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The prevalence of high/risk HPV viruses in Najaf city

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Abstract

Cancer is a serious public health problem, and one of the main causes of cervical cancer in women is the infection with high/risk Human papillomavirus (HPV). Human papillomaviruses are DNA viruses that have specific tropism for squamous epithelia. More than 120 different HPV types have been isolated to date; of these Low-risk HPV types, such as HPV6 and HPV11, induce benign hyperproliferations of the epithelium such as papilloma or warts. By contrast, high-risk oncogenic types (HPV16, -18, -31, -33, -35, -39, -45, - 52, - 56, - 58, - 59, - 66) are defined to have strong epidemiologic association with cervical cancer. Multiplex-PCR analysis was used for the detection and genotyping of high/risk HPV DNA in the cytological samples of cervical squamous intraepithelial lesions.

The results show that the proportion of High/risk HPV genotypes in cervical smears of patients with cervical squamous intraepithelial neoplasia was 59%. In details, the genotypes were: HPV16 (15.3%), HPV18 (15.3%), HPV33 (7.7%), HPV31 (7.7%), HPV35 (30.7%), HPV56 (7.7%), HPV45 (7.7%), HPV58 (7.7%) and HPV52 (7.7%). In the other hand, none of the DNA isolated from 10 healthy group showed high/risk HPV amplicons.

Keywords: *Molecular virology, Cervical cancer, High/risk human papillomaviruses, Genotypes of HPV in cervical squamous intraepithelial lesions.*

1. Introduction

High-risk oncogenic types of Human papillomaviruses (eg. HPV16, -18, -31, -33, -35, -39, - 45, - 52, - 56, - 58, - 59, - 66,) are defined to have strong epidemiologic association with cervical cancer [1]. Worldwide the massive toll of papillomaviruses - associated cancer was 20% of all cancer death in women [2]. In addition, this virus is responsible for 500,000 new cases of cancer per year and billions of dollars in medical expenses [3]. Cervical cancer is from five most common cancer in women globally, in fact approximately 570,000 new cases and 311,365 deaths were recorded in 2018. Over 85% of new cases and 80% of mortality take place in low and middle -income countries [4]. In cervical carcinomas, where HPV is found in over 90% of cancer specimens [5]. Knowledge of HPV status is becoming increasingly important as a triage screen after detection of atypical cells of undetermined significance [6] and as a primary screen for cervical cancer detection [7]. Therefore, HPV typing has an important prognostic and therapeutic value, as it can distinguish between HPV types of high and low oncogenic risks. Identification of high-risk HPV genotypes may permit selection of those patients who are at increased risk for disease and may therefore provide additional clinical value. An important requirement for this approach is that HPV testing and identification of high-risk HPV types have been highly sensitivity and specificity [8]. Iraq has a population of 8.21 million women ages 15 years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 311 women are diagnosed with cervical cancer and 212 dead from the disease. Cervical cancer ranks as the 10th most frequent cancer among women in Iraq, and the 7th most frequent cancer among women between

15 and 44 years of age, Data is not yet available on the HPV burden in the general population of Iraq. However, in Western Asia, the region Iraq belongs to, about 2.2% of women in the general population are estimated to harbor cervical HPV infection at a given time [9]. In Najaf , and up to our knowledge, it is the first study that use Multiplex-PCR technology for detection and genotyping High risk HPV infection in cytological specimens from different lesion affected the cervix uteri.

2. Materials and Methods

This study was conducted in Najaf, in order to establish the occurrence of high/risk HPVs cervical infection and determine the high/risk/HPV genotypes. Cervical pap smears screening applied on one hindered patient referring to AL-Zahra teaching hospital in AL-Najaf province who suspected to harbor high/risk human papillomaviruses, two samples from each patient were taken the 1st sample for cervical pap smear and the 2nd sample for detection and genotyping of High-risk Human papillomaviruses using PCR based technique.

Among these 100 cervical specimens, twenty-two cases with different grades of cervical intraepithelial neoplasia, sixty-eight different types of cervicitis, and ten healthy cases without any significant pathological changes (apparently healthy). the age of all these groups were ranged between 20 and 65 years.

2.1. Materials used for DNA Extraction of HPV

DNA Extraction Kit of Human papillomaviruses from cervical scrapings specimens was purchased from Geneaid Biotech Ltd. USA (Cat. Number. GT100).

