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Micronucleus and FTIR spectroscopy analysis as screening tests for HPV vírus detection

Análises de micronúcleo e de espectroscopia FTIR como testes de triagem para a detecção do vírus HPV

Análisis de micronúleos y espectroscopia FTIR como pruebas de tamizaje para la detección del virus HPV

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Abstract
Human Papillomavirus (HPV) infection is the main precursor of cervical cancer. Pap Smear is the main diagnostic method of this neoplasm by cytological analysis, however presents a high rate of false-negative cases. This study aimed to verify if micronucleus (MN) frequency test and the Fourier Transform Infrared (FTIR) Spectroscopy analysis may be alternative useful techniques to detect HPV in cervical fluid. Fifty samples with normal cytology obtained from patients after cytopathological examination were divided into two groups based on the presence or absence of HPV by previous analysis of molecular biology. Of the fifty samples analyzed, 46% showed the presence of MN in the cells. The frequency of MN observed was higher in the samples with HPV positive and was related with age, use of oral contraceptives and use of alcoholic beverages ($p < 0.001$). FTIR analysis spectra showed high peak DNA and proteins in samples with a high frequency of MN (1170cm-1; 1516cm-1; 1404cm-1; 1473cm-1) compared with samples with absence of MN, probably due to chromosomal histones and MN constituents. However, no statistically significant difference was observed between these groups considering that the FTIR is a screening test and not specific. Thus, by the FTIR technique it was not possible to classify the samples by the spectral differences presented. The MN test can be considered a screening test for the detection of HPV, being possible to use it in clinical practice because it is a practical, simple, low-cost and non-invasive method. However, it is important to carry out studies in larger sample groups to investigate the application of the MN test and FTIR spectroscopy in the diagnosis of this infection and in the application in the prevention of cervical cancer.
Keywords: Micronucleus; Fourier Transform Infrared Spectroscopy; HPV.

Resumo
A infecção pelo Papilomavírus Humano (HPV) é o principal precursor do câncer do colo do útero. O Papanicolau é o principal método diagnóstico dessa neoplasia pela análise citológica, porém apresenta alto índice de casos falso-negativos. Este estudo teve como objetivo verificar se o teste de frequência de micronúcleos (MN) e a análise por espectroscopia de infravermelho transformada de Fourier (FTIR) podem ser técnicas alternativas úteis para detectar o vírus HPV no líquido cervical. Cinquenta amostras com citologia normal obtidas de pacientes após exame citopatológico foram divididas em dois grupos com base na presença ou ausência de HPV pela análise prévia de

biologia molecular. Das cinquenta amostras analisadas, 46% apresentaram a presença de MN nas células. A frequência de MN observada foi maior nas amostras com HPV positivo e relacionou-se com a idade, uso de anticoncepcional oral e uso de bebida alcoólica (p<0,001). As análises dos espectros de FTIR mostraram altos picos de DNA e proteínas em amostras com alta frequência de MN (1170cm-1; 1516cm-1; 1404cm-1; 1473cm-1) em comparação com amostras com ausência de MN, provavelmente devido a histonas cromossômicas e constituintes do MN. No entanto, não foi observada diferença estatisticamente significativa entre esses grupos considerando que o FTIR é um teste de triagem e não específico. Assim, pela técnica FTIR não foi possível classificar as amostras pelas diferenças espectrais apresentadas. O teste MN pode ser considerado um teste de triagem para detecção do HPV, sendo possível utilizá-lo na prática clínica por ser um método prático, simples, de baixo custo e não invasivo. Contudo, é importante a realização de estudos em grupos amostrais maiores para investigar a aplicação do teste de MN e da espectroscopia FTIR no diagnóstico dessa infecção e na aplicação na prevenção do câncer do colo do útero.
Palavras-chave: Micronúcleo; Espectroscopia de Infravermelho Transformada de Fourier; HPV.

