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Original Article

Simultaneous Detection of Seven Pathogens of Cervicitis Among Young Female Sex Workers by Multiplex Real Time PCR in Dhaka, Bangladesh

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Abstract

The prevalence of STIs related cervicitis in Bangladesh among female sex workers (FSWs) is quite high and among them young (≤ 24 years) FSWs are more sufferers. The aim of this study was to detect infectious agents of cervicitis including *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum* and *Ureaplasma parvum* in SWs of aged 10-24 years from endocervical swabs by multiplex real time PCR. A cross sectional study was done in collaboration with department of Microbiology, BSMMU, Dhaka and Save the Children, Bangladesh between March to December 2017 among sex workers enlisted to receive HIV prevention services at different drop in centers (DICs) in Dhaka.Total 105 SWs of aged between 10-24 years and clinically suspected as cervicitis, were enrolled for the study. Endo-cervical swabs were collected during examination and tested in dept of Microbiology, BSMMU by multiplex PCR and other tests for aforementioned pathogens. Data were collected by face to face interview using semi-structured questionnaire and clinical examinations were done using Casco's vaginal speculum. Among the study population, 87 (82.9%) were between 20-24 years of age. On examination, out of 105, 67 (63.8%) patients had no cervical discharge, only 8 (7.6%) had muco-purulent discharge. Out of total, 95 (90.5%) patients were mPCR positive for at least one pathogen and only 3 (2.9 %) *N. gonorrhoeae* isolated by culture, 8(7.6%) cases of *C. trachomatis* were detected by DFA and 8 (7.6%) cases of *T. vaginalis* were detected by wet film. Among the mPCR positive (95) cases, 63(66.3%) patients had mixed infections and among them, M. hominis was the highest (76.2%) followed by U. urealyticum (49.2%). In the patients having 'no' (67) cervical discharge, 32 (48%) had *M. homini* sinfection followed by *U. parvum* (40%). Majority of FSWs had mixed infection and *M.hominis* was the highest. A high number of patients hadno cervical discharge though it is one of the diagnostic criteria for cervicitis in current syndromic management. In comparison to other available diagnostic tests, organisms were detected efficiently by multiplex PCR and could be advised routinely in such cases of mixed infection.

Key words: Female sex workers, cervicitis, multiplex PCR

Introduction

Cervicitis, mostly caused by sexually transmitted pathogens, remains an important public health problem among female sex workers (FSWs) in developing countries like Bangladesh.[1] Untreated or undiagnosed cervicitis may cause ascending infection such as endometritis[2], salpingitis.[3,4] pelvic inflammatory disease[5] andinfertility.[6,7] It is also related to chorioamnionitis and other pregnancy related complications.[2,8,9,10,11,12,13] It enhances the risk of HIV transmission[14,15] and causes cervical cancer.[16,17] It serves as a reservoir that facilitates widespread transmission among multiple sexual partners.[18] There are estimated 90,000 to 150,000 FSWs operating in Bangladesh and the number of FSWs between the ages of 10-24 years old (adolescent and young group) is estimated to be 31,101.[19] Higher rates of STIs related to cervicitis (83.2%) in young people have been reported in Bangladesh.[1]

In most cases, etiologies of cervicitis are polymicrobial in nature. It is estimated that annually there are 131 million new cases of *Chlamydia trachomatisand* 78 million of *Neisseria gonorrhoeae* infections among people aged 15-49 years all over the world[20]. Other organisms including Mycoplasma genitalium[21,22,23,24,25,26], *Mycoplasma hominis, Ureaplasma urealyticum*[27], *Trichomonas vaginalis*[28,29], viruses like Herpes simplex virus, Human Papilloma Virus and *Ureaplasma parvum* also causes cervicitisand termed as nonspecific cervicitis (NSC) or nonchlamydial-nongonococcal cervicitis. Nonspecific cervicitis (NSC) is increasing day by day.[30,31,32,33,34,35,36,37]

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WHO approaches syndromic management (SM) for the diagnosis of cervicitis and other sexually transmitted infections.But cervicitis in FSWsis mostly asymptomatic or having mild or nonspecific symptoms.[1,38,39,40] So syndromic approach led to many cases untreated or over diagnosed.[41,42]

Multiplex PCR are being developed to diagnose multiple organisms in a single clinical samplefrom endocervical specimen in both symptomatic and asymptomatic cervicitis patients which might be helpful to diagnose all the microbes in a single test and also to the clinicians to prescribe appropriate antibiotics for patients according to results. Therefore, this study was designed to detect *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum, Ureaplasma parvum* and *Trichomonas vaginalis* from endocervical swabsamong FSWs aged of 10-24 years by multiplex real time PCR and compare the results of PCR with that of other conventional methods.

