Diagnosis of herpes keratitis by PCR technique

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ABSTRACT

Herpes simplex virus causes variety of human diseases and is prone to hide in nervous system. There are, much more technique for HSV diagnosis such as serological test, cell culture and molecular tools which there is problems in front of each others. In this study, illustrated diagnosis Herpes Keratitis by PCR technique. DNA of 100 samples suspected to viral superficial stromal keratitis, were extracted using SINAGEN DNA kit. Two primers for PCR technique in diagnosing HSV infection were investigated. Among the 100 eye samples, only 35 cases were PCR positive and The PCR sensitivity up to 50 particles was observed. It brings about we, had just 35 positive cases, because of the failure in differential diagnosis of corneal edema as one of the manifestation of herpetic keratitis. In one hand, this study showed a 100% specificity of PCR technique for early diagnosis and prevention of the HSV infection. PCR is a cost effective and fast diagnostic procedure in detecting herpetic ocular infection.

Key Words: Herpes, PCR, Keratitis, Molecular Diagnostics

INTRODUCTION

Herpes simplex viruses are essentially transmitted through direct contact from the person having mucous secretions or wastages. HSV₁ is basically secreted in saliva and might be transmitted directly through kissing or indirectly through infected hands, vessels, etc. Self-insemination is performed using fingers and the virus might infiltrate into the eyes or genital tract [1]. On the other hand, HSV₂ is mainly, but not exclusively, transmitted through sexual intercourse. The neonates get infected via passing through the birth canal, or scarcely, via the infected couple [2 & 3]. Probability and age of HSV infection strongly depend on economic-social conditions. In developing countries or poorer communities of the developed countries, this state is undoubtedly reflective of factors such as high population, poor hygiene, and human contact paradigms including social customs and traditions [4]. In addition, genital herpes infection is a highly significant factor in transmission and acquisition of HIV infection [4]. It can be generally stated that the difference between genital and orolabial herpes types pertains to their occurrence and is not almost associated with their viral types. It means that both HSV-1 and HSV-2 types might be observed in upper and lower regions of body. Nonetheless, despite remarkable analogy of HSV-1 and HSV-2, there are also discrepancies between them, including: different composition of nucleic acids of these two serotypes. Sum of cytosine and guanine bases is 68% and 69% in HSV-1 and HSV-2, respectively [5].

Using novel diagnostic techniques, it was demonstrated that HSV₂ can also act as agent of benign meningitis aseptic in adults but this disease is not accompanied with mental alterations or severe cerebral disorders whereas virus
proliferation in herpes simplex encephalitis severely results in involvement of cerebral cortex [7-10]. Nearly everybody has encountered type 1 of this virus during early stages of their lives i.e. almost all people are seropositive against HSV1 but the level might have been lowered by the course of time. Such individuals differ from seronegative persons who have no kind of counter-herpes antibodies [11]. Yet, it is noteworthy that most people suffering from recurrent herpes have often high level of antibody against type 1, leading to their protection against type 2 [12 & 13]. In fact, several studies have shown that virus attack occurs less intensely in persons with recurrent and severe herpes record or the patients suffering from genital herpes 2 compared to those having low antibody level (i.e. those who are seldom infected by herpes). Since many laboratory tests use a part of herpes virus as antigen for detection of antibodies, it is actually difficult (if not impossible) to state whether the increase in antibody titer is associated with type 1 or results from a new infection of type 2. The acquired results must be attached together with the whole clinical evidences of the patient and cannot be utilized as an absolute indicator [14, 15, 16, & 17].

ELISA is another serologic test for diagnosis of HSV virus. The principle of the test is detection of special virus antibodies attached to the solid phase through marking the secondary anti-IgM and anti-IgG antibodies. This test is fast, sensitive, automatic, and currently commercial. Among the restrictions of the test are false positive and negative responses. Positive responses are confirmed by presence of IgG and IgM antibodies [18 & 19]. The diagnostic methods based on antibody and biopsy cannot diagnose the disease at early stages and only become capable of diagnosing the pathogens long time after the infection. Thus, acquisition of false positive and negative results is highly likely. For these reasons, molecular diagnostic techniques are currently applied at a greater extent thanks to advances in science and technology [20, 21, & 22]. Disadvantages of aforementioned diagnostic methods include: failure to diagnose HSV-1 and HSV-2 from each other, time-consuming procedures, and incapability of diagnosis at early stages of disease, and also, likelihood of acquiring false positive and negative responses [23]. Therefore, application of molecular diagnostic techniques is inevitable [24]. PCR test not only can be employed for diagnosis of herpes simplex virus disease but also might be used for tracking and analyzing the result of treatment by anti-virus medications [25].

The objective of the current study is to analyze and assess the PCR molecular technique in diagnosis of herpes keratitis and for prevention from herpes of the eye.

MATERIALS AND METHODS

Samples under study: 100 samples of surface herpes stromal keratitis taken through scraping method and benefitting from ANESTOCAINE were used in the present research. The samples were prepared from Eye Emergency Department of FÁRÁBI Hospital.

