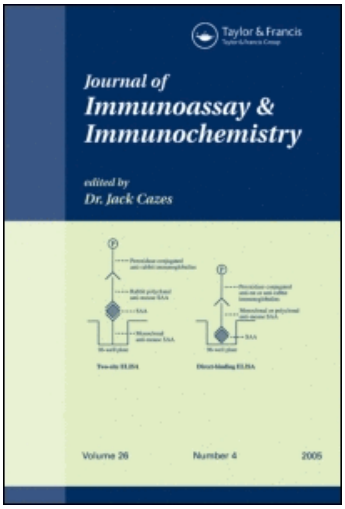

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Antibody Response for L1, E6 and E7 HPV 16 and HPV 18 Antigens in Tunisian Women with Cervical Cancer and Controls

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Abstract: Results obtained in the present work indicated that the Luminex assay is more sensitive than ELISA. The reactivity to the early antigens E6 and E7 was 37% versus 42% for HPV 16 and 21% versus 20% for HPV 18 among cervical cancer cases using ELISA. However, these ratios were 44% and 61%, respectively, for E6 and E7 HPV 16 versus 28% and 21% for E6 and E7 HPV 18 when using the Luminex technique. Data also indicated that HPV 16 and HPV 18 showed distinct profiles for the different antigens tested. Finally, the differences in antibody responses between cervical cancer cases and benign cases toward the different antigens were significant.

Keywords: ELISA; HPV 16; HPV 18; Luminex; Serology

INTRODUCTION

Human papillomaviruses (HPVs) cause one of the most common sexually transmitted infections in the world. A subset of ''high-risk'' HPV

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genotypes is unequivocally associated to cervical cancer, the second main cause of death from cancer in women worldwide.[1,2] To date, more than 100 HPV genotypes have been identified and at least 50 are known to infect the female anogenital tract.[3,4] Among these, thirteen mucosotropic HPVs (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66) have been recently classified as class I carcinogens to human beings.[5] Several others types, however, need further studies, as high risk viruses on the basis of (*i*) molecular phylogenetic relatedness to carcinogenic genotypes;[3,6] (*ii*) epidemiological studies on the association with cervical cancer worldwide;[2] and (*iii*) the *in vitro* biological properties.[7]

The prevalence of HPV genotypes in cervical cytological samples varies greatly in different geographical regions and shows a strong correlation with the incidence of cervical cancer.[8–11,12]

On a global level, human papillomavirus (HPV) is estimated to cause almost half a million cases and more than 270,000 deaths from cervical cancer, corresponding to more than 2.5 million years of life lost (YLL) annually.[13] HPV type 16 (and to a lesser degree HPV type 18) is linked with more rare cancers, namely cancer of the vulva, vagina, penis, anus, oropharynx, and larynx. Effective prophylactic vaccines have been developed.[14]

Less than 50% of women affected by cervical cancer in developing countries survive longer than 5 years, whereas, in developed countries, the 5-year survival rate is about 66%.[15,16] Every year, an estimated 190,000 deaths from cervical cancer occur worldwide, with more than 75% of them in developing countries, where mortality from this disease is the highest, among deaths caused by neoplasm. In the United States each year, there are approximately 9,800 new cases of invasive cervical cancer with 3,700 deaths due to this disease.[17] The highest risk areas for cervical cancer are in Central and South America, Southern and Eastern Africa, and the Caribbean, with average incidence rates of approximately 40 per 100,000 women per year. Other mucosal HPVs are differently distributed in various geographical regions.[11,18,19] HPV can persist in a chronic way without being detected and without being eradicated by the immune system; however, this property is related primarily to its whole genotype HPV.[20] The first immune defence mechanism is related to the intracellular and non-lytic replication of the HPV in the keratinocyte. The virus "hides" in the cell and the cellular lysis pulled by other cytolytic viruses resulting in the release of a great quantity of viral antigens and intracellular proteins, which will give warning of a danger to the immune system. During HPV infection, these alarm signals are missing; consequently, the absence of viremy in HPV infection is another mechanism which limits the contact of the virus with the immune system cells.[20]