Materials and methods

This cross sectional study was done at the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh in between March, 2017 to February, 2018 and was approved by the Institutional Review Board of BSMMU. It had enrolled about 105 FSW saged between 10-24 years enlisted to receive services at different drop in centers (DICS) in Dhaka implemented by Save the Children, Bangladesh based on the inclusion criterias.

Patients who had any of the following findings per speculum examination were included in the study[43,39,35] a) visible mucopurulent or mucoid or creamy discharge from the cervix or on endocervical swab, b) easily induced cervical bleeding by gentle passage of cotton swab, c) cervical tendernessobserved on bimanual examination, d)red, edematous and hypertrophied cervix, e) signs of ectropion (The condition when lips of the cervix curl upwards and outwards to express the red looking endocervix).[44] Menstruating women, pregnant and known case of vesico-vaginal fistula and stress incontinencewere excluded from the study. After taking informed consent a pretested semi-structured data sheet gathering socio-demographic data, risk behavior and genitourinary complaints was administered.

Sample collection procedure

A pelvic examination was carried out by the attending gynaecologist. With all aseptic precautions, pre-moistened (warm water) sterile Casco's self retaining bivalve vaginal speculum was introduced into the vagina. Ectocervical mucus was adequately removed with sterile cotton ball soaked with normal saline and the cervix was then inspected for the presence ofcervicitis and endocervical swabs were then collected. Cotton tipped wooden sticks were introduced deeply (2-3cm) into the cervical canal and rotated gently against the endocervical wall for 15-30 seconds before removal. Total 3 cotton-tipped wooden swabs were collected and one sample was also collected using a small, nylon bristled cytobrush. First swab was used for direct inoculation into culture mediaat room temperature immediately after collecting the sample. Second one was immediately used for smear preparation for Gram staining and for preparing wet film. After air dry, smear for Gram staining was fixed by 99% ethanol and placed in a slide box for carrying. Saline wet mount microscopy was performed on cervical samples for detection of *T. vaginalis*. Third swab was kept in an empty screw capped test tube (5ml) for multiplex real time PCR and the cytobrush was immersed in a tube with 5ml PBS. All the tubes containing swab sticks and the slide box were transported at ambient temperature to the laboratory in a sample carrying box.

Culture and isolation of *N. gonorrhoeae*

Culture for *N. gonorrhea* was carried out by inoculating onto modified Thayer- Martin media (Himedia, Himedia Laboratories Limited, Mumbai, India) blood agar, and chocolate agar media and incubated under micro-aerophilic conditions (5% O_2) at 37°C for at least 48 hours and confirmed by Gram staining, oxidase, catalase, and sugar fermentationtests. Antimicrobial susceptibility test was done for penicillin, tetracycline, ciprofloxacin, cefuroxime, ceftriaxone and cefixime following CLSI guideline.[45] For wet film preparation, the test tubes were slightly heated and swabs were agitated vigorously in the PBS and we film was prepared.For DFA microscopy, smear was prepared as per the instruction of manufacturer (VIRCELL, Spain). Slides were stored at 2°C to 8°C for maximum 2-3 days in a tight slide box.

Molecular detection of the organisms by multiplex real time PCR

Real-time PCR amplification for seven microorganisms (*C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium, M. hominis, U. urealyticum,* and *U. parvum*) was performed using the FTD urethritis plus kit (Fast track Diagnostics, Luxembourg) in accordance with the manufacturer's protocol, in a CFX96 real- time thermocycler (Bio-Rad, Hercules, CA, USA). The assay used murine Cytomegalovirus (mCMV) as an internal control which was introduced into each sample and the negative control at the lysis buffer stage of the extraction process. Extraction of nucleic acids from specimen was done according to the

manufacturer's instruction (RTP pathogen kit, STRATEC Molecular, Berlin, Germany. Cat no. 104050 0200).

It contains sets of primers and Taqman probes that are specially designed from highly conserved regions of genetic sequences for the 7 pathogens. The kit has a detection limit of 10^3 copies/ml for all pathogens except *T. vaginalis* which could be detected upto 10^2 copies/ml. Amplification of DNA was performed on real-time PCR platform (CFX96™ Bio-Rad, USA). Two reaction mixes were prepared in two 1.5ml Eppendorf tube and labeled 'tube 1' and 'tube 2'. Total reaction volume was 15 µl in each tube and prepared with 12.5 µl buffer, 1 µl enzyme and 1.5 µl of URscreen Primer-Probe mix in tube 1 and 1.5 µl UTriMyc Primer-Probe mix in tube 2. Complete reaction mix was vortexed briefly and spun down for few seconds.The amplification was performed under the following conditions: initial denaturation at 50˚c for 15 minutes followed by 94˚c for 1 min hold and 40 cycles (94˚c for 8 sec and 60˚c for 1 min).