Resume

La infección por el Virus del Papiloma Humano (HPV) es el principal precursor del cáncer de cuello uterino. La prueba de Papanicolaou es el principal método diagnóstico de esta neoplasia por análisis citológico, pero tiene una alta tasa de casos falsos negativos. Este estudio tuvo como objetivo verificar si la prueba de frecuencia de micronúcleos (MN) y el análisis de espectroscopia infrarroja transformada de Fourier (FTIR) pueden ser técnicas alternativas útiles para detectar el virus VPH en el fluido cervical. Cincuenta muestras con citología normal obtenidas de pacientes después del examen citopatológico se dividieron en dos grupos según la presencia o ausencia de HPV mediante análisis de biología molecular previo. De las cincuenta muestras analizadas, el 46% mostró la presencia de MN en las células. La frecuencia de MN observada fue mayor en las muestras con HPV positivo y se relacionó con la edad, el uso de anticonceptivos orales y el consumo de bebidas alcohólicas (p<0,001). El análisis de espectros FTIR mostró picos elevados de DNA y proteína en muestras con una alta frecuencia de MN (1170 cm-1; 1516 cm-1; 1404 cm-1; 1473 cm-1) en comparación con las muestras que carecían de MN, probablemente debido a los cromosomas de histonas y los constituyentes de MN. Sin embargo, no se observó una diferencia estadísticamente significativa entre estos grupos considerando que el FTIR es una prueba de tamizaje y no específica. Así, por la técnica FTIR no fue posible clasificar las muestras por las diferencias espectrales presentadas. La prueba de MN puede considerarse una prueba de tamizaje para la detección del HPV, siendo posible su uso en la práctica clínica por ser un método práctico, sencillo, de bajo costo y no invasivo. Sin embargo, es importante realizar estudios en grupos de muestra más grandes para investigar la aplicación de la prueba de MN y la espectroscopia FTIR en el diagnóstico de esta infección y en la aplicación en la prevención del cáncer de cuello uterino.
Keywords: Micronúcleo. Espectroscopía Infrarroja por Transformada de Fourier; HPV.

1. Introduction

Invasive cervical cancer is the second most common cancer among females in countries with low Human Development Index, with approximately 570,000 new cases and more of 310,000 deaths in 2018 worldwide (Bray et al., 2018). In Brazil, this malignancy is the second with the highest incidence among women, making this disease an important health problem (INCA, 2020). Several studies address that the development of this pathological condition is intrinsically related to infection by high-risk oncogenic variants of the Human Papillomavirus (HPV), which highlights the importance of an early diagnosis of this infection for the prevention of cervical cancer (Nascimento et al., 2018).

The main method for diagnosing cellular and tissue changes is the cytological analysis provided by the Pap smear. However, cytopathological and morphological methods currently used have some disadvantages, such as the high number of false negative cases (Koonmee et al., 2017). Another method that can help prevent cervical cancer is the gene amplification promoted by molecular biology, as it has high specificity and sensitivity in the detection of HPV (Melo et al., 2018). It can be applied when there are financial resources and qualified professionals, which is not a reality in developing countries (Lyng et al., 2015). Thus, it is very important to use alternative and accessible diagnostic techniques to improve the quality of cervical cancer screening and detect with greater precision and reliability the changes that HPV cause in the cervical cells.

The micronucleus (MN) test has been highlighted as a valid technique to assess the occurrence of chromosomal damage, when there is a failure in the mitotic process or defect in DNA repair (Em; Aires, 2008; Vilanova et al., 2012; Soeteman-Hernández et al., 2016). This method is an endogenous dosimeter of tissues that are specific targets for carcinogenic and

genotoxic agents, presenting the advantages of been simple, practical, reliable, sensitive, low cost and identify abnormalities in the target tissue of agents where carcinomas develop, what makes it an important tool in monitoring people exposed to risk for cancer development (Stich, 1987; Nersesyan et al., 2016). Thus, considering that a neoplastic process is characterized by genomic instability that can result in chromosomal damage, it is extremely important to study if there is a correlation between the HPV infection and the cellular alterations that may involve specific genes and structural or numerical chromosomal changes that promote the onset of the malignant process (Khanmohammadi et al., 2018).

In addition, some studies speculates that the presence of HPV may cause vibrational changes in the biological cervical fluid spectra, which is detectable by Fourier Transform Infrared (FTIR) spectroscopy technique (Conti et al., 2008). This technique have been used in scientific research for allowing reliable measurements, quickly and enabling quality control and dynamic analysis, to measure the biochemical information in the tissue composition caused by viruses, such as increased proteins and polypeptides, allowing the analysis of small amounts of samples (Kong & Yu, 2007). It is relevant to declare that these techniques are of quick result, have no high cost and can be developed in a clinical analysis laboratory, prepared with the necessary equipment for this purpose.

In this way, this study aimed to verify whether MN frequency test and the FTIR Spectroscopy analysis may be indirect techniques useful in detecting HPV in cervical fluid from patients undergoing cytopathological examination, so that it is possible to reduce the cost of this analysis and make it accessible more.

