ORIGINAL ARTICLE

Changes in the diversity of local cervical bacteria in women with cervical cancer receiving antineoplastic treatment

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Background: Some studies show changes in the microbiota in people undergoing antineoplastic treatment. Currently, there is not enough evidence of this effect in the treatment of cervical cancer (CC). The objective was to determine changes in the diversity of local cervical bacteria in women with CC receiving chemotherapy, radiotherapy, and brachytherapy. **Materials and Methods:** A descriptive, longitudinal, and prospective study was conducted in 68 women with locally advanced CC with a treatment plan based on the administration of chemotherapy, external beam radiotherapy, and brachytherapy. Cervical-vaginal fluid samples were taken during antineoplastic treatment. The samples were used to isolate bacterial strains. The bacteria were identified at the molecular level by comparing sequences of the 16S ribosomal RNA gene. **Results:** The bacteria identified belonged to three phyla: Firmicutes, Proteobacteria, and Actinobacteria. Nine genera and 25 species of bacteria were identified. The most frequent species were *Staphylococcus epidermidis, Corynebacterium amycolatum*, and *Enterococcus faecalis*. There were statistically significant differences when comparing bacterial diversity found in the different stages of treatment (≤0.05). Bacterial diversity decreased as antineoplastic treatment progressed and increased at the end of therapy. **Conclusion:** Antineoplastic treatments generate changes in the diversity of local cervical bacterial communities of women with CC.

Key words: Aerobic-culturable bacteria, antineoplastic treatment, cervical cancer, cervicovaginal bacteria

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INTRODUCTION

In recent years, evidence that bacterial microbiota plays an essential role in the carcinogenesis and pathophysiology of human cancer has increased. Currently, it has been shown that infection with *Helicobacter pylori* stimulates the development of gastric carcinoma,[1-3] *Fusobacterium nucleatum* is involved in colorectal tumorigenesis and modulation of the tumor microenvironment.[4] However, what is the role of the bacterial communities in the context of cancer treatment?

Some studies have published the impact of intestinal commensal bacterial species on the metabolism, efficacy, and toxicity of chemotherapeutic drugs, showing that the microbiota regulates the response to different types of cancer chemotherapy by affecting their mechanism of action and toxicity.[5-7]

The knowledge of the impact of the bacterial microbiota in the treatment of gastrointestinal cancer is widely studied. However, there is still a deficiency in the analysis of the impact of bacterial microbiota on

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the treatment of other types of cancer such as cervical cancer (CC).

In Mexico, CC represents the second cause of death in women.[8] This figure is because 6 out of 10 women are diagnosed in advanced stages of the disease. This stage of the disease implies the need to administer treatments based on chemotherapy, radiotherapy, and brachytherapy. These treatments are not always effective.[9,10] The public health system subsidizes most treatments. This financial contribution represents a considerable expense for the government.

Recently, Tsakmaklis *et al*.[11] showed that cervical microbiota has a considerable reduction after chemoradiation. However, they did not find modifications in the composition of the cervical microbiota under antineoplastic treatment. At this point, we do not know if quantitative changes could be a synergistic factor for better treatment outcomes or vice versa. More studies are needed to evaluate both quantitatively and qualitatively the changes in the cervicovaginal microbiota and its role in response to antineoplastic treatment.

Therefore, we established the aim of evaluating the changes in the composition of the local cervicovaginal microbiota of women with CC under antineoplastic treatment. We hypothesized that the studied cervicovaginal microbiota would change during chemotherapy, radiotherapy, and brachytherapy.

MATERIALS AND METHODS

This study was conducted at the National Institute of Cancerology of Mexico (INCan) and the Autonomous University of the State of Mexico, from February 2016 to February 2018. The study protocol was reviewed and approved by the ethics and research committees of INCan, with the code 016/11/ICI-CEI/1016. Eligible women signed their informed consent. All procedures were carried out following regulations governing the protection of human subjects.

