Evaluation of Major Outer Membrane Protein-based PCR and Plasmid-based PCR in Diagnosis Chlamydia trachomatis Endocervicitis

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ABSTRACT

Background: *The World Health Organization has stated that sexually transmitted diseases (STDs) rank second in importance after cancer as treatable diseases in women. Chlamydia trachomatis is one of the most important sexually transmitted diseases in terms of incidence, prevalence of serious complications. The nucleic acid amplification techniques (NAATs), which have become the method of choice have high sensitivity and specificity and are considered to be a more suitable method either for screening or diagnosing chlamydial infections.* ***Aim of the study:*** *compare 2 sets of primers in the diagnosis of C. trachomatis endocervicitis by performing PCR with primers directed against the MOMP and endogenous plasmid.* ***Materials and Methods:*** *endocervical swabs from 50 patients (patients group) and 25 females (control group), the Total DNA was extracted and multiplex PCR technique was done to diagnose C. trachomatis by using 2 different sets of primers to amplify genes of against the MOMP and endogenous plasmid.* ***Results:*** *58% of the patients group had C. trachomatis infection by plasmid –based PCR assay and 50% had C. trachomatis by MOMP-based PCR assay; while only 12% of the control group had C. trachomatis by the 2 technique. IUD was the most important risk factor of C. trachomatis endocervicitis and the infection usually tend to occur in the females between 20-30years old.* ***Conclusion:*** *PCR directed against the endogenous plasmid is superior sensitivity in comparison with PCR directed against Major Outer Membrane Protein (MOMP) gene of C. trachomatis; so plasmid- based PCR the best candidate for use in the detection of C. trachomatis in cervical smears.*

Key Words: *Chlamydia trachomatis, plasmid- based PCR , MOMP- based PCR.*

INTRODUCTION

Urogenital tract infections are a major cause of morbidity in sexually active individuals worldwide, therefore, the World Health Organization has stated that sexually transmitted diseases (STDs) rank second in importance after cancer as treatable diseases in women. In particular, *Chlamydia trachomatis* (*C. trachomatis*) is the leading cause of bacterial STD, with an estimated 5 million new cases annually worldwide[1–3]. *C. trachomatis* endocervical/urethral infection, caused by serotypes D to K is the most common bacterial, treatable sexually transmitted infection worldwide[4,5]. The term "mucopurulent cervicitis" arose from these authors observations that most women with STDs that caused cervicitis in those study (namely, had *C. trachomatis* & N. gonorrhea) displayed endocervical mucopurulent discharge. Despite increasing documentation during the ensuing decades that easily induced endocervical bleeding (sometimes referred to as "friability") was equally, if not more common among infected women with these pathogens, use the term "mucopurulent" has persisted [6].

C. trachomatis mucopurulent cervicitis is usually complicated by endometritis[7] ductal obstruction, pelvic inflammatory disease, and spontaneous abortion or preterm labour[8,9] tubal occlusion and extrauterine pregnancy, infertility[10,11].

The cell culture which was the only method that detects live *Chlamydia* was the most specific test (100%) but, the sensitivity is low (70-85%) in the best experienced laboratory), and it requires specialized handling and laboratory service; it is thus reserved for specialized research laboratories and has not been generally implemented as a routine diagnosis method[12]. The DFA have been used to detect *C. trachomatis* in every type of specimen. One important advantage of DFA is that the quality of the specimen can be objectively assessed and remains one of the most accessible and useful diagnosis techniques available. This test is sensitive, specific and rapid, but microscopic evaluation of each specimen is laborious and requires highly trained and experienced personnel[13]. The

nucleic acid amplification techniques (NAATs), which have become the method of choice have high sensitivity and specificity and are considered to be a more suitable method either for screening or diagnosing chlamydial infections [14, 15].

NAATs are based on the amplification, of specific nucleotide sequences in a sample, by means of specific primers and Taq DNA polymerase. PCR methods may overcome the shortcomings of the more conventional techniques for detection of *C. trachomatis*. Several studies have been reported on the use of PCR in *C. trachomatis* detection[16-18]. In these studies, 3 sets of primers were used that had been derived from three different *C. trachomatis* -specific DNA sequences-the gene coding for the major outer-membrane protein (MOMP), the 16s ribosomal RNA gene and the 7.5 kb plasmid[19]. It is clearly important to know whether these three PCR methods differ in sensitivity and specificity in diagnosis of *C. trachomatis* endocervicitis. So in this study we aimed to compare 2 sets of primers in the diagnosis of *C. trachomatis* endocervicitis by performing PCR with primers directed against the MOMP gene (the one universally present) and highly conserved endogenous plasmid.

