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ORIGINAL ARTICLE

Detection of *Chlamydia trachomatis* in Women Experiencing Miscarriage and Molecular Insights in AL-Anbar Region

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Key words:
Miscarriages, Chlamydia trachomatis, genes identification, vaginal, bacteria

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ABSTRACT

Background*: Chlamydia trachomatis is the primary cause of many sexually transmitted diseases. This infection is widespread globally and can last a long time, traveling upward through the genital system. This quiet infection within cells is linked to harmful effects on pregnancy, such as the risk of miscarriage.* ***Objective****: Evaluation of gene expression of Chlamydia trachomatous among women who experienced miscarriage and seronegative for the bacteria.* ***Methodology:*** *The research was conducted at several hospitals and laboratories between December 2023 and August 2024. 55 patients and 110 controls who had undergone previous legal abortion due to intrauterine death were included in the study. Vaginal swabs were taken from each case and analyzed using Chlamydia Antigen ELISA technique, and PCR for Chlamydia trachomatis.* ***Results:*** *All patients were seronegative for Chlamydia trachomatis. The successful amplification of the Chlamydia trachomatis gene through multiplex conventional PCR was considered a favorable result for PCR Chlamydia trachomatis. Among the 55 DNA samples taken from vaginal samples, zero was positive for OMP A, 30 (54.55%) were positive for CPAF gene, and 33 (60%) were positive for TOX gene, 25 (45%) were positive for Tarp gene indicating the presence of Chlamydia trachomatis p-value < 0.001* ***Conclusion:*** *Polymerase chain reaction has proven to be a more effective and efficient method for diagnosing Chlamydia using nucleic acid amplification tests. Our findings suggest a connection between Chlamydia trachomatis infection and miscarriage, underlining the importance of early detection and treatment of this infection in pregnant individuals to potentially prevent miscarriages.*

INTRODUCTION

Recurrent spontaneous abortion, known as RSA, is a frequent issue during pregnancy, impacting 2-5% of individuals. The unknown causes make it difficult to manage and treat many patients effectively. Typical reasons for RSA consist of chromosomal defects, hormonal and metabolic issues, autoimmune diseases, and infections [1] More than 80% of miscarriages happen in the first 3 months [2].

Chlamydia trachomatis is a germ that spreads through sexual contact and has two distinct stages in its life cycle. The elementary body can cause infection, while the reticulate body is active in its metabolism but does not spread infection [3]. Numerous investigations have linked *Chlamydia trachomatis* to pregnancy problems, such as recurrent spontaneous abortion (RSA), in serum, endometrial tissue, and urine, nevertheless, the pathogen's mechanism of abortion-inducing activity is still being actively investigated [4].

The female genital tract's columnar epithelial cells are the target of *Chlamydia trachomatis* and involve the barrier of mucosa consists of epithelial cells, mucus, and an immune response that is innate, humoral, and cell mediated[5]. When *Chlamydia trachomatis* infects women, it can lead to chorioamnionitis, which can result in an abortion or an unviable fetus. Particularly, infections have been connected to 66% of late births and 15% of early births [6].

Chlamydia trachomatis is a pathogen that lives inside cells and has a unique two part development cycle. In this cycle, the bacteria can be in the form of infectious elementary bodies (EBs) or as active reticular bodies (RBs) that are metabolically active [7].

Time expression *Chlamydia trachomatis* corresponds to the stages of the Chlamydial development cycle [8]. *Chlamydia* protease-like activity factor (CPAF; CT858), translocated actin-recruiting phosphoprotein (Tarp, CT456), and chlamydial cytotoxin (CT166) are some of the most well-researched chlamydial effector proteins. The Chlamydial type lll secretion system transports the tarp into the host cell, where host kinases phosphorylate its tyrosine [9].

The tarp gene participates in signaling processes that attract actin, causing the EB to internalize and support Chlamydia's intracellular survival [3].A strong type ll-secreted protease called the CPAF gene was found in the cytoplasm of the host cell [10]. Although many of the effects of CPAF that have been previously reported have been questioned [11]. CPAF appears to have a role in

the remove of EBs[12]. The Chlamydial cytotoxin causes the host cell's actin to depolymerize by glycosylating Rho GTPase Rac1[13].