According to Meshede et al.,[21] HPV serology is complex for several reasons: (i) HPV antibody analysis is not evident but seems to be very

complicated due to the large number of HPV types which can infect humans; (ii) many assays can be used to assess the serology of HPVs; (iii) peptide enzyme-linked immunosorbent assays (ELISAs) which use small, linear epitopes of the proteins for the antibody detection have low levels of sensitivity and specificity; and (iv) radioimmunoprecipitation with whole native proteins is complex.

Actually, two methods of antibody detection can use the mono-test ELISA assay: (a) GST capture which improves the sensitivity as well as the specificity but they allow the analysis of sera to only one antigen per well and (b) luminex immunoassay developed by Waterboer et al.[22] This paper is a report on our serological study, which is important to improve our understanding of HPV seroconversion in Tunisian patients, with or without cervical lesions.

EXPERIMENTAL

Human Sera

Seventy-one blood samples were collected from patients with cervical cancer in the Salah Azaeiz Institute, and 64 cases of women with cervical inflammation from the Center of Maternity and Neonatology. Seventy apparently healthy adult Tunisian women were randomly selected and used as controls. The status of patients with inflammation or cancer were clinically determined based on the cytology or biopsy analysis. Blood samples (5 mL) were taken, centrifuged, and sera were kept at $-20^{\circ}C$ until use.

GST Capture ELISA Test

GST capture ELISA test was carried out according to the procedure described by Sehr et al.[23] In brief, the polysorp plates were coated with glutathione casein (2 μg/mL) in coating buffer overnight then the coated plates were blocked with casein blocking buffer (180 μL per well) and incubated for 1 h at 37°C. The wells were incubated for 1 h at room temperature with the cleared lysates from *E.coli* over expressing GST fusion proteins diluted in casein blocking buffer (0,2% w/v casein in phosphate buffer saline) to 0,25 μg/μL total lysate protein. After washing, the ELISA plates were incubated with the diluted serum (1/200 in buffer). The bound human antibodies were detected by immunoglobulin G goat anti-Human biotinylated at a dilution of 1/105 and the streptavidine conjugated to horseradish peroxydase (HRP) diluted at 1/104 and incubated for 1 h at room temperature.

Tetramethylbenzidine was used as substrate and the reaction was stopped after 7 minutes by adding 50 μL of 1 M sulphuric acid/well and the absorbance was measured at 450 nm. The absorbance in wells with GST alone as an antigen defined the background reactivity of the serum and was subtracted from the absorbance with the GST fusion protein to calculate the specific reactivity of the serum against the fused antigen. The cut-off was calculated for each antigen as the median of the specific absorbance values of all control sera (n = 70) plus three standard deviations excluding positive outliers. All plates were tested in duplicate. Using the cut-off value for each antigen, we were able to judge if a sera was negative or positive. To absorb antibodies directed against bacterial proteins and GST, sera were incubated in blocking buffer containing lysate from bacteria expressing GST alone.

Production of Glutathione-Casein

Glutathione-Casein has been produced as previously described.[23] Casein was reacted with the heterobifunctional cross linker sulfo-succinimidyl-4-(p-maleimidophenyl)-butyrate to yield thio-reactive casein-maleimidophenyl-butyrate (casein-MPB). The casein-MPB was reacted with glutathione (GSH) to yield glutathione-casein (GC).

Production of Glutathione-Beads

To produce glutathione-beads, the terminal amines of the GC have to be coupled to the carboxyl groups of the beads. Following a standard activation procedure, the beads'carboxyls forms an acyl amino ester that reacts with the primary amines of the glutathione molecules in the GC, yielding a stable amine bond. After this coupling procedure, at least one of the Glutathione molecules acts as cross linker between the casein and the beads. Since the chemical activation procedure takes place on the beads, unreacted amines of the glutathione are unaffected and are still able to interact with the GST.

