Diagonosis of Cervical Cancer with Real Time PCR and HPV-16

Anil Kumar
Department of Biotechnology, Engineering and Technology,
Rama University, Uttar Pradesh, Kanpur, India.
anil.biotech@gmail.com

***Abstract*:** Cervical cancer is a deadly disease of women. Excessive vaginal bleeding, such as bleeding after sex, bleeding after menopause, bleeding and spotting between periods, and having longer or heavier (menstrual) periods than usual are common symptoms of cervical. The main causative agent for this disease is Human Papilloma Virus-16 (HPV-16).We have screened several patients of cervical cancer with the use of real time PCR and HPV-16 kit Genesig (UK). The data of real time PCR has shown huge viral load of HPV-16 gene in the case of some patients. There is slight up-regulation of HPV gene is also recorded in these cases. The viral load in these cases has exceeded around the $3x10^8$. For the authentication of our result we have taken two controls along with the two duplicated samples in each case. The positive control was used to get cut off value of viral load. The negative control was used to check the working status of the kit (Genesig). The entire analysis was screened with HPV-16 probe.
Keywords: Cervical cancer, Primer probe mix, Viral load, gene regulation and HPV-**16.**

I. INTRODUCTION

Cancer is uncontrolled growth of cells which becomes tumour in the body later. Most of the cancer is generated due to mutation in the DNA, or uncontrolled growth of cells but certain types of cancer are caused by the infection of occuloviruses, such as Human papilloma virus, is responsible for causing cervical cancer in human. The carcinomas arising from cervix is called as the cervical cancer i.e., the growth of cells is abnormal and invades the other cells. It is the most common and serious types of cancer in the women population in the 21th century in the world. In present time approximately 500,000 deaths each year occur in the world due to different type of cancers (1). Cervical cancer starts in cells lining the cervix. The cervix is the lower part of the uterus (womb). Cervical cancers and cervical pre-cancers are classified by how they look under a microscope. There are 2 main types of cervical cancer: *squamous cell carcinoma* and *adenocarcinoma*. About 80% to 90% of cervical cancers are squamous cell carcinomas. These cancers form from cells in the exocervix, and the cancer cells have features of squamous cells under the microscope. Most of the remaining types of cervical cancers are adenocarcinomas. Adenocarcinomas are cancers that develop from gland cells. Cervical adenocarcinoma develops from the mucus producing gland cells of the endocervix. Cervical adenocarcinomas seem to have become more common in the last 20 to 30 years (2).

It is the most common cancer in women of the developing countries where screening facilities are inadequate. At present throughout the globe, there are nearly 1 million women each year having cervical cancer. Major factors affecting the prevalence of carcinoma cervix in a population are economic factor, sexual behavior and degree of effective mass screening. First of all, there are no symptoms seen but later symptoms like abnormal vaginal bleeding, pelvic pain or pain during sexual intercourse may arise. On examination and investigation, we can find a large mass which indicates the presence of malignancy. The disease may be

advanced i.e., it may be meta statitised in the abdomen, lungs or elsewhere, then it may present with the symptoms like; loss of appetite, weight loss, fatigue, pelvic pain, swollen legs, heavy vaginal bleeding, bone fractures and/or (rarely) leaking of urine or faces from the vagina. Bleeding after douching or after a pelvic exam is a common symptom of cervical cancer (3).

The mode of spread of disease may be through different routes like; by direct extension (surrounding structures vagina, uterus), by lymphatic's, by blood stream (lungs, liver or bone), by direct implantation (4). The main cause of cervical cancer is the infection due to some types of Human Papilloma Virus (HPV). HPV types 16 and 18 are the cause of 75% of cervical cancer, while 31 and 45 for another 10%. Beningn tumour of epithelial cells like genital warts is also caused by various strains of HPV. HPV infection is strongly implicated in the etiology of cervical cancer. Besides HPV, a few other viruses may adversely affect the prognosis but do not have etiologic relationship are HIV, HTLV-1 and EBV infection. For simultaneous quantification of Human Papilloma Virus types associated with high-risk of cervical cancer a real-time PCR based system is used (5).

In order to study the relationship between viral DNA amount and risk of cervical carcinoma for the HPV types most commonly found in cervical tumours, a real- time PCR assay is developed. Cervical cancer is more common in women of 40 year of age or older and those who smoke or take contraceptive drugs for more than 5 years or the women which have the history of multiple sex partners (6). It is considered by the scientists and Researches that certain Human papilloma virus (HPV) is the most common cause for growth of cervical cancer. HPV infection is more commonly sexually transmitted disease worldwide and more than 50% of sexually active women will become infected by HPV in the genital tract (7).