METHODOLOGY

Study setting:

An analytical cross-sectional study was conducted at AL-Ramadi Teaching Hospital for Maternity and Children, Fallujah Teaching Hospital, and two private Medical Laboratories from December 2023 till August 2024 on a total of 55 women and 110 controls who were legally allowed for abortion because of intrauterine death.

Inclusion Criteria:

The study included all cases of medically induced abortion due to intrauterine death of unknown cause.

Exclusion Criteria:

All cases with eclampsia gravidarum, diabetic mothers or those with any history of chronic disease that can lead to intrauterine death of the fetus were excluded. Any case related to chromosomal abnormality, and congenital abnormality were also excluded from this study.

Sample collection:

The patients in the case group were selected right after being diagnosed with missed abortion. The reference group participants provided samples at the time they chose to end their pregnancy. Each participant underwent a vaginal discharge examination, and samples were taken by a gynecologist after their basic medical history was reviewed. To gather enough vaginal secretion, one sterile vaginal swab was turned three times in the same area of the posterior vaginal fornix.

Once collected, the sample was placed in clean test tubes containing a preservation solution from Sangon Biotech, Co. Ltd. , in Shanghai, China. These tubes were held vertically for ten minutes at room temperature and then stored at -80 Celsius until testing. Swabs were tested using PCR for Chlamydia trachomatis. Serum samples were obtained in sterile containers and analyzed for anti Chlamydia trachomatis IgG using an ELISA kit.

Molecular method

DNA extraction:

The entire genetic material of the bacterial genome was separated via Genomic DNA extraction Kit. The company Geneaid offers a quick protocol for following. DNA quality was assessed following the manufacturer's guidelines. Confirmed through electrophoresis in a 1.5% agarose gel. A UV transilluminator was used for observing. The musical groups. The Nanodrop spectrophotometer was used to measure Gel Documentation and DNA concentration. Following the extraction of DNA, it was stored at a temperature of -20 degrees Celsius for additional purposes research.

PCR test:

Four specific primers were designed for *Chlamydia trachomatis* genome. The primer sequences were show in table (1):

Table 1: Sequence of forward and reverse primer 5-3

Gene	Sequence of forward and reverse (Primer 5 - 3)	Product(pb)
CPAF	F 5-ATGGTTCCGCATTTTTGGGC-3	339 bp
	R 5-ACCACCTGGGTTGTTCGTTT-	
TOX	F- 5-GCCTCCGGATGGTATCGAAG-3	668bp
	R 5-CTGTTGCGGCTGCATAGTTC-3	
Tarp Ct	F 5-GGATGATGTTGGGGGCTTGA-3	384bp
	R 5-GTGCCGGGAATTGCGTTATC-3	
OMPA	F 5-TTTGCCGCTTTGAGTTCTGC-3	584bp
	R 5-CCACATTCCCACAAAGCTGC-3	

and the length of PCR target. PCR reaction was done in a total volume of 25ml PCR master mix.

PCR amplification program:

Starting with an initial denaturation step at 94°C lasting 5 minutes, then continuing with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 min Carp gen,59C for 45 seconds Tarp Ct gene, 56C for 30 mint Tox gen and extension at 72°C for 1 min; finally, a final extension at 72°C for 7 minutes.

Serological assays:

Serum specimens were analyzed using MOMP based ELISA kits (Euroimmun, Germany) to identify anti CT IgA and IgG antibodies. Each procedure was carried out following the guidelines provided by the manufacturer. The IgA kit utilized in this study demonstrated 100% sensitivity and 97. 4% specificity, while the IgG kit showed 78. 2% sensitivity and 97. 1% specificity. The

kits did not show cross reactivity with samples that tested positive for other Chlamydia pneumoniae.

Statistical analysis:

The information was inputted into SPSS software version 20 for analysis, assuming an effect size of 0.5, chi-square test was utilized to compare quantitative and qualitative variables, with a significance level of $p<0.05$, and a statistical power of 0.80. The analysis indicated that a minimum of 55 participants was required.