2.2. Materials used for Multiplex Amplification of High-Risk HPV DNA

HPV High Risk Typing Kit was purchased from Sacace Biotechnologies Srl. Italy (Cat. Number. V-25-50F), for qualitative detection and genotyping of Human Papillomavirus (16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66,) in the cervical scrapes and biopsies. HPV High Risk Typing Test is based on three major processes sample preparation, multiplex amplification of DNA using specific HPV primers and detection of the amplified products on agarose gel.

3. Results and Discussion

The diagnosis of HPV infection that based on cytological criteria has underestimated the number of women with HPV infection [10]. furthermore, abnormal cells could also be missed either by the cytologist or due to sampling error giving rise to false negatives, the presence of false-negative smears varies from 15% to 20% [11]. This mean that the sensitivity of cytology for cancer and CIN screening is quite low (50%-85%) and it is specificity high (about 90%) [14]. Acetowhitening of the cervix as a predictor of subclinical HPV infection had low sensitivity (22%) for detecting HPV DNA in young women and the specificity was 90% this means that there was a missed positive woman and falsely labeled other women as being HPV infected by a percentage remained from these sensitivity and specificity. Further, the combination of acetowhitening and cytology did not improve the diagnostic value [15]. In addition, antibodies against HPV antigens are generally not type specific, and can't detect HPV antigens in dysplastic or neoplastic cells since these antigens are poorly expressed in undifferentiated cells. Furthermore, in situ DNA hybridization

can detect HPV but suffers from lack of sensitivity in dysplastic cervical lesions as the amount of viral DNA is reduced with increasing dysplasia (Mohamed Ali.,2001). Therefore, the view of the drawbacks of the previous methods, the PCR technology, among many other methodologies, has been used for HPV DNA detection. This is because the number of cells obtained from the remnant of cervical scrapes was small and since it is possible by PCR, detect 10^{-10} copies of HPV DNA per cell, which correspond to a single HPV 16 gene (Shimada *et al.*,1990) therefore PCR is the most sensitive and specific molecular method capable of resolving extremely low copy numbers of HPV DNA in cervical smears [16]. In addition, the PCR was used for HPV DNA-analysis of cell samples because it is a simple technique convenient for everyday use. Moreover, replication of HPV takes place in the superficial layers of the keratinocytes, a fact which favors the possibility of detecting HPV DNA by cell sampling [17].

PCR should allow an absolute prevalence of HPV infection to be estimated and also clarify the roles of these agents in cervical oncogenesis [12].

3.1. The detection and genotyping High/risk HPVs in cervical squamous intraepithelial lesions group by Multiplex PCR-technique

The twenty-two cases of cervical intraepithelial neoplasia were diagnosed by using Cervical Pap smear screening constituted 5 % of these cases were High grade (HSILs), 63% were Low grade (LSILs) and atypical cell of undetermined significance constituted 32%.

After application and analysis of PCR, the results in table (1) showed that the percentage of detection of HPV DNA in the cervical scrapes of Squamous intraepithelial lesions was 59% (13 out of 22).

In comparison to percentage of HPV detection in healthy group, the differences between the percentage of DNA in each of SILs and Healthy groups (as control) are statistically highly significant ($P<0.01$).

Table 1 The percentage of HPV-DNA Multiplex PCR technique results in the studied groups

Studied groups		HPV DNA		Total	Statistical Analysis	
		PCR-Positive	PCR-Negative		P-value	X^2
Apparently healthy (as control)	N	0	10	10	0.002	9.952
	%	0	100	100		
Squamous intraepithelial lesion	N	13	9	22		
	%	59.09	40.90	100		
Total	N	13	19	32		
	%	40.62	59.37	100		

The potential of molecular techniques like Multiplex- PCR can be noted when compared to cytological examination results. Out of 22 cytologically diagnosed as typical cervical Squamous intraepithelial lesion only 13 samples revealed infection with High/risk HPV, representing 59% of the total group. In comparison with other

similar reported studies, the results of the present study showed an agreement with results reported by Mohamed Ali [12]. he found 62.5%, (10 0ut of 16 cases) and different results were shown by Hilman *et* al. [18] (36%, 4 out of 11 cases) were reported as positive cases by using PCR. The presence of HPV DNA – negative cases with Cervical SILs is not surprising since cellular alterations, morphologically observed as perinuclear clear zone in exfoliated cells from the human cervix, have been reported for long time.

Table 2 Genotyping of HPV-DNA using Multiplex PCR technique in cervical scrapes with squamous intraepithelial lesion.

