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: 1 approved with reservations, 2 not approved]

Hajir Mohammed Hussien Omer ¹, Khalid Eltahir Khalid², Elhadi Ibrahim Miskeen³, Madiha Yousif Taha¹, Eylaf Yasir Saleh¹, Elhadi A. Ahmed ¹, Omaima Hassan Abdelwahid¹, Mohammed Abdelssalam Hassan⁴, Adam Dawoud Abakar⁵

¹Department of Medical Microbiology, University of Gezira, Wad Madani, Gezira State, 11111, Sudan

²Department of Basic Medical Sciences, University of Albaha, Albaha, Saudi Arabia

³Department of Obstetrics and Gynecology, University of Bisha, Bisha, Saudi Arabia

⁴Center for Biomedical Research, Huazhong University of Science & Technology, Wuhan, China

⁵Department of Medical Parasitology, University of Gezira, Wad Madani, Gezira state, 11111, Sudan

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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

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- Jayanti Mania-Pramanik**, Department of Infectious Diseases Biology, ICMR-National Institute for Research in Reproductive Health, Mumbai, India
- Phillip M. Giffard**, Charles Darwin University, Darwin, Australia
- Fernando Guerra** , Instituto Nacional de Perinatología, Mexico City, Mexico

value, and negative predictive value of Giemsa stain in detecting chlamydia compared to PCR were 86.4%, 100%, 100%, and 83.6%, respectively.

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.

Any reports and responses or comments on the article can be found at the end of the article.

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Corresponding author: Hajir Mohammed Hussien Omer (wajankamal@gmail.com)

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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 tubes⁴. 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 technique³. Due to the asymptomatic nature of many chlamydia infections, screening is recognized as the most effective approach for reducing the sequela 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 worldwide¹.

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: $n = (z)^2 p (1 - p) / d^2$, 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*⁶) 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 Mohammed⁷. 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'-TAGTAAGTgCCACTTCATCA-3') and CT reverse primer (5'-TTCCCCTTgTAATTCgTTgC-3'), to produce 201 bp length end product. For the PCR setups; 25 µl 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; Maxime™ 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. 2DNA fragments were visualized in 1.5% agarose gels under UV light using gel documentation system (Ingenuis, 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% (35/50) 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

Table 1. Demographic data of study subjects.

		Primary infertility N (%)	Secondary infertility N (%)
Age group, years	0–20	25 (9.4)	5 (3.3)
	21–30	90 (34)	70 (46.7)
	31–40	115 (43.4)	50 (33.3)
	41–50	35 (13.2)	25 (16.7)
	Total	265 (100)	150 (100)
Occupation	Employee	110 (41.5)	25 (16.7)
	Housewife	155 (58.5)	125 (83.3)
	Total	265 (100)	150 (100)
Residence	Urban	190 (71.7)	105 (70.0)
	Rural	75 (28.3)	45 (30.0)
	Total	265 (100)	150 (100)
Education level	High	100 (37.7)	40 (26.7)
	Medium	95 (35.8)	80 (53.3)
	Low	70 (26.4)	30 (20.0)
	Total	265 (100)	150 (100)
Socioeconomic status	High	10 (3.8)	0 (0)
	Moderate	180 (67.9)	115 (76.7)
	Low	75 (28.3)	35 (23.3)
	Total	265 (100)	150 (100)

Table 2. Types of specimens and techniques used in the study.

	Urine sample		High vaginal swab	
	Giemsa	PCR	Giemsa	PCR
Valid	415	415	130	130
Missed	0	0	285	285
Total	415	415	415	415

Table 3. Frequencies and percentages of positive and negative samples for *Chlamydia trachomatis* using Giemsa and PCR.

	Urine sample		High vaginal swab sample	
	Giemsa N (%)	PCR N (%)	Giemsa N (%)	PCR N (%)
Positive	140 (33.7)	185 (44.6)	95 (73.1)	110 (84.6)
Negative	275 (66.3)	230 (55.4)	35 (26.9)	20 (15.4)
Total	415 (100)	415 (100)	130 (100)	130 (100)

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^{2,7-9}. Screening of the causative pathogen is important, not only to identify symptomatic individuals for the management of the infection and preventing the more serious

sequela 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 4. Frequencies of *Chlamydia trachomatis* infection in infertile women using Giemsa and PCR.