Ethical approval

This study was granted approval by the Medical Ethics Committee 2024/02/22 at the University of AL-Anbar Governorate in Ramadi, Iraq. Parents also provided signed informed consent for the research**.**

RESULTS

Table (2) PCR *Chlamydia trachomatis*: successful amplification of the *Chlamydia trachomatis* gene via multiplex conventional PCR was deemed a positive outcome. Among the 55 DNA samples taken from vaginal samples, zero was positive for OMP A gene, 30 (54.55%) were positive for CPAF gene, and 33 (60%) were positive for TOX gene, 25(45%) were positive for Tarp gene indicating the presence of *Chlamydia trachomatis*. Most of the cases were recorded in the older age group between 16 and 44 years (figure 1). A control group of 110 non-pregnant women who are in good health was included in the study, with ages ranging from 16 – 42 years. (Figure 2) The link between age and miscarriage peaks between the ages of 20-27, with the highest risk occurring in the final trimester of pregnancy, but no direct association was found between the two factors. In our study, we discovered that *Chlamydia trachomatis* was most commonly found in females between the ages of 20 and 27, and we also observed a notable association between Chlamydia and miscarriage. All patients were seronegative for ELISA *Chlamydia trachomatis*.

Table 2: *Chlamydia trachomatis* identification by TOX, CPAF, TARP CT genes:

		Chlamydia		Control		Chi-Square	Exact p-value
		N	%	N	%		
CPAF	**Positive**	30	54.5%	0	0.0%	73.33	0.001*
	Negative	25	45.5%	110	100.0%		
Total		55	100.0%	110	100.0%		
Tox	**Positive**	33	60.0%	0	0.0%	82.50	0.001*
	Negative	22	40.0%	110	100.0%		
Total		55	100.0%	110	100.0%		
Tarp Ct	**Positive**	25	45.5%	0	0.0%	58.93	0.001*
	Negative	30	54.5%	110	100.0%		
Total		55	100.0%	110	100.0%		