PATIENTS, MATERIALS & METHODS

This study was conducted in the department of Medical Microbiology & Immunology & included 50 patients (patients group) and 25 females (control group) attending the gynecologic outpatient clinic in Kasr Al-Aini Obstetric and Gynecology Hospital Faculty of Medicine, Cairo University during the period from August 2011 to April 2012.

Patients group: Included 50 patients; 29 patients suffering from mucopurulent cervicitis (10 patients using IUD for contraception, 9 patients using hormonal contraceptive methods and 10 patients without any contraceptive method) and 21 patients suffering from infertility (12 patients with primary infertility and 9 patients with secondary infertility). Those females who were non-married, used antibiotics or local vaginal antiseptics within 2 weeks before sampling, menstruating or pregnant were excluded from the study.

Control group: Included 25 females attending the outpatient clinic in Kasr Al-Aini Obstetric and Gynecology Hospital for any other causes and they are subjected to the same exclusion criteria as the patients group.

After obtaining the informed consent, all females participating in this study were subjected to:

1. Full history taking including name, age, parity, method of contraception and its duration (if present), symptoms suggestive of cervicitis or infertility, review of investigations as hysterosalpingiography and laparoscopy.
2. Gynecological examination of the cervix for signs of cervicitis or infertility. According to CDC definition of cervicitis [20]. Only mucopurulent discharge or friability (bleeding induced by gentle passage of the cervical swab through the cervical os) was searched for.
3. Endocervical swabbing: using endocervical Dacron swab.

Sample taking:

1. Endocervical swabbing was done using endocervical Dacron swab which was inserted in the endocervical canal until the tip of the swab was no longer visible and the swab was then rotated 20 – 30 seconds with scraping of the mucosa so as to collect as many as columnar or squamocolumnar cells as possible (as *Chlamydia* are intracellular organism).
2. The swab was then withdrawn avoiding contact with the vaginal surfaces.
3. The swab was then inserted in 2ml sterile phosphate buffered saline (PBS) (Dulbecco. U.K.) and was vigorously swirled in the liquid for 15-30 seconds and then the liquid was expressed from the swab by pressing the swab against the side wall of the tube. The swab was removed and discarded.
4. The tube was capped after proper labeling and stored at 4C during transport to the laboratory where it was frozen at -70c until processing.

DNA extraction: the specimens were allowed to thaw at room temperature then volumes of 500µl of the specimens were used to extract DNA using QIAamp DNA Mini Kit through the swab spin protocol which is recommended by the manufacture for the used Dacron swabs.

DNA amplification: a multiplex PCR technique was done using two sets of oligonucleotide primers were used one was for cryptic plasmid and the other for MOMP of *C. trachomatis*.

Table 1: Sequences of the oligonucleotide primers and Amplified fragment lengths.

	Sequences of the primers set	Amplified fragment length
Cryptic Plasmid primers [21]	CtPl+ 5'-TAGTAACTGCCACTTCATCA-3' CtP2-5'-TTCCCCTTGTAATTCGTTGC-3'	201-bp fragment
MOMP primers [22]	CtM1+ '5-GCCGCTTTGAGTTCTGCTTCCTC-3' CtM2-5'-CCAAGTGGTGCAAGGATCGCA-3'	129-bp fragment

- PCR was performed by adding l0µl of DNA sample to 40µ1 of a PCR master mix solution containing one µl of each primer, 50 mM KCl, 10 mM Tris-HC1, pH 8.3, 1.5 mM MgCl, 200 pµ of each dNTP, 1 unit of Taq DNA polymerase (Ampfitaq, Cetus. USA) to reach a final amplification PCR volume of 50µ1, and the mixture was overlaid with several drops of paraffin oil to prevent evaporation and incubated for 4 min at 95 "C for DNA denaturation. Forty cycles of amplification were performed with T-personal Thermal cycler (Biomtra-Germany) Each PCR cycle consisted of a denaturation step at 95°C for 1 min, a primer-annealing step at *55°C* for 2 min and a chain-elongation step at 72°C for 1.5 min. After 40 cycles a temperature-delay step of 4 min at 72°C was done to complete elongation [19].

