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Association of Vaginal *Lactobacillus* Status with Cervical Intraepithelial Neoplasia

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Abstract

Background: Female cervix and vagina maintain a microecology, that have a symbiotic relationship with the host and serve a crucial role in cervico-vaginal health. The cervical microbial flora has a prevalence of *Lactobacillus* species, which produce lactic acids that maintain an acidic environment and may inhibit pathogenic growth.

Objective: To assess the association between vaginal *Lactobacillus* status with cervical intraepithelial neoplasia (CIN).

Methodology: This cross-sectional analytical study was carried out at the Department of Gynecological Oncology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh. Study population comprised of 66 women, of them 33 had cervical intraepithelial neoplasia (CIN) and 33 were women with healthy cervix. Women with healthy cervix and women with CIN were confirmed by colposcopy and histopathology. Cervico-vaginal *Lactobacillus* was analyzed by culture in De Man Rogosa Sharpe (MRS) agar media and conventional polymerase chain reaction (PCR). Data were analyzed and compared by statistical tests.

Results: The mean age of the women with CIN was 37.56 ± 10.59 years and most of them (72.7%) were belonged to the age of >35 years. Regarding *Lactobacillus* status among the groups showed that, low level of *Lactobacillus* (0-1000 copy/cmm) was in 90.9% of women with CIN and 6.1% in women with healthy cervix group. A significant decrease of *Lactobacillus* was found in women with CIN compared to women with healthy cervix group ($p<0.05$). It was observed that, a low *Lactobacillus* status has 4.034 times increased risk of developing CIN (95% CI=0.047-36.199, $p<0.05$).

Conclusion: It could be assumed that vaginal low *Lactobacillus* status is associated with cervical intraepithelial neoplasia (CIN).

Keywords: Cervical Intraepithelial Neoplasia (CIN); Bacterial Vaginosis; Dysbacteriosis; *Lactobacillus* Status; Microecology.

Introduction

Cervical intraepithelial neoplasia (CIN) is a precancerous condition and potentially progress to invasive cervical cancer [1]. Cervical cancer is the 4th most common cancer affecting women around the world [2]. The reported prevalence of CIN range from 2.7% to 6.6% among women in reproductive age [3, 4]. Persistent infection with high-risk human papilloma virus (hrHPV) 16 and 18 are the main causative agent for the development of invasive

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cervical cancer and its precursor cervical intraepithelial neoplasia (CIN), that has been found in 99.7% of cervical cancer samples [5]. Approximately 90% of infections by hrHPV are transient and regress spontaneously [6]. Woman's risk of acquiring an infection by any type of HPV during the lifetime is approximately 80%, whereas risk of developing CIN and cervical cancer is only 0.6% [7]. As cervical cancer is caused by persistent infection of hrHPV and a lot of attention is being focused on HPV control and vaccination but a little is known about the mechanisms associated with clearance or persistence of HPV infection [8]. Most of the HPV infection is cleared off, only small portion of women who develop persistent infection leading to premalignant lesions with two specific HPV types (16 and 18) being the most common agents [9]. The vaginal micro-environment plays an important role in reproductive health. A healthy vaginal microbiome dominated by *Lactobacillus* species may have a protective effect against pathogens and have therapeutic potential [7]. In addition, lactic acid has been recognized as a component of the immune defense system, as it has been demonstrated to potentiate the production of protective proinflammatory cytokines by vaginal epithelial cells, to promote the activation of T helper lymphocytes, also stimulate dendritic cell maturation and induce interferon production [10]. Vaginal dysbacteriosis, characterized by low level of vaginal *Lactobacillus* species and predominance of *Gardnerella Vaginalis* alone or in complex with other anaerobic bacteria, aerobic vaginitis and other sexually transmitted vaginal pathogens may be an HPV-dependent cofactor for cervical neoplasia development [11]. Next generation sequencing (NGS) based studies have facilitated detailed characterization of the healthy vaginal microbiomes and shown that 5 major community-state types (CSTs) exist; CST I, II, III and V are dominated by *Lactobacillus Crispatus, L. Gasseri, L. Iners and L. Jensenii* respectively, whereas CST IV has characteristically low numbers of *Lactobacillus* species and increased diversity of anaerobic bacteria [12]. It was found that; a *Lactobacillus* species depleted, *Atopobium* species enriched (CST IV) community structure is associated with slowest regression of HPV where as a *Lactobacillus Gasseri* dominated microbiome (CST II) is associated with the most rapid regression rates for HPV [13]. It was reported that, the risk for occurrence of CIN in patients with vaginal dysbacteriosis is twice as large compared to healthy population [14]. Previous research has shown that the abundance of vaginal microbiota such as *Mycoplasma Genitalium,* aerobic *Lactobacilli, Staphylococcus Epidermidis, Enterococci, Escherichiacoli,* and *Bacteriodes* species in patients with CIN and cervical cancer is different from that in healthy controls [15]. There is emerging evidence that increased diversity of vaginal microbiota combined with reduced relative abundance of *Lactobacillus* species is involved in HPV acquisition and persistence and the development of cervical precancerous lesions and cancer [4, 16, 17]. Some experimental studies found that *Lactobacillus* and its metabolites inhibit the proliferation of cancer cells by regulating cancer-related genes or through an immunological mechanism [18, 19]. These studies provide a theoretical basis for further clinical application of *Lactobacillus* in development of CIN. The concept of manipulation of vaginal *Lactobacillus* communities by using probiotics may be an exciting prospect in the field of preventive measure in CIN and cervical cancer development. In this background current study was aimed to evaluate the association between vaginal *Lactobacillus* status with cervical intraepithelial neoplasia.