2. Methodology

2.1 Casuistry

This study was approved by the Research Ethics Committee of University Center UNINOVAFAPI, number 2.434.076 and authorized by the Ethics and Research Committee of the Municipal Health Foundation of Teresina. All study participants were duly informed about the research objective and signed the Informed Consent Form, as established by Resolution 466/2012, of the National Health Council.

Fifty samples were obtained of cervical fluids from participants who underwent gynecological examination, aged between 18 and 65 years, sexually active and with clinical suspicion or not of HPV. The patients answered a questionnaire to obtain information on risk factors for cervical cancer. The patients' clinical and behavioral data, such as age, age at first sexual intercourse, partners, use of oral contraceptives, abortion, smoking, and alcohol consumption were obtained and related with the presence or absence of MN by non-parametric Mann-Whitney test at a significance level of 95% ($p < 0.05$).

2.2 Sample collection

Two cervical scrapes were collected, one representative of the ectocervical scraping (Ayre's spatula) and one of the endocervical scraping (cytobrush), were used to make the cytological smear slides. The scrape of the endocervical brush was placed in a Neogene tube without any solution and stored at -20°C for later analysis by FTIR. Finally, a scraping of the ectocervix was performed with the cytobrush, which was cut and packed in a Neogene tube, with 100% alcoholic solution and stored at -20°C, to perform the MN test.

2.3 Micronucleus Test

Two drops of saline solution (0,9% NaCl) were added to the glass slide smear, in a sequence fixed with Sacomanno Fixative, in a concentration of methanol/acetic acid solution 3:1 and after 24hours hydrolysis in hydrochloric acid solution was performed for 15 minutes, washing in distilled water was performed three times. The preparations were stained with Schiff's reagent, counterstained with 1% Fast Green, in absolute alcohol, for 1 min, and dried at room temperature.

The cytological analysis of the MN was performed under optical microscopy (100x) and in a blind test in relation to the data obtained in the questionnaire. 2,000 cells were analyzed per patient. The MN identification criteria adopted were those described by Tolbert et al. (1992), considering MN rounded structures and distinctly separated from the nucleus, with well-defined limits, measuring about 1/3 to 1/5 of the size of the nucleus and presented in relation to it, chromatin structure and similar coloring, besides being visualized in the same plane.

2.4 FTIR spectroscopy

Samples for FTIR were divided into two groups based on a previous study using molecular analysis by PCR [16], being a group consisting of seven positive HPV samples and another group of 43 HPV negative samples. Each sample was initially diluted in 0.5 ml of saline and centrifuged for 2 min at 4000 RPM, 1 ml of SLH buffer (red cell lysis solution) was added and centrifuged for 15 min at 5000 rpm. After this procedure, an additional 1 ml of saline was added and centrifuged for an additional 15 minutes at 5000 rpm and then the supernatant was removed. This step was repeated twice.

It was used to collect the spectra, a Spectrum 400 system (Perkin Elmer) equipped with a microscope (Spotlight Perkin Elmer 400, USA) and Spectrum Image, Spotlight 400, in software version 3.6.2. The system was purged with dry air without CO2 (Parker Purifier, USA) for 2 hours before spectral acquisition. The 20 µl of each purified sample was deposited in calcium fluoride (CaF2) windows and dried to form a thin film under vacuum for 30 min. The analysis was performed at ten random points on the surface of the thin film, each point averaging 32 scans in the spectral range between 750 to 4000 cm^{-1} using 4 cm^{1} resolution. The main components were calculated using a range of FTIR spectra and a covariance matrix. In addition, in order to standardize all data, vector normalization was applied, followed by centralization of the mean. To classify the data of the PCs of the normal and HPV groups, they were analyzed by discriminant analysis (Linear and Quadratic), according to the classification of the PCR previously performed.