Coupling of Antigens to Beads

The production of viral proteins as antigens for immunoassays was carried out as described previously.[22,23] Briefly, viral antigens were expressed as GST fusion proteins in *E coli* using pGEX vectors and inducing over expression by adding isopropyl-B-D-thiogalactoside (IPTG) to the bacterial culture. Bacterial cells were harvested by

centrifugation and the cells were lysed using a French Press. GC-beads per serum were loaded with antigen directly in the diluted lysate. Afterwards, the beads were washed three times with the blocking buffer.

Luminex Assay

Preincubated sera and antigen-labelled beads were mixed and incubated in 96-well plates with filter bottoms. Each well was washed using a vacuum manifold. Biotinylated secondary antibody (goat-anti-human IgG) was incubated with the beads. Detection conjugate (Streptavidin-R-Phycoerythrin) was reacted with the beads, washed, and measured with the Luminex analyser.

Cut-Off Value for Luminex Assay

For the Luminex assay, cut–off values were determined using control sera from apparently healthy women. The cut-off represents the mean value of these sera plus three standard deviations. This cut-off definition allows the discrimination between positive and negative sera.

Statistics

We used a *t* test to determine p values to estimate differences of positivity of the antibody response between cases and controls. Differences were considered significant for $P < 0{,}005$.

RESULTS AND DISCUSSION

The results reported in Table 1 clearly show a comparison between the percentages of seropositivity toward the six antigens (L1, E6, and E7) of both HPV genotypes 16 and 18. However, the late antigen L1 of HPV 18 was tested only with Luminex. The results also indicated that, in the Luminex procedure, elevated percentages of seropositivity were noted, especially for the two early proteins E6 and E7, compared to the late antigen L1 of HPV 16 (44% and 61% versus 21%, respectively).

Differences in positivity obtained by ELISA and Luminex for L1, E6, E7 HPV 16, and E6 HPV 18 antigens were significant ($P = 0.001$, $P < 0.0001$, $P < 0.0006$, $P = 0.001$ respectively); however, this difference was not significant for E7 HPV 18 ($P = 0.28$). Moreover, for HPV 18, the important percentage of positivity was observed for E6 followed by L1,

Table 1. Distribution of percentages of seropositivity toward the different antigens as detected by LUMINEX and ELISA

	Antigens	16 E6	16 E7	16 L1	18 E6	18 E7	18 L1
Luminex	Cancers	44%	61%	21%	28%	21%	24%
	Controls	6%	10%	3%	3%	9%	17%
P values*		***	***	***	***	**	**
ELISA	Cancers	37%	42%	13%	21%	20%	ND
	Controls	3%	3%	0%	1%	3%	ND
P values*		***	***	***	***	***	

ND: not done.

*P values for the difference between cervical cancer cases and controls Differences are significance for $P < 0.05$.

***$P < 0.0001$.

**$P = 0.001$.

then E7 (28%, 24%, and 21%, respectively). High significant differences were found between cancer cases and controls regarding all antigens but not for L1 HPV 18 which was less significant and showed a rate of positivity, i.e., 17% in controls compared to 24% among cervical cancer cases ($P = 0.001$). Data obtained with the ELISA technique showed, albeit, the same profile found using the Luminex procedure. In fact, the increased percentages were noted for E6 and E7 HPV 16 compared to the late antigen L1 with 37% and 42% versus 13% for E6, E7, and L1, respectively. On the other hand, comparative percentages were obtained for the two early antigens E6 and E7 (21% and 20% respectively) for HPV 18.