Materials and methods

Study design

This cross-sectional study was conducted at the Department of Gynaecological Oncology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh from April 2021 to March 2022. The study protocol was approved by the Ethical Review Committee, BSMMU, Dhaka, Bangladesh.

Study population

A total of sixty-six (66) participants were included as study population by purposive sampling technique following selection criteria. Women who were visual inspection of the cervix with acetic acid (VIA) positive and were referred to the colposcopy clinic, BSMMU, for evaluation by colposcopy were enrolled as cases. Women with colposcopically proven CIN, women without use of any oral or vaginal probiotics/ antibiotic for 30 days prior to the taking cervicovaginal swab and women having no history of genital tract infection during last 30 days prior to enrolment was included as study subject. Apparently healthy women with colposcopically proven no cervical lesion were taken as control group. Women with carcinoma cervix, pregnant women, smoker, women having active vaginal bleeding of unknown etiology, women who were not sexually active yet, women taking corticosteroids/ antibiotics/imidazoles/probiotics, women who used any types of vaginal preparations in the last 30 days, women using oral contraceptive pill or in hormone replacement therapy, women with any types of cancer and taking radio or chemotherapy, women with known systemic diseases, immune compromised patients, women with autoimmune diseases, women having diabetes mellitus, thyroid or other endocrine disorders were excluded from the study.

Study procedure

After selection, all study participants were informed regarding the purpose, procedure and benefits of the investigation with conditions of selection. An informed written consent was obtained for participation in the study. Detailed obstetric, medical history and clinical information had obtained and recorded accordingly. Then per speculum

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examination of each patient had been performed to evaluate vaginal secretion according to the following specifications: quantity, consistency, color, and odor. During per speculum examination from the cervix, a sample from cervico-vaginal swab was collected aseptically with a dry sterile swab stick for microbiological testing from the study subjects before doing colposcopic examination and sent it for bacteriological evaluation at Microbiology laboratory. Then the colposcopic examination were conducted and findings were recorded accordingly. Biopsy was taken from the patients having cervical acetowhite area and colposcopically diagnosed CIN was leveled. *Lactobacillus* genus detected by conventional polymerase chain reaction (PCR) by Applied Bio System® Simpli AmpTM Thermal Cycler, origin, Singapore; according to the protocol of Heilig *et al*., [20]. Colposcopic findings, final histopathologic reports and microbiology reports were recorded accordingly. Data were taken and analyzed from those women who had healthy cervix and those who had CIN detected by colposcopy and biopsy findings. Data were collected from the study population on variables of interest by interview, observation, clinical examination, colposcopic findings and histopathological reports. Data were collected by face-to-face interview and all data were recorded in a predesigned data collection sheet. Separate data collection sheet was used for each study subject. Highest level of confidentiality was maintained.