2.5 Statistical Analysis

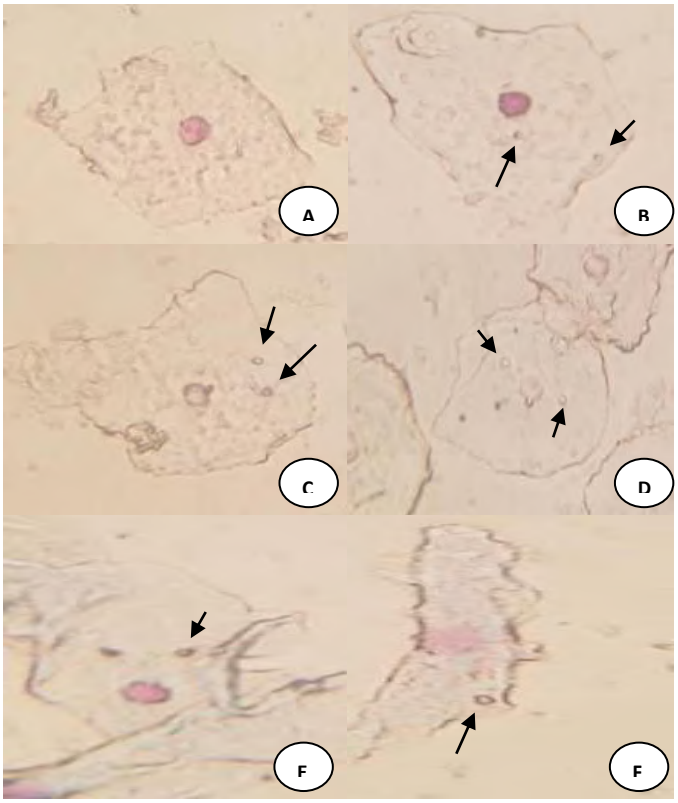
Statistical analysis was performed using the chi-square test (X^2), Shapiro-Wilk and the U-test or Mann-Whitney, as a non-parametric test for comparing independent samples the value of statistical significance was set at 95% ($p < 0,05$).

3. Results

3.1 Micronucleus Analysis

All samples were evaluated by cytological analysis as normal. 23 samples (46%) shown cells with MN formation, while 27 samples (54%) did not have these nuclear changes (Figure 1).

Figure 1 - Cervical sample, cytology of liquid medium, 1000X. (A) Superficial cell of patient without MN; (B, C, D) Superficial cell of patient with MN; (E, F) Basal and parabasal cell with MN. The arrows indicate the presence of MN.



Source: Authors.

In the comparison between presence and absence of MN in cells of cervical fluid of the patients with clinical and behavioral data was observed a relationship with the age of the patient and the use of alcoholic beverages ($p < 0.05$). The age of first sexual intercourse number of sexual partners, use of oral contraceptives and smoking were not statistically significant when compared with MN frequency (Table 1).

Table 1 - Comparison between MN frequency in cells of cervical fluid with clinical and behavioral characteristics of 50 patients.

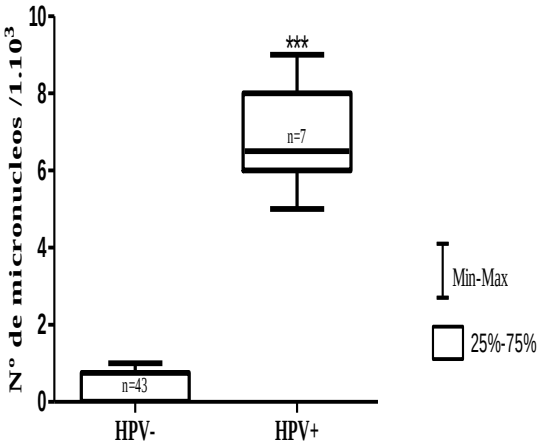
Factors		**Samples**	**MN Frequency**	**MN (%)**	**(Average ± DP)**	**p value**
Age	≤ 30 years	20	120	85,7	(70 ± 70,7)	**0,0001*** **
	> 30 years	30	41	25,4	(35,5 ± 7,7)	
Age of first sexual intercourse	≤ 16 years	36	160	68,6	(98 ± 87,6)	0,73
	> 16 years	14	73	31,3	(43,5 ± 41,7)	
Partners	1	26	76	40,8	(26 ± 35,3)	0,10
	2	23	90	48,3	(23 ± 47,3)	
	3 or +	1	20	10,7	(1 ± 13,4)	

Oral contraceptive	Yes	**7**	8	4,4	(7,5 ± 0,70)	0,23
	No	43	173	95,6	(108 ± 91,9)	
Smoking	Yes	22	92	51,6	(57 ± 49,4)	0,4237
	No	28	86	48,3	(57 ± 41,0)	
Alcoholism	Yes	10	10	5,7	(10 ± 0,0)	**0,0039*** **
	No	40	165	94,2	(102,5 ± 88,3)	

Significance level of 5% *** $P \leq 0,05$. Source: Authors.

