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Research Article

DETECTION OF HUMAN HERPES SIMPLEX VIRUS INFECTIONS IN THE LEBANESE POPULATION USING CONVENTIONAL PCR

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ABSTRACT

Extremely widespread, Herpes infections are in most cases benign and even completely asymptomatic. When the disease is expressed, cutaneous and mucous clinical signs are highly variable, often disturbing, sometimes disabling because of the pain and are even hopeless due to their recurrent infections. The genital Herpes, which affects the sexual organs and their proximity, is caused by Herpes simlex virus type 2 (HSV-2). In some cases, the symptoms are intense and painful, especially in female patients, yet these symptoms are very limited and even absent in other cases depending mostly on the physiology of the patient.

However, when the infection reaches the orofacial region, it appears in most cases as "cold sores" outside the mouth and around the lips but can also affect the eyes which may lead to altered vision in the long term. Oral Herpes is caused by the Herpes simplex virus type 1 (HSV-1).

Currently, Pap Smears are crucial in order to prevent HSV infections. The detection of the viral genome of HSV is possible using molecular biology tools, displaying a wide range of detection strategies.

The aim of this paper is to perform an epidemiologic study to evaluate the prevalence of Human Herpes simplex virus infection in the Lebanese population and it's variation within 10 consecutive years between 2006 and 2015.

To process this study, we've analyzed data provided by the Lebanese National institute of pathology (INP) using Food & Drug Administration (FDA) approved HSV genotyping kits, which allow the detection of both HSV-1 and HSV-2. These data consisted of reports revealing HSV infectious status in addition to patients personal information of different types of samples (vaginal secretions, genital lesions, oral lesions, vulvar vesicles, cerebrospinal fluid).

Statistical analysis has shown that Herpes infections occurred in variable rates ranging between 9% and 30% of infected subjects within the time course of the study (2006-2015).

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INTRODUCTION

An infection occurs when a living organism is invaded by germs, more precisely microscopic pathogens, such as bacteria, viruses, fungus or even parasites. A viral infection can either be acute (influenza virus) or persistent since the immune response was by itself insufficient for eliminating infected cells and completely blocking the viral replication. A persistent infection can either be chronic (chronic hepatitis B) or latent (Herpes virus) [1].

Herpes simplex virus (HSV) is a common human pathogen, causing infections of orofacial mucosal surfaces (HSV-1) and

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genital mucosal surfaces (HSV-2). Productive infection results in the formation of vesicular lesions in the mucosal epithelia, followed by spread of the virus to sensory neurons and establishment of a latent infection that may remain for the life of the host. Reactivation of dormant virus results in recurrent disease at or adjacent to the site of primary infection. The common cold sores caused by HSV-1 and the genital Herpes lesions caused by HSV-2 are not life-threatening conditions, but serious pathology can result from infections of the cornea (keratitis) or central nervous system (encephalitis), and infection of newborns or immunocompromised individuals can result in severe disseminated disease [2].

Of all sexually transmitted diseases (STD), genital Herpes progresses the most rapidly. It's in fact one of the most widespread STDs in the world. In France, studies have shown that approximately two million patients suffered from genital Herpes. However, nowadays, eight of ten people don't even know they have the infection until after they consult a physician, meaning that these people risk transmitting the disease to their sexual partners. The current epidemiologic status highlights the fact that this infection is under diagnosed, mostly due to the lack of knowledge and the under evaluation of the disease. The particular aspect of the infection that can take asymptomatic or atypical forms, the spontaneous regression of the outbreaks as well as the taboo aspect of the disease are major contributors to its current epidemiology [1].

The viral replication cycle in the host is characterized by the establishment of a latency state, which allows the virus to escape the immune control. The infections caused by Herpes simplex virus are characterized by a primary infection that is usually asymptomatic, happening early during childhood in case of HSV-1 and during the period of sexual activity when caused by HSV-2. Reactivation of dormant viruses can take place throughout the entire life, with or without clinical signs. Besides the physical pain, Herpes can alter greatly life quality of those who have it. Misguided, lots of those who have the disease live their lives in shame, guilt and suffering.