Cellular culture: The cellular culture used for analysis in the current study was acquired from Pasteur Institute Bank, Tehran/Iran. DNA was extracted from VERO cellular class (cells of African green monkey’s kidneys) infected by DNP kit (cinna gene) and was used as molecules.

Preparation of samples: Each scraping eye herpes sample was dissolved into 100 microliters of double-distilled water.

DNA Extraction: Using DNA extraction kit, genomic DNA of herpes species were extracted and designated as “DNA”. 100 ml of serum sample was mixed with 5 ml of protease K, and after undergoing vortex for 5 seconds, was placed on dry plate apparatus for 10 minutes at 72 ºC for proteolysis. Then, adding 400 ml of DNG, the acquired lysate underwent vortex for 5 seconds. 300 ml of isopropanol was added to lysate in the pipette containing the suspension and the pipette was inverted 10 times. The pipette was subsequently centrifuged at room temperature for 5 minutes at a rotation speed of 12000 rpm. The upper liquid was removed and 1 milliliter of 70% ethanol was added to the residual precipitation. The mixture was well stirred through inverting the pipette for another 10 times. Again, the pipette was centrifuged at rotation speed of 12000 rpm for 5 minutes, the upper liquid was removed, and the remaining alcohols were extracted by emptying the pipette on a tissue. The pipette containing the residues was placed at 65 ºC for 5 minutes in order to dry the DNA. The resulting residue was dissolved in 30 microliters of deionized sterilized water and then placed at 65 ºC for 5 minutes. Accordingly, DNA had a completely aqueous state, ready for PCR.
PCR Reaction

"---" ml of both extracted PCR Buffer X, "---" ml of DNA in each reaction consisting of "---" ml of 10 mM dNTP, "---" of MgCl2, "---" of 10 mM "???”; the front and rear primers in the final volume were 25 ml. 0.4 ml of 5 u/µl Taq DNA Polymerase at 70 °C for 20 seconds and adhesion temperature of 93°C; temperature schedule of the reaction was performed in the form of denaturation for 40 minutes and with 40 cycles.; Finally, polymerization was carried out at 72 °C for 20 seconds with nucleotide sequence as follows. Two sets of special front and rear primers of HSV were used in this reaction.

HSVF 5′ acc tac cgg cat aca agc tca-3′
HSVR 5′ aag tgg ctc tgg cct atg tcc-3′

Observation of PCR product: PCR product was electrophoresed in 2% Agarose gel containing ethidium bromide in 0.5 X TBE buffer.

Determining sensitivity of PCR test

To determine sensitivity of the optimized PCR test, it is necessary to prepare dilutions of HSV DNA from 1,000,000 to 5 particles.

Confirmation of PCR test exclusivity:

In order to confirm exclusivity of the optimized PCR test, DNA of HSV was analyzed simultaneously with DNAs of several other microorganisms. In this section, DNAs of viruses such as cytomegalovirus (CMV), varicella zoster virus (VZV), hepatitis B virus, mycobacterium tuberculosis, saccharomyces, and human were used. The optimized PCR test was analyzed on these DNAs and DNA of herpes simplex virus as well as the control negative sample.

Research Findings

PCR product was loaded on 1.5% Agarose gel. The size of the acquired segment is based on the special 454 primers (Figure 1). It was also mentioned in the present research that PCR test features great exclusivity in detection of this virus. Through sensitivity determination of PCR test, the test was shown to have an sensitivity of 50 virus particles. Out of 100 keratitis samples, 35 samples were reported as positive using PCR technique. Test sensitivity was reported to be 50 viruses and number of thermal profile cycles equalled 40.

DISCUSSION

Herpes simplex virus causes different diseases in human and herpes infection of eye is known as the most widespread cause of cornea-associated blindness in USA since this virus tends to enter the nervous system. Herpes

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They took use of nested PCR technique in their research. The test was conducted at optimal annealing temperature morbidities [26]. If initial infection with HSV-1 occurs in the eye, a severe corneal conjunctivitis (herpes keratitis) will arise, which is normally limited to one eye. Infection might be new or a result of recurrence. Recurrent ocular morbidities are prevalent and appear as serrated keratitis, corneal ulcers, and/or vesicles on the eyelids [27 & 28]. Therefore, it is vitally inevitable to take use of an appropriate diagnostic technique for identification of this pathogen. In spite of remarkable advances made in serologic HSV diagnosis methods at the present time, but these methods are not considered as suitable means for detection of the respective infection owing to their pitfalls in diagnosis. Hence, for precise and timely diagnosis of infective agents, alternative molecular techniques, such as PCR, need to be applied to quickly determine the sequence of herpes gene. Additionally, having determined the specific genetic sequences in herpes, the corresponding primer can be designed for such infections, and also, skin, blood, spinal liquid samples can be specified through PCR test [29-33].