When comparing the results obtained from the two assays, the Luminex assay showed elevated percentage values for E6, E7, L1 HPV 16, and HPV 18 compared to those obtained with ELISA. Furthermore, differences between the two assays were noted, especially for HPV 16 antigens. In the Luminex assay, analysis of median fluorescence intensity (MFI) values obtained for the different antigens in the three tested groups showed different profiles (Figure 1). Among cancer cases, elevated values of MFI were found for E6 and E7 HPV 16 antigens compared to HPV 18 antigens. In fact, 10% of cervical cancer cases showed MFI values that exceed 6,000 but only 5% among cervical cancer cases have MFI values exceeding this value for E6 antigen HPV 16 and HPV 18, respectively. MFI values reached 13 317 units for E6 HPV 16. The same profile was observed for E7 antigen and elevated values of MFI were also noted for HPV16 compared to HPV18. However, for the late antigen L1, less important MFI values were noted for L1 HPV 16 and L1 HPV 18; these values did not exceed 6,000 units. Moreover, the discrimination between

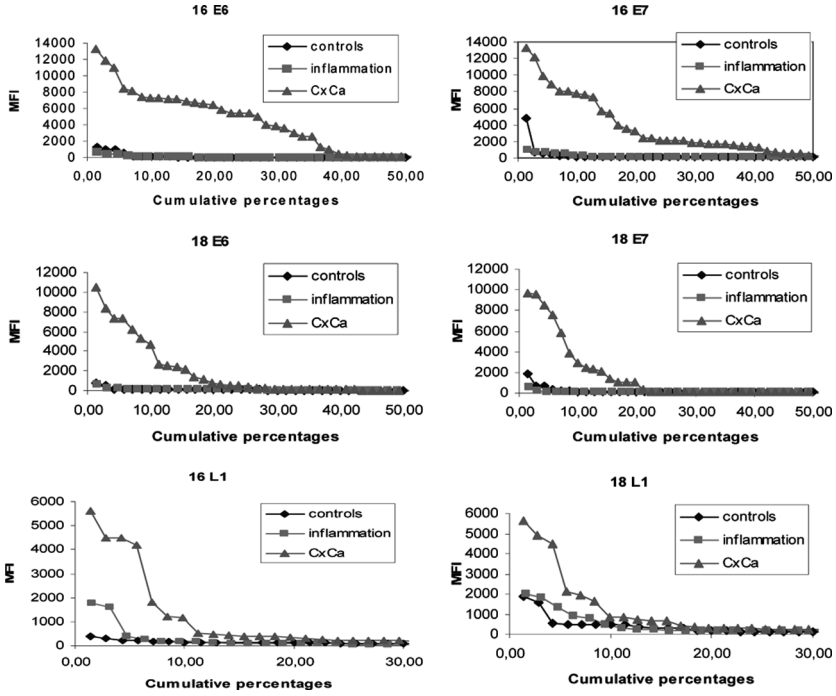

Figure 1. Repartition of mean fluorescence intensity (MFI) for the different antibodies anti-HPV 16 and anti-HPV 18 among the three groups of patients.

controls and inflammation among the benign group was clearer for L1 HPV 16 and HPV 18 than for E6 and E7 antigens.

Data presented in Figures 2 and 3 indicate a direct comparison between the performance of multiplex serology and GST capture ELISA in cancer patients and the control group towards the different antigens. The correlation coefficient allowed us to appreciate the degree of correlation between the two analyses. The data showed that the best concordance between ELISA and Luminex for all markers was observed in women with cervical cancer, compared to controls (Figure 2). Furthermore, the best correlation between the two assays was noted for E7 HPV 16 antigen with a correlation coefficient (R^2) of 0.754 followed by L1 HPV 16, then E6 HPV 16, E6 and E7 HPV 18. In addition, data in Figure 3 represents the comparison between the correlation coefficient values R^2 for the different antigens among the two groups of women (cancer cases and benign cases including controls and inflammation cases). The current results showed a heterogeneous distribution of R^2 values for the different antigens. In this latter group, the values obtained for the control group were different from those in the inflammation