Many are the patients that don't consult a specialist when encountering Herpes outbreaks, however when genital Herpes is diagnosed in an early stage and correctly supported, the risk of transmitting the disease is reduced as well as being able to prevent recurrences. Nowadays, thanks to treatment and some basic hygienic rules, it's possible to learn when to anticipate the outbreaks and reduce the risk of contagion. Supporting the disease begins with a simple yet correct education of the patients, allowing an easier physician/patient dialogue as well as improving earlier diagnostic [3]. This study was launched in order to determine the prevalence of Human Herpes simplex virus type 1 and 2 infections in the Lebanese population and its variation within 10 consecutive years between 2006 and 2015.

MATERIALS AND METHODS

In this section, we are going to be screening for HSV infections in patients originating from different Lebanese areas. Molecular diagnostics will be performed on products of oral or genital secretions submitted by the patients themselves, in order to isolate the viral genomic DNA, amplify the extracted DNA via PCR multiplex and finally detect/analyze PCR products migrated on Agarose gel.

A statistical analysis will also be performed in order to determine the prevalence of HSV infection in the studied population as well as its variation between the years 2006 and 2015.

Workflow

Each sample, in order to achieve the molecular diagnostic test properly, must follow a certain procedure described below.

Viral DNA isolation

Isolating nucleic acids is usually the first step in most molecular biology studies. In this study we used specialized kits (kit High pure ROCHE), which contain ready-to-use reagents allowing rapid and efficient viral DNA extraction.

The employed kit aims towards extracting DNA found in cervical cellular specimen, under specific denaturation conditions and at high temperatures. First of all, samples are put into Eppendorf tubes which are centrifuged in order to discard the supernatant. The pellet is resuspended in a lysis buffer (LB), Internal Control (IC) and universal sorbent will be added. The mixture is then incubated at 65°C followed by a simple vortex step and the DNA is purified by adding a washing buffer (WB). Finally, DNA elution buffer (EB) is added, the mixture is then centrifuged and the supernatant containing targeted DNA is placed in a new tube ready for PCR amplification.

DNA quantification

Extracted DNA should be subject to spectrophotometric evaluation using a NanoDrop 2000® prior to the amplification step. This step will determine the DNA concentration in 2 µl of the extracted solution which is an important determinant for PCR parameters; it will also give the ratio of absorbance (optical density) 260/280 (Fig.1). This ratio is used to assess the purity of DNA. A ratio of ~ 1.8 is generally accepted as pure DNA. If the ratio is appreciably lower, it may indicate the contamination with proteins (since proteins have a maximum absorbance at 280 nm) or other contaminant reagents associated with the extraction protocol that absorb strongly at or near 280 nm. However, a ratio higher than 2 indicates other impurities that absorb strongly at 260 nm (such as compounds with carboxylic acid groups). An optical density (OD) of 1 corresponds to a 50µg/µl of double stranded DNA.

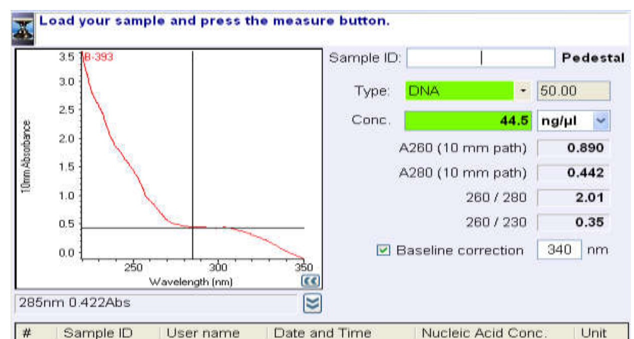

Figure 1 Example of the NanoDrop 2000® Result Display [4]

PCR amplification

After being spectrophotometrically quantified, the target DNA is then amplified via PCR multiplex. This step is possible due

to specific primers that are chosen either from the literature or researched using bioinformatic tools (primer3, primerblast…).

Polymerase Chain Reaction (PCR) is performed in a reaction mastermix including 3 µl of extracted DNA, DNA Taq polymerase, specific sense and anti-sense primers (0.1 µM of each), the four types of deoxynucleotide triphosphates (dNTPs) in excess in a buffer solution and 17 µl of sterile water.