Detection of the amplified PCR products: Ten µl of the amplified PCR products were analyzed by1.5 *%* agarose gel electrophoresis.

Statistical study: All data were analyzed using SPSS17 program.

RESULTS

Our study included 50 females (patients group) and 25 females (control group).

Twenty nine patients out of 50 (58%) of the patients group had *C. trachomatis* infection by plasmid –based PCR assay and 25 patients (50%) had *C. trachomatis* by MOMP-based PCR assay; while only 3 females (12%) of the control group (25 females) had *C. trachomatis* by the 2 technique (table 2).

In our study a correlation between the results of the used laboratory tests and the patients' clinical presentation denoted that:

- Out of 29 patients presented with mucopurulent cervicitis 15 (51.7%) patients had *C. trachomatis* proved by plasmid –based PCR assay and the percentage was higher among patients of used IUDs than those who did not use any contraceptive method with statistical significant difference (P< 0.05), while the percentage of *C. trachomatis* infection in patients used hormonal contraceptive method was 55.5% without statistical significant difference than those who did not use any contraceptive method. Meanwhile; 3 (44.7%) patients out of 29 patients presented with mucopurulent cervicitis had *C. trachomatis* infection proved by MOMP – based PCR assay and the percentage was also higher among patients of used IUDs than those who did not use any contraceptive method with statistical significant difference (P< 0.05), while the percentage of *C. trachomatis* infection in patients used hormonal contraceptive method was 33.3% without statistical significant difference than those who did not use any contraceptive method (table 3 and table 4).
- In our patient group and 21 patients were presented with infertility 14 of them (66.7%) had *C. trachomatis* infection proved by plasmid –based PCR assay and 12 of them (57.1%) had *C. trachomatis* infection proved by MOMP –based PCR assay. Without statistical significant differences between patients with secondary infertility (75% & 58.3%) than those primary infertility (55.5%) (table 3 and table 4).

C. trachomatis infection was significantly higher among young aged patients (20 - 30 years) by both plasmid –based PCR assay & MOMP –based PCR assay (78.9%) than middle aged patients (30 – 40 years) and old patients (>40 years) (table 5).

C. trachomatis were present in 28 females by both plasmid –based PCR & MOMP-based PCR 25 cases & 3 control females) while 4 cases were positive by plasmid –based PCR only. The plasmid –based PCR had 100% sensitivity, 92.16% specificity, 87.5% positive predictive value, 100% negative predictive value and 98.3% accuracy, while; the MOMP-based PCR technique had 87.7% sensitivity, 100% specificity, 100% positive predictive value, 92.16% negative predictive value and 96.6% accuracy (table 6).

Table 2: Results of the Plasmid-based PCR and MOMP-based PCR among the studied groups.

Test	Patients (50)		Controls (25)		X^2	P
	No.	%	No.	%		
Plasmid-based PCR						
- **Positive**	29	58%	3	12%	12.38	< 0.001
- **Negative**	21	42%	22	88%		
MOMP-based PCR						
- **Positive**	25	50%	3	12%	9.19	< 0.001
- **Negative**	25	50%	22	88%		

Table 3: Correlation between results of Plasmid-based PCR and the patients' clinical pictures

Patients (50)	Plasmid-based PCR				P*
	Positive		Negative		
	No.	%	No.	%	
I- Mucopurulent cervicitis (29):	15	51.7%	14	48.3%	> 0.05
- Mucopurulent cervicitis only (10)	1	10%	9	90%	
- Mucopurulent cervicitis with IUD (10)	9	90%	1	10%	< 0.05
- Mucopurulent cervicitis with hormonal contraceptive (9)	5	55.5%	4	44.5%	> 0.05
II- Infertility (21):	14	66.7%	7	33.3%	> 0.05
- Primary (9)	5	55.5%	4	44.5%	
- Secondary (12)	9	75%	3	25%	

Table 4: Correlation between results of MOMP-based PCR and the patients' clinical pictures.