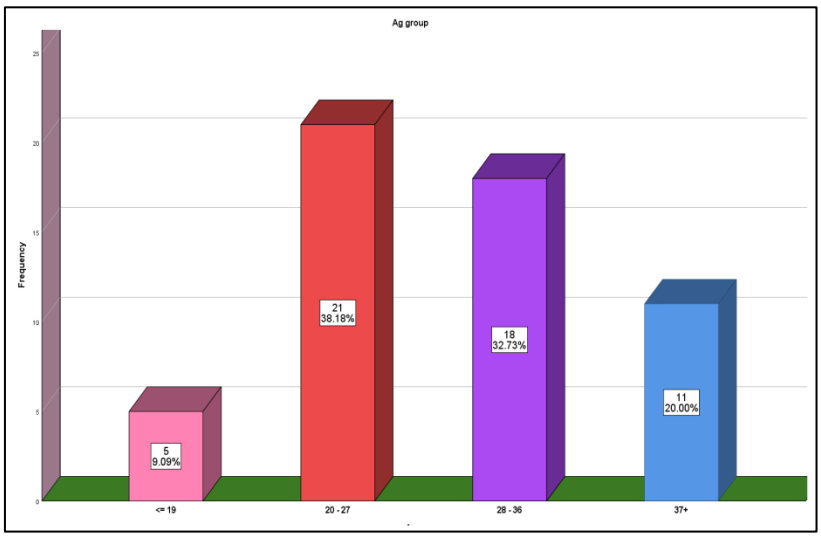

Fig. 1: Age of abortion

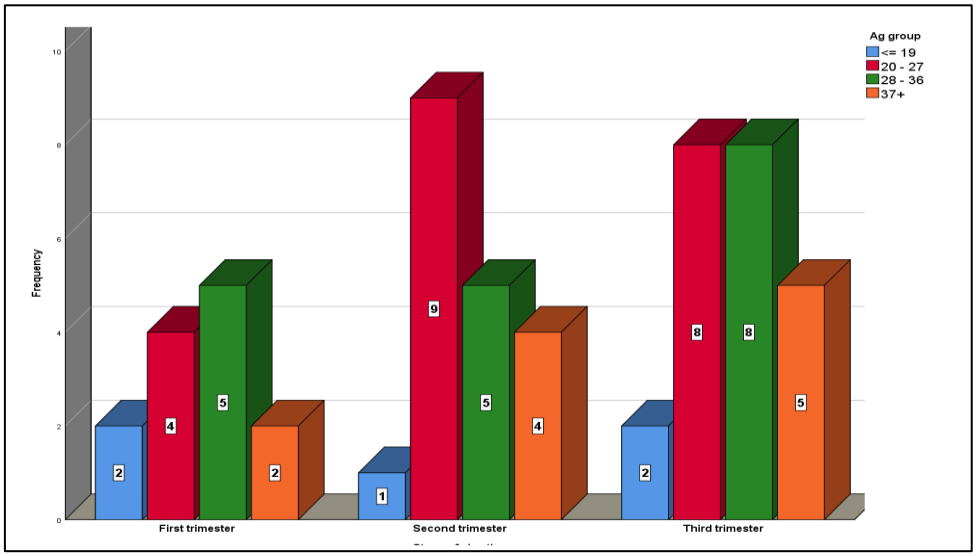

Fig. 2: Frequency of abortion according to the trimester and age

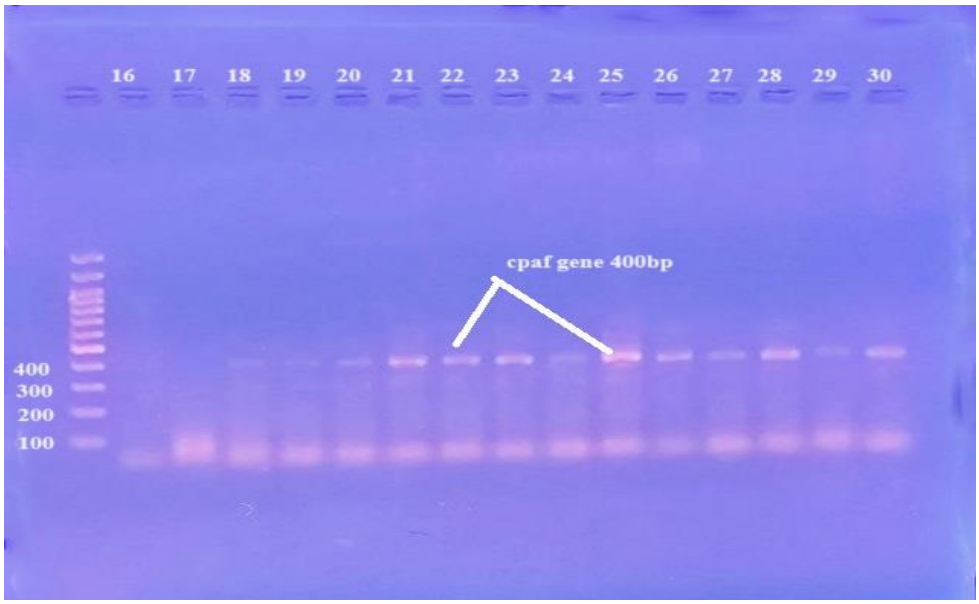

Fig. 3: Agarose gel for genotypes of polymerase-producing bacteria bp) 400 (using the starter pair CPAF (for bacteria isolates) (M = molecular parameter bp 100-700). Lane M: Contains a 100 base pair ladder for size reference. Lanes 18 to 30: Represent positive samples for *Chlamydia trachomatis*, showing PCR amplification products, likely confirming the presence of the target genes CPAF. The PCR products from these lanes should appear at the expected sizes corresponding to the amplified regions of interest. Lanes 16 and 17: Contain negative samples, where no amplification should be visible if no *Chlamydia trachomatis* DNA was present.To properly interpret the gel, you'd compare the PCR product sizes to the ladder in Lane M. The presence or absence of bands at specific locations will confirm the identity of the samples as positive or negative for Chlamydia.