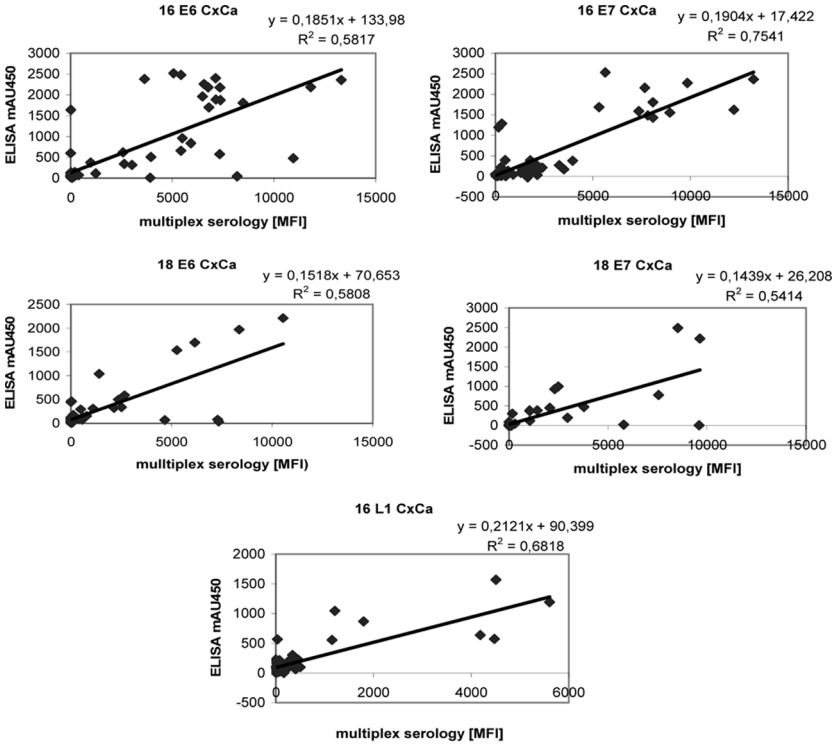

Figure 2. Correlation between ELISA and Luminex analysis. The x-axis represents data of Luminex and the y-axis shows ELISA data among cervical cancer cases toward the different antigens. R^2 : Correlation coefficient.

group. Elevated R^2 values were found among control cases (0.9) for both antigens E7 HPV 16 and HPV 18, however, and very low R^2 values were found in the inflammation group for the two early antigens E6 and E7 (0.03 and 0.0015, respectively).

The Luminex system is an open platform for the analysis of molecular interaction and has been used for the detection of bacterial rRNA,[24] cytokines,[25] single-nucleotide polymorphisms,[26] and antibodies against viral antigens.[22,27] In the present study, we used this novel method for HPV serologic analysis, which combines fluorescent bead array with a generic method allowing in situ affinity purification of any glutathione S transferase (GST) fusion protein developed for conventional ELISA. Antigens were expressed as GST fusion proteins in *E. coli* and directly purified from bacterial lysates via GC. The Luminex technology showed a high degree of sensitivity and allowed the simultaneous analysis of a very large number of targets, but was

