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Detection of *Actinomyces* spp. in cervical exudates from women with cervical intraepithelial neoplasia or cervical cancer

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Abstract

Purpose. Under certain circumstances, *Actinomyces* behaves as an opportunistic microorganism and can cause actinomycosis, a chronic and inflammatory granulomatous infection. The purpose of this project was to detect the presence of *Actinomyces* in cervical exudates from women with cervical intraepithelial neoplasia (CIN) and women with cervical cancer.

Methodology. Cervical samples from 92 women were divided into three groups: CIN, cervical cancer and healthy women. Metagenomic DNA extraction was performed following the Qiagen QIAamp Mini Kit protocol. A specific fragment (675 bp) was amplified by PCR in order to detect the presence of *Actinomycetales*. Samples in which *Actinomycetales* was detected were subjected to separate amplification reactions with primer pairs for *A. israelii, A. viscosus, A. meyeri* and *A. odontolyticus.* Amplified products were observed by 2 % agarose gel electrophoresis.

Results. *Actinomyces* were found in 10 % of women with CIN, 36.6 % of women with cervical cancer and 9 % of healthy women. The species identified in this study were *A. meyeri* in 14/92 samples (15.2 %), *A. viscosus* in 10/92 samples (10.8 %), *A. odontolyticus* in 4/92 samples (4.3 %) and *A. israelii* in 6/92 samples (6.5 %).

Conclusion. Patients with cervical cancer had a higher prevalence of the presence of *Actinomyces* compared to the CIN and control groups. This is the first study in which a deliberate search of this genus has been performed in women with cervical pathologies. The use of specific primers for each species facilitated their detection in comparison with traditional isolation methods. More information is necessary to understand the molecular mechanisms involved in the complex role that bacterial communities may play in the development of cancer (and vice versa).

INTRODUCTION

Actinomyces is a bacterial genus that belongs to the order *Actinomycetales* and the phylum *Actinobacteria*, species of which are characterized by having 69–78 % guanine-cytosine (GC) content in their DNA [1, 2]. *Actinomyces* species are Gram-positive anaerobic or microaerophilic bacteria, which are non-motile and non-spore-forming and present filamentous growth [3]. Saprophytic *Actinomyces* species [1] form part of the microbiotas of the oropharynx, gastrointestinal tract and urogenital tract [4]. Under certain circumstances, *Actinomyces* species become opportunistic and can cause actinomycosis, which is an inflammatory and chronic suppurative granulomatous infection that forms abscesses and fistulas [5] and often mimics malignant tumours, tuberculosis and nocardiosis [4]. These bacteria have a very low virulence potential linked to their fimbriae, which require interruption of the normal mucosal barriers [6] by trauma, a neoplasia, surgery, or an infection [7, 8]. It has been proven that they can infect the cervicofacial, thoracic, abdominal and pelvic areas [4, 9].

Currently, there is a shortage of epidemiological studies that provide information about the prevalence of pelvic actinomycosis, given the difficulties of the standard culture and identification techniques, which result in the use of molecular

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Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; IUD, intrauterine device; MSA, microorganisms similar to *Actinomyces*.

techniques instead [8, 10]. There are, however, retrospective studies that have reported 'microorganisms similar to *Actinomyces*' (MSA) in a number of women, as revealed through Papanicolaou (Pap) tests [3, 11–13]. As the Pap test lacks specificity and sensitivity for bacterial identification, *Actinomyces* spp. are not reported at the species level [14]. The identification of *Actinomyces* species is complicated, and clinical and chemical findings from cases of pelvic actinomycosis are non- specific [8, 15, 16]. Therefore, a technique that allows for the quick and specific detection of *Actinomyces* species in samples of cervical exudates is required.

Given the opportunistic nature of these bacteria, it would be interesting to study their behaviour in conditions such as cancer and premalignant lesions, which are bodies that involve immunosuppression [17]. For this reason, in this study we analysed the frequency of *Actinomyces* in women with cervical intraepithelial neoplasia (CIN, a premalignant condition that is divided into three stages, CIN 1, CIN 2 and CIN 3, depending on the thickness of the compromised cervical epithelium) [18] and women with cervical cancer, and compared the results with those for women without cancer or CIN.

METHODS

Study participants

A cross-sectional study was carried out on a total of 70 cases from two hospitals in the central area of Mexico from 2015 through 2016, after approval by the local ethics and research committees at both hospitals. Twenty-two healthy women were included as controls. The characteristics of the participants in the three study groups were as follows.