Human papilloma virus is a small non enveloped DNA virus measuring 52-55 nm in diameter. For non enveloped viruses, the proteinaceous coat encases and protects the viral nucleic acid and provides the initial interaction between the viral particle and host cell (8). Various methodologies have been used in other studies for the detection of HPV including; PCR, in-situ hybridization, sequencing, DNA chip technology, immune histochemistry (IHC) and Southern blot. The majority of studies have utilized conventional PCR (23/24 studies) as the method of detection.

Seegene™ introduced the first Real Time (RT) multiplex quantification test for Human papilloma virus in Gaithersburg MD and Seoul, South Korea. The detection kit (Seegene™) was launched in the third quarter of 2012. This detection assay is based on newly developed TOCE™ (Tagging Oligonucleotide Cleavage & Extension) technology which makes it possible to detect multiple pathogens in a single fluorescent channel on RT PCR instruments (9). The Anyplex™ II HPV28 Detection is a multiplex RT PCR assay that permits simultaneous amplification, detection and differentiation of target nucleic acids of 19 high-risk HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70) as well as internal control (IC). This system is an innovative HPV detection/genotyping assay system which amplifies only specific targets without any cross reactivity and, using RT- PCR methods, detection is automated (10).

II. REVIEW AND LITERATURE

Cervical cancer is considered to be a slow-growing reproductive disease that affects thousands of women in the United States annually (11). This particular type of cancer forms within and/or on the tissue of the cervix. The cervix is part of the female reproductive tract, which connects the uterus to the vagina. Cervical cancer begins when surface level cells on the cervix divide uncontrollably causing cervical lesions, which if left untreated can progress into cancer. If not found in the early stages, cervical cancer can spread from the surface of the cervix, deep into the tissue of the cervix as well as the surrounding tissues within the vagina and uterus . It is estimated that there is an incidence rate, or rate of new cases, of approximately twelve thousand women

per year (12). Research shows that while although all women are at risk for developing cervical cancer, the majority of women that actually die from the disease are low-income women and/or women of minority groups, such as African Americans and Hispanics. Due to various social, political, and cultural factors, as well as disparities in the healthcare system, such as access to resources and socioeconomic status, thousands of low income and primarily minority women die unnecessarily from cervical cancer. Several risk factors are involved when looking at various causes for cervical cancer. Some of these include, the HPV virus, smoking, a weak immune system, sexual history and number of sexual partners, and the lack of regular PAP smears (13). The HPV infection, also known as the Human Papilloma virus, is one of the most common sexually transmitted infections in the United States today. It is contracted through sexual contact from person to person, and is known to be associated with almost all cases of cervical cancer due to the fact that the virus has the capability to alter the cells on the cervix (14).

The most important risk factor for cervical cancer is infection by the human papilloma virus (HPV). HPV is a group of more than 150 related viruses, some of which cause a type of growth called *papillomas*, which are more commonly known as *warts* (15*)*. HPV can infect cells on the surface of the skin, and those lining the genitals, anus, mouth and throat, but not the blood or internal organs such as the heart or lungs. HPV can be spread from one person to another during skin-to-skin contact. One way HPV is spread is through sex, including vaginal, anal, and even oral sex. Different types of HPV cause warts on different parts of the body. Some types cause common warts on the hands and feet (16). Other types tend to cause warts on the lips or tongue. Certain types of HPV may cause warts to appear on or around the genital organs and in the anal area. These warts may barely be visible or they may be several inches across. These are known as *genital warts* or *condyloma acuminatum*. HPV 6 and HPV 11 are the 2 types of HPV that cause most cases of genital warts. These are called *low-risk* types of HPV because they are seldom linked to cervical cancer. Other types of HPV are called *high-risk types* because they are strongly linked to cancers, including cancers of the cervix, vulva, and vagina in women, penile cancer in men, and anal and oral cancer in men and women. The high-risk types include HPV 16, HPV 18, HPV 31, HPV 33, and HPV 45, as well as some others (17). There might be no visible signs of infection with a high-risk HPV until pre-cancerous changes or cancer develops. Doctors believe that a woman must be infected by HPV before she develops cervical cancer. Although this can mean infection with any of the high-risk types, about two-thirds of all cervical cancers are caused by HPV 16 and 18. Infection with HPV is common, and in most people the body is able to clear the infection on its own. Sometimes, however, the infection does not go away and becomes chronic. Chronic infection, especially when it is with high-risk HPV types, can eventually cause certain cancers, such as cervical cancer. Although HPV can be spread during sex − including vaginal intercourse, anal intercourse, and oral sex doesn't have to occur for the infection to spread. All that is needed to pass HPV from one person to another is skin-to-skin contact with an area of the body infected with HPV. Infection with HPV seems to be able to be spread from one part of the body to another for example; infection may start in the cervix and then spread to the vagina. Completely avoiding putting the areas of your body that can become infected with HPV (like the mouth, anus, and genitals) in contact with those of another person may be the only way to truly prevent these areas from becoming infected with HPV (18).