Study design and population

A descriptive, longitudinal, prospective study was carried out. A total of 68 untreated women who were recently diagnosed with locally advanced CC (I-B2 to IV-A FIGO stages) were included.[12] The diagnosis was histologically confirmed.

Women over the age of 18 were included, subdivided for analysis into women of fertile age (18–44 years) and women in menopause (older than 45 years). All had as a treatment plan the administration of concomitant chemotherapy; radiotherapy from external beam to the whole pelvis and high or low rate brachytherapy.[13,14]

Exclusion criteria included pregnant women; women who used systemic or local antimicrobials or antifungals for at least 4 weeks before taking the sample; women who had sexual intercourse 48 h before taking the sample; those who douched; and those who were menstruating on the day of sampling. Women with concomitant diseases were also not included. Participants were eliminated from the study if they presented any criteria for exclusion in the follow-up measurements.

Characteristics of the study group

A questionnaire was applied to obtain sociodemographic and gynecological-obstetric data. The symptoms reported by the patients at the end of each treatment, including pruritus, abnormal vaginal discharge, foul-smelling discharge, colored discharge, pelvic pain, dysuria, and tenesmus, were obtained from the clinical histories. In the same way, the data related to the disease and the administration of the treatment were obtained.

Antineoplastic treatment

Total external radiotherapy was applied to the pelvis with doses of 45–50.4 Gy covering the uterus, parametrium, vagina, common iliac nodes, internal and external, obturators, and presacrum. Concomitant chemotherapy based on cisplatin was administered weekly 40 mg/m^2. Chemotherapy and radiotherapy treatment lasted for 5 weeks.

Subsequently, brachytherapy was administered in one of the two available forms: low rate 2 Gy/h for a final dose of 30–55 Gy or high rate 12 Gy/h for a final dose of 30–55 Gy. The treatment with brachytherapy lasted 4 days.

Response to treatment was assessed 6 weeks after the completion of brachytherapy. The evaluation was based on changes in tumor size, observed by computed tomography scan and Response Evaluation Criteria In Solid Tumors criteria.[15] It was taken as a cure or complete response to the disappearance of all lesions and pathological nodes. It was taken as a persistent disease when the criteria for complete response were not met.[15]

If the disease persisted, patients took 8 weeks off before starting second-line chemotherapy. For the cure response to treatment, patients took 12 weeks off as a control period before being discharged.

Sampling

From each of the participants, four samples of cervicovaginal fluid were taken by swab throughout the antineoplastic treatment, using the following scheme:

- Initial assessment (IA): The initial sample was taken at the time of establishing the diagnosis of the presence of locally advanced CC

- Postchemotherapy and radiotherapy assessment (P-QRT): The second sample was taken at the end of the concomitant chemotherapy and radiotherapy treatment
- Postbrachytherapy assessment (P-BT): The third sample was taken at the end of the brachytherapy treatment
- Final assessment (FA): The fourth sample was taken after the rest period before second-line chemotherapy or after the period of monitoring before discharge, as appropriate.

Sample processing

The procedures for microbiological analysis from sample processing to genetic identification were modified from previous publications and are described below.[16-19] Under sterile conditions, the fibers were separated from the swab, placed in a 2.0 ml Eppendorf tube; 1 ml of saline was added and centrifuged at 14,000 rpm for 15 min. The remaining suspension was used to isolate bacteria.

Culture and isolation media

An aliquot of 200 µl of the suspension obtained from the sample was inoculated on a brain heart infusion (BHI; Becton Dickinson, Cat. No. 214700) agar plate, and 200 µl was inoculated in a Petri dish with blood agar (Becton Dickinson, Cat. No. 220150). The plates were incubated at 37°C for 48 h under aerobic conditions. Subsequently, the predominant colonies were isolated and purified.

Extraction of DNA

Pure cultures growing for 48 h at 37°C on IHB agar were used to obtain the biomass. The biomass was recovered by centrifugation, and DNA extraction was carried out using the Promega Wizard® genomic kit (Cat. No. A1120) according to the manufacturer's instructions.