Group 1. Patients with CIN 1–2. The 40 patients (age range, 21–63 years) in this group were referred by their family doctor to the dysplasia unit of hospital 1, following abnormal Pap screening results. The different stages of CIN were verified by colposcopy and Pap tests.

Group 2. Patients with cervical carcinoma in situ *(CIN 3) or locally advanced cervical cancer of stages 1B2–3B and 4B.* The 30 patients (age range, 27–65 years) in this group, diagnosed with different stages of cervical cancer, were treated at hospital 2.

Group 3. Healthy women. The 22 women (age range, 24–65 years) in this group had no alterations in the cervix, as verified through Pap screening.

Sample collection

The objective of the project was explained to all the participants and they were asked to sign an informed consent form. Thereafter, they were given a questionnaire about their obstetrical and gynaecological history. The samples of cervical exudates taken were transported in screw-top test tubes in thioglycolate solution. In the case of patients with cervical cancer, both *in situ* and locally advanced, the samples were taken before the women had received any cancer treatment.

Metagenomic DNA extraction

The DNA extraction was carried out following the protocol of the QIAamp UCP Pathogen Mini Kit from Qiagen (Cat. no. 50214). The quality of the extracted DNA was evaluated through 1 % agarose gel electrophoresis.

Detection of the order *Actinomycetales*

To detect the presence of bacteria of the order *Actinomycetales* from the metagenomic DNA, a specific fragment (675 bp) was amplified through PCR using a pair of primers designed specifically for *Actinomycetales* by Kaya *et al.* [8], who also designed primer pairs for species of *Actinomyces* (*A. israelii*, *A. viscosus*, *A. naeslundii*, *A. meyeri* and *A. odontolyticus*) from their 16S rRNA sequences. The sequences of the specific primers and the expected length of the PCR products are shown in Table 1.

Detection of species of *Actinomyces*

The samples with an enhanced band of expected size (675 bp) for *Actinomycetales* underwent separate amplifications with the primer pairs for each species of *Actinomyces*. The PCRs for the *Actinomycetales* and *Actinomyces* species were carried out in a total volume of 25 µl containing PCR buffer, 10 pmol of each of the forward and reverse primers, 1 U of DNA *Taq* polymerase and 10 µl of DNA sample. All of the PCRs included a negative control containing nuclease-free water.

The PCR was carried out in a thermal cycler using the following conditions: initial denaturation for 10 min at 94 °C,

Table 1. Primers used in this study

Species	Primer pairs (5′–3′)	Amplicon size, in base pairs (Position of the bases)
Actinomycetales	GGCKTGCGGTGGTACGGGC	675 pb
	GGCTTTAAGGGATTCGCTCCRCCTCAC	(632–1306)
A. meyeri	TCTGCGATTACTAGCGACTCC	519 pb
	CCACCCGTGGTTTTCTGCG	(818–1337)
A. viscosus	TCTGCGATTACTAGCGACTCC	785 pb
	TCGTAGGCGGCTGGTCGC	(538–1323)
A. odontolyticus	TCTGCGATTACTAGCGACTCC	353 pb
	CGGCACTGCAGAGATGTYGTGG	(888–1241)
A. israelii	TCTGCGATTACTAGCGACTCC	339 pb
	GGGCCTCCCCTTTTTGGGCC	(783–1122)

followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 90 s at 72 °C. After the main cycle programme was complete, post-elongation was carried out for 10 min at 72 °C [8]. The amplified products were observed through 2 % agarose gel electrophoresis.

RESULTS

The most important clinical aspects of each group are as follows.

Group 1. In this group of patients, the average age was 37.5 years, 55 % were married, 50 % did not use any family-planning method during the time of the study and 52.5 % had used an intrauterine device (IUD) at some point in their lifes. The most common stage of the pathology was CIN 1, occurring in 55 % of the patients. The specific fragment of *Actinomycetales* was amplified in 10 % of the cervical samples, suggesting the presence of this order. The amplified species-specific fragments of *Actinomyces* had the following distribution: *A. meyeri* in one sample, *A. viscosus* in two samples, *A. odontolyticus* in one sample, and *A. israelii* in three samples.

Group 2. In this group of patients, the average age was 50.2 years, 40 % were married, 53.3 % did not use any family-planning method during the time of the study and only 23.3 % had used an IUD at some time in their lifes. The most common stage of the pathology was 2B (i.e. a tumour with parametrial invasion without reaching the pelvic wall), occurring in 56.6 % of the patients. The specific fragment for *Actinomycetales* was amplified in 36.6 % of the samples. At the species-specific level, *A. meyeri* was found in 11 samples, *A. viscosus* in six samples, *A. odontolyticus* in two samples and *A. israelii* in two samples.