A real-time polymerase chain reaction (RTPCR) is a molecular biology technique which is based on the polymerase chain reaction. During PCR it monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR (5).

There are two common methods for the detection of PCR products in real-time:-

1. Non-specific fluorescent dyes that intercalate with any double-stranded DNA,
2. Sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence.

Women with early cervical cancers and pre-cancers usually have no symptoms. Symptoms often do not begin until a pre-cancer becomes a true invasive cancer and grows into nearby tissue (20). When this happens, the most common symptoms are: Abnormal vaginal bleeding, such as bleeding after sex (vaginal intercourse), bleeding after menopause, bleeding and spotting between periods, and having longer or heavier (menstrual) periods than usual. Bleeding after douching, or after a pelvic exam is a common symptom of cervical cancer but not pre-cancer. An unusual discharge from the vagina the discharge may contain some blood and may occur between your periods or after menopause (21).

A. Human Papilloma virus:

HPV 16 is a non enveloped virus containing double stranded DNA molecule. It belongs to family Papillomaviridae. Its size is 52-55 nm in diameter. For non enveloped viruses the proteinaceous coat protects the viral nucleic acid and provides the initial interaction between the viral particle and the host cell. Papilloma virus have a unique process of infection (25) . Research into this has suggested that its uniqueness is related to adaptations in the viral life cycle which includes restriction of the production life cycle and the lack of effective immune response for an extended time period (26).

The HPV genome as shown in fig. consists of circular double stranded DNA of about 7900 base pairs, associated with cellular histones to form a chromatin like substrate. Histones are simple proteins derived from cell nuclei which yield certain amino acids resulting from hydrolysis. The HPV genome can be functionally divided in two regions (27):-
1. LCG (Long control region): Also called URR (upstream regulatory region) necessary for regulation of gene expression and for DNA replication, it occupies about 10% of the genome.

2. ORF (Open reading frame): Occupies over 90% of the genome and is divided into the early region (E1 to E7), which is necessary for the replication, cellular transformation and control of viral transcription, and the late region, that codes for the capsid proteins (L1 and L2).

B. HPV and cervical cancer:

Some types of HPV can increase the risk of developing cervical cancer, particularly types 16, 18, 31, 33 and 45. They are called high risk types. Almost all women with cervical cancer have at least one of these types of HPV in the cells of their cervix. Different types of HPV, types 16 and 18 cause about 7 out of 10 (70%) cancers of the cervix. The other types cause most of the remaining 30% of cervical cancers (32). There is no treatment that can get rid of the HPV virus. The body normally clears the virus from the body on its own after sometime. But treatment can get rid of any visible signs of HPV infection, such as warts. Treatment can also get rid of changes in the cervical cells that may develop into cancer.

Some types of HPV can increase risk of developing cancers in other parts of the body, not just the cervix. Not everyone with these types of HPV will go on to develop cancer. These cancers are rare and other factors are necessary before cancer will develop (33).

C. Real time PCR (qPCR):

Real time quantitative PCR (q PCR) is most similar to normal PCR. The major difference is that with qPCR the amount of PCR product is measured after each round of amplification while with normal PCR, the amount of PCR product is measured only at the end point of amplification (34). The concept of qPCR is simple; amplification products are measured as they are produced using a fluorescent label (35). During amplification the fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, to the accumulating DNA molecules, and the fluorescence values are recorded during each cycle of the amplification

Process (36). The fluorescence signal is directly proportional to DNA conc. Over a broad range, and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. The point at which fluorescence is first detected as statistically significant above the baseline is called the threshold cycle or Ct value (37).