The tubes containing the PCR mix are submitted to repeated heat cycles several tens of times in a thermocycler. The PCR apparatus allows programming of the period as well as the succession of the thermal plateau cycles. Each cycle of amplification involves a step of DNA denaturation at 94°C for 30 seconds, followed by a step of primers annealing to their specific target sequence at 60°C for 1.5 minutes and finally a step of elongation by the Tap polymerase at 72°C for 1 minute. PCR amplification finishes with a step ending all elongations at 72°C for 10 minutes and the temperature is lowered to 4°C.

Gel electrophoresis

PCR products are analyzed by electrophoresis on Agarose gel (2%) after adding Ethidium bromide (EtBr) intercalating agent which generates an orange-red fluorescence when exposed to ultraviolet (UV) light (~300 nm).

When an electric field is applied to the gel in the presence of a buffer solution (TAE or TBE), DNA fragments, negatively charged, migrate towards the positive electrode at a speed depending on their molecular weight, the Agarose concentration, the size of the gel as well as the applied voltage. Molecular weight markers are also deposited in the gel in order to estimate the weight of the DNA fragments.

Once the migration is done, DNA bands are visualized under UV light, photographed and registered.

Data analysis

Gel analysis is performed by software "SCION IMAGE" in order to facilitate the interpretation. This software allows DNA bands analysis by calculating the integrated pixels density constituting the band. Screening and classifying HSV in PCR products is done by migration on Agarose gel using specific markers of HSV-1 and HSV-2.

Clinical Cases

Amplicon Size

Table 1 Molecular weight (bp) of the different targets

Target	Size on Agarose gel (bp)
Internal control (IC)	981
HSV-2	473
HSV-1	300

Clinical application

Figure 2 Results obtained after migration of PCR amplicons on Agarose gel

M: HSV specific markers
1-6: Clinical cases
NEG: Negative control
IC: Internal control

Results interpretation

Table 2 Clinical cases 1-6

Clinical case	Result
1	HSV-1
2	-
3	HSV-2
4	HSV-1 and HSV-2
5	-
6	HSV-2

RESULTS

Statistical analysis has shown variable rates of HSV infection between the years 2006 and 2015.

Note that the distinctive genomic screening of HSV-1 Versus HSV-2 was introduced as of 2011.

2006

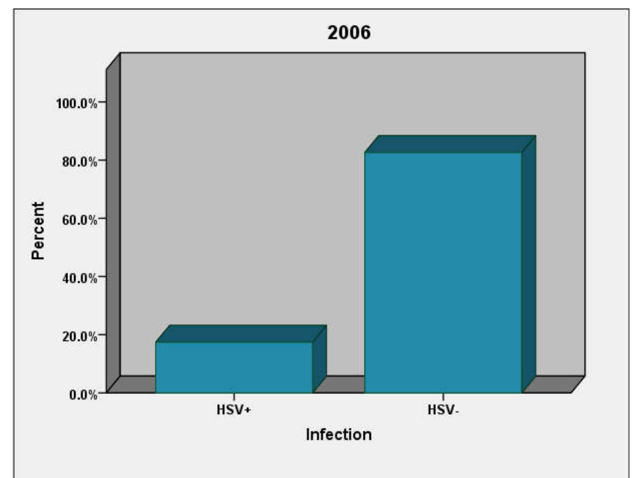

Figure 3 Percentage of HSV infected and non-infected subjects, 2006.

Screening for HSV infections in 2006 has shown that, out of 23 subjects, 4 (17%) were infected by the HSV while 19 (83%) were not.

2007

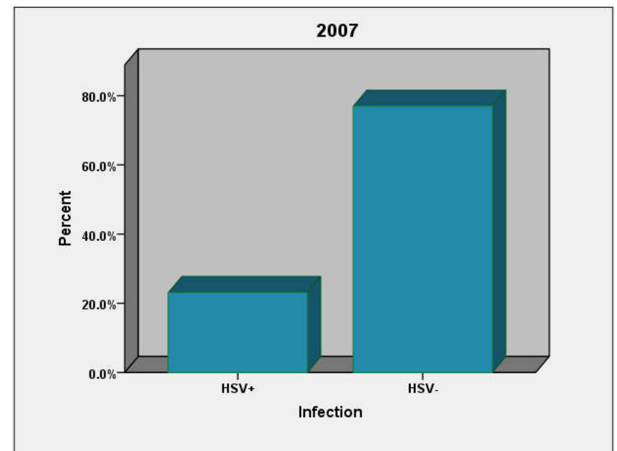

Figure 4 Percentage of HSV infected and non-infected subjects, 2007.