Squamous intraepithelial lesion Grading	No. of HPV +ve	HPV genotype	No. of cases with this Genotype	% of Total
High grad squamous intraepithelial lesion	1	16&45	1	100
Low grad squamous intraepithelial lesion	9	16	2	22
		35	4	44
		16&31&56	1	11
		18&52&58	2	22
Atypical cells of undetermined significance	3	18	2	66
		16&35	1	33
Any Grade	13	16&45	1	7.7
		16	2	15.3
		35	4	30.7
		16&31&56	1	7.7
		16&35	1	7.7
		33&58&52	2	15.3
		18	2	15.3

However, numerous explanations of the cause of koilocytosis have been offered. This could be referred to a mechanically produced artifact caused by scraping process of cervical epithelium. Electron microscopic studies have also depicted koilocytosis as an abnormal karyopyknosis, possibly caused by abnormal physic-chemical dynamics of the cells. In addition, perinuclear halo was associated with acute and chronic Cervicitis, especially due to *Trichomonas vaginalis.* Whereas, a koilocytotic atypia that not associated with trichomoniasis, was a manifestation of marked dysplasia, possibly of viral origin [12]. Furthermore, the presence of morphological signs of HPV in cytological smears of HPV DNA-negative cases could assume that HPV was the necessary cause and that other variables may act as co-factors [19].

The genotyping results of HPV positive of cervical Squamous Intraepithelial Lesion group of study are shown in Table (2) and figure (1), (2), (3).

The result revealed 9 different HPV genotypes. These were HPV 16, HPV18, HPV56, HPV35, HPV31, HPV45, HPV58, HPV52, and HPV33. These genotypes were shown in figures (1), (2), (3).

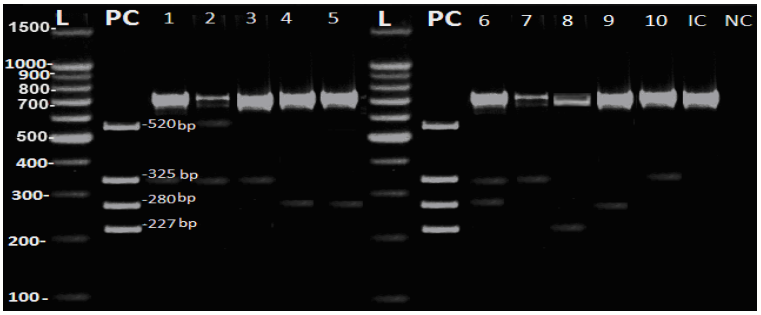

Figure (1): Agarose gel electrophoresis of PCR product amplified from HPV isolates. L: - Leder, PC: - positive control of HPV16(325bp), HPV31(520bp), HPV33(227bp), HPV35(280bp) and Lane 1-10 show positive results with High risk / HPV16, HPV31, HPV33, HPV35.

The amplification of the viral DNA of High/risk HPV could be noticed by the presence of the typical DNA band of molecular weight 520 bp for HPV 31, 325 bp for HPV16,227 bp for HPV33 and 280 bp for HPV35. From this figure, the presence of single genotype of HPV DNA were detected in lane 4, 5, and 9 this was HPV35. In lane 2 and 6 double HPV infection was reported these are HPV16 plus HPV31 in lane 2, and HPV16 plus HPV33 in lane 6. While in lane 1,3,7, and lane 10 there are single infection with HPV16.

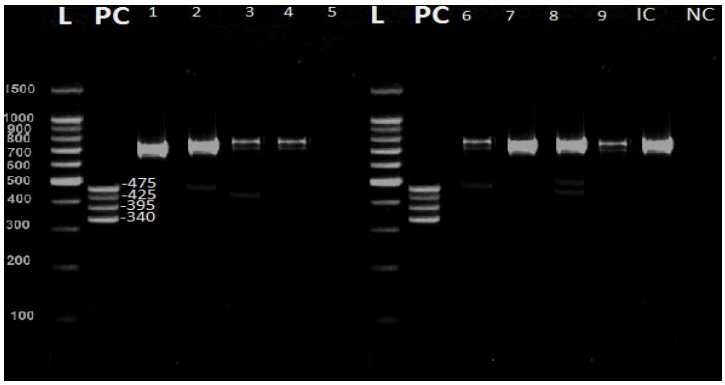

Figure (2): 3%Agarose gel electrophoresis of PCR product amplified from HPV isolates. L: DNA Leder.
PC: positive control amplification of HPV18(425 bp), HPV39(340bp), HPV45(bp475), HPV 59(395bp). and Lanes 1-10 show positive results with High risk / HPV18, HPV45.