Sub grouping

Sample was categorized as following two groups.

Group- 1: Apparently healthy women having healthy cervix (colposcopically)

Group- 2: Women who had CIN on colposcopy and biopsy.

Data analysis

The data were coded and preserved accordingly with ensuring all sorts of safety, security and confidentiality. Data were processed and analyzed by using windows-based computer software "Statistical Packages for Social Sciences (SPSS)" version- 26. The quantitative data were presented as mean with standard deviation (SD) and qualitative data were expressed as frequency with percentage. The Chi-square test/Unpaired 't' test was used to assess the significance of difference between the groups. Odd ratio obtained from the logistic regression were used to estimate the risk of developing CIN. A p value <0.05 was considered as statistically significant.

Results and Observations

Total 66 participants were included in this study, among them 33 subjects had healthy cervix, 33 subjects had CIN. Presence of cervicovaginal *Lactobacillus* was evaluated in these two groups. It was observed that almost three fourth 24(72.7%) of women belong to the age of >35 years in CIN and 18(54.6%) women with healthy cervix belong to that age, which was statistically significant (p<0.05). More than 35 years age had 2.83 times increased risk to develop CIN (OR=2.83; 95% CI=1.02-7.90) (Table- 1). The mean age was 37.56±10.59 years in CIN and that was 37.85±7.31 years in healthy cervix group. In the distribution of educational background, it was observed that almost two third (66.7%) of patients were illiterate in CIN group and 12(36.4%) women were illiterate in healthy cervix group, which was statistically significant (p<0.05) between the groups. Illiterate patients had 3.50 times increased risk to develop CIN (OR=3.50; 95% CI=1.27-9.64) (Table- 1). The distribution of monthly income showed that, monthly family income level was ≤20000 BDT (Bangladeshi currency) in majority (69.7%) women with CIN and that was 4(12.2%) in women with healthy cervix group, which was statistically significant (p<0.05) between the groups. Low monthly income had 22.6 times increased risk to develop CIN (OR=22.6; 95% CI=4.63-60.11) (Table- 1).

Table 1: Distribution of socio-demographic factors in CIN and healthy cervix group (N= 66)

Variables	CIN (n= 33)		Healthy cervix (n= 33)		OR (95% CI)	p-value
	n	%	n	%		
Age (years)						
>35 years	24	72.7	18	54.6	2.83 (1.02-7.90)	0.043[s]
≤35 years	9	27.3	15	45.4		
Mean±SD	37.56±10.59		37.85±7.31			
Range (Minimum-Maximum)	23-70		28-56			
Education						
Illiterate	22	66.7	12	36.4	3.50 (1.27-9.64)	0.014[s]
Literate	11	33.3	21	63.6		
Monthly income						
≤20000 BDT	23	69.7	4	12.2	22.60 (4.63-60.11)	<0.001[s]
≥20000 BDT	10	30.3	29	87.8		

BDT= Bangladeshi Taka (Bangladeshi currency), s= significant, p value reached from Chi-square test

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Distribution of body mass index (BMI) in healthy cervix and CIN group showed that, majority (87.9%) of women with healthy cervix had normal body weight (BMI was between 18.5-24.9 kg/m^2), on the other hand 28(84.8%) women with CIN had normal body weight (BMI was between 18.5-24.9 kg/m^2). The mean BMI was 21.75±2.41 kg/m^2 in women with healthy cervix group and that was 22.01±2.5 kg/m^2 in women with CIN group. The difference of BMI was not statistically significant between the groups (p>0.05) (Table- 2).