Sample	Technique		Primary infertility Frequency (%)	Secondary infertility Frequency (%)
Urine	Giemsa stain	Positive	95 (35.8)	45 (30)
		Negative	170 (64.2)	105 (70)
		Total	265 (100)	150 (100)
	PCR	Positive	135 (50.9)	50 (33.3)
		Negative	130 (49.1)	100 (66.7)
		Total	265 (100)	150 (100)
High vaginal swab	Giemsa stain	Positive	60 (75)	35 (70)
		Negative	20 (25)	15 (30)
		Total	80 (100)	50 (100)
	PCR	Positive	60 (75)	50 (100)
		Negative	20 (25)	0 (0.0)
		Total	80 (100)	50 (100)

Table 5. Risk factors associated with Chlamydia infection in study subjects.

		Positive	PCR Negative	Total	P Value
Age group, years	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		Total	Sensitivity	Specificity	PPV	NPP	P value
		Positive	Negative						
Giemsa (urine)	Yes	140	0	140	75.7%	100.0%	100%	83.6%	0.000
	No	45	230	275					
	Total	185	230	415					
		PCR	Negative	Total	Sensitivity	Specificity	PPV	NPP	P value
		Positive	Negative						
Giemsa (swab)	Yes	95	0	95	86.4%	100.0%	100%	57.1%	0.000
	No	15	20	35					
	Total	110	20	130					

and chlamydia infection¹⁰. This is supported by results reported by Kucinskiene *et al.*¹¹ and Navarro *et al.*¹⁰, and other studies performed in Sudan⁷, Brazil¹², China¹³, and Peru¹⁴. 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)¹⁵. 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%)^{7,16}.

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 recently²⁰. Some researchers reported the link between chlamydia and both primary and secondary infertility^{4,9,21}. 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%)²² and Gorini *et al.* (31.8%)²¹. 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, respectively²³. 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 State²⁴. 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 methods³⁵. Giemsa staining is used and accepted by many authors to detect intracellular inclusion bodies of CT in cell lines used for culturing techniques^{30,35,36}. 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 sequelae 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>⁶.

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

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Reviewer Report 15 November 2022

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Fernando Guerra

Instituto Nacional de Perinatología, Mexico City, Mexico

Cytological and molecular screening of Chlamydia trachomatis in infertile women attending a maternity teaching hospital in Gezira State, Sudan: a cross-sectional study.

After reading this manuscript, I have some questions. The objective of the manuscript is confusing because the aim, according to the manuscript, is the screen CT urogenital infection using cytology and molecular methods in women who have infertility. If the intention is to evaluate the cytology method is necessary to change the title of the manuscript to Evaluation of Giemsa stain for screening of Chlamydia trachomatis in urine and vaginal samples from infertile women. Afterward, they concluded that Giemsa stain could be used as a screening test for detecting urogenital chlamydia in urine and vaginal samples where PCR is difficult to perform (Background).

Introduction section. This section does not describe the sensitivity and specificity of the Cytology method for screening Chlamydia infection, much less the still of Giemsa stain in Chlamydia detection. It is imperative to beef up their results with a good introduction and discussion.

Material and Methods section. In this section, there is no good description of the cytology assay and no cytological description for considered as positive urine or vaginal sample; there are no images of the cytology of these samples.

Results section. In this section, the patients are grouped into primary and secondary infertility. However, women up to 40 years old are considered infertile. If so, it is necessary that the material and methods section put the definition of infertility. I saw a better classification as women with and without lower abdominal pain. If you change the infertility classification, you must make a new statistical analysis.

Discussion section. There is no information about cytology methods in the discussion, such as an assay to screen Chlamydia infection. There are no described sensitivity and specificity of this test. There is no comment about of utility of Giemsa stain; there are no images of positive urine and vaginal samples. It is necessary to put these images. What experience has the technique to

identify Chlamydia positive urine smear? Is it straightforward to locate positive urine smears? What is the weakness of the study, and what are its strengths?

The authors must remember that the description of the methodology must be well described if someone wants to reproduce this methodology.

References

1. Woods GL, Walker DH: Detection of infection or infectious agents by use of cytologic and histologic stains. *Clin Microbiol Rev.* 1996; **9** (3): 382-404 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Sexual transmission diseases, Chlamydia, HPV, Gardnerella vaginalis, Mycoplasma spp.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 06 Dec 2022

Hajir Omer

Dear reviewer:

Please find enclosed the response to your comments.