The amplification of the viral DNA of High/risk HPV could be noticed by the presence of the typical DNA band of molecular weight 475bp for HPV45, 425 bp for HPV18,395 bp for HPV59 and 340bp for HPV39.In lane 8 showed double infection with HPV 45 plus HPV18.
While in lane 2, 6, 3 showed single infection with HPV45 and HPV18 respectively.

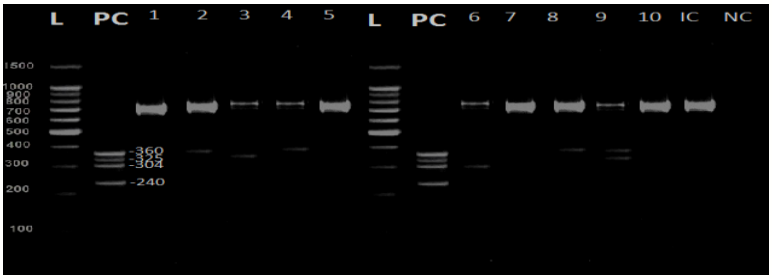

Figure (3): 3% Agarose gel electrophoresis of PCR product amplified from HPV isolates. L :- DNA Marker; Lanes 1 and 9 positive control amplification mix of HPV52(360bp), HPV56(325bp), HPV58(240bp), HPV66(304bp), and Lanes 2-14 show positive results with High risk / HPV52, HPV56, HPV58.

Figure (3) showed the amplification of the viral DNA of High/risk HPV could be noticed by the presence of the typical DNA band of molecular weight of 360 bp for HPV52, 325 bp for HPV56, 304bp for HPV66 and 240bp for HPV58. In lane 9 there were double infection with HPV52 plus HPV56, while in lane 2, 3, 4, 6 and 8 showed single infection with HPV52, HPV56 and HPV58 respectively. From these figures, multiple HPV genotypes infection could be noticed, these are HPV16+HPV45, HPV16+HPV33+, HPV16+HPV35 and HPV18+HPV52+HPV58. The detection of multiple HPV genotypes infection comprised 30.7% of total cases. the percentage of HPV genotypes in the total group of cervical squamous intraepithelial lesion are found as follow:

HPV16(15.3%), HPV18(15.3%), HPV33(7.7%), HPV31(7.7%), HPV35(30.7%), HPV56(7.7%), HPV45(7.7%), HPV58(7.7%) and HPV52(7.7%). The distribution of these genotypes according to the association of different grades of cervical Squamous intraepithelial lesion are shown in table (2).

The detection of high/risk HPV DNA in cervical smear of these premalignant cases versus normal smear group (as will be shown later) are in accordance that these HPV genotypes playing a role in the development of these Squamous intraepithelial lesion.

In the view of these result, it is possible to speculate that Multiplex PCR technology may be recommended as a good tool to screening these patients with cervical Squamous intraepithelial lesion. In comparison, the prevalence of multiple High/risk HPV infection (30.7%) in the present study was lower than the prevalence's (68.8% and 48%) reported by Griffin *et al.* [20] and Hillman *et al.* [18], respectively. The recent results indicate that multiple HPV infection is relatively uncommon. This study confirms the previous finding that the relationships between carriage of a "high" and "intermediate" HPV types and cervical neoplasia is not as clear cut as previously thought. This might be as a consequence of the higher sensitivity of PCR –based techniques .in addition, this may indicate that high copy numbers of "high risk" HPV types are necessary for oncogenesis [20]. Furthermore, the occurrence of multiple HPV types- infection could be related to a state of localized or generalized deficient immune responses to HPV. This was supported by the finding of increased risk for multiple HPV infection in these patients infected with HIV [19]. However, the role of such multiple HPV infection in cervical neoplasia remains to be established. The results of cases with typical criteria of Squamous intraepithelial lesion in this study are consistent with those result obtained by Mohamed Ali [12]. Since HPV types 16, 18, and less frequently HPV types 13, 33 and 35 were associated with all grades of Cervical Squamous intraepithelial lesion and invasive carcinomas [21], these results