PCR technique is among the most common and important methods of target proliferation; this technique enjoys many advantages and applications such as numerous replicates of a gene, checking presence or absence of a certain gene in a DNA segment, pre-natal diagnosis of genetic diseases, determination of embryo's gender, and also, assessment of bacterial and viral infections. Using Random PCR during their researches at UK Dentistry Department for diagnosis of herpes simplex from saliva and oral and orolabial samples, Robinson et al. (1992) benefitted from special primers of glycoproteins B and D (gB and gD) genes and also Thymidine Kinase gene. Consequently, size of the proliferative segment for Thymidine Kinase (TK) gene was equal to 110 open pairs. Test sensitivity was reported equal to 10 viruses, which is excellent [43]. In comparison with the optimized method, the abovementioned test was more precise and sensitive than the current research; however, DNA extraction procedures and number of the analyzed samples have not been mentioned. Besides, features of both studies were 100% expressed.

Mitchell et al. (1997) took advantage of nested-PCR technique and Thymidine Kinase gene for diagnosis of herpes virus. In this test, PCR product for Thymidine Kinase gene equaled 290 bp and there were 2100 samples for analysis. In the respective study, the analyzed sensitivity was reported to be less than 10 particles [44]. The aforementioned method is better and more precise than the current optimized test but has not been verified in terms of feature. A group of researchers in Mayo Clinic in 1996 used primers of polymerase DNA gene and Real-Time PCR technique for diagnosis of herpes simplex of the extracted Mollaret’s meningitis samples. The number of samples under study was 6607. Sensitivity of PCR test in the respective study was determined to be less than 300 virus particles [45]. Compared to the latter method, the current optimized technique is superior and more precise in terms of sensitivity but there are fewer tested samples than the abovementioned study. In addition, number of cycles and size of the proliferated segment were not mentioned; both tests are 100% precise in terms of feature.

Through another experiment on ocular and dermal herpes samples in 1999, Madhavan benefitted from the primers already adopted by Lakeman in 1996. Number of tested samples was not mentioned but test sensitivity was below 10 viruses and feature of this diagnosis was reported to be 100% [46]. In the same year, Therese et al utilized PCR technique for identification and diagnosis of herpes simplex virus in diseases of the retina. It was stated in this study that minimal increase of anti-HSV antibody was observed in serum of patients who received appropriate dosage of acyclovir [47]. Van Doornum applied nested-PCR technique and primers of gD and gG genes for diagnosing non-genital herpes cases (ocular, dermal, labial, CSF). There were 688 herpes samples and test sensitivity equaled 10-100 viruses [48].

To diagnose ocular keratitis, Chichili et al. (2003) deployed multiplex-PCR. Following determination of consecutive dilutions, test sensitivity was approximately 4 particles for HSV1 and 12 particles for HSV2 [49]. Although this test was more precise than the current study based on the acquired results but DNA was extracted through boiling, which is not efficient considering the kit used in the respective test. Also, nothing was mentioned about the observation band and the designed primer. Furthermore, number of samples was smaller than that of the present research. Nevertheless, both tests are identical in terms of feature.

A group of physicians in Carolina’s Hospital in 2004 performed molecular diagnosis using in-house PCR technique on polymerase DNA gene and its designed primers on herpes infection. The shortcomings of their study included uncertainty of its sensitivity and exclusivity [50]. In another study, Grenier et al. (2009) benefitted from two pairs of internal primers and two pairs of external primers for diagnosing herpes from GCF liquid of periodontal patients. They took use of nested PCR technique in their research. The test was conducted at optimal annealing temperature.
of 55 °C. Also, DNA was extracted using kit. There were 300 tested samples and test sensitivity was reported to be 25 viruses. This sensitivity was observed with thermal profile of 25 cycles [51]. The aforementioned method was superior to the technique applied in the current research in terms of number of samples. In addition, feature test was not verified in the respective paper.

RESULTS AND CONCLUSION

17 negative samples were reported in an analogous research on 55 patients. The authors also mentioned the problem of lack of comparison between molecular and clinical techniques.

On the other hand, the primers used in the present research were verified by the website “http://www.cypalleles.ki.se/cyp3a4.htm”; also, enzyme section and distance of SNPs from the primer was more favorable than the 55-patient research carried out by Noriku Koizumi et al.

Another research was conducted by “Inou Y”, in which 55% positive response was achieved using Real-Time PCR [53] while the research analyzed in the present paper reported 35% positive response. The reason can be attributed to better diagnosis of Real-Time PCR compared to PCR.

During the present project, the authors realized the failure of technique to differentiate keratitis from corneal edema. Thus, the test encountered problems for obtaining percentage of positive response and recording the statistics of samples. Nonetheless, the samples are recommended to be taken by a skilled clinician so as to alleviate this problem. In the current study, the target gene was part of polymerase DNA gene of herpes simplex virus. 35 out of 100 samples were reported positive in the current study using PCR technique. According to the results acquired from PCR technique, it can be acknowledged that the respective technique is more sensitive compared to other techniques such as cellular culture and serologic tests, and also, takes less time despite of its high precision and sensitivity.

REFERENCES