The Ct Value is the most important parameter for quantitative PCR. This threshold must be established to quantify the amount of DNA in the samples. It is inversely correlated to the logarithm of the initial copy number. The threshold should be set above the amplification baseline and within the exponential increase phase (which looks linear in the log phase). Most instruments automatically calculate the threshold level of fluorescence signal by determining the baseline (background) average signal and setting a threshold 10-fold higher than this average (38). In theory, an equal number of molecules are present in all of the reactions at any given fluorescence level. Therefore, at the threshold level, it is assumed that all reactions contain an equal number of specific amplicons. The higher the initial amount of sample DNA, the sooner the accumulated product is detected in the fluorescence plot, and the lower the Ct value (39).

Dye based detection is performed via incorporation of a DNA binding dye in the PCR. The dyes are non specific and bind to any double stranded DNA generated during amplification resulting in the emission of enhanced fluorescence. This allows the initial DNA conc. To be determined with reference to a standard sample (41).

DNA binding dyes bind reversibly but tightly to DNA by intercalation, minor groove binding, or a combination of both. Most real time PCR assays that use DNA binding dyes detect the binding of the fluorescent binding dye SYBR Green. Some examples of DNA binding dyes are SYBR Green, SYBR Gold, BOXTO, YOYO^{TM}-1, BEBO, Amplifluor, Quencher labeled primers etc (42).

Prior to binding DNA, these dyes exhibit low fluorescence. During amplification, increasing amounts of dye bind to the double stranded DNA products as they are generated. For SYBR Green, after excitation at 497 nm, an increase in emission fluorescence at 520 nm results during the polymerization step followed by a decrease as DNA is denatured. Fluorescence measurements are taken at the end of the elongation step of each PCR cycle to allow measurement of DNA in each cycle (43).

D. Probe-Based Detection:

Probe-based quantization uses sequence specific DNA-based fluorescent reporter probes. Sequence specific probes result in quantification of the sequence of interest only and not all dsDNA. The probes contain a fluorescent reporter and a quencher to prevent fluorescence. Common fluorescence reporters included derivatives of fluoresce in, rhodamine and cyanine (44). Quenching is the process of reducing the quantum yield of a given fluorescence process. Quenching molecules accept energy from the fluorophore and dissipate it by either proximal quenching or by Fluorescence Resonance Energy Transfer (FRET) Most

reporter systems utilize FRET or similar interactions between the donor and quencher molecules in order to create differences in fluorescence levels when target sequences are detected. The fluorescent reporter and the quencher are located in close proximity to each other in order for the quencher to prevent fluorescence. Once the probe locates and hybridizes to the complementary target, the reporter and quencher are separated. The means by which they are separated varies depending on the type of probe used (45). Separation relieves quenching and a fluorescent signal is generated. The signal is then measured to quantitate the amount of DNA. The main advantage to using probes is the specificity and sensitivity they afford. Their major disadvantage is cost (46).

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 30 cycles (47)

1. Denaturation: High temperature incubation is used to "melt" double-stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

2. Annealing: During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

3. Extension: At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

III. MATERIALS AND METHODS

The following materials were used to perform the screening the cervical cancer. Cervical cancer can be defined as the formation of malignant tumor in the cervix, the lowermost part of the uterus. Causes and risk factors for cervical cancer include human papillomavirus (mainly HPV16) infection. HPV infection may cause cervical dysplasia, or abnormal growth of cervical cells. Regular pelvic examinations and pap testing can detect precancerous changes in the cervix. With the help of Real time PCR we confirm the cervical cancer. Precancerous changes in the cervix may be treated with cryosurgery, cauterization, or laser surgery. The most common symptoms and signs of cervical cancer are abnormal vaginal bleeding, increased vaginal discharge, bleeding after going through menopause, pain during sex and pelvic pain (23).

Cervical cancer can be diagnosed using a pap smear test and confirmed by Real time PCR. Chest X-ray, CT scan, MRI, and a PET scan may be used to determine the stages of cervical cancer. Treatment options for cervical cancer include Radiation therapy, surgery and chemotherapy. Two vaccines Gardasil and cervarix are vaccines that are available to prevent HPV infection (24).

IV. RESULTS AND DISCUSSIONS

The following steps have been followed to screen the cervical cancer.

A. Isolation of DNA from sample cervical fluid:

For the isolation of DNA we use Qiagen kit method and isolate the DNA of samples with the help of DNA isolation kit. The protocol for the isolation of DNA of cervical fluid from Quigen kit is given below:

(1) Took 3ml of infected saline solution and add 50µl of lysis buffer.