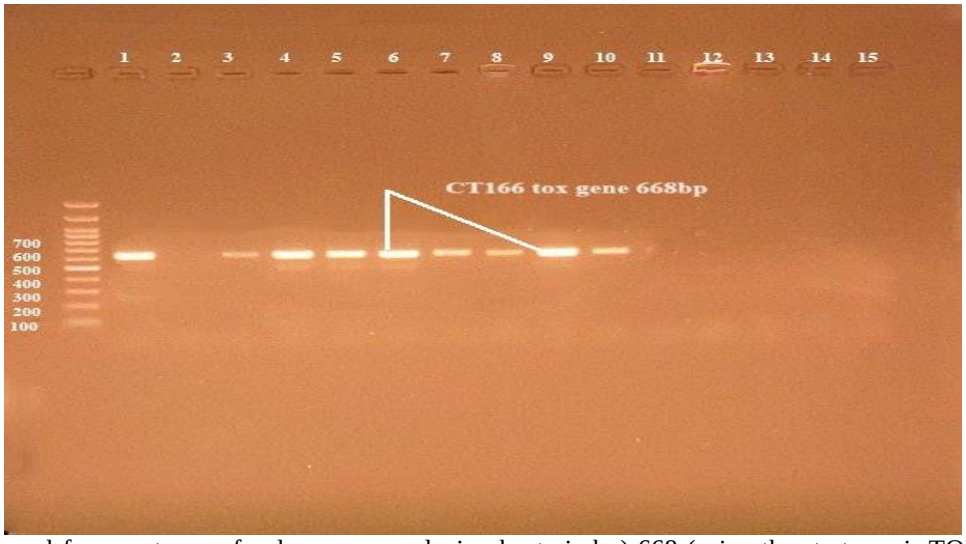

Fig. 4: Agarose gel for genotypes of polymerase-producing bacteria bp) 668 (using the starter pair TOX (for bacteria isolates) (M = molecular parameter bp 100-700). Lane M: Contains a 100- 700 base pair ladder for size reference. Lanes 1,3,4,5,6,7,8,9 and 10: Represent positive samples for *Chlamydia trachomatis*, showing PCR amplification products, likely confirming the presence of the target genes Tox. The PCR products from these lanes should appear at the expected sizes corresponding to the amplified regions of interest. Lanes 2,11,12,13,14 and 15: Contain negative samples, where no amplification should be visible if no *Chlamydia trachomatis* DNA was present. To properly interpret the gel, you'd compare the PCR product sizes to the ladder in Lane M. The presence or absence of bands at specific locations will confirm the identity of the samples as positive or negative for Chlamydia.