Patients (50)	MOMP-based PCR				P*
	Positive		Negative		
	No.	%	No.	%	
I- Mucopurulent cervicitis (29):	13	44.7%	16	55.3%	> 0.05
- Mucopurulent cervicitis only (10)	1	10%	9	90%	
- Mucopurulent cervicitis with IUD (10)	9	90%	1	10%	< 0.05
- Mucopurulent cervicitis with hormonal contraceptive (9)	3	33.3%	6	66.7%	> 0.05
II- Infertility (21):	12	57.1%	9	42.9%	> 0.05
- Primary (9)	5	55.5%	4	44.5%	
- Secondary (12)	7	58.3%	5	41.7%	

Table 5: Comparison between the results of Plasmid-based PCR and MOMP-based PCR among the different ages of the patients group.

Age (years)	Plasmid-based PCR		MOMP based PCR	
	No.	%	No.	%
20 – 30 * (19)	15	78.9%	15	78.9%
31 – 40 (19)	11	57.9%	9	47.3%
□ 40 (12)	3	25%	1	8.3%
Total (50)	29	58%	25	50%

* P < 0.05 (significant) by fisher exact test.

Table 6: Consensus results among the studied groups versus validity of the two laboratory tests (Plasmid-based PCR and MOMP-based PCR).

	Plasmid-based PCR	MOMP-based PCR
Positive results (32)		
- Positive by both tests	28	28
- Positive by one test	4	0
Sensitivity	100%	87.5%
Specificity	92.16%	100%
Positive predictive value	87.5%	100%
Negative predictive value	100%	92.16%
Accuracy	98.3%	96.6%

***Consensus results**: any case giving positive results by the two laboratory methods used in this study.

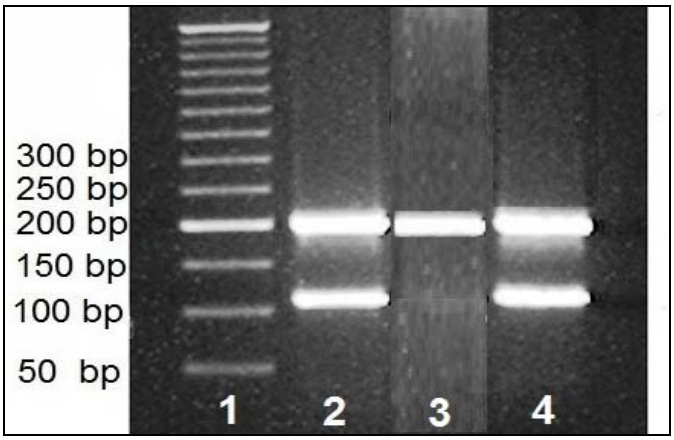

Figure 1: Agarose gel shows the results of multiplex PCR for detection of amplified products of Plasmid-based PCR & MOMP-based PCR techniques: lane 2 and 4 show positive results by the two techniques while lane 3 shows positive result by Plasmid-based PCR only.

DISCUSSION

Urogenital tract infections are a major cause of morbidity in sexually active individuals worldwide, therefore, the World Health Organization has stated that sexually transmitted diseases (STDs) rank second in importance after cancer as treatable diseases in women. In particular, *C. trachomatis* is the leading cause of bacterial STD, with an estimated 5 million new cases annually worldwide [(1-3)].

C. trachomatis is one of the most important sexually transmitted diseases in terms of incidence, prevalence of serious complications and associated health care costs such as infertility and ectopic pregnancy[(23)].

Diagnosis of *C. trachomatis* infection remains a challenge because *C. trachomatis* infection is usually asymptomatic in up to 80% of infected women and 50% of infected men [(8)].

The standard technique for diagnosis of *C. trachomatis* infection is isolation of Chlamydiae in cell culture. However, the viability of *C. trachomatis* may be compromised by collection, transportation and various steps in the culture procedure which can affect the sensitivity of culture methods. Therefore, several alternative methods have been developed. Antigen detection methods such as direct immunofluorescence (DIF) and enzyme-immunoassays (EIAs) are the most widely used tests that do not rely on growth of *C. trachomatis* and recently, DNA hybridisation techniques have been introduced[(19)]. However, none of these tests shows optimal sensitivity and only cell culture is 100 **%** specific[(24)]. Furthermore, DIF is a laborious technique that requires great experience, and EIAs may lack specificity, due to the detection of cross-reactiveantigens [(25)].