(2) Kept sample for 30 min. at room temp Digested sample was filled into the column provided in the kit.

(3) Centrifuged the samples at 4000 rpm for 2 min.

(4) Washed the sample with 50µl of wash solution 1.

(5) Washed the column with wash solution 2.

(6) After centrifuged eluted the samples of DNA with elution buffer approx of 50µl.

(7) Collected DNA was electrophoresis with the use of Agarose gel electrophoresis.

(8) We obtained the DNA.

B. Agarose gel Electrophoresis:

1. The extracted DNA was carried out with Agarose gel electrophoresis in a horizontal submarine electrophoresis unit.
2. Measure out 0.3 g of agarose powder.
3. Pour agarose powder into flask with 10ml of diluted 1x Tris borate buffer (TBF).
4. Transfer the mixture onto the hot plate until the Agarose was completely dissolved.
5. The solution was allowed to cool at room temp and then added 0.5µl of EtBr.
6. Pour the agarose gel into casting tray.
7. Allow the gel to solidify and removed the comb and tape.
8. The gel was transferred to 1x TBE buffer filled electrophoresis tank.
9. Loading dye was added to the sample DNA, mix well and then loaded into agarose gel.
10. Power card terminals were connected at respective to run the gel.
11. The separated DNA bands were visualized under ultraviolet trans-illuminator.

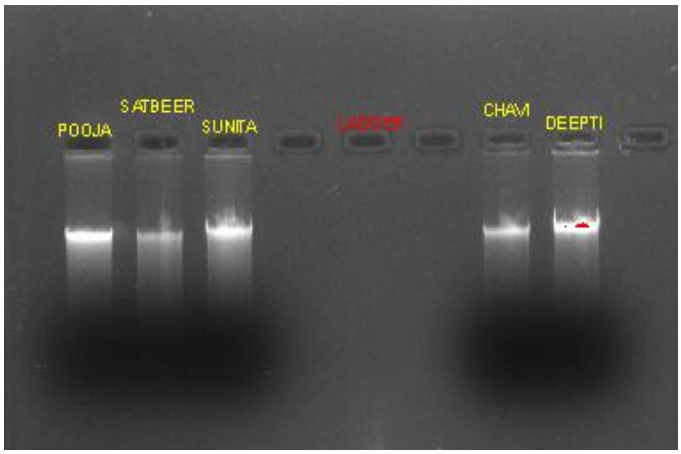

Fig. 1, Isolated DNA

We have isolated the DNA from the five suspecting patient's samples and out of five we have checked the gene expression of two patients named as Pooja and Deepti. After the real time PCR we have got the large up-regulation of HPV-16 gene in the case of Pooja. There is slight up-regulation of HPV gene is also recorded in the case of Deepti, but Deepti is not severely suffered. The viral load in the case of Pooja was around the $3x10^8$ and the viral load in the case of Deepti is around $6x10^7$. For the

authentication of our result we have taken two controls along with the two duplicated samples. The control was positive for the getting of the cut off value of viral load. The negative control was taken to check the working status of the kit (Genesig). One standard was also run parallel to the test to get the accurate result. HPV-16 probe kit was purchased from the Genesig (UK). The master mix contained the fluorescence cyber green dye. In the case of primer we have used primers probe for HPV-16.

From the above results we have concluded that the patient Pooja was suffered with HPV-16.