could also support a causal role for these viruses in the carcinogenesis of the associated cervical neoplasia. The detection of high and moderate –risk oncogenic HPV types in cases of cervical SIL constitutes a real threat for these patients. Griffin *et al*. [20] and Bollen *et al.* [22] followed a similar case had found a persistence of HPV infection as well as a rapid progression of their associated CIN to high grad CIN and cervical cancer,[16]. Therefore, the patients in this group necessitate to have a follow up by cytological screening and HPV PCR test at short interval periods for the early detection and treatment of progressive lesions. In addition, these results confirmed both the observation of Strand *et al*. [17] that "high-risk" HPV can usually be traced for longer periods in the epithelium than the "low –risk; and those benign genital warts and low –grad cervical dysplasia caused by HPV 6 or HPV-11 are almost transient and regressive.

The importance of knowing the genotypes of HPV in such patients would help clinicians to address the proper recommendation regarding the treatment and / or the follow up of such cases. for example, those patients who bearded SIL in here cervix uteri but its negative for high/risk HPV is expected to have benign cervical lesion of transient or regressive nature. therefore, they need a less frequent follow up and at a longer interval period [22].

Table 3 Detection of HPV DNA by PCR in various grad of squamous intraepithelial lesion

Grade/Squamous intraepithelial Lesion	No tested	% Total	HPV DNA detection		
			State of PCR	No.	%
High grade squamous intraepithelial lesion	1	4.54	Positive PCR	1	100%
			Negative PCR	0	00.0
Low grade squamous intraepithelial lesion	14	63.63	Positive PCR	9	64.28
			Negative PCR	5	35.72
Atypical cells of undetermined significance	7	31.81	Positive PCR	3	42.85
			Negative PCR	4	57.15
Total	22	100	Positive PCR	13	100

From these results, it was noticed that in women with dysplasia, 13 out of 22 sample (59%) showed concordant results between cervical cytology and PCR. The discrepancy between cervical cytology and PCR in detection of High /risk HPV infection is probably due to the high sensitivity of PCR as well as the unique ability of PCR methods to detect latent and subclinical infections in addition, the variability in the definition of cytological criteria of HPV infection may also explain the observed differences. Furthermore, these results are in line that HPV PCR is a more sensitive method to document the precise prevalence of HPV infection in these patients. In comparison, it was found that, the prevalence of HPV DNA in the patients of this study was relatively lower than the results of many other studies. earlier prevalence studies yielded a range of percentages from 15% to 90% of HPV positivity in a range of cervical lesions from low grad SIL to cervical cancer [21]. This was explained to be related to differences in the sensitivity of the used HPV-detection methods as well as the difference in the prevalence of HPV in their relevant population.

The results revealed 9 different HPV genotypes. These were HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 45, HPV52, HPV56, and HPV58.the distribution of these genotypes according to the grad of cervical SIL is shown in table (3) These HPV genotyping results are in concordance with the results obtained by Gjoen *et al.* [10]. The majority of HPV types observed in this study corresponded to the "intermediate" and "high –risk " HPV types (HPV 31, -33, -35, -45, -52, -56, -58, -16, and 18). It was shown that the determination of oncogenic HPV genotypes in precancerous lesions was used an important early marker of cancer since specific HPV types differ in their association with cervical cancer [23].

The present study indicates that using cervical cytology alone is less reliable method than HPV-PCR technique for the detection of HPV infection in women with dysplasia. Therefore, we can conclude from these results that the addition of the molecular analysis of HPV to cytological screening improved the detection level of these high/risk types.

3.2. The Detection of High-risk HPVs in cervical smear of healthy group by Multiplex PCR -technique

A total number of 10 cervical smears from women with healthy cervices were taken as the control group in this study. following Multiplex – PCR analysis of DNA isolated from this group, none of these DNA sample showed High/risk HPV DNA amplicons as shown in figures (4), (5) and (6). The results of the present study were similar to the results obtained by Shen *etal*.[24] in those studies no HPV DNA, of similar types used in this study, was detected in smears of normal cervical epithelium. A detectable HPV infection that precedes development of these premalignant lesions detectable by cytology would lengthen the total detectable phase. therefore, in such setting, women with a cytomorphological normal high risk –negative cervical smear may need less frequent screening, whereas women with a high – risk HPV –positive smear could be offered a more intensive screening scheme or clinical management [14].