Amplification of the 16S ribosomal RNA gene

The 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using the obtained DNA and *Taq* DNA polymerase (MyTaq; Bioline, Cat. No. BIO-21105). The following universal primers were used:

- 27f: 5′-AGAGTTTGATCMTGGCTCAG-3′
- 1492r, 5′-TACGGYTACCTTGTTACGACTT-3′.

The following thermal cycling conditions were used for the amplification of the gene: initial denaturation at 94°C for 5 min, followed by 29 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 30 s, and extension at 72°C for 1 min. The final extension was carried out for 10 min at 72°C.

The amplified fragments were separated by 1% agarose gel electrophoresis (CondaPronadisa, Cat. No. 8100.10) in TAE buffer (Invitrogen, Cat. No. 24710-030) at 120 V for 40 min and then stained with ethidium bromide (Sigma, Cat. No. E7637-1G).

Ribotyping of 16S ribosomal RNA by polymerase chain reaction-restriction fragment length polymorphism

In a sterile Eppendorf tube, 10 µl of a 16S rRNA amplicon was digested with the *Msp*I enzyme (Promega, Cat. No. R6401) according to the manufacturer's specifications. The same procedure was performed with the *Rsa*I enzyme (Promega, Cat. No. R6371).

The restriction products were separated by electrophoresis in a 1.5% agarose gel (CondaPronadisa, Cat. No. 8100.10) in TAE buffer (Invitrogen, Cat. No. 24710-030) at 120 V for 80 min and stained with ethidium bromide (Sigma, Cat. No. E7637-1G), with 1-kb DNA ladder used as a molecular weight marker (Thermo Scientific, Cat. No. 5M0311).

The restriction patterns generated were analyzed by the number of bands and their sizes concerning the molecular weight marker. For grouping analysis, profiles containing at least two bands were included. A group of strains with identical restriction enzyme profiles was defined as a ribotype.

Identification of isolated strains

The selection of strains for genetic identification was based on the established ribotypes. The second round of PCR amplification of the 16S rRNA gene was performed, as described previously, using the DNA of each strain selected. The products of this amplification were purified using an Amicon Ultra® centrifugal filter unit (Millipore, Cat. No. UFC500308) and sent to a sequencing service (Macrogen, Rockville, MD, USA).

The obtained sequences were analyzed and edited using ChromasPro version 2.6.4 and BioEdit version 5.0.9. Consensus sequences were constructed and compared using the Basic Local Alignment Search Tool program, with sequences deposited in GenBank of the National Biotechnology Information Center as well as in the public database EzBiocloud. The obtained sequences were deposited in the GenBank of the National Center for Biotechnology Information with access numbers MH108987-MH109026, MK131292-MK131312.

Data analysis

The sociodemographic, gynecological-obstetric, the disease, and isolated strains characteristics were analyzed using descriptive statistics. We compared the proportion of women who presented the identified bacterial species, by stratified subgroup by fertile age and menopause, as well as the proportion of women who presented the species identified through antineoplastic treatments by Fisher's

exact test. Stata version 15 was used for the analysis of all evaluated data.

RESULTS

At the start of the study, 68 women with locally advanced CC were included. Throughout the study, there were losses to follow-up due to participants dropping out of the study, changing treatments, or death. The sample size at follow-up stages was $n = 68$ in IA, $n = 57$ in P-QRT, $n = 54$ in P-BT, and $n = 50$ in FA. The loss to follow-up was 26%.

Study group characteristics

The mean age of the patients was 45 years, and the mean education was six years; 64% had no family history of cancer, 55% were menopausal, 53% had more than three children, and 52% did not use contraception. According to the clinical stage of the disease, 15% of patients were in Stages IB2 to IIA2 (without parametrial invasion); the remaining 85% were in Stages IIB to IVA (with parametrial invasion). An epidermoid tumor was diagnosed in 89% of the participants, with an average size of 5 cm.