During recent years, a new sensitive and specific technique for the detection of micro-organisms, the polymerase chain reaction (PCR), has become available [(26)]. Several authors have described a PCR method for the detection of *C. trachomatis* with primers against the MOMP[(27-29)] the one universally present and

highly conserved endogenous plasmid[29,30] and the 16s rRNA gene [30].

In the current study PCR was used as a diagnostic method for diagnosis of

C. trachomatis infection in females and we used 2 sets of primers targeting different genes of *C. trachomatis* MOMP-gene and endogenous plasmid.

The present study include two groups of females; the patients' group which contained 50 patients (29 with mucopurulent cervicitis and 21 with infertility) and the control group that included 25 apparently healthy females.

In our study the percentage of *C. trachomatis* infection in the patients' group by the two used PCR technique was 58% and 50 % by plasmid –based PCR and MOMP-based PCR respectively; these results are in agreement with ***Haggerty et. al.***[8] and ***Paavonen***[4] where they found the prevalence of *C. trachomatis* infection in endocervical specimens were 39% and 30% respectively.

The percentage of *C. trachomatis* infection in our control group was 12% by the two PCR techniques and this result is in agreement with ***Mohamed et. al.***[31] and ***Badary et. al.*** [32] who found that the percentage of *C. trachomatis* infection in apparently healthy females was 10% and 12.5% respectively.

Intrauterine contraceptive device (IUD) was found to be one of the most important risk factors for *C. trachomatis* mucopurulent cervicitis as we found in our study that *C. trachomatis* was detected by the two PCR techniques in 90% of patients presented with mucopurulent cervicitis and using IUD and this was statistically significantly higher than those patients who presented with mucopurulent cervicitis and using hormonal contraceptives (55.5% plasmid–based PCR and 33.3% by MOMP-based PCR), this finding may be due to that the IUD acts as a reservoir for *C. trachomatis* and preventing the uterine mucosa from cleaning itself from infection. In agreement with these results ***Marrazzo*** [33] reported that the incidence of *C. trachomatis* infection in females used IUD was 46% higher than the incidence in females using hormonal contraceptives which was 11%. But ***Omar***[34] detected *C. trachomatis* infection by plasmid–based PCR in females higher in those who used oral contraceptives (31%) than those who used IUD (4%).

Infection of the female genital tract of which the cervix is the "gate-way" not only predispose to ectopic pregnancy but also to the risk of tubal infertility [35]. In our study 66.7% & 57.1% of patients presented with infertility had Chlamydia trachomatis infection proved by plasmid –based PCR assay and MOMP –based PCR assay respectively. In agreement to this results ***Aspock et. al.***[36] reported that Chlamydia trachomatis infection was present in 55% in infertile females by plasmid –based PCR assay.

We found that *C. trachomatis* infection was higher in secondary infertility than in primary infertility but without any statistical significant differences and these results were in agreement with the results of ***Debattista et. al.***[37] and ***Haggerty et. al.***[8].

In the presented study the percentage of *C. trachomatis* infection was higher among young aged patients (20 - 30 years) than middle aged patients (30–40 years) and old patients (>40 years) by both plasmid –based PCR assay & MOMP–based PCR assay and this was statistically significant. In agreement with these results ***Eggleston et. al.***[38] and ***Corbeto et. al.***[39] reported that the percentage of *C. trachomatis* infection was higher in females below the age of 25 years and ***Land et. al.*** [40] reported that *C. trachomatis* infection was significantly common in younger females (<30 years).

Four cases in our study were positive by plasmid –based PCR only while 28 females by both plasmid –based PCR & MOMP-based PCR 25 cases & 3 control females). Statistically these results proved that plasmid –based PCR assay is more sensitive (100%) and is a better negative predictive value (100%) than MOMP-based PCR assay (87.7% & 92.16% respectively) but less specific (92.16%) and less positive predictive value (87.5%) than MOMP-based PCR (100% for both). In agreement with these results ***Marions et. al.*** [41] reported that the plasmid –based PCR may have a higher sensitivity than MOMP-based PCR. Also ***Shepetiuk et. al.***[42] mentioned that a higher sensitivity of plasmid –based PCR which was able to detect DNA corresponding to 1-10 Elementary bodies (EBs), compared to MOMP-based PCR which detected only 10 -100 EBs. This may be explained by the presence of multiple copies (6 -10) of the plasmid in every *Chlamydia* organism in comparison to a single chromosomal MOMP gene[41].