With Regards,

The corresponding author: Hajir Omer

Author comments in italics

After reading this manuscript, I have some questions. The objective of the manuscript is confusing because the aim, according to the manuscript, is the screen CT urogenital infection using cytology and molecular methods in women who have infertility. If the intention is to evaluate the cytology method is necessary to change the title of the manuscript to Evaluation of Giemsa stain for screening of Chlamydia trachomatis in urine and vaginal samples from infertile women. Afterward, they concluded that Giemsa stain could be used as a screening test for detecting urogenital chlamydia in urine and vaginal samples where PCR is difficult to perform (Background).

Answer:

Yes, the aim of this study was to screen CT using Giemsa stain and molecular method. And this was achieved by the result of positive cases using urine and swab samples.

Further analysis was performed for the feasibility and of screening using Giemsa stain due to some reasons:

- 1. CT is neglected infection in the study area.*
- 2. Molecular detection was confirmed and well established but with cost and applicability restriction.*
- 3. Instead, Giemsa stain is simple, cheap and available in study area.*
- 4. The cytology was confirmed and accepted by many authors but, this is the first study conducted in Sudan and Gezira State, so the need to notify the gynecologists for the test feasibility to screen CT using both simple test and non-invasive sample*

Introduction section. This section does not describe the sensitivity and specificity of the Cytology method for screening Chlamydia infection, much less the still of Giemsa stain in Chlamydia detection. It is imperative to beef up their results with a good introduction and discussion.

The sensitivity and specificity of Giemsa stain was 38% and 97% respectively as described by Asadi and colleague in 2015.

(Asadi-Amoli F, Nozarian Z, Mehrtash V, et al.: Comparison of Direct Immunofluorescence and Giemsa Staining in Chlamydia trachomatis Follicular Conjunctivitis. Zahedan Journal of Research in Medical Sciences. 2015; 17(10).)

An extra description will be added to the new version of the introduction and discussion about Giemsa stain

Material and Methods section. In this section, there is no good description of the cytology assay and no cytological description for considered as positive urine or vaginal sample; there are no images of the cytology of these samples.

The following citation will be added to the references:

Omer, Hajir (2021): Giemsa stain for *C.trachomatis*. figshare. Figure.
<https://doi.org/10.6084/m9.figshare.14406413.v1>

Results section. In this section, the patients are grouped into primary and secondary infertility. However, women up to 40 years old are considered infertile. If so, it is necessary that the material and methods section put the definition of infertility. I saw a better classification as women with and without lower abdominal pain. If you change the infertility classification, you must make a new statistical analysis.

The infertility definition was mentioned in methodology section.

Discussion section. There is no information about cytology methods in the discussion, such as an assay to screen Chlamydia infection. There are no described sensitivity and specificity of this test. There is no comment about utility of Giemsa stain; there are no images of positive urine and vaginal samples. It is necessary to put these images. What experience has the technique to identify Chlamydia positive urine smear? Is it straightforward to locate positive urine smears? What is the weakness of the study, and what are its strengths?

The description of Giemsa stain will be mentioned in more details in methodology section.

The description of Giemsa sensitivity will be mentioned in discussion section.

The image (figshare) will be added in the references as follow:
Omer, Hajir (2021): Giemsa stain for *C.trachomatis*. figshare. Figure.
<https://doi.org/10.6084/m9.figshare.14406413.v1>

Thank you.

Competing Interests: No competing interest

Reviewer Report 11 November 2022

<https://doi.org/10.5256/f1000research.25923.r154077>

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Phillip M. Giffard

Global and Tropical Health Division, Menzies School of Health Research, Charles Darwin University, Darwin, Australia

The authors compared PCR and a cytology approach to screening infertile women for Chlamydia trachomatis.

The cytology was significantly less sensitive than the PCR, but apparently quite specific.

Although this study does not make use of modern concepts/genetic methods, it succeeded in answering a clear question, and has some translation potential. The finding was that there is a high prevalence of C trachomatis in infertile women, and that screening with a low cost cytology assay, while somewhat insensitive, has some value.

A problem with the study design is that there was no validation of the PCR method against a e.g. a fully validated molecular STI diagnostic platform. It would have been OK if such a validation had been done elsewhere/as part of another study. However, unless a critical reference has been omitted, this seems to be a new method, with an unknown performance in regard to a recognized gold standard such as a commercial diagnostics system. I guess total lack of false positive cytology assays lends some credibility to the assay.