At the IA, 76% of the patients reported abnormal vaginal discharge with a foul odor and pruritus. At the P-QRT, 4% of the patients reported pruritus, 6% presented with abnormal vaginal discharge, 3% reported vaginal discharge with a foul odor and no changes in coloration, and 11% reported dysuria. During the P-BT, only one patient reported abnormal vaginal discharge with a foul odor. Finally, during FA, only one patient presented with dysuria, whereas the rest of the evaluated symptoms were absent in all the members of the study.

Treatment characteristics

For chemotherapy, 98% of patients received Cisplatin, 2% of patients received Vinorelbine because of their low tolerance to Cisplatin. The average dose of chemotherapy was 65.07 mg. The average dose of external radiation was 50.45 Gy. For brachytherapy, 68% of the patients received high rate brachytherapy, and 32% received low rate brachytherapy with total dose averages of 23 Gy and 35 Gy, respectively. Finally, 94% of the patients had a complete response to the treatment, and 6% had persistence of the disease.

Isolated strains and ribotyping by polymerase chain reaction-restriction fragment length polymorphism of 16S ribosomal RNA

From the initial samples, 116 strains were isolated, which were divided into 12 ribotypes. From postchemotherapy and radiotherapy, 71 strains were isolated, nine ribotypes were obtained. From the postbrachytherapy samples, 44 strains were isolated and grouped into nine ribotypes. Finally, from the samples collected at the FA, 66 strains were isolated, which were divided into 15 ribotypes.

Identification of isolated strains

The identification by sequencing of the 16S rRNA gene was performed on sixty strains. The consensus sequences obtained were between 1339 bp and 1421 bp, with percentages of similarity between 97% and 100%. The bacteria identified belonged to three phyla: Firmicutes, Proteobacteria, and Actinobacteria. A total of nine bacterial genera and 25 bacterial species were identified [Table 1].

Bacterial diversity

The most frequent bacterial species in the different treatment stages were in the IA *Staphylococcus epidermidis*, found in 51% of the women evaluated, and *Corynebacterium amycolatum*, present in 41% of the women evaluated. In the P-QRT evaluation, the most frequent bacterial species were *S. epidermidis*, found in 49% of the women evaluated, followed by *Enterococcus faecalis*, found in 40% of the women studied. In the P-BT evaluation, *S. epidermidis* was found in 20% of the women evaluated, followed by *E. faecalis* in 16%. In FA, 36% of the women presented *S. epidermidis*, and 16% presented *Streptococcus agalactiae*. The frequency of the remaining bacteria identified is presented in Table 1. There were statistically significant differences when comparing the proportion of women who presented the bacterial species found in the different stages of antineoplastic treatment [Table 1].

Bacterial diversity decreased as antineoplastic treatment progressed. In IA, 12 species were detected, representing 48% of all identified bacteria. In the P-QRT and P-BT assessments, nine of the 25 species identified in the study were found presenting 36% of the identified bacteria. Finally, in FA, 15 bacterial species were detected, representing 60% of all bacteria identified. When evaluating the differences in proportions in this area, no statistically significant data were found.

According to the clinical stage (evaluated before antineoplastic treatment), the stages without parametric invasion (IB2, IIA, IIA1, and IIA2) presented eight species out of the 12 identified in the initial assesment (IA): *S. epidermidis, C. amycolatum, E. faecalis, Bacillus safensis, Lactobacillus rhamnosus, Escherichia fergusonii, Corynebacterium, Jeikeium* and, *Bacillus malikii*. That is, 66% of the total number of species identified at that stage. The parametric invasion stages (IIB, IIIA, IIIB and, IVA) presented the 12 species identified (100%): *S. epidermidis, C. amycolatum, E. faecalis, E. fergusonii, S. agalactiae, C. jeikeium, B. safensis, Streptococcus urinalis, L. rhamnosus, B. malikii, Escherichia coli*, and *Corynebacterium striatum*. Statistically significant differences were found in the presence of