So plasmid –based PCR assay is a good screening test for detection of *C. trachomatis* endocervical infection and thus plasmid –based PCR assay is a very important technique for screening and early detection and treatment of uncomplicated *C. trachomatis* infection, the control of the overall prevalence of the infection in the population and thus the reduction of transmission.

It would be interesting to know whether a positive plasmid PCR reflects only the presence of plasmids of dead *Chlamydiae* or the presence of low copy numbers of still viable *Chlamydiae*. In the latter case, the patient might still be infectious. This has to be evaluated further.

In conclusion; PCR directed against the endogenous plasmid is the best candidate for use in the detection of *C. trachomatis* in cervical smears. This conclusion is based on its superior sensitivity in comparison with PCR directed against Major Outer Membrane Protein (MOMP) gene of *C. trachomatis*. The plasmid PCR needs to be evaluated further on clinical samples in comparison with current detection techniques such as culture, ELISA or DIF.

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تقييم تفاعل متسلسلة البلمرة المستندعلى جين بروتين الغشاء الخارجي الرئيسي والمستند على جين البلازميد فى تشخيص التهاب باطن العنق الرحم بالمتدثرة الحثرية

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مقدمة : منظمة الصحة العالمية ذكرت أن الأمراض المنقولة جنسيا تقع في المرتبة الثانية من حيث الأهمية بعد سرطان والأمراض التي يمكن علاجها في النساء. المتدثرة الحثرية هو واحد من الأمراض المنقولة جنسيا أهم من حيث الانتشار، حدوث مضاعفات خطيرة. التضخيم تقنيات الحمض النووي التي أصبحت الطريقة المفضلة لديك حساسية عالية وخصوصية وتعتبر وسيلة أكثر ملاءمة لتشخيص العدوى الناجمة عن المتدثرة الحثرية .

الهدف من الدراسة: مقارنة ٢ مجموعات من مبتدأت تفاعل متسلسلة البلمرة في تشخيص التهاب باطن العنق بالمتدثرة الحثرية . الموجهة ضد البلازميد و بروتين الغشاء الخارجي الرئيسية (MOMP) .

المواد والطرق المعملية: مسحات من باطن عنق الرحم ٥٠ مريضا (مجموعة المرضى) و ٢٥ من الإناث (مجموعة المراقبة)، تم استخراج الحمض النووي، ٢ تم عمل تفاعل متسلسلة البلمرة بستخدام مبتدأت موجهة ضد البلازميد و بروتين الغشاء الخارجي الرئيسية MOMP في تشخيص التهاب باطن العنق بالمتدثرة الحثرية

النتائج: ٥٨٪ من المرضى كان لديهم العدوى المتدثرة الحثرية عن طريق الفحص تفاعل متسلسلة البلمرة المستندة إلى البلازميد وكان٥٠٪ من المرضى كان لديهم العدوى المتدثرة الحثرية عن طريق الفحص تفاعل متسلسلة البلمرة المستندة إلى بروتين الغشاء الخارجي الرئيسية MOMP ، بينما ١٢٪ فقط من مجموعة المراقبة كان لديهم العدوى المتدثرة الحثرية عن طريق الفحص تفاعل متسلسلة البلمرة بواسطة الطريقتان . وكان اللولب الرحمى عامل الخطر الأكثر أهمية في التهاب باطن العنق بالمتدثرة الحثرية . وتميل العدوى عادة ان تحدث في الإناث بين سن ٢٠ ـ ٣٠ سنة.

ومن هدا نستخلص ان : الفحص تفاعل متسلسلة البلمرة المستندة إلى البلازميد لديه حساسية متفوقة بالمقارنة مع الفحص تفاعل متسلسلة البلمرة المستندة إلى البلازميد الموجهة ضد بروتين الغشاء الخارجي الرئيسية (MOMP) من الجين المتدثرة الحثرية ، لذا تفاعل متسلسلة البلمرة المستندة إلى البلازميد أفضل طريقة في فحص الكشف عن المتدثرة الحثرية في مسحات عنق الرحم .