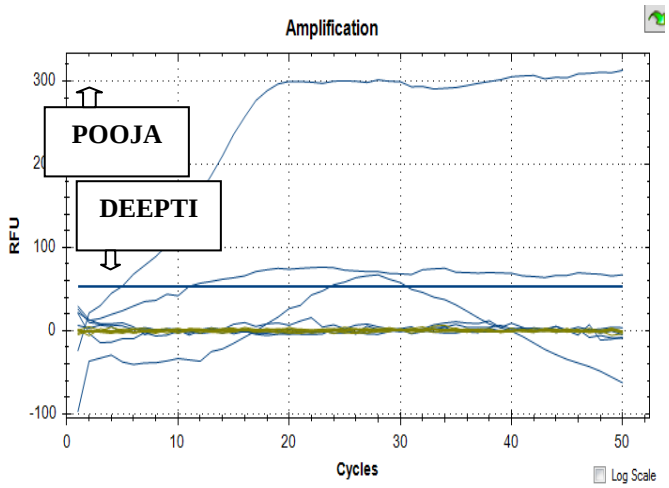

Fig. 2 Real time PCR, amplification of HPV-16 gene

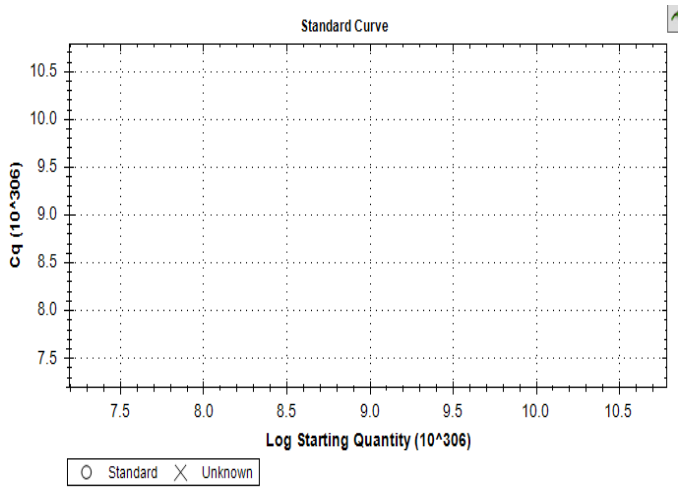

Fig. 3 Real time PCR, log of the viral load

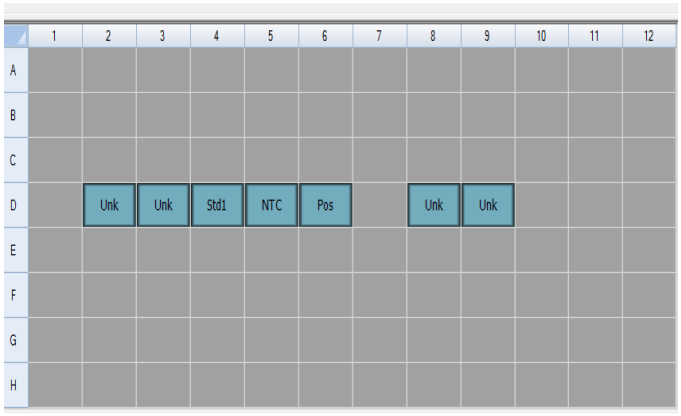

Fig4 Real time PCR, Occupied wells in the PCR machine)

Well	Fluor	Target	Content	Sample	Cq	SQ
D02	FAM	1	Unkn	unk11	N/A	N/A
D03	FAM	1	Unkn	unk12	11.02	N/A
D04	FAM	1	Std-1	control1	N/A	1.000E+02
D05	FAM	1	NTC	control	N/A	N/A
D06	FAM	1	Pos Ctrl	control3	N/A	N/A
D08	FAM	1	Unkn	unk13	23.75	N/A
D09	FAM	1	Unkn	unk14	4.97	N/A
D02	VIC	2	Unkn	unk11	N/A	N/A
D03	VIC	2	Unkn	unk12	N/A	N/A
D04	VIC	2	Std-1	control1	N/A	1.000E+02
D05	VIC	2	NTC	control	N/A	N/A
D06	VIC	2	Pos Ctrl	control3	N/A	N/A
D08	VIC	2	Unkn	unk13	N/A	N/A
D09	VIC	2	Unkn	unk14	N/A	N/A

(Fig. 5, Real time PCR, Cq and SQ values)

V. CONCLUSIONS

Although, real time PCR detection has developed efficient mechanisms for the detection of HPV-16. HPV-16 has induced high risk cervical cancer. This observation has been reported in association with high and sustained detection against HPV-16 infection.

In recent years, gene expression with real time PCR has got attention high efficacy against persistent HPV-16 infection and associated precancerous lesions. HPV-16, has also been confirmed. Large, integrated safety analyses show that cervix has a favorable safety profile in diverse populations of women.

The ultimate proof of vaccine reliability and long-term consistency will be continual maintenance of efficacy and minimal or lack of breakthrough cases following vaccination. Ongoing studies and post marketing surveillance evaluations will reveal the full impact of vaccination in reducing HPV infections and precancerous lesions.

ACKNOWLEDGMENTS

Author (Anil Kumar) is thankful to the Principal, Rama Medical College & Research Centre, Kanpur (India) for the financial support.

REFERENCES

[1]. Ryan KJ.”Kistner’s Gynecology & women’s Health’’ 1999, page 100-109.