All the data were entered into an electronic database and analysed using SPSS software (Version-20).

Results

Out of 105 FSWs (street, residence and hotel based) with clinical signs of cervicitis, 46 werefrom streets, 53 in residences and 6 were in the hotels. Table I shows the sociodemographic and behavioral characteristics of these FSWs.

Table-I: Sociodemographic and behavioral characteristics in FSWs (n=105)

Indicators	Total (%)	Street based FSWs (n=46)	Residence based FSWs (n=53)	Hotel based FSWs (n=6)
Age (year)				
14-19	18 (17.1)	11 (23.9)	7 (13.2)	0
20-24	87 (82.9)	35 (76.1)	46 (86.8)	6 (100)
Current marital status				
Married*	46 (43.8)	21 (45.7)	23 (43.4)	2 (33.3)
Unmarried	6 (5.7)	0	06 (11.3)	0
Divorced	16 (15.2)	9 (19.6)	07 (13.2)	0
Separated	32 (30.5)	14 (30.4)	14 (26.4)	4 (66.7)
Widow	5 (4.8)	2 (4.3)	3 (5.7)	0
Education (year)				
No education	42 (40)	25 (54.3)	14 (26.4)	3 (50)
1-5	27(25.7)	16 (34.8)	11 (20.8)	0
6-10	32 (30.5)	5 (10.9)	24 (45.3)	3 (50)
11-12	4 (3.8)	0	4 (7.5)	0
Income in last month (BDT)				
3,000-5,000	32 (30.5)	17 (37)	15 (28.3)	0
5,001-10,000	27 (25.7)	11 (23.9)	13 (24.5)	3 (50)
10,001-20,000	36 (34.3)	16 (34.8)	18 (34)	2 (33.3)
20,001-30,000	7 (6.7)	2 (4.3)	5 (9.4)	0
More than 30000	3 (2.9)	0	2 (3.8)	1 (16.7)
Age of first intercourse (year)				
7-12	44 (41.9)	25 (54.3)	17 (32.1)	2 (33.3)
13-18	58 (55.2)	21 (45.7)	33 (62.3)	4 (66.7)
More than 18	3 (2.9)	0	3 (5.7)	0
Number of pregnancy*				
None	19 (18.1)	9 (19.6)	9 (17.0)	1 (16.7)
Upto 5	83 (79.0)	35 (76.1)	44 (83.0)	4 (66.7)
More than 5	3 (2.9)	2 (4.3)	0	1 (16.7)
Duration of sex work (year)				
Less than 5	48 (45.7)	15 (32.6)	31 (58.6)	2 (33.3)
5 or more	57 (54.3)	31 (67.4)	22 (41.5)	4 (66.7)
No. of days in sex work in last week				
Mode:3; Min:1; Max :7				
No. of total clients in last week				
Upto 10	71 (67.6)	25 (54.3)	41 (77.4)	5 (83.3)
More than 10	34 (32.4)	21 (45.7)	12 (22.6)	1 (16.7)
Used condoms with new clients in last week (n=84)				
Irregularly	41 (48.8)	16 (34.8)	23 (43.4)	2 (33.3)
Consistently	40 (47.6)	24 (52.2)	14 (26.4)	2 (33.3)
Never	03 (3.6)	0	2 (3.8)	1 (16.7)
Used condoms with regular clients in last week (n=56)				
Irregularly	27 (48.2)	9 (19.6)	18 (34)	0
Consistently	21(37.5)	8 (17.4)	12 (22.6)	1 (16.7)
Never	8 (14.3)	3 (6.5)	5 (9.4)	0
H/O previous vaginal discharge				
Yes	98 (93.3)	44 (95.7)	48 (90.6)	6 (100)
No	7 (6.7)	2 (4.3)	5 (9.4)	0
No. of previous STIs (n=98)				
Less than 5	59 (60.2)	24 (52.2)	33 (62.3)	2 (33.3)
5 or more	39 (39.8)	20 (43.5)	15 (28.3)	4 (66.7)
H/O taking antibiotic before (>2 weeks) (n=98)				
Yes	80 (81.6)	35 (43.8)	40 (50)	5 (6.2)
No	18 (18.4)	9 (50)	8 (44.4)	1 (5.6)

*Married means currently living with husband.
Number of pregnancy includes live birth, MR (men strual regulation) and abortion.

