   | 200 (48.2)| 0.000 |
| No                          | 70 (37.8) | 145 (63.0)| 215 (51.8)  |      |
| Total                       | 185 (100) | 230 (100) | 415 (100)   |      |

| Table 6. Sensitivity, specificity, positive predictive value and negative predictive value of cytology using Giemsa. |
|---------------------------------------------------------------|----------------|----------------|----------------|---------------|---------------|
| Sample                          | PCR Positive | Total | Sensitivity | Specificity | PPV | NPP | P value |
| Giemsa (urine)                  | Yes          | 140   | 75.7%       | 100.0%      | 100% | 83.6% | 0.000   |
|                               | No           | 45    | 230         | 275          |      |      |         |
| Total                          | 185           | 230   | 415         |              |      |      |         |
| Giemsa (swab)                  | Yes          | 95    | 86.4%       | 100.0%      | 100% | 57.1% | 0.000   |
|                               | No           | 15    | 20          | 35           |      |      |         |
| Total                          | 110           | 20    | 130         |              |      |      |         |
and chlamydia infection\(^{10}\). This is supported by results reported by Kucinskiene et al.\(^{11}\) and Navarro et al.\(^{10}\), and other studies performed in Sudan\(^{1}\), Brazil\(^{12}\), China\(^{1}\), and Peru\(^{16}\). In a previous study, many women who tested positive for CT reported increased risk of PID, ectopic pregnancy, and infertility (adjusted hazard ratio was 2.36, 1.87, and 1.85 respectively)\(^{13}\). For the current study, 81.1% of women positive for chlamydia were suffering from PID.

This study attempted to use the Giemsa staining technique as a non-invasive sample to detect CT in order to be used as an approach for screening program in Gezira state, where PCR is difficult to be applied. In our study, participant’s swab samples tested by PCR showed that 84.6% of participants were positive, which is higher than that reported by Ortashi et al. (7.3%)\(^{16}\), Mohamed Elawad (49%)\(^{17}\), and Mohammed (22.5%)\(^{16}\).

Infertility is a medical problem that affects more than 80 million people worldwide\(^{18,19}\). Although few data exist on infertility in Sudan, 68.9% of Sudanese couples suffering primary infertility, due to various causes, was reported recently\(^{20}\). Some researchers reported the link between chlamydia and both primary and secondary infertility\(^{1,3,11}\). In this study, using PCR, the percentage of positive cases among primary infertile women was 75%, which is higher than that reported by Malik et al. (27%)\(^{21}\) and Gorini et al. (31.8%)\(^{22}\). In addition, in our study, all women with secondary infertility were found to be suffering from chlamydia infection by PCR, which is also higher than that reported by Malik et al. (30.6%)\(^{22}\). This might suggest the role of chlamydia in the infertility cases seen in our study.

Traditionally, detection and diagnosis of CT depends mainly on culturing cervical and urethral swab samples taken from women and men, respectively\(^{23}\). The culture technique is time consuming and requires well trained personnel and access to specialized facilities, which is not easy to be offered as a routine screening technique in Gezira State\(^{24}\). On the other hand, the sampling of both cervical and urethral swab have not been accepted by most patients due to the patients reported pain caused by the invasive nature of the spatula and the swab. In addition, there is resistance due to traditional beliefs (especially in communities that advocate circumcision in women). In order to overcome these drawbacks, we used urine samples in this study as a non-invasive sample to detect CT, which had been reported, evaluated and validated for use in most new techniques directed towards the detection of chlamydia trachomatis\(^{25-30}\).

Cytology was used as one of the non-cultural methods to detect intracellular inclusion bodies of CT\(^{31-34}\). This technique is especially useful and easy to perform and is accessible. Ultimately, this technique has established itself as a primary methods\(^{35}\). Giemsa staining is used and accepted by many authors to detect intracellular inclusion bodies of CT in cell lines used for culturing techniques\(^{36,35,36}\). In the current study, the Giemsa staining cytology reported high specificity and sensitivity when compared to PCR, which may suggest this technique as a reliable screening test for use in Gezira State.

**Conclusions**

From the results of this study, chlamydia may be one of the causes of infertility in women at Gezira State. The sensitivity and specificity of a cytology technique using Giemsa stain from a urine or vaginal sample can be used to screen urogenital chlamydia infection where PCR may be difficult to perform.

CT screening, especially for Sudanese women, is of the utmost importance and should be performed through local or national screening programs. Screening programs have been shown to significantly decrease CT prevalence in some regions of the United States and Sweden\(^{37,38}\), and subsequently the severe sequel of the infection, including genital damage and infertility.

**Data availability**

**Underlying data**

Figshare: Cytological and Molecular Screening of Chlamydia trachomatis in infertile women-Sudan.xlsx, https://doi.org/10.6084/m9.figshare.11871303.v2\(^{39}\).

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Extended data**

Figshare: Extended data. Cytological and molecular screening of Chlamydia trachomatis in infertile women- Sudan, https://doi.org/10.6084/m9.figshare.12307331.v2\(^{40}\).

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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**References**

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