[2]. Wallin, K.L.; Wiklund, F.; Angstrom, T.; Bergman, F.; Stendahl, U.; Wadell, G.; Hallmans, G.;Dillner, J. Type-specific persistence of human papillomavirus DNA before thedevelopment of invasive cervical cancer. *N. Engl. J. Med.* 1999, *341*, 1633–1638.

[3]. Gudleviciene Z, Didziapetriene J, Suziedelis K, Lapkauskaite L , Investigation of human papillomavirus, its types and variants. Medicina (Kaunas) 2005, 910-5.

[4]. Moberg M, Gustavsson I, Gyllensten U, Real-Time PCRBased System for Simultaneous Quantification of Human Papillomavirus Types Associated with High Risk of Cervical Cancer. J Clin Microbiol 2003, 3221-3228.

[5]. Mortazavi S, Zali M, Raoufi M, Nadji M, Kowsarian P, Nowroozi A ,The Prevalence of Human Papillomavirus in Cervical Cancer in Iran. Asian Pac J Cancer Prev, 2002, 69-72.

[6]. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R, The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol, 1989, 4417 4421.

[7]. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders P J, Meijer CJ ,Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003, 518-527.

[8]. Murray PR, Rosenthal KS, and Kobayashi GS, In: Medical Microbiology. Mosby Inc, 4th ed. USA, 2007, pp. 459-62.

[9]. Peedicayil A, Abraham P, Sathish N, John S, Shah K, Sridharan G, Gravitt P ,Human papillomavirus genotypes associated with cervical neoplasia in India. Int J Gynecol Cancer, 2007, 1591-5.

[10]. Qiu AD, Wu EQ, Yu XH, Jiang CL, Jin YH, Wu YG, Chen Y, Chen Y, Shan YM, Zhang GN, Fan Y, Zha X, Kong W ,HPV prevalence, E6 sequence variation and physical state of HPV16 isolates from patients with cervical cancer in Sichuan, China. Gynecol Oncol. 2007, 77-85.

[11]. Shobeiri F, Nazari M, A prospective study of genital infections in Hamedan, Iran. Southeast Asian J Trop Med Public Health 37 (Suppl 3), 2006, 174-7.

[12]. Silins I, Wang X, Tadesse A, Jansen KU, Schiller JT, Avall- Lundqvist E, Frankendal B, Dillner J , A populationbased study of cervical carcinoma and HPV infection in Latvia., 2004, 484-92.

[13]. Simbar M, Tehrani FR, Hashemi Z , Reproductive health knowledge, attitudes and practices of Iranian college students. East Mediterr Health J, 2005, 888-97.

[14]. Sotlar K, Selinka H C, Menton M, Kandolf R, Bultmann B , Detection of human papillomavirus type 16 E6/E7 oncogene transcripts in dysplastic and nondysplastic cervical scrapes by nested RT-PCR. Gynecol Oncol, 1998, 114-121.

[15]. Syrjanen K, Syrjanen S." Papillomavirus infections in human pathology" 2005, 41-48.

[16]. The Center for Women's Participation (CWP) Chapter 3, Women and Health. In: National Report on Women's Status in the Islamic Republic of Iran. Olive Leaf Publishing, Tehran, Iran. 2005, 33-45.

[17]. Tornesello ML, Duraturo ML, Botti G, Greggi S, Piccoli R, De Palo G, Montella M, Buonaguro L, Buonaguro FM; Italian HPV Working Group Prevalence of alphapapillomavirus genotypes in cervical squamous intraepithelial lesions and invasive cervical carcinoma in the Italian population. J Med Virol 2006, 1663-72.

[18]. Vizcaino AP, Moreno V, Bosch FX, Muñoz N, Barros-Dios XM, Borras J, Parkin DM International trends in incidence of cervical cancer: II. Squamous-cell carcinoma. Int JCancer 2000, 429-435.

[19]. Von Knebel Doeberitz M New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. Eur J Cancer 2007, 2229-2242.

[20]. You K, Liang X, Qin F, Guo Y, Geng L High-risk human papillomavirus DNA testing and high grade cervical intraepithelial lesions. Aust N Z J Obstet Gynaecol 2007, 141-4.

[21]. Zaeimi Yazdi J, Khorramizadeh MR, Badami N, Kazemi B, Aminharati F, Eftekhar Z, Berahme A, Mahmoudi M Comparative Assessment of Chlamydia trachomatis Infection in Iranian Women with Cervicitis: A Cross-Sectional Study. Iranian J Publ Health 2006, 69-75.