2008

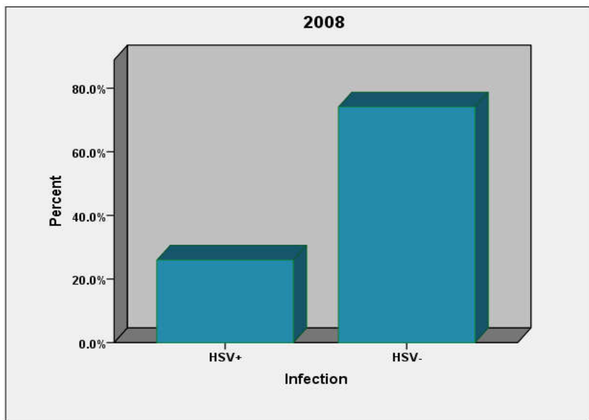

Figure 5 Percentage of HSV infected and non-infected subjects, 2008.

Screening for HSV infections in 2008 has shown that, out of 27 subjects, 7 (26%) were infected by the HSV while 20 (74%) were not.

2009

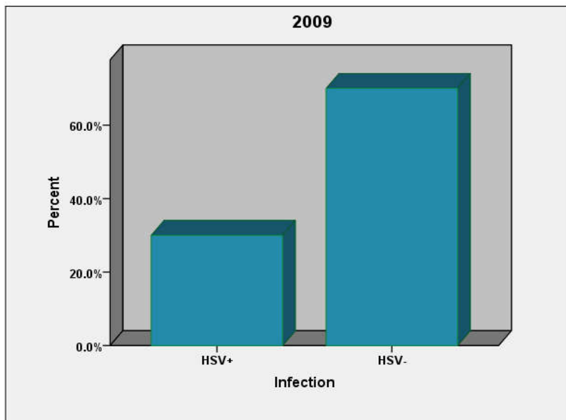

Figure 6 Percentage of HSV infected and non-infected subjects, 2009.

Screening for HSV infections in 2009 has shown that, out of 20 subjects, 6 (30%) were infected by the HSV while 14 (70%) were not.

2010

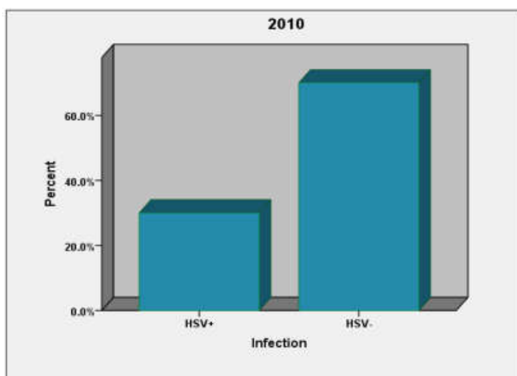

Figure 7 Percentage of HSV infected and non-infected subjects, 2010.

Screening for HSV infections in 2010 has shown that, out of 30 subjects, 9 (30%) were infected by the HSV while 21 (70%) were not.

2011

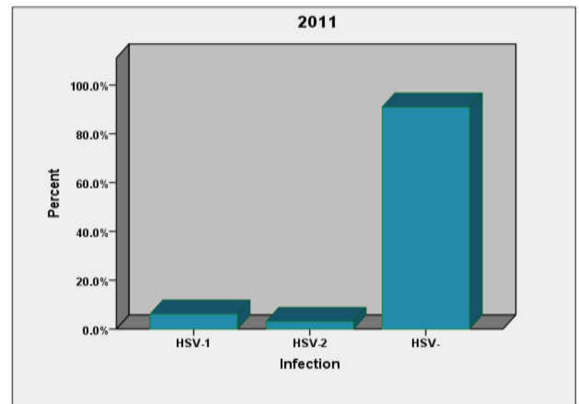

Figure 8 Percentage of HSV (HSV-1 Vs HSV-2) infected and non-infected subjects, 2011.