According our previous study [16], that detected seven samples (14%) positive for HPV by molecular analysis, the samples were divided in two groups (HPV positive and HPV negative). The statistically analysis shown a significant increase in MN frequency in the HPV positive group when compared with the HPV negative group ($p < 0.001$) (Figure 2).

Figure 2 - Frequency of MN in cervical fluid samples of HPV positive and HPV negative patients.



Source: Authors.

The data were represented by mean ± SEM (standard error of the mean). U-Mann-Whitney test (non-parametric data). ***p <0.05 positive group vs HPV negative group.

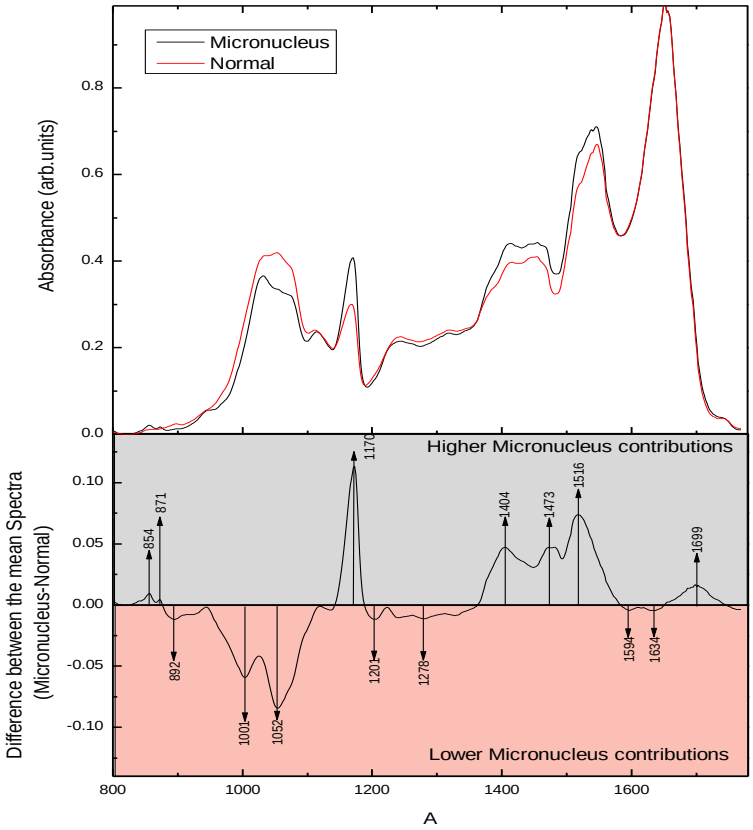
3.2 FTIR Analysis

This study used the spectral region between 800 and 1800 cm^{-1}, as this region presents bands with biochemical compounds found in biological material, such as proteins, nucleic acids and lipids (Movasaghi et al., 2008) despite the spectra being acquired in the region between 750 cm^{-1} to 4000 cm^{-1}.

Through FTIR spectra of the normal and MN groups and after spectral subtraction it is possible to identify and relate some bands of the amino acid and their assignments, with the most representative bands: in 854cm^{-1} representing the $\nu(PO_2)$ bond, in 871cm^{-1} a $\nu(PO_2)$, in 1170cm^{-1} and in 1516cm^{-1} to which a strong bond is attributed between ν(CO) and ν (NH)

respectively, in 1404 cm^{-1} and in 1473 cm^{-1} a deformation of δ (CH_2) is attributed and in 1699 cm^{-1}, which represents a strong link between ν(C = O) (Figure 3). Besides that, it is possible to verify the absorption peaks (cm^{-1}), the vibrational mode and the biological constituent in samples that presented a high frequency of MN (Table 2). However, through this analysis and after application of statistical methods, it was not possible to differentiate the sample groups based on the frequency of MN in the cervical cells.

Figure 3 - FTIR mean spectra of the normal and MN groups and spectral subtraction.



Source: Authors.

Table 2 - Distribution of the absorption peak in relation to the vibrational mode and biological constituent in samples with a high frequency of MN.

Absorption peak (cm^{-1})	Vibrational Mode	Biological Constituent
854	C-H folding out of plane	Nucleic acids
871	C-H folding out of plane	Nucleic acids
1170	C-O Stretch	Proteins
1516	N-H angular deformation	Proteins
1404	CH_2 angular deformation	Proteins
1473	CH_2 angular deformation	Proteins
1699	C=O stretch	Nucleic acids

Source: Authors.