Analyzing different reproductive factors in CIN and healthy cervix group revealed that, more than one third (39.4%) of women age during 1st intercourse was ≤15 years in CIN group and that was 5(15.2%) in healthy cervix group, which was statistically significant (p<0.05). Age during 1st intercourse ≤15 years had 3.64 times increased risk to develop CIN (OR=3.64; 95% CI=1.12-11.84). The mean age during 1st intercourse was 16.62±2.97 years in CIN group and 15.85±3.1 years in healthy cervix group. In CIN group, majority (90.9%) of the women had duration of sexual life span was >10 years and 21(63.6%) women in healthy cervix group had similar duration of sexual life span, which was statistically significant (p<0.05). Duration of sexual life span level >10 years had 5.71 times increased risk to develop CIN (OR=5.71; 95% CI=1.43-22.77). The mean duration of sexual life was 20.47±10.41 years in CIN group and that was 22±6.76 years in healthy cervix group. Regarding parity of the study population, it was observed that majority (90.9%) of the women had multipara in CIN group and 32(97.0%) women in healthy cervix group were multipara, the difference of parity was not statistically significant (p>0.05) between CIN and healthy cervix groups (Table- 3).

It was observed that, majority (90.9%) women in CIN group had *Lactobacillus* level 0-1000 copy/cmm and only 2(6.1%) women in healthy cervix group had similar level of *Lactobacillus* species, which was statistically significant (p<0.05). Women with low level (0-1000 copy/cmm) of *Lactobacillus* had 155 times increased risk to develop CIN (OR=155; 95% CI=24.2-939.0) (Table- 4).

Table 2: Distribution of the study population in body mass index (BMI) categories (N= 66)

Body mass index (BMI) Categories (kg/m^2)	Healthy cervix (n=33)		CIN (n=33)		p-value
	n	%	n	%	
Underweight (BMI <18.5 kg/m^2)	0	0	0	0	
Normal body weight (BMI 18.5-24.9 kg/m^2)	29	87.9	28	84.8	
Overweight (BMI 25-29.9 kg/m^2)	4	12.1	5	15.2	
Mean±SD	21.75±2.41		22.01±2.50		0.279^{ns}
Range (Minimum-Maximum)	19-28		19-29		

ns= not significant, p value reached from Unpaired t- test

Table 3: Distribution of different reproductive factors in women with CIN and healthy cervix group (N= 66)

Reproductive factors	CIN (n= 33)		Healthy cervix (n= 33)		OR (95% CI)	p-value
	n	%	n	%		
Age during 1st intercourse (years)						
≤15 years	13	39.4	5	15.2	3.64 (1.12-11.84)	0.027^{s}
>15 years	20	60.6	28	84.8		
Mean±SD	16.62±2.97		15.85±3.1			
Range (Minimum-Maximum)	Dec-26		Dec-26			
Duration of sexual life						
>10 years	30	90.9	21	63.6	5.71 (1.43-22.77)	0.008^{s}
≤10 years	3	9.1	12	36.4		
Mean±SD	20.47±10.41		22±6.76			
Range (Minimum-Maximum)	Sep-56		14-40			
Parity						
Multipara	30	90.9	32	97	0.31 (0.03-3.17)	0.302^{ns}
Grand multipara	3	9.1	1	3		

s= significant, ns= not significant, p value reached from Chi-square test

Table 4: Distribution of study population according to *Lactobacillus* status in women with CIN and healthy cervix group (N= 66)

Lactobacillus status (copy/cmm)	CIN (n=33)		Healthy cervix (n=33)		OR (95% CI)	p value
	n	%	n	%		
0-1000 (copy/cmm)	30	90.9	2	6.1	155 (24.2-939.0)	0.001^{s}
>1000 (copy/cmm)	3	9.1	31	93.9		

s= significant, p value reached from Chi-square test

We performed univariate logistic regression analysis to observe any association in age, monthly income, duration of sexual life and *Lactobacillus* status between healthy cervix and CIN groups. In univariate analysis, age, monthly income, duration of sexual life and *Lactobacillus* status were found statistically significant association between healthy cervix and CIN. These variables were also selected for multivariate analysis by logistic regression model to find out the *Lactobacillus* status between healthy cervix and CIN (Table-5). We observed the predictor for cervical intraepithelial neoplasia (CIN) patients with different variables in multivariate logistic regression. Low *Lactobacillus* status had 4.034 times increased risk of developing CIN (95% CI=0.047-36.199) which was statistically significant (p<0.05). But, age, monthly income and duration of sexual life were not significantly (p>0.05) associated with CIN (Table- 5).