Group 3. In this group of women, the average age was 40.3 years, 40.9 % were married, 45.45 % did not use any family-planning method at the time of the study and only 9 % had used an IUD at some point in their lifes. The presence of *Actinomycetales* was detected in 9 % of the samples. *A. meyeri* was found in two samples, *A. viscosus* in two samples, *A. odontolyticus* in one sample and *A. israelii* in one sample.

Table 2 shows the general characteristics of the three study groups.

Table 3 shows the clinical characteristics of the patients whose samples showed the presence of *Actinomyces*.

DISCUSSION

This study shows the relative frequency of *Actinomyces* in the vaginal tract of women with precancerous lesions, women with locally advanced and metastatic cervical cancer, and healthy women. *Actinomyces* was found in 10 % of the women with CIN, 36.6 % of the women with cervical cancer and 9 % of the healthy women. Although the results coincided with those of some studies carried out with healthy women, our study observed that the prevalence of *Actinomyces* was highest in women with cervical cancer.

In a study carried out in Mexico, Ramos *et al.* [19] identified a 7 % prevalence of *Actinomyces* in cervicovaginal secretions; all the women had used an IUD, and only two of them had symptoms of infection. Kaya *et al.* [8] identified an 8 % prevalence of *Actinomycetales* among 200 samples, of which eight (4 %) were found to be positive for species of *Actinomyces*.

However, there is a discrepancy between the results of different studies with regard to the prevalence of *Actinomyces* in the vaginal tract. For example, Kim *et al.* [20] found a 7 % rate of positive MSA in cervical smears from women using IUDs, with the occurrence varying according to the type and duration of use of the device. From 20 390 cervical samples, examined through Pap tests, they found MSA in 52 samples (0.26 %), of which 42 women (80.8 %) were IUD users. Demirezen *et al.* [3] observed MSA in 19 (0.83 %) of a total of 2290 cervicovaginal samples, also through Pap tests. This variation in results may be due to the different methods used for detecting *Actinomyces*, as well as the different sample sizes.

The identification of bacteria through molecular biology techniques is an efficient method, but it is more costly than culturing or Pap testing. In the case of *Actinomyces* species, which are slow-growing bacteria with strict growth conditions, molecular techniques are found to be more efficient and much more sensitive than culturing or Pap testing. Culturing may fail for various reasons, such as previous treatment with antibiotics or poor methodology [10]. Likewise, Kaya *et al.* [8] showed the advantages of PCR for Pap smears and demonstrated the low sensitivity of anaerobic bacteria culturing, mentioning that analysis through PCR appears to be a sensitive, precise and highly discriminatory diagnostic and classification method for *Actinomyces* species of clinical origin.

In our study, *A. meyeri* was found in 14 (15.2 %), *A. viscosus* in 10 (10.8 %), *A. odontolyticus* in four (4.3 %) and *A. israelii* in six (6.5 %) of the 92 samples tested. Of the species reported in this study, *A. odontolyticus* is generally found in the mouths of babies as well as in early dental bacterial plaque. Other species of *Actinomyces*, such as *A. georgiae*, *A. gerencseriae*, *A. israelii*, *A. naeslundii* and *A. meyeri*, have been found in gingival crevices, both in individuals with full periodontal health and in those without. In general, *Actinomyces* species have been isolated from supragingival and subgingival plaques, tonsils, dentin, root surfaces, carious processes, periodontal pockets and infected root canals [21].

A. israelii is one of the most commonly reported species in cases of pelvic actinomycosis, as shown by the study by Schaal *et al.* [22], in which 588 (56.4 %) of 1042 examined cases of actinomycosis carried the pathogen. Although in our study *A. israelii* was found in six of the 17 samples in which the presence of *Actinomyces* was detected, it was not the most common species. This leads us to question whether *A. israelii* is the most virulent species for humans, given that its mere presence is not indicative of actinomycosis, despite the fact that it has been found more frequently in