Table 6 is difficult to understand, mainly because of using "Yes" and "no" to mean positive and negative for the cytology assays.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular microbiology, genetic analysis methods, bioinformatics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Nov 2022

Hajir Omer

Dear reviewer:
Please find enclosed the response to your comments.

With Regards,
The corresponding author: Hajir Omer

Author comments are underlined.

The authors compared PCR and a cytology approach to screening infertile women for Chlamydia trachomatis. (Yes)

The cytology was significantly less sensitive than the PCR, but apparently quite specific.

Although this study does not make use of modern concepts/genetic methods (the limited resources, cost, availability of PCR as a routine test for Chlamydia may influence the author to use the simple method in detection, as the reviewer noticed that one of the study objective was to find more simple and reliable screening test), it succeeded in answering a clear question, and has some translation potential. The finding was that there is a high prevalence of *C. trachomatis* in infertile women (and this was so important findings because genital *C. trachomatis* infection is neglected at study area), and that screening with a low cost cytology assay, while somewhat insensitive, has some value.

A problem with the study design is that there was no validation of the PCR method against a e.g. a fully validated molecular STI diagnostic platform. It would have been OK if such a validation had been done elsewhere/as part of another study. However, unless a critical reference has been omitted, this seems to be a new method, with an unknown performance in regard to a recognized gold standard such as a commercial diagnostics system. I guess total lack of false positive cytology assays lends some credibility to the assay. (the study depend on previous study conducted by Sudanese scientists ((Mohammed A. Molecular detection of Chlamydia trachomatis among gynecological patients attending Khartoum Teaching Hospital. Journal of Bacteriology Research. 2012;4(4):42-5.)) in the molecular method and on several studies approved the use of Giemsa and cytology to detect the inclusion bodies of CT ((such as: Asadi-Amoli F, Nozarian Z, Mehrtash V, Beheshtnejad H, Shabani A. Comparison of Direct Immunofluorescence and Giemsa Staining in *Chlamydia trachomatis* Follicular Conjunctivitis. Zahedan Journal of Research in Medical Sciences. 2015;17(10.)). Some modifications was introduced to suit the local used materials)

Table 6 is difficult to understand, mainly because of using "Yes" and "no" to mean positive and negative for the cytology assays. (Yes, and will be replaced by "positive" and "negative")

Competing Interests: no competing interest.

Reviewer Report 19 October 2020

<https://doi.org/10.5256/f1000research.25923.r71310>

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Jayanti Mania-Pramanik

Department of Infectious Diseases Biology, ICMR-National Institute for Research in Reproductive Health, Mumbai, Maharashtra, India

1. This a very old ref: ---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).
2. Any reference for the following?
The study sample size (415) was calculated according to the following equation: $(n = (z)^2 p (1 - p) / d^2)$, 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.
3. What is SDG? Write the full form.
4. Why vaginal samples have been collected? Endo Cervical samples are the best for testing CT.
5. For the PCR setups; 25 ml PCR?
Any reference for this PCR is followed?
6. There is no figure to suggest that Giemsa staining can identify CT.
7. There is also no figure on PCR results.
8. Both Giemsa staining and PCR used seems to be showing false positives, may be due to contaminations.
9. Even for interpretation of Giemsa staining and PCR for CT diagnosis, very trained personnel and technical experts are required.
10. There were no control groups used in the study.
11. In-house PCR should have been compared with a commercially available CT diagnostic kit to evaluate its sensitivity and specificity.
12. What is the prevalence of CT in fertile women?
13. Infertility may be due to several factors, these are not evaluated in these women.
14. What is the infection rate among the spouses of these women
15. There many tables, which could have been represented only in 2 tables

16. Evaluation of the methods used for the study needs to be verified, before its application in this study population.
17. What other infections are detected in Giemsa staining?
18. How the authors have interpreted that the urine samples are adequate and useful for PCR or Giemsa? What denominator was used to test the quality of urine samples for testing?
19. Any treatment was given to these women and what were the outcomes?

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunodiagnostics of sexually transmitted infections , Immunogenetics and Immunology of related to Gynecological complications and cancers

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 13 Apr 2021

Hajir Omer

Dear reviewer:

Please find enclosed the response to your comments.