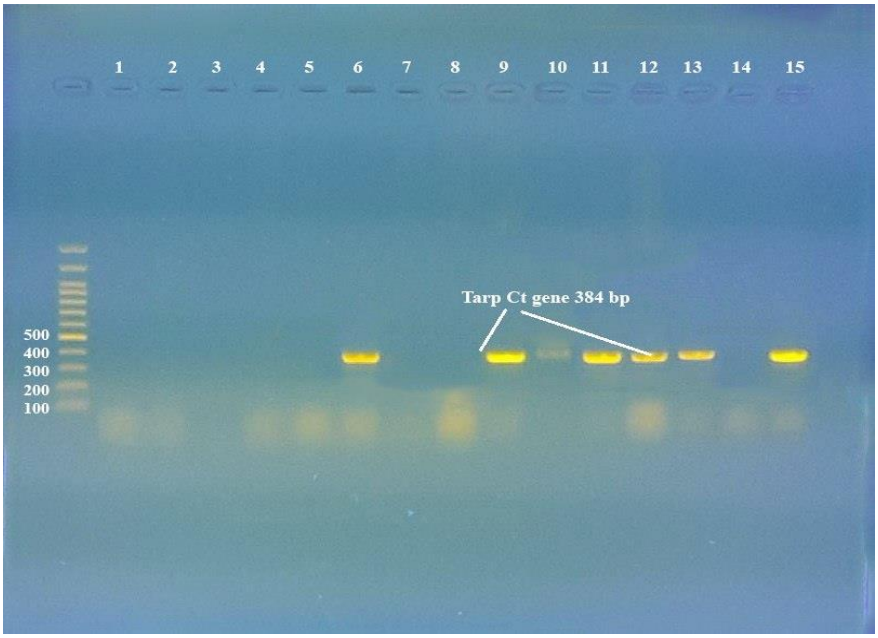

Fig. 5: Agarose gel for genotypes of polymerase-producing bacteria bp) 384 (using the starter pair Tarp (for bacteria isolates) (M = molecular parameter bp 100-700). Lane M: Contains a 100-700 base pair ladder for size reference. Lanes 6,9,10,11,12,13 and 15: Represent positive samples for *Chlamydia trachomatis*, showing PCR amplification products, likely confirming the presence of the target genes tarp. The PCR products from these lanes should appear at the expected sizes corresponding to the amplified regions of interest. Lanes 1,2,3,4,5,7,8 and 14: Contain negative samples, where no amplification should be visible if no *Chlamydia trachomatis* DNA was present. To properly interpret the gel, you'd compare the PCR product sizes to the ladder in Lane M. The presence or absence of bands at specific locations will confirm the identity of the samples as positive or negative for Chlamydia.

DISCUSSION

In our study, *Chlamydia trachomatis* DNA was detected in 54.55% (CPAF), 60% (tox), and 45.5% (tarp) of vaginal samples using multiplex conventional PCR, confirming the presence of the bacteria. However, no sample tested positive for the OMPA gene, which is commonly used for *Chlamydia trachomatis* detection and typing. The absence of OMPA about strain variation, potential gene deletions, or alternative genetic adaptations that allow these strains to evade detection by conventional PCR assays. Some *Chlamydia trachomatis* strains undergo genomic rearrangements or mutations affecting OMPA, potentially altering pathogenicity and immune evasion. The complete absence of OMPA in our study suggests that these strains may belong to an atypical genetic variant, requiring further investigation through whole-genome sequencing (WGS) or comparative genomic analyses.

A significant association was found between *Chlamydia trachomatis* infection and miscarriage ($p = 0.001$) as shown in (Table-2). *Chlamydia trachomatis* infection is often a hidden illness, leading many women to be asymptomatic and unaware that they are infected. Consequently, women with *Chlamydia trachomatis* may face the risk of abortion and related complications. The PCR test is effective in identifying Chlamydia trachomatis infections in females, as it is more sensitive, specific, affordable, and faster compared to standard microbiological methods used to examine infections in the genital area.

In our study, the relationship between Chlamydia infection and abortion was significant p-value 0.001. *Chlamydia trachomatis* is a common cause of sexually transmitted diseases that can have a significant impact on public health. It is crucial to have proper and sensitive diagnostic methods in place for effective epidemiological control [14]. [A] study in Baghdad found that Chlamydia trachomatis might be linked to spontaneous abortion, with 6.4% of women who had abortions testing positive for its antibodies. This underscores the need for accurate diagnosis and monitoring to prevent reproductive complications [15]. Research in Sanandaj, Iran, found that women with spontaneous abortions had a higher prevalence of *Chlamydia trachomatis* (22.9%) compared to those with normal pregnancies (11.9%). This suggests a significant association between the infection and spontaneous abortions. The study advocates for screening and treatment of Chlamydia during pregnancy to potentially reduce miscarriage risks.[16]

The infection was most common in women aged 20–27 years, indicating that recurrent miscarriage is a distinct clinical entity compared to sporadic miscarriage. This distinction is supported by the observation that recurrent miscarriages often occur with chromosomally normal fetuses. Despite rapid advances in genetic knowledge of diseases, progress in understanding the genetic causes of female recurrent miscarriage has been slower, aligning with studies that found a high occurrence of *Chlamydia trachomatis* infection in sexually active young adults. [17] Previous study suggested that age played a significant role in the risk of missed abortions due to the decline in ovarian function and the corpus luteum with advancing age. However, other studies indicated that age did not greatly impact the occurrence of spontaneous abortions [18]. Thi Qar's previous study by Fatima et al[19]. shows that most of the samples aged 20 to 30 years with missed abortions were for unknown reasons. This age group also showed the highest rate of miscarriage, particularly in the second trimester, raising concerns about the potential role of *Chlamydia trachomatis* in late pregnancy complications. While some studies suggest that maternal age influences miscarriage risk due to declining ovarian function [20].