[22]. ZUR HAUSEN H. Papillomaviruses in the causation of human cancer: a brief historical account. *Virology Journal*, 2008, 260 - 265

[23]. Salz, T., Gottlieb, S. L., Smith, J. S., & Brewer, N. T, the Association between Cervical Abnormalities and Attitudes toward Cervical Cancer Prevention. *Journal of Women's Health (15409996)*, 19(11), 2011-2016. doi:10.1089/jwh.2009.17

[24]. Park, Y.; Lee, E.; Choi, J.; Jeong, S.; Kim, H.S. Comparison of the Abbott Real Time High-Risk Human Papillomavirus (HPV), Roche Cobas HPV, and Hybrid Capture 2 assays to direct

[25]. Sequencing and genotyping of HPV DNA. *J. Clin. Microbiol.* 2012, *50*, 2359–2365.

[26]. Sarma U, Mahanta J, Borkakoty B, et al (2015). Distribution of human papilloma virus infections of uterine cervix among women of reproductive age--a cross sectional hospital-based study from North East India. *Asian Pac J Cancer Prev*, 16, 1519-23. Sherigar B, Dalal A, Durdi G, et al.

[27]. Ghosh C, Baker JA, Moysich KB, et al. Dietary intakes of selected nutrients and food groups and risk of cervical cancer. 2008; 60(3):331−341.

[28]. American Cancer Society. Cervical Cancer Last Revised: 31 January 2014. Availableonline:http://www.cancer.org/cancer/cervicalcancer/detailedguide/cervical-cancer-prevention (accessed on 2 May 2014).

[29]. Anderson, clinical Microbiology Newsletter, 2002, 113.

[30]. Zhengz-M and Baker C " Papilloma virus genome st. expression and post transcriptional regulation" 2010,2286-2302.

[31]. Frazer et al.''Interaction of human papilloma virus (HPV) type 16 Capsid proteins with HPV DNA" 1991, 619-625.

[32]. Zur Hausen H "Viruses in Human cancers science'' 1991, 1167-1173.

[33]. Zur Hausen "Papilloma viruses in the causation of human cancer a brie historical account" virology journal, 2008, 260-265.

[34]. Schiffman et. Al, Human Papilloma virus to cervical cancer obstet, Gynecology'' 2010, 177-185.

[35]. Steverwald et al. "Analysis of gene expression in single oocytes and embryos by real time rapid cycle fluorescence monitored RT- PCR" 1999, 1034-1039.

[36]. Whitcombe et al. "Detction of PCR products using self probing amplicons and fluorescence" 1999, 804-807.

[37]. Lattora et al. "Design consideration and effects of LNA in PCR primers molecular and cellular probes" 2003, 253-259.

[38]. Lattora et al." Multiplex allele specific PCR with optimized locked nucleic acid primers" 2003, 150-158.

[39]. Bustine S.A." Quantification of m RNA using real time reverse transcription PCR" 2002, 23-29.

[40]. Higuchi et al. "Kinetic PCR analysis, Real time monitoring of DNA amplification reactions" 2003, 1026.

[41]. Pals et al." A rapid and sensitive approach to mutation detection using real time polymerase chain reaction and melting curve analyses" 1999, 241-246.

[42]. Selvin , P.R. et al." Fluorescence resonance energy transfer methodology Enzymology" 1995, 300-334.

[43]. Perlette et al. " Real time monitoring of intracellular m RNA hybridization inside single living cells" 2001, 5544-5550.

[44]. Mhlanga et al. "Molecular beacons to detect single nucleotide polymorphism with real time methods" 2001, 463-471.

[45]. Nolan et al." Quantification of mRNA using Real Time PCR'' 2006, 1559-1582.

[46]. Huggett et al. "Guidelines for Minimum information for publication of Quantitative Digital PCR Experiments'' 2013, 392-402.

[47]. Gita Elasmi et al." PCR detection and high risk typing of Human Papilloma virus DNA in Cervical cancer in Iranian women'' 2008, 361-366.

[48]. Benes v. et al. "Expression profiling of micro RNA using real time PCR'' 2010, 244-249.

[49]. PCR Technologies, current innovations, 3rd ed., Nolan and Bustin, CRC press, 2013.

[50]. Broomer et al. "Real Time quantification of Micro RNA_S" 2005, 33-35.

[51]. Bustin, S.A., Benes, V. Garson et al. "The Miqe guideline, minimum information for

[52]. Publication of Quantitative real time PCR experiments'' 2009, 611-622.