With Regards,

The corresponding author: Hajir Omer

1. This a very old ref: ---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).

This estimation was cited to a reference published in 2018 Hascoet JL, Dahoun M, Cohen M, *et al.*: Clinical diagnostic and therapeutic aspects of 221 consecutive anorectal *Chlamydia trachomatis* and *Neisseria gonorrhoeae* sexually transmitted infections among men who have sex with men. *Int J Infect Dis.* 2018; **71**: 9–13. [PubMed Abstract](#) | [Publisher Full Text](#)

2. Any reference for the following?

The study sample size (415) was calculated according to the following equation: $(n = (z)^2 p (1 - p) / d^2)$, 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.

Jaykaran Charan and Tamoghna Biswas (2013), How to Calculate Sample Size for Different Study Designs in Medical Research? . *Indian J Psychol Med.* 2013 Apr-Jun; 35(2): 121–126. doi: [10.4103/0253-7176.116232](#)

3. What is SDG? Write the full form.

SDG will be replaced by SDP which used to abbreviate the Sudanese Pounds, and will be corrected in the text (highlighted by the pink color).

4. Why vaginal samples have been collected? Endo Cervical samples are the best for testing CT.

The study aimed to use more easier and also simple sample such as vaginal swab samples and urine samples rather than the endo-cervical sample which is usually not accepted by most of Sudanese women due to some traditional/social beliefs.

5. For the PCR setups; 25 ml PCR?

Any reference for this PCR is followed?

This was a typing mistake and corrected to 25ml (highlighted by the pink color).

6. There is no figure to suggest that Giemsa staining can identify CT.

[10.6084/m9.figshare.14406413](#)

7. There is also no figure on PCR results.

[10.6084/m9.figshare.14406494](#)

8. Both Giemsa staining and PCR used seems to be showing false positives, may be due to contaminations.

No false positive results in the study. The PCR was performed by an expert under qualified and accurate conditions to avoid carryover and contamination.

The false negative results showed by Giemsa staining method may be due to the low shedding of the epithelial cells in both urine and vaginal swab samples (low number of epithelial cells 'the cells which carry the inclusion bodies of chlamydia' in the sample).

9. Even for interpretation of Giemsa staining and PCR for CT diagnosis, very trained

personnel and technical experts are required.

The authors are experts in the field of: *Microbiology (Hajir Omer and Alhadi Abdalla)
*Molecularbiology (Khalid Eltahir and Hajir Omer) and
*Cytology (Mohammed Abdelsalam)

10. There was no control groups used in the study.

The study designed as cross sectional study aimed to detect the feasibility to use both technique and sample for screening, so, no need to use control group.

11. In-house PCR should have been compared with a commercially available CT diagnostic kit to evaluate its sensitivity and specificity.

CT is one of the neglected infections in Gezira state, so absence of screening test prompted us to establish the use of cytology and PCR as screening test. Then, research will be followed further to include other objectives and evaluations.

12. What is the prevalence of CT in fertile women?

Using urine sample and PCR, CT was detected in 44.6% of the infertile women.in pilot study conducted before this one, including pregnant women, CT was detected in 9.2% of them. Lack of symptoms makes clinical diagnosis of chlamydial infection difficult. Screening of infertile women for CT is therefore recommended so far early therapeutic interventions.

13. Infertility may be due to several factors, these are not evaluated in these women.

The study subjects were selected in routine clinic after being evaluated and suggested for chlamydia infection on the basis of clinical symptoms.

14. What is the infection rate among the spouses of these women

Not included and not tested in this study.

15. There many tables, which could have been represented only in 2 tables

The tables were presented in this way to be more clear that the study included different techniques and different samples in addition to the table of sensitivity and specificity which need to be presented separately.

16. Evaluation of the methods used for the study needs to be verified, before its application in this study population.

(A): The methods used in this study were evaluated and verified by many authors 1.PCR was used by different authors to detect CT.

***Ortashi OM, El Khidir I, Herieka E.** Prevalence of HIV, syphilis, Chlamydia trachomatis, Neisseria gonorrhoea, Trichomonas vaginalis and candidiasis among pregnant women attending an antenatal clinic in Khartoum, Sudan. Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology. 2004;24(5):513-5.

***Mohammed A.** Molecular detection of Chlamydia trachomatis among gynecological patients attending Khartoum Teaching Hospital. Journal of Bacteriology Research. 2012;4(4):42-5.