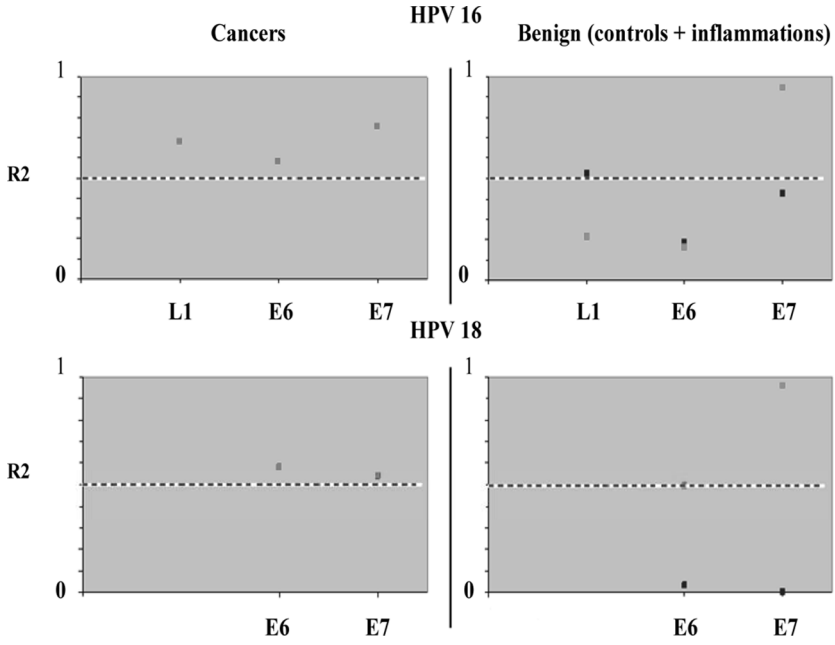

Figure 3. Distribution of correlation coefficient values between Luminex and ELISA upon groups of patients for different antigens.

limited in the number of samples that could be analyzed in a reasonable time frame at acceptable costs.[22,28,29]

Using this method, we were able to detect antibodies directed against the six antigens L1, E6, and E7 of HPV 16 and 18 and in parallel to study the dynamics of antigen-specific HPV antibodies in relation to the clinical outcome of the viral infection. Luminex assay showed increased percentages of positivity, especially among cervical cancer cases for the different antigens (44%, 61%, and 21% versus 37%, 42%, and 13% for E6, E7, and L1 HPV 16, respectively). The profile obtained for the two HPV genotypes studied were not exactly identical. For HPV 16, the majority of cancer cases reacted against the E7 antigen (61% with Luminex and 42% with ELISA); however, for HPV 18, elevated percentages of positivity were found for the E6 antigen. The correlation between the two assays used in this study was more important in the analyses of cervical cancer cases than controls; this concordance is likely due to the fact that the seroprevalence and the magnitude of sero-response were presumably higher in this group of patients.

Increased MFI values were noted for E6 as well as E7 antigens HPV 16, compared to those of HPV 18. However, the late antigen L1 MFI values did not exceed 6,000 units for HPV 16 and for HPV 18. These

results may be explained by the fact that expression of low-molecular-weight proteins in bacteria is much better than that of larger proteins, as shown for GST fusion proteins of HPV E6 and E7 versus L1. In this regard, previous studies indicated that GST fusion proteins were 46.8 kDa for HPV 16 E6, 38.6 kDa for E7, and 82.7 kDa for L1. Bound antigen was quantified by anti-tag-PE and the data showed that, at a lysate concentration of 1 g/L, all proteins reached saturation on the GC beads, with plateaus for E6 and E7 approximately twice as high as that for L1. The 3-fold difference in lysate concentration at half-maximal binding indicated that the L1 lysate contained up to 3-fold less fusion protein than the E6 or E7 lysates. There are two possible reasons for the 2-fold lower plateau for the L1 protein (a) the expression of larger GST fusion proteins may produce more N-terminal fragments that compete for glutathione binding but lack the C-terminal tag and/or (b) the higher molecular weight may produce a lower molar density of fusion proteins on the bead surface.[23,30] The differences in HPV 16 and 18 profiles can be explained by the fact that these two viruses have different biological properties. In fact, HPV 18 is more strongly associated with adenocarcinomas or adeno-squamous carcinomas than with pure squamous cell carcinomas, and there is evidence of more aggressive behaviour and a higher recurrence rate of HPV-positive cancers.[9,31] Differences in their integration sites may have an influence on the states of infection and, thus, presents different immune responses.[32]