Table 1: Local cervical bacteria of women with cervical cancer identified in the different stages of antineoplastic treatment

Ribotype	Identified species	IA (*n*=68), *n* (%)	P-QRT (*n*=57), *n* (%)	P-BT (*n*=54), *n* (%)	FA (*n*=50), *n* (%)	*P**
1	*S. epidermidis*	35 (51)	28 (49)	12 (20)	18 (36)	<0.01
2	*E. faecalis*	14 (20)	23 (40)	9 (16)	6 (12)	<0.01
3	*E. coli*	3 (4)	5 (9)	4 (7)	10 (2)	0.04
4	*C. amycolatum*	24 (41)	5 (9)	5 (9)	2 (4)	<0.01
5	*S. agalactiae*	6 (9)	-	-	8 (16)	<0.01
6	*E. fergusonii*	8 (11)	-	-	-	<0.01
7	*S. urinalis*	4 (6)	-	-	-	0.01
8	*B. safensis*	6 (9)	-	-	-	<0.01
9	*B. malikii*	4 (6)	-	-	-	0.01
10	*C. jeikeium*	6 (9)	-	-	-	<0.01
11	*C. striatum*	1 (1)	-	-	-	1.00
12	*L. rhamnosus*	5 (7)	-	-	-	<0.01
13	*E. durans*	-	5 (9)	7 (13)	7 (14)	<0.01
14	*S. anginosus*	-	1 (2)	-	-	0.70
15	*S. pneumoniae*	-	1 (2)	1 (2)	-	0.58
16	*S. pasteurianus*	-	2 (3)	4 (7)	4 (8)	0.70
17	*C. lipophiloflavum*	-	1 (2)	-	-	0.70
18	*S. dysgalactiae* subsp dysgalactiae	-	-	1 (2)	2 (4)	0.70
19	*O. urethralis*	-	-	-	1 (2)	0.04
20	*S. oralis*	-	-	1 (2)	2 (4)	<0.01
21	*C. coyleae*	-	-	-	1 (2)	0.05
22	*S. mitis*	-	-	-	3 (6)	0.01
23	*M. luteus*	-	-	-	1 (2)	0.21
24	*C. lactis*	-	-	-	1 (2)	0.21
25	*S. dysgalactiae* subsp equisimilis	-	-	-	1 (2)	0.21

**P*≤0.05. *P*: Significance of Fisher's exact test for comparison of proportions of women who presented bacterial species identified by assessment stage. IA=Initial assessment; P-QRT=Postchemotherapy and radiotherapy assessment; P-BT=Postbrachytherapy assessment; FA=Final assessment; -=Absent; *S. epidermidis=Staphylococcus epidermidis; E. faecalis=Enterococcus faecalis; E. coli=Escherichia coli; C. amycolatum=Corynebacterium amycolatum; S. agalactiae=Streptococcus agalactiae; E. fergusonii=Escherichia fergusonii; S. urinalis=Streptococcus urinalis; B. safensis=Bacillus safensis; B. malikii=Bacillus malikii; C. jeikeium=Corynebacterium jeikeium; C. striatum=Corynebacterium striatum; L. rhamnosus=Lactobacillus rhamnosus; E. durans=Enterococcus durans; S. anginosus=Streptococcus anginosus; S. pneumoniae=Streptococcus pneumoniae; S. pasteurianus=Streptococcus pasteurianus; C. lipophiloflavum=Corynebacterium lipophiloflavum; S. dysgalactiae=Streptococcus dysgalactiae; O. urethralis=Oligella urethralis; S. oralis=Streptococcus oralis; C. coyleae=Corynebacterium coyleae; S. mitis=Streptococcus mitis; M. luteus=Micrococcus luteus; C. lactis=Corynebacterium lactis; S. dysgalactiae=Streptococcus dysgalactiae*

S. epidermidis (P < 0.01), which occurred in 100% of the women with invasive parametric cancer and only 44% of women with non-parametric invasive cancer.