(B): In order to overcome the samples' drawbacks, the urine (as non-invasive sample) had been reported, evaluated and validated by different authors for use in most new techniques directed towards the detection of Chlamydia trachomatis.

- *Paul ID**, Caul EO. Evaluation of three Chlamydia trachomatis immunoassays with an unbiased, noninvasive clinical sample. *Journal of clinical microbiology*. 1990;28(2):220-2.
- *M Palmer H**, B Gilroy C, J Thomas B, E Hay P, Gilchrist C, Taylor-Robinson D. Detection of Chlamydia trachomatis by the polymerase chain reaction in swabs and urine from men with non-gonococcal urethritis 1991. 321-5 p.
- *Jensen IP**, Fogh H, Prag J. Diagnosis of Chlamydia trachomatis infections in a sexually transmitted disease clinic: evaluation of a urine sample tested by enzyme immunoassay and polymerase chain reaction in comparison with a cervical and/or a urethral swab tested by culture and polymerase chain reaction. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. 2003;9(3):194-201.
- *Haugland S**, Thune T, Fosse B, Wentzel-Larsen T, Hjelmevoll SO, Myrmel H. Comparing urine samples and cervical swabs for Chlamydia testing in a female population by means of Strand Displacement Assay (SDA). *BMC Women's Health*. 2010;10(1):9.
- *Mouton J**, Verkooyen R, Meijden W, Rijsoort-Vos T, Goessens W, Kluytmans J, et al. Detection of Chlamydia trachomatis in male and female urine specimens by using the amplified Chlamydia trachomatis test. *Journal of clinical microbiology*. 1997;35:1369-72.
- (C):** Cytology was used as one of the non-cultural methods to detect intracellular inclusion bodies of CT in different studies
- *Szarewski A**, Pompey A, Bertrand J, Bradbeer C. Use of the Cytobrush for concurrent endocervical cytology and chlamydia sampling. *Genitourinary medicine*. 1990;66(3):205-7.
- *Vinette-Leduc D**, Yazdi HM, Jessamine P, Peeling RW. Reliability of cytology to detect chlamydial infection in asymptomatic women. *Diagnostic cytopathology*. 1997;17(4):258-61.
- *Levi AW**, Beckman D, Hui P, Schofield K, Harigopal M, Chhieng DC. Comparing Two Methods of Detection for Chlamydia trachomatis in Liquid-Based Papanicolaou Tests. *American journal of clinical pathology*. 2012;138(2):236-40.
- (D):** Giemsa staining cytology was found used and accepted by many authors to detect intracellular inclusion bodies of CT in cell line used for culture technique.
- *Koumans EH**, Black CM, Markowitz LE, Unger E, Pierce A, Sawyer MK, et al. Comparison of Methods for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Using Commercially Available Nucleic Acid Amplification Tests and a Liquid Pap Smear Medium. *Journal of clinical microbiology*. 2003;41(4):1507-11.
- *Asadi-Amoli F**, Nozarian Z, Mehrtash V, Beheshtnejad H, Shabani A. Comparison of Direct Immunofluorescence and Giemsa Staining in Chlamydia trachomatis Follicular Conjunctivitis. *Zahedan Journal of Research in Medical Sciences*. 2015;17(10).
- *Cornetta MdCdM**, Gonçalves AKdS, Bertini AM. Efficacy of cytology for the diagnosis of Chlamydia trachomatis in pregnant women. *Brazilian Journal of Infectious Diseases*. 2006;10:337-40.

17. What other infections are detected in Giemsa staining?

No others were detected, the cases already were selected in the basis of testing profile in the clinic.

18. How the authors have interpreted that the urine samples are adequate and useful for PCR or Giemsa? What denominator was used to test the quality of urine samples for testing?

1. Urine sample was approved to be useful in screening most of infectious agents using

PCR.

2. Urine sample was used widely in detection most of microorganisms using cytological techniques.

3. The positive predictive value was 100% and the negative predictive value was 83.6% using the urine sample, and the positive predictive value was 100% and the negative predictive value was 57.1% using the swab sample (table 6).

19. Any treatment was given to these women and what were the outcomes?

Azithromycin 1 g daily for 7 days was administered to positive cases and they were suggested to be followed by the doctor.

Competing Interests: no competing interests I am the corresponding author: Hajir Omer

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