Table-II: Results of PCR, culture for *N. gonorrhoeae*, DFA for *C. trachomatis*, wet film for *T. vaginalis* and Gram's stain for gram negative diplococci in endocervical swabs among the FSWs with clinically suspected cervicitis (n=105)

Tests	No. of positive patients	Percentage
PCR*	95	90.5
Culture for *N. gonorrhoeae*	3	2.9
DFA for *C. trachomatis*	8	7.6
Wet film for *T. vaginalis*	8	7.6
Gram's stain for gram negative diplococci	0	0

*PCR for *C. trachomatis, N. gonorrhoeae, T. vaginalis, M.genitalium, M. hominis, U. urealyticum* and *U. parvum*

In table II, out of 105 FSWs, 90.5% patients were PCR positive for at least one pathogen and 2.9 % patients were culture positive for *N. gonorrhoeae*, 7.6% patients were DFA positive for the detection of *C. trachomatis* and 7.6% patients with *T. vaginalis* were detected by wet film. None was positive by Gram's stain for gram negative diplococci.

Table-III: Different infectious agents detected from endocervical swabsby PCR (n=95)

Infectious agents	No. of patients	Percentage
Chlamydia trachomatis	24	25.3
Neisseria gonorrhoeae	18	18.9
Trichomonas vaginalis	25	26.3
Mycoplasma genitalium	16	16.8
Mycoplasma hominis	53	55.8
Ureaplasma urealyticum	34	35.8
Ureaplasma parvum	43	45.3

***Sum exceed 100% because of multiple organisms were present.**

Among 95 PCR positive patients, 55.8% patients were *M. hominis* positive followed by *U. parvum* (45.3%). (Table III)

Table-IV: Rate of single and mixed infections in PCR positive patients (n=95)

No. of organisms	Organisms detected by PCR							
	CT n(%)	NG n(%)	TV n(%)	MG n(%)	MH n(%)	UU n(%)	UP n(%)	Total (%)
Single	2(6.2)	1(3.1)	3(9.4)	1(3.1)	5(15.6)	3(9.4)	17(53.1)	32(33.7)
Mixed	22(35)	17(27)	22(35)	15(23.8)	48(76.2)	31(49.2)	26(41.3)	63(66.3)

CT:Chlamydia trachomatis; NG:Neisseria gonorrhoeae; TV; Trichomonas vaginalis; MG:Mycoplasma genitalium ; MH:Mycoplasma hominis; UU:Ureaplasma urealyticum; UP:Ureaplsma parvum

Majority of the patients (66.3%) had mixed infections and among them, *M. hominis* was present in 76.2% of mixed infection. (Table IV)

Table-V: Distribution of per speculum findings in relation to PCR results (n=105)

Per speculum findings	n%	Single organism (n=32) n%	Mixed organisms (n=63) n %	No organism (n=10) n%
Cervical discharge				
Mucopurulent	8 (7.6)	3 (9.4)	5 (7.9)	0
Thick	9 (8.6)	4 (12.5)	5 (7.9)	9 (90)
Thin	21 (20)	4 (12.5)	14 (22.2)	3 (30)
No	67 (63.8)	21 (65.6)	39 (62)	7 (70)
Ectropion	27 (25.7)	10 (31.2)	16 (25.4)	1 (10)
Easily induced cervical bleeding	60 (57.1)	20 (62.5)	37 (58.7)	3 (30)
Cervical tenderness on movement	18 (17.1)	6 (18.6)	8 (12.7)	4 (40)
Red & swollen cervix	33 (31.4)	9 (28.1)	21 (33.3)	3 (30)

*Sums exceed 100% because of multiple signs were present.

TableV shows, most of the patients (63.8%) had no cervical discharge followed by easily induced cervical bleeding (57.1%) per speculum examination.