Table 2. General characteristics of the study groups

Study group	Average age *SD (range)	Marital status	Average gestations (SD)	Average no. of sexual partners (SD)	Family-planning method	Previous use of †IUDs	Time of use for IUDs, average years (range)	Disease stage	Patients with the presence of *Actinomyces*	Sample code
Group 1 *N*=40	37.5±10.7 (21–63)	Single 10	2.4 (2.08)	1.9 (1.07)	None 20	Yes 21	3.5 (1 week to 16 years)	‡HPVI 7	4	305
		Married 22			Condom 4			§CIN1 22		323
		Unmarried couple 4			Hormonal 0			CIN2 6		337
		Divorced 3			IUD 0			CIN3 3		
		Widowed 1			Salpingoclasia 16	No 19		Invasive cancer 1		338
					Other 0			Other 1		
Group 2 *N*=30	50.2±10.7 (27–65)	Single 6	4.1 (2.2)	2.2 (0.9)	None 16	Yes 7	9 (4–21 years)	IB2 1	11	P1
										P4
		Married 12			Condom 0			IIA1 6		P7
										P9
		Unmarried couple 8			Hormonal 7			IIB 17		P18
										P19
		Divorced 1			IUD 7			IIIA 1		P20
										P21
		Widowed 3			Salpingoclasia 0	No 23		IIIB 2		P22
										P23
					Other 0			IVB 3		P26
Group 3 *N*=22	40.3±9.5 (24–65)	Single 5	2 (1.3)	2.6 (2)	None 10	Yes 2	–	–	2	
		Married 9			Condom 4					C2
		Unmarried couple 5			Hormonal 1					
		Divorced 3			IUD 2	No 20				
		Widowed 0			Salpingoclasia 4					C13
					Other 1					
Total *N*=92	41.81±11.16 (21–65)	Single 21	2.8 (2.07)	2.19 (1.33)	None 46	Yes 30	4.7 (1 week to 21 years)	–	17	
		Married 43			Condom 8					–
		Unmarried couple 17			Hormonal 8					
		Divorced 7			IUD 9	No 62				
		Widowed 4			Salpingoclasia 20					
					Other 1					

*SD, standard deviation.

†IUD, intrauterine device.

‡HPVI, human papillomavirus infection.

§CIN, cervical intraepithelial neoplasia, IB2: tumour confined to the cervix, clinically visible lesion greater than 4 cm, IIA1: tumour beyond the cervix without reaching the pelvic wall, less than 4 cm tumour, IIB: tumour with parametrial invasion without reaching the pelvic wall, IIIA: tumour involving the lower third of the vagina not reaching the pelvic wall, IIIB: tumour spread to the pelvic wall, IVB: distant metastasis.

Table 3. Clinical characteristics of patients with *Actinomyces*

Study groups	Sample code	Detected *Actinomyces* species	Age	Disease stage	Gestations	Sexually active	No. of sexual partners	Previous use of *IUDs	Time of use for IUDs	Signs and symptoms	PAP
Group 1	305	*A. israelii*	46	†CIN1	3	Yes	2	Yes	3 months	–	CIN1, ‡HPV, mixed bacteria
	323	*A. meyeri* *A. viscosus* *A. odontolyticus* *A. israelii*	63	Invasive cancer	6	No	1	Yes	8 years	Bleeding	–
	337	*A. viscosus*	38	CIN1	3	Yes	2	No	–	–	–
	338	*A. israelii*	42	Other (bacterial condilomatosis)	3	Yes	2	Yes	1 year	Pruritus	Negative class II, inespecific inflammatory alterations
Group 2	P1	*A. meyeri* *A. viscosus* *A. israelii*	56	6: IVB: distant metastasis	2		2	No	–	–	–
	P4	*A. meyeri*	27	IIB	1	Yes	3	No	–	Abnormal vaginal discharge, metrorrhagia, dyspareunia, postcoital bleeding	–
	P7	*A. meyeri* *A. viscosus*	53	IIB	4		1	No	–	Abnormal vaginal discharge, metrorrhagia	–
	P9	*A. meyeri* *A. viscosus* *A. odontolyticus*	43	IIIB	7		3	No	–	Abnormal vaginal discharge	–
	P18	*A. meyeri*	65	IVB	7	Yes	2	No	–	Abnormal vaginal discharge, metrorrhagia, postcoital bleeding	–
	P19	*A. meyeri* *A. viscosus* *A. odontolyticus*	43	IIB	3	Yes	2	Yes	21 years	Metrorrhagia, dyspareunia, postcoital bleeding	–
	P20	*A. meyeri*	55	IB2	4		1	No	–	Abnormal vaginal discharge, metrorrhagia	–
	P21	*A. meyeri* *A. viscosus*	63	IIIB	9	Yes	2	No	–	Abnormal vaginal discharge, metrorrhagia, dyspareunia	–
	P22	*A. meyeri*	40	IIB	3	Yes	1	No	–	Abnormal vaginal discharge, metrorrhagia, dyspareunia, postcoital bleeding	–
	P23	*A. meyeri* *A. viscosus*	27	IIB	4		3	Yes	8 years	Abnormal vaginal discharge, metrorrhagia	–
	P26	*A. meyeri* *A. israelii*	45	IIA1	2	Yes	2	Yes	4 years	Abnormal vaginal discharge, metrorrhagia, postcoital bleeding	–
Group 3	C2	*A. meyeri*	40	–	1		1	No	–	–	–

Table 3. cont.