Our data indicate that *Chlamydia trachomatis* may act as an independent risk factor for pregnancy loss, particularly in younger women. This aligns with research from Iran and Iraq showing that *Chlamydia trachomatis* infections are more common in reproductive-age women and can contribute to adverse pregnancy outcomes. Anwar et al[21]. in Baghdad conducted previous studies focusing on individuals aged 16 to 45 and reported that a woman's age did not affect the likelihood of having an abortion. The latest findings contradicted the previous research by Ahmadi et al.[22] which investigated the relationship between genital infection caused by *Chlamydia trachomatis* and miscarriage. Then results showed that most women experiencing spontaneous abortion were in the age range of 35-39 years. A study in Basra found that 60% of participants aged 20-40 were at increased risk for Chlamydia infection, linked to higher sexual activity. Similarly, research in Al-Najaf indicated that 52.38% of pregnant women with *Chlamydia trachomatis* were within the 20-30 age range. These findings highlight the need for targeted screening and educational efforts to reduce Chlamydia transmission among young adults.[23,24,25]

The study underscores the limitations of serological testing for *Chlamydia trachomatis*, as all positive cases were seronegative by ELISA, suggesting that the absence of detectable antibodies does not imply a lack of immune response. It highlights the role of cell-mediated immunity and immune evasion mechanisms, particularly in OMPA-negative strains, in infection outcomes. Research reveals a significant association between *Chlamydia trachomatis* infections and abortion, particularly among women aged 16-25, with a 17.5% positivity rate in affected participants. Various studies in different regions indicate differing prevalence

rates, with molecular detection showing notable links between Chlamydia infection and adverse pregnancy outcomes, including miscarriages. The importance of genetic factors, such as TNFa polymorphisms, in relation to recurrent spontaneous abortion alongside Chlamydia infection is also emphasized. Overall, the research suggests a critical connection between *Chlamydia trachomatis* and reproductive complications, indicating the need for further investigation and improved detection methods.[26,27,28,29,30,31,32,33,34,35,36,37,38]

The findings underscore the significance of PCR-based diagnostics for accurately detecting Chlamydia trachomatis, especially in reproductive health to prevent pregnancy complications. The study reveals that OMPA may not be present in all strains, and additional gene sequencing identified a distinct genetic lineage linked to potential host cell damage and inflammation, possibly causing placental dysfunction. Key proteins like CPAF and TARP disrupt maternal immune responses and alter trophoblast function, increasing miscarriage risk. PCR detection in vaginal swab samples is essential for evaluating infection impacts on reproductive health. Future research should focus on identifying genetic markers in both the pathogen and host, enhancing diagnostics, therapies, and potential vaccines against virulent strains.

Limitations

The test used in this study was implemented as a screening test to detect previous exposure, and no additional measures were taken for individuals who tested positive for chlamydia antibodies and for women whose antibodies were negative. Further studies are needed to clarify the issue of *Chlamydia trachomatis* infection in adult women.

CONCLUSION

The study detected *Chlamydia trachomatis* DNA in various vaginal samples, with 54.55% (CPAF), 60% (tox), and 45.5% (tarp) testing positive, yet none had the OMPA gene typically used for detection. The absence of OMPA suggests potential strain variation that evades conventional PCR detection. A strong association between *Chlamydia trachomatis* infection and miscarriage was found ($p = 0.001$), emphasizing the need for effective screening, particularly in asymptomatic women who could face reproductive complications. Chlamydia infections were most prevalent in women aged 20–27 and may act as an independent risk factor for pregnancy loss. Previous studies corroborate the link between these infections and spontaneous abortion, highlighting the need for targeted screenings in reproductive-age women. Despite no positive ELISA results, the study indicated that immune responses may vary, as OMPA-negative strains could evade serological detection. Future research should focus on genetic markers for both the pathogen and host to improve diagnostic and therapeutic strategies for managing *Chlamydia trachomatis* infections and related adverse pregnancy outcomes.

Recommendation

We recommend screening for *Chlamydia trachomatis* in fertile women who have experienced miscarriages in order to reduce the risk of spontaneous abortion and miscarriage during early pregnancy.

Funding source

No funds received.

Data availability

Study data will be available upon request to the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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