4. Discussion

The present study is the first in the literature that describes the association of screening techniques, MN analysis and FTIR spectroscopy in detecting the presence of the HPV virus in cervical fluid samples. Both techniques do not require prolonged sample processing, they are fast and from the moment they were implemented they are cheap, both having the potential to be implemented in the clinical routine.

We demonstrated the potential of cellular analysis by MN test in the detection of uterine injury. From this perspective, Shi et al. (2015) demonstrated the effectiveness of MN analysis in screening for cervical cancer through the detection of high-grade lesions when comparing with the hybrid capture technique (Shi et al., 2015). Similarly, Adam et al. (2015) described significant associations between the presence of MN, HPV viral load and uterine lesions, highlighting the importance of this analysis in monitoring cervical cancer risks (Adam et al., 2015).

Regarding the relationship between HPV infection and the incidence of MN, our results reveal a significant increase in the MN frequency of the positive HPV group when compared to the negative HPV group samples analyzed in our previous study by PCR (Viana et al., 2020). These findings allow suggesting a possible association between the cellular lack of control provided by HPV infection and the formation of MN. Using this approach, Yıldırım et al. (2019) reported in their studies the formation of MN as a possible result of the genomic instability promoted by HPV infection. Cortés-Gutiérrez et al. (2010) compared the number of MN in 1.000 cells among women without cervical lesions (HPV negative) and women with squamous intraepithelial lesions infected with low-risk HPV (LR-HPV) and high-risk HPV (HR-HPV) and their results demonstrated the associations between HR-HPV infection and a higher MN frequency ($p= 0.0001$). Subsequently, Cassel et al (2014) performed genomic amplification of HR-HPV and demonstrated that MN was significantly higher in HPV-DNA positive samples ($p = 0.016$), which allowed these authors to propose the analysis of MN as a possible test for monitoring exposure of population to HPV infection.

Among the clinical characteristics evaluated, only young age and alcoholism showed to be statistically associated with the higher frequency of MN in cervical samples, considering the samples referring to patients under the age of 30 years showed a significant increase in the formation of MN. Ambroise et al. (2013) related the association of these factors and viral infections with the presence of this nuclear abnormalities and our results disagree with those presented by Wojda et al. (2007), Kažimírová et al. (2009), Nefic and Handzic (2013) and Franzke et al. (2020), as they relate that increasing age associates to chromosomal instability and, consequently, promotes the formation of nuclear abnormalities, including MN. In our study, samples from patients who reported not drinking alcohol had a higher frequency of these chromosomal fragmentations and this association disagree of results pointed out by Dickemann et al. (2018), who related absence of association between frequency of MN in oral mucosa cells of individuals exposed to mouthwashes containing alcohol. The discrepant results found can be explained by the low number of samples included in our study.

The characteristics observed in our study that did not show a significant association with the presence of MN were: age of first sexual intercourse, the number of sexual partners, the use of oral contraceptives, and the habit of smoking, the latter being highlighted. The absence of clear association between smoke and MN frequency is in disagreement with the appointments of Geus et al (2015), who related a significantly higher frequency of MN in buccal cells of smokers' patients. This discordance can be explained considering the active effect of genotoxic chemical substances upon buccal cells, which these authors studied. In other hand, cervical cells analyzed in our study receives an indirect effect of this malefic molecules and its necessary a deep analysis considering the exposure frequency.

The spectroscopy analysis provided by FTIR, evidenced in our study an increase in protein and nucleic acid peaks founded in the same samples that present a high MN frequency and that are HPV-DNA positive, but were statistically poor to demonstrate the potential of this technology in sample differentiation. Some studies, as Rymsza et al. (2018) and Viana et al.

(2020), also evidenced low sensibility and specificity of FTIR in HPV detection. The absence of statistical difference between the analyzed groups by FTIR denotes that the spectra is based in all molecules of sample, such as lipids, fatty acids, and water and so we conclude that the MN presence at cells do not promotes significates spectral differences to this screening test.

5. Conclusion

The MN test can be considered a screening test for HPV detection as a preventive exam, being possible to use it in clinical practice. Otherwise, the use of the FTIR technique was not able to demonstrate a spectral difference to classify the samples, because it does not show a high sensitivity in detecting the presence of the HPV virus in cervical fluid samples. However, we highlight the importance of carry out studies in larger sample groups for validate the results.

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