Discussion

In this study, about 90.5% cases, at least one pathogen was detected by PCR from the collected endocervical swabs. A similar finding was observed by Sylverken et al in 2016.[38] In that, 86.5% of symptomatic women infected with at least one pathogen, were detected by PCR. Another study conducted on patients with STDs also showed higher detection (80.7%) rate of pathogens on swab and urine samples by PCR.[46]

Among 95 PCR positive patients, 55.8% cases were *M. hominis* positive followed by *U. parvum* (45.3%) and *U. urealyticum* (35.8%). Other organismsincluding *T. vaginalis, C. trachomatis, N. gonorrhoeae* and *M. genitalium* were detected in 26.3%, 25.3%, 18.9% and 16.8 % patients respectively. A similar study was conducted in patients with cervicitis in Australia where the detection rate was *M. hominis* (13.7%), *U. parvum* (57%), *U. urealyticum* (6.1%), *T. vaginalis* (3.4%), *C. trachomatis* (0.4%), *N. gonorrhoeae* (0) and *M. genitalium* (1.3%) by PCR.[27] In Surat, another study conducted among FSWs showed that gonorrhoeae was 16.9%, chlamydial infection was 8.5% and trichomoniasis was 14.4%.[40] In Bangladesh, Nusrat had found only 16% *C. trachomatis*, 10% *N. gonorrhoeae* and 2% *M. hominis* by PCR among cervicitis patients receiving services from outdoor of Dhaka Medical

College.[47] The lower detection rate might be due to the fact that the participants were not from sex trade.

In the present study, most (55.8%) of the cases were found to be positive for *M. hominis* followed by *U. parvum* (45.3%). Similar results were found by McKechnie *et al* in 2011where 47.7 % of *U. parvum* was detected in endocervival swabs from women attending sexual health clinics[48]. On the contrary, Sylverkenet al found higher rates of *M. hominis* positive (67.5%) and *U. parvum* positive (62.5%) among the study group.[38]

Recently, cervical infection with *M. genitalium* is dramatically increasing among the high risk people including female with multiple sexual partners.[49,50,51] In the current study, out of total 105 sex workers, *M. genitalium* was found in 16.8 % patients. Similar higher prevalence rates of *M. genitalium* were reported in different parts of the world in symptomatic and high risk populations ranging between 13%-25%.[52,24] A study conducted on sex workers in Ghana reported the prevalence rate of *M. genitalium* to be 26.3% in endocervical swabs whereas Mckechnie and co workers found < 5% of *M. genitalium* among non sex workers in Australia.[38,48]

Detection rate of *T. vaginalis* (26.3%) by PCRin the present study as single or mixed infections among the study population was comparable with the results of the studies in Surat (14.4%) and in Uganda (18.9%).[53,40] Variable rates of microscopically *T. vaginalis* positive were found in street based and hotel based sex workers in Dhaka city that were reported to be 45.5 % and 4.3% respectively.[54,55] The higher rate of detection of *T. vaginalis* in the present studymay be due to the use of most sensitive method of detection like PCR.

In present study, *C. trachomatis* was present in 25.3% of study patients. Similar finding was reported by Desai *et al.*[40] In another study, higher prevalence of *C. trachonmatis* (43.5%) than that of present study was found among hotel based sex workers.[54] Rate of Chlamydial infection among symptomatic and asymptomatic FSWs was 6.3% in Bangladesh[1] whereas the rate was higher (25.3%) in the present study. The high result may be due to the enrollment of symptomatic patients in this study.

N. gonorrhoeae was found only in 18.9% of study patients which was much lower than the rates (35.5%)among the sex workers reported by Nessa *et al*[54] and Rahman *et al.*[55] But the result of present study was much higher than that of a previous study (5.4%) in Bangladesh.[1]

In this study, among PCR positive cases, the rate of mixed infections (66.3%) was higher than that of single infection (33.7%). In contrast, Sylverken *et al* found higher rate of single infection (57.1%) than that of the mixed infections (42.9%).[38] But similar result of higher prevalence of infection with mixed organisms (67%) was found by Pereyre *et al* in screening of genital infection among symptomatic and asymptomatic women in France.[56] In the present study, out of the total single infections, *U. parvum* was the commonest (53.1%) which was followed by *M. hominis* (15.6%).

On the other hand, *M. hominis* was found as the most common pathogen (76.2%) among the mixed infections followed by *U. urealyticum* (49.2%) in the present study. The infection with two organisms was the most frequent (49.2%) co infection among mixed infections. The combination of *M. hominis U. urealyticum* was found to be higher in the infections in this study. This finding was similar to the result of a recent study described by Capoccia *et al* in 2013 with the rate of 38.7%.[57] The different patterns of co infection were supposed to establish a chronic infection among the cervicitis patients as well as making a threat of developing antibiotic resistance which may be a evaluated by extensive research.[58]

Conclusion

Majority of FSWs had mixed infection and among the pathogens *M. hominis* was the highest. A high number of patients had no cervical discharge though it is one of the diagnostic criteria for cervicitis in current syndromic management. In comparison to other available diagnostic tests, organisms were detected efficiently by multiplex PCR and could be advised routinely in such cases of mixed infection.

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