Screening for HSV infections in 2011 has shown that, out of 33 subjects, 3 (9%) including 2 HSV-1 and 1 HSV-2 were infected by the HSV while 30 (91%) were not.

2012

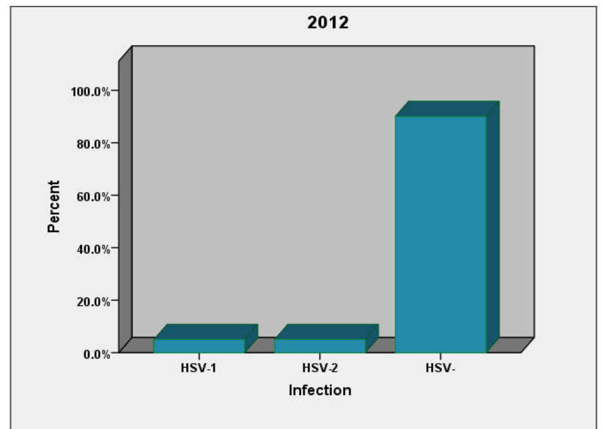

Figure 9 Percentage of HSV (HSV-1 Vs HSV-2) infected and non-infected subjects, 2012.

Screening for HSV infections in 2012 has shown that, out of 20 subjects, 2 (10%) including 1 HSV-1 and 1 HSV-2 were infected by the HSV while 18 (90%) were not.

2013

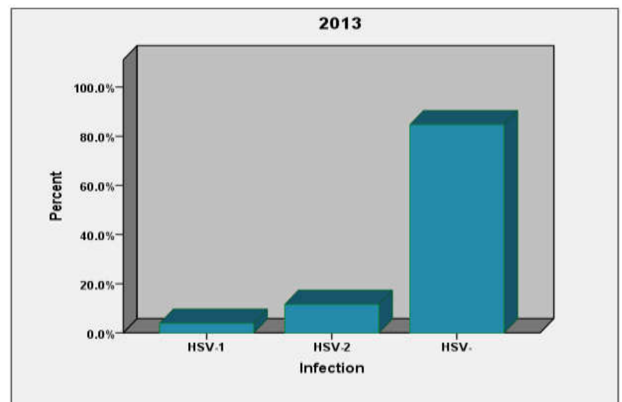

Figure 10 Percentage of HSV (HSV-1 Vs HSV-2) infected and non-infected subjects, 2013.

Screening for HSV infections in 2013 has shown that, out of 26 subjects, 4 (15.38%) including 1 HSV-1 and 3 HSV-2 were infected by the HSV while 22 (84.62%) were not.

2014

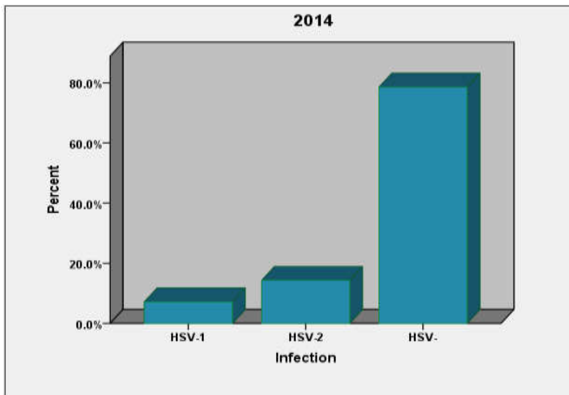

Figure 11 Percentage of HSV (HSV-1 Vs HSV-2) infected and non-infected subjects, 2014.

Screening for HSV infections in 2014 has shown that, out of 28 subjects, 6 (21.43%) including 2 HSV-1 and 4 HSV-2 were infected by the HSV while 22 (78.57%) were not.

2015

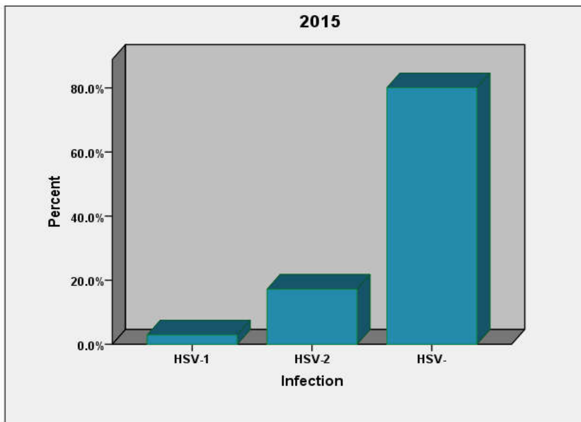

Figure 12 Percentage of HSV (HSV-1 Vs HSV-2) infected and non-infected subjects, 2015.