Study groups	Sample code	Detected *Actinomyces* species	Age	Disease stage	Gestations	Sexually active	No. of sexual partners	Previous use of *IUDs	Time of use for IUDs	Signs and symptoms	PAP
		A. viscosus *A. odontolyticus* *A. israelii*									
	C13	*A. meyeri* *A. viscosus*	46	–	0		2	No	–	Abnormal vaginal discharge	–

*IUD, intrauterine device.
†HPV, human papillomavirus.
‡CIN, cervical intraepithelial neoplasia, IB2: tumour confined to the cervix, clinically visible lesion greater than 4 cm, IIA1: tumour beyond the cervix without reaching the pelvic wall, less than 4 cm tumour, IIB: tumour with parametrial invasion without reaching the pelvic wall, IIIA: tumour involving the lower third of the vagina not reaching to pelvic wall, IIIB: tumour spread to pelvic wall, IVB: distant metastasis.

established infections. In contrast to the study by Schaal *et al.* [22], *A. meyeri* was the species with the highest prevalence in our study. Whether the differences are due to biogeographical or racial effects or originate from socioeconomic conditions merits further study.

The virulence and pathogenicity of these microorganisms may increase as a result of two important factors: one is the addition of the colonies in a cohesive way, and the other is the adherence of the bacteria to the host cells, which can be achieved through the surface or through proteins secreted by *Actinomyces* species, such as neuraminidase (sialidase) or superficial bacterial lectins. These factors protect bacteria from being swept away by normal cleaning and physiological functions, such as menstrual flow [3].

The relationship between the use of IUDs and the presence of *Actinomyces* is well documented in the literature. In this study, because neoplasia treatments required the patients to stop using IUDs, the time since the patients had stopped using the devices was a very prolonged period. For this reason, no relationship between the use of IUDs and the bacteria was found.

There is currently not enough information about the changes that occur in the microbiota of patients with cervical cancer, and it is not known how this varies with time. However, the cellular changes that are associated with this disease, in addition to environmental changes, could favour the presence of different microorganisms, both aerobic and anaerobic. From the results of this study, it is evident that there are significant changes in patients with cervical cancer, at least in the population of *Actinomyces*. The meaning of these findings is not yet understood on either the microbiological or the molecular level.

On the other hand, there are indeed reports about changes in the microbiota of human papillomavirus (HPV)-positive and HPV-negative patients. As Gao *et al.* [23] found, the bacterial diversity and composition are more complex in HPV-positive women than in HPV-negative women. *Gardnerella vaginalis* and *Lactobacillus gasseri* have been detected significantly more frequently in those positive for HPV. With regard to intracervical neoplasia, Oh *et al.* [24] reported that a microbial cervical composition characterized by the predominance of *Atopobium vaginae*, *G. vaginalis* and *Lactobacillus iners*, with a concomitant lack of *Lactobacillus crispatus*, is associated with a high risk of CIN. Mitra *et al.* [25] suggested that *Lactobacillus jensenii* and *Lactobacillus coleohominis* could be particularly protective in preventing the progression of dysplasia and, ultimately, a carcinogenic process, given that they were detected with higher prevalence in women with low-grade squamous intraepithelial lesions compared to women with high-grade lesions.

However, more information is needed to understand the molecular mechanisms involved in the complex role that the bacterial communities may play in the development of cancer (and vice versa), and what influence cancer could have on the prevalence of the different bacterial communities. Moreover,

further studies may allow us to consider the presence or increase of *Actinomyces* (or one of the species in particular) in the microbiota population as an aid in the early diagnosis of some of these pathological conditions.

In conclusion, in our sample, which included women with CIN or cervical cancer, the presence of *Actinomyces* was detected in 18.47 % with the use of specific primers for each species of this genus, which facilitated their detection in comparison with traditional isolation methods. This is the first study in which the presence of *Actinomyces* species has been detected in cervical samples from women, with and without neoplastic changes, using species-specific primers.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This project was approved by the Ethics and Research Committee of the Gyneco-Obstetrics Hospital of the Mexican Social Security Institute (IMSS) of the State of Mexico and was approved by the Ethics and Research Committee of the National Institute of Cancerology. All participants were informed about the project and the sampling procedure, and all of them signed a letter of informed consent.

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