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Table 5: Predictor for CIN patients with different variables in logistic regression analysis

Variables	p value	OR	95% CI	
			Lower	Upper
Age	0.150^{ns}	0.646	0.356	1.172
Monthly income	0.139^{ns}	0.336	0.079	1.428
Duration of sexual life	0.168^{ns}	1.543	0.832	2.861
Lactobacillus status	0.024^{s}	4.034	0.047	36.199

ns= not significant, s= significant

Discussion

Total 66 participants were included in this study, among them 33 subjects had healthy cervix and 33 subjects had cervical intraepithelial neoplasia (CIN). Presence of cervicovaginal *Lactobacilli* species was evaluated in these two groups. Evaluating different socio-demographic factors between women with healthy cervix and CIN revealed that, almost three fourth (72.7%) study women belonged to the age group >35 years in CIN and 54.6% women with healthy cervix was in that age group, which was statistically significant ($p<0.05$). More than 35 years age had 2.83 times increased risk to develop CIN (OR=2.83; 95% CI=1.02-7.90). In this study the mean age of the women with CIN was 37.56±10.59 years. Regarding educational status, it was observed that almost two third (66.7%) of the study population were illiterate in CIN group and that was 36.4% in healthy cervix group, which was statistically significant ($p<0.05$). Illiterate patients had 3.50 times increased risk to develop CIN (OR=3.50; 95% CI=1.27-9.64). In accordance, Nessa *et al*., reported that in the age group of less than 29 years, 19.01% women developed high grade pre-cancerous state, this condition increased to 45.42% between 30 -39 years, however that declined to 25.06% during 40-49 years age and further declined to 10.51% after 50 years of age [21]. In another study the mean age of the women with high-grade squamous intraepithelial lesions (HSIL) was found 26 years [22]. It was reported that low socio-economic condition is one of some reasons for the high burden of CIN [23]. In the context of monthly family income, we found that majority (69.7%) of the study participant's monthly family income was less than 20,000 BDT (BDT=Bangladeshi Taka/ Bangladeshi currency) in CIN group and that was 12.2% in healthy cervix group, which was statistically significant ($p<0.05$). It was observed that, low monthly family income had 22.6 times increased risk to develop CIN (OR=22.6; 95% CI=4.63-60.11). Nessa *et al*., also reported that low monthly family income was significantly associated with CIN, which was an agreement to our study [21]. In our study, distribution of study population in different body mass index (BMI) categories showed that majority (87.9%) of women with healthy cervix and most (84.8%) of the women with CIN had normal body weight (BMI between18.5-24.9 kg/m^2). The mean BMI was 21.75±2.41 kg/m^2 in women with healthy cervix group and that was 22.01±2.50 kg/m^2 in women with CIN group, the difference of BMI was not statistically significant ($p>0.05$) between the groups. It was reported that the risk of cervical intraepithelial neoplasia (CIN) was positively associated with BMI and inversely associated with physical activity [24]. In this study, the distribution of different reproductive factors in CIN and healthy cervix group showed that, more than one third (39.4%) of women age during 1st intercourse was ≤15 years in CIN group and 15.2% in healthy cervix group, which was statistically significant ($p<0.05$). Age during1st intercourse ≤15 years had 3.64 times increased risk to develop CIN (OR=3.64; 95% CI=1.12-11.84). The mean age during 1st intercourse was 16.62±2.97 years in CIN group and that was 15.85±3.1 years in healthy cervix group. We found majority (90.9%) of the women with CIN had duration of sexual life span was >10 years and only 21(63.6%) women with healthy cervix group had such duration of sexual life span, which was statistically significant ($p<0.05$). It was observed that duration of sexual life span >10 years had 5.71 times increased risk to develop CIN (OR=5.71; 95% CI=1.43-22.77). The mean duration of sexual life was 20.47±10.41 years in women with CIN and 22±6.76 years in women with healthy cervix group. In the distribution of parity, it was observed that majority (90.9% and 97.0%) of women had multipara in both CIN group and women with healthy cervix group. The difference of parity was not statistically significant ($p>0.05$) between CIN and healthy cervix groups. These findings were consistent with a couple of previous studies [21-22, 25-26].