There were no statistically significant differences when evaluating differences in high rate versus low rate brachytherapy. There were no statistically significant differences in bacterial diversity when stratified by reproductive age and menopause or by chemotherapeutic agents administered.

DISCUSSION

The present report shows an analysis of the changes in the cervicovaginal bacterial composition evaluating the local species present. Only two reports about cervicovaginal microbiota and its changes under antineoplastic treatment have been published at this time. Mubangizi *et al*.[20] determined the aerobic cervical bacteria prevalent in patients with advanced CC before and after external beam radiotherapy. Bacterial identification was carried out by biochemical methods only. In the study, it was reported that the bacterial microbiota was increased after 4 weeks of radiotherapy.[20]

On the other hand, Tsakmaklis *et al*.,[11] in 2020, evaluated by molecular methods, quantitative and qualitative changes in cervical microbiota in 8 patients undergoing chemotherapy and radiotherapy. In this study, the authors reported a considerable reduction in cervical bacterial load after chemotherapy and radiotherapy. The quantitative changes are of great importance and provide new knowledge on the subject; however, they do not report changes in the composition of the microbiota evaluated.

Our study complements the little evidence that has been described to date. We evaluated a significant number of women (n = 68). Changes were observed in the different stages of locally advanced cancer, indicating that, when the cancer is established, the more advanced stages present a higher number of local bacterial species. This observation is

consistent with the findings of Tango *et al*.,[21] who concluded that the variation in diversity might be associated with atypical health conditions. The greater diversity found may show a higher probability that the bacterial community present is not healthy. Kwasniewski *et al*.[22] suggest that there may be an association between HPV infection, a decrease in the abundance of *Lactobacillus* species, and an increase in other bacterial species.

The changes in bacterial composition were analyzed at four stages of treatment, in contrast to what was reported by Tsakmaklis *et al*.[11] and Mubangizi *et al*.[20] Our results show that chemotherapy and brachytherapy decrease local bacterial diversity, in contrast to previous reports. We also show that diversity increases again over time, after the completion of the antineoplastic therapy.

Previous data about the cervical bacterial community of healthy women generally define it as a community dominated by lactobacilli. However, it has now also been reported that a high percentage of Latina women are characterized by a combination of nonlactobacillary communities.[23,24] In our study, the bacterial communities evaluated throughout the treatment showed only the presence of one species of Lactobacillus. This finding does not prove that the *Lactobacillus* genus is not present in the population group evaluated. However, our results could show that there is a numerical prevalence of the other bacterial species, forming a community with a low lactobacillary presence, which differs widely from the concept of a healthy cervical bacterial community. These results are in line with what has been described by Di Paola *et al*.[25]

We also showed that, when patients received chemotherapy and radiotherapy, they had a different bacterial community than that found when receiving brachytherapy. Finally, the bacterial community found at the beginning of the study was also different from the bacterial community found at the end of the treatments.

Within the limitations of the study, we can consider that only the culture-dependent aerobic bacterial community was evaluated. The analysis of the culture-dependent anaerobic bacterial community is proposed, as well as the analysis of the nonculture-dependent bacterial community. A control group with healthy women was not evaluated either. The aim of the study was not to compare women with CC and women without CC but to compare the change in microbiota in the presence of antineoplastic treatment. For this reason, this study was established longitudinally, so the initial measurement worked as a control group to evaluate the bacterial community present before the treatments. Future studies may consider the option of including a healthy control group.

CONCLUSION

Our results support the hypothesis that antineoplastic treatments change the composition of the cervicovaginal microbiota. With this study, it is possible to raise new hypotheses and proposals to evaluate if these changes influence the response to treatment and the improvement of the quality of life of patients with CC.

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Conflicts of interest

There are no conflicts of interest.

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