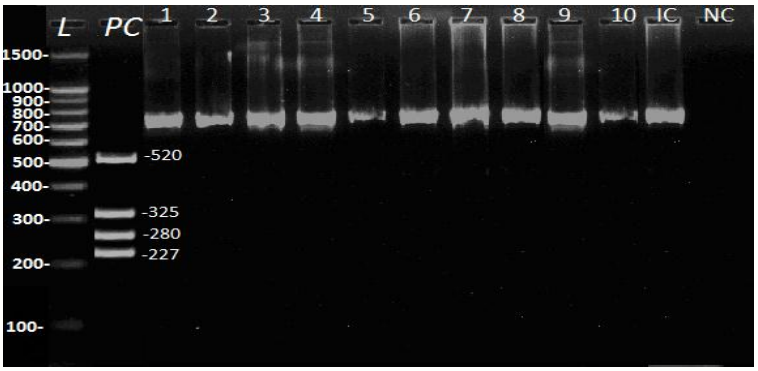

Figure (4): 3% Agarose gel electrophoresis of PCR product amplified from cervical smear of Healthy patients (control group). L: Leder, PC: positive control amplification of HPV16(325bp), HPV31(520bp), HPV33(227bp), HPV35(280bp), Lanes 1-10 showed negative results with High risk / HPV16, HPV31, HPV33, HPV35,
IC: Internal Control and NC: negative Control.

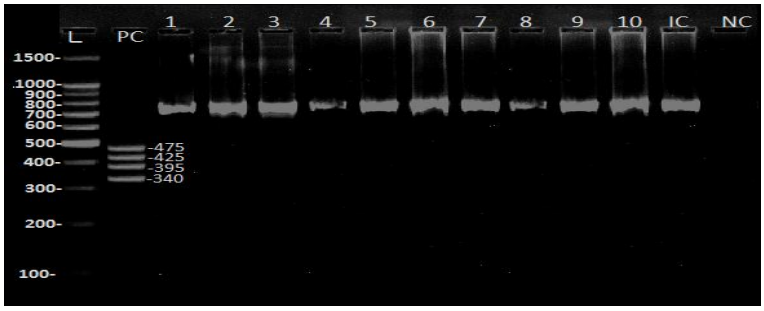

Figure (5): 3% Agarose gel electrophoresis of PCR product amplified from cervical smear Healthy patients (control group). L: Leder, PC: positive control amplification of HPV18(425bp), HPV45(475bp), HPV39(340bp), HPV59(395bp), Lanes 1-10 showed negative results with High risk / HPV18, HPV45, HPV39, HPV59, IC: Internal Control and NC: negative Control

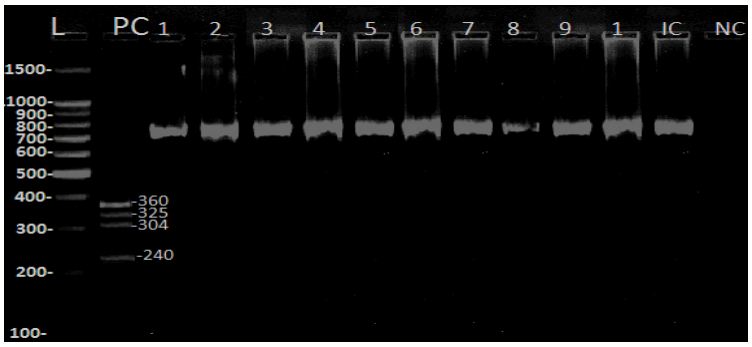

Figure (6): 3% Agarose gel electrophoresis of PCR product amplified from Healthy patients (control group).
L: Leder, PC: positive control amplification of HPV52(360bp), HPV56(325bp), HPV58(240bp), HPV66(304bp), Lanes 1-10 showed negative results with High risk / HPV52, HPV56, HPV58, HPV66,
IC: Internal Control and NC: negative Control.

4. Conclusions

The cytological examination of the cellular scrapes alone is not sufficient in common clinical practice for screening HPV in premalignant lesions of the cervix. alternatively, the addition of molecular technique, on top of them is the highly sensitive PCR test, to cytological examination is to amplify but not replace cytology in screening of these cases. While, Testing for High/risk HPV genotypes was of crucial value for general population or those with earlier Low grades cervical abnormalities on Pap test to identify and follow up women at increased risk for developing High grades cervical SILs or invasive cervical cancer. Authors recommend to studying the actual prevalence of HPV infection among Iraqi general population and attendants of dermatological and sexually transmitted diseases clinics for better implicates of HPV role in many important lesions and cancers.

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