In our study, the distribution of study population according to *Lactobacillus* status in CIN and healthy cervix showed that, majority (90.9%) study women had *Lactobacillus* level between 0-1000 copy/cmm in CIN group and only 2(6.1%) women with healthy cervix group had *Lactobacillus* level between 0-1000 copy/cmm, which was statistically significant ($p<0.05$). It was revealed that, low *Lactobacillus* level (0-1000 copy/cmm) had 155 times increased risk to develop CIN (OR=155; 95% CI=24.2-939.0). It was documented that, changes in the vaginal micro ecological environment leads to multiple gynecological diseases including CIN and cervical cancer [27]. Previous research has shown that *Lactobacillus* may play an important role in the occurrence and development of CIN and cervical cancer [28]. Recent study revealed that, the bacteria in the vagina maintain a dynamic balance under physiological conditions, but the imbalance of vaginal flora leads to multiple gynecological diseases, such as colitis, high-grade cervical intraepithelial neoplasia (CIN), and cervical cancer [28-30]. On a similar note, Curty *et al*., stated that the cervicovaginal microbiome

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is a dynamic network of microbes able to modulate a host's immune responses and promote an environment susceptible to viral infection acquisition and development of CIN [30]. They also emphasized that the association between high-diversity cervical microbiota and HPV infection, CIN and cervical cancer [30]. Thus, specific bacteria or the high diversity microbiota may function as biomarkers for cervical alterations, and can as well as be used to identify women at high risk to develop persistent HPV infection, CIN, and cervical cancer [30]. By univariate logistic analysis, age, monthly income, duration of sexual life and *Lactobacillus* status were found statistically significant association between healthy cervix and CIN. These variables were also selected for multivariate analysis by logistic regression model. In multivariate logistic regression the predictor for CIN patients with different variables showed that, low *Lactobacillus* level had 4.034 times increased risk of developing cervical intraepithelial neoplasia (CIN) (OR=4.034; 95% CI=0.047-36.199) which was statistically significant ($p<0.05$). However, age, monthly income and duration of sexual life were not significantly ($p>0.05$) associated with CIN. This result was supported by a couple of related studies [30-32]. In a similar study, Plummer *et al*., reported that early age at first sexual intercourse (AFI) is an important risk factor for CIN and other risk factors include lifetime number of sexual partners, young age at first full-term pregnancy and number of full-term pregnancies [25]. Champer *et al*., emphasized that a healthy vaginal microbiome dominated by *Lactobacillus* species may have a protective effect against pathogens and have therapeutic potential [7]. Vaginal dysbacteriosis, characterized by low level of vaginal *Lactobacillus* species and predominance of *Gardnerella Vaginalis* alone or in complex with other anaerobic bacteria, aerobic vaginitis and other sexually transmitted vaginal pathogens may be an HPV-dependent cofactor for cervical neoplasia development [11]. It was documented that *Lactobacillus* species markedly decreased in CIN compared to healthy individuals [31]. Therefore, it is noteworthy that current study provides a theoretical basis for further clinical application of the role of *Lactobacillus* in CIN.

Conclusion

This study documented that *Lactobacillus* status in CIN is significantly low. Low level of *Lactobacillus* has 4.034 times increased risk to develop CIN. Therefore, the relative paucity of *Lactobacillus* is important causative factor for the development of CIN. Moreover, there are ample data which emphasized that women with CIN have a high diversity in vaginal microbiota and depletion of *Lactobacillus* species. Thus, it can be inferred that the concept of manipulation of vaginal *Lactobacillus* by using probiotics may be an exciting prospect in the field of preventive measure in CIN development.

Limitations of the study

It was a single centre study with a relatively small sample size. Moreover, the present study was conducted at a very short period of time.

Recommendation

A large population based multicentre study should be done to confirm the results of this current study.

Conflicts of interest

All authors declared that they have no conflict of interest regarding this publication.

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