Screening for HSV infections in 2015 has shown that, out of 35 subjects, 7 (20%) including 1 HSV-1 and 6 HSV-2 were infected by the HSV while 28 (80%) were not.

DISCUSSION

Since we lacked statistical data, highlighting the prevalence of Herpes infections in the Lebanese population, it was essential to launch an epidemiological study concerning this frequent, disturbing but yet benign infection.

The epidemiology of a disease indicates how prevalent and impacting this disease is within a population. When it comes to Herpes, it is difficult to identify the exact number of infected subjects, due to the various asymptomatic forms of the disease as well as the uneasy diagnostic. Herpes is a disease found in almost every part of the globe at unequal rates. The lower the socio-economic status is, the higher the infectious rate is for HSV-1, whereas the prevalence of the infection by HSV-2 is elevated in subjects having multiple active sexual partners.

Studies have shown that the highest Herpes infectious rates are found in Africa, where the socio-economic status in most regions is deteriorated (prevalence 60-80%), whereas the lowest rates of Herpes infection are found in countries with higher hygienic profiles such as the united states (prevalence 20%) and Europe (prevalence 17%) [5].

Our launched statistical study reported variable rates of Herpes infection within the time course of the study (2006-2015). Results have shown an increase in the percent of infected subjects between the years 2006 (17%) and 2010 (30%).

This spreading of the infection is supported by many factors: the lack of a rapid and reliable diagnostic, a clinical diagnostic sometimes difficult, inaccurate in 30% of the cases, the possibility of asymptomatic viral excretions (when the virus affects the mucosa where its detection is delicate, the underutilization of anti-viral therapies that aren't curative anyways. Studies were performed in order to apprehend risk factors which predisposed to infections by HSV-1 and HSV-2, the majority of these studies emphasized a correlation with the age, sex, sexual habits of the subject as well as the surrounding socio-economic status.

The development of a specific diagnostic test allowing a distinctive identification of both HSV-1 and HSV-2 as of 2011, has lead to lower infectious rates ranging between 9% (2011) and 21.43% (2014).

The observed decrease of Herpes infectious rates is related to higher hygienic and preventive measures as well as the development of more rapid/accurate diagnostic tests.

CONCLUSION

Statistical analysis that was performed as part of this study has shown variable Herpes infectious rates between the years 2006 and 2015.

Results, within the Lebanese population, have shown a constant increase in the prevalence of the disease between the years 2006 (17%) and 2010 (30%). The observed increase is most likely correlated with the presence of various asymptomatic forms of the disease as well as the lack of rapid/accurate diagnostics.

Concurrently with the development of a specific diagnostic test allowing a distinctive identification of both HSV-1 and HSV-2, the analysis has shown lower infectious rates ranging between 9% (2011) and 21.43% (2014).

Herpes infections induced by either HSV-1 or HSV-2 are persistent and sometimes unbearable contributing to an anti-social behavior, a low self-esteem as well as feelings of depression and anxiety.

Prognosis of Herpes infection depends on an early diagnostic and an immediate support. Despite the impressive progress made in understanding the biology of the HSV virus as well as diagnosing and treating Herpes infections, a lot of questions concerning the pathogenesis and efficient therapy of the disease, remain unanswered.

The role of cytotoxic lymphocyte T cells (LTc) in preventing the infection, the effect of acquired immunity on vaccine efficiency and the action of immunity adjuvants are all

important considerations highlighting the need to develop efficient therapeutic vaccines activating helper lymphocyte T cells (LTh).

Despite being able to greatly reduce the mortality of Herpes infections, patients still suffer from a neurological discomfort. A deeper knowledge of the biology of the infection as well the diagnostic and prevention/treatment of the disease is required in order to reduce the morbidity associated with Herpes infections.

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