A previous Tunisian study was undertaken to determine epidemiological and pathological profiles of cervical cancer in Tunisia and it showed that the incidence of cervical cancer in Tunisia is relatively low, in spite of the absence of a screening program. Moreover, the authors reported that squamous carcinoma was more frequent than adenocarcinoma.[33] In fact, the incidence of cervical cancer was inferior to 5/100,000 women and the viral positivity type HPV16 is the most common from all the studies done in Tunisia.[34] The incidence of cervical cancer is estimated to be 11.2 per 100,000 women in developed countries and 18.2 per 100,000 women in developing countries and the incidence is different when studying developing countries individually.[35] There is no Tunisian serological study which has analysed the type of HPV in cervical cancer patients.

Tunisian studies have only determined the prevalence of cervical infection in Tunisia for detection and molecular typing of human papillomaviruses using the polymerase chain reaction. In a previous study in our laboratory, we noted that, among cervical cancer patients, 55% were HPV 16 positive and 30% were HPV 18 positive.[36]

Previous studies have reported that these two viruses have a similirarity in the fact that their antibody responses were more likely to persist than HPV6 throughout follow-up.[37] Moreover, other studies showed

that the difference between cervical cancer cases and control cases for seroreactivity to HPV-16 VLPs (virus like particles) was highly significant when comparing either distributions of values or antibody prevalence, indicating that VLP antibodies are markers of cervical cancer while those targeting the viral oncoproteins E6 and E7 are markers of HPV-associated cancer.[21,38] The prevalence of 21% for HPV 16 and 24% for HPV 18 obtained in the current study with the Luminex assay are different from those reported in previous study of cervical cancer around the world, where the prevalence was 59%.[39] This difference noted in the prevalence's values may be due to the method of detection used for groups of patients analysed in the current study. Previous studies have reported that, although the highest seroprevalence was observed among women who had HPV-16 DNA in the genital tract, a high seroprevalence was also seen in women who had other HPVs in the genital tract. The most plausible explanation for the high seroprevalence in women without a current HPV-16 infection is that the serological markers reflect a history of HPV exposure, and women with cervical cancer associated with HPV types other than 16 commonly have been exposed to HPV 16 in the past.[19] The early antigens E6 and E7 are constitutively expressed in HPV-16-induced cervical cancer.[40] Probably, there is a viral reactivation in tumour cells. However, in the benign group, the profiles obtained are more heterogeneous and complex; this may be due to the viral cycle, a slow cellular proliferation stage, and a viral reactivation, as well as inflammatory cytokines.[41,42] In the benign group, we have noted different profiles between controls and the inflammation group. This may be explained by the fact that inflammation of the cervix, under the effect of inflammatory cytokines, the virus modulates its biology to get in the lytic stage and, so, to produce viral particles. Previous studies have reported that hallmarks of the inflammatory response include migration of natural killer cells and phagocytes that release inflammatory mediators. Inflammation, often in response to chronic infection, results in the production of non-specific protective antimicrobial oxidants that can also cause oxidative damage to host DNA, and predispose to cancerogenis.[43] This emphasizes the need to investigate anti-HPV antibodies in addition to HPV DNA to evaluate the overall HPV infection more effectively in a population.

CONCLUSIONS

The use of the ELISA and Luminex techniques in the present study confirm the elevated prevalence of HPV 16 compared to HPV 18 in Tunisian cervical cancer patients with high expression of early antigens E6 and E7, as well as the late antigen L1.

In addition, heterogeneous results were obtained in the benign group. Finally, the profile of HPV 16 with higher seropositivity to E7 antigen, followed by E6 then L1, is different from HPV 18 with elevated seropositivities to E6 followed by L1 then E7, showing probably different biological evolution of these two viruses. The Luminex assay can be a useful tool when undertaking large epidemiological studies to determine antibody response toward different antigens of HPVs or against other infectious agents as well as vaccination and follow up in a target population.

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