Co-prevalence of Human Papillomavirus and Herpesviruses in Anal Infection of Asymptomatic Men Who Have Sex with Men in Khon Kaen, Thailand

Kanisara Proyrungroj[1,4], Saowarop Hinkan[1,4], Jiratha Budkaew[2], Bundit Chumnvorathayee[3,4], Chamsai Pientong[1,4], Tipaya Ekalaksananan[1,4,5]

[1] *Department of Microbiology, Faculty of Medicine, KhonKaen University*

[2] *Department of Social Medicine, Khon Kaen Center Hospital*

[3] *Department of Obstetrics and Gynecology, Faculty of Medicine, Khon Kaen University*

[4] *HPV & EBV and Carcinogenesis Research Group, Khon Kaen University*

Background and Objective: Men having sex with men (MSM) remains to be the largest high-risk group involved in on-going transmission of sexually transmitted infections (STI). Asymptomatic MSM with anal infection of HPV, HSV and EBV could substantiate the importance of transmission between partners and also has been associated with anal cancer.

The objective of this study was to determine the prevalence of HPV, HSV and EBV infection and co-infection of them in anus of asymptomatic MSM in Khon Kaen, Thailand.

Methods: The anal swabs were collected from 200 MSM. DNA was extracted and quantitated by detecting GAPDH gene using real-time Polymerase Chain Reaction (PCR). HPV, HSV and EBV DNA were investigated by real-time PCR using specific primers. HPV positive samples were genotyped by reverse line blot hybridization.

Results: The prevalence of HPV infection in asymptomatic MSM showed the highest (51%) and followed by EBV (39%) and HSV (4%). High-risk and low-risk HPV types were found in 83.3% and 23%, respectively, and 20.6% of cases were infected with both of high-risk and low-risk HPV types. In high-risk HPV group, HPV 16, was the most common followed by HPV 58 and HPV 18. Multiple HPV type infections were found in 52% of HPV positive cases. The co-infection with these three viruses were also detected especially HPV and EBV were the most common (23%).

Conclusion: The anal infection of HPV and herpes viruses were commonly found in asymptomatic MSM. The asymptomatic MSM can spread these viruses to other persons. Co-infection of high-risk HPV with herpesviruses in anus may be an important risk factor to promote anal cancer development.

Key Words: Men who have sex with men (MSM), HPV, HSV, EBV

Proceeding

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Introduction

Since the late 1990s, there has been an increase in notifications of newly diagnosed sexually transmitted infections (STIs) in men having sex with men (MSM) in north America, western Europe, Australia and the developed countries of east Asia (Hong Kong, Singapore, Taiwan and Japan)[1, 2]. This includes *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, herpes simplex virus type 1, 2 (HSV-1,-2) and human papillomavirus (HPV).

HPV is the most common sexually transmitted virus and causes an important problem of disease in men and women[3]. Although many HPV infections in men are usually subclinical and asymptomatic and most often clears spontaneously within two years, similar to HPV infection in women, a small percentage of HPV infection is persist and can develop to genital warts, preneoplastic and malignant lesions of the anus, penis, and orophar-

[5]Corresponding author: Tipaya Ekalaksananan, Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand 40002. E-mail: tipeka@kku.ac.th

ynx; and recurrent respiratory papillomatosis[4]. HPV infection is highly predominant and is accountable for important disease in men, especially MSM[5]. The incidence of HPV infection is low in women older than 30 years but remains high in all age ranges of MSM[6,7]. The prevalence of HPV infection is high among young sexually active MSM, with the anal canal being the most common site of infection. The high prevalence of anal HPV in MSM is associated with an increased anal cancer with the incidence estimated to be 44 times higher than that among the general population[8]. More recent studies have indicated that anal cancer is now more common in men than cervical cancer in women[9].

HSV infection is one of the most common sexually transmitted diseases worldwide and is also the cause of most genital ulcer disease and neonatal herpes. In symptomatic infections, the virus causes painful ulcerative lesions that can take two to four weeks to heal in primary outbreaks, and recurrences can be frequent. The prevalence of HSV-2 infection in the general population ranges from 10 to 60%, with higher prevalence in female sex workers, men who have sex with men (MSM), and certain regions of the world[10].

Epstein-Barr virus (EBV) or human herpesvirus 4 (HHV4) is a double-stranded DNA gamma herpes virus which establishes a life-long persistent infection in over 90% of the human adult population world-wide[11]. EBV can infect B-cells and epithelial cells[12]. Although the infection is often asymptomatic, it causes infectious mononucleosis and is associated with a number of human diseases, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD), oral hairy leukoplakia (OHL) and gastric carcinoma. EBV can be transmitted via oral route and infect B-lymphocytes in vivo. In addition, it can be transmitted via a different route, through sexual contact.

In 1999, Lowhagen et al.[13] studied the PCR detection of EBV, HSV and HPV from the anal mucosa in HIV-seropositive and HIV-seronegative homosexual men. EBV and HSV were demonstrated in 32% and 16% of anal samples from HIV-infected homosexual men. HPV was found almost regularly in samples from the anus in HIV-positive men, more often infected with multiple HPV type (HPV 16, HPV18), and in more than half of the HIV-negative men[13]. In 2016, Gianella et al. found that presence of seminal EBV shedding was the strongest factor associated with detectable HR-HPV infection of anal mucosa in MSM population[14]. However, the prevalence of HPV, HSV and EBV infection in asymptomatic MSM in Thai population are still limited. The objectives of this study were to evaluate the prevalence of HPV, HSV and EBV infection among asymptomatic MSM in KhonKaen, Thailand and to investigate the co-infection of HPV, HSV and EBV.

Methods

Samples

Anal cell samples were collected by swab technique from MSM who visited Mreach STDs clinic in Chatapadung contracting medical unit, Khon Kaen Center Hospital and were recruited in an analytic cross sectional study of a project entitled "Factors associated to *Neisseria gonorrhoeae* infection by anatomic distributions among men who have sex with men, and multidrug resistant patterns of *Neisseria gonorrhoeae*"

The samples had been transported in 3 ml of 0.1% formal saline and vortexed before the swab was discarded. Then, the samples were centrifuged at 1,500 rpm for 5 min. Anal cell pellets were washed with sterile phosphate buffer saline (PBS) and stored in absolute ethanol at -20°C until DNA extraction.

DNA extraction

The anal cell pellets were incubated in lysis buffer (1M Tris-base, 0.5M EDTA, 5M NaCl and 10% SDS) at 60°C for 30 min. Protein precipitation solution was added. The samples were centrifuged at 13,000 rpm for 5 min at 4°C. Supernatant was transferred to a new tube. Isopropanol was added and incubated at -70°C for 1 h. DNA was washed with 70% ethanol by centrifugation at 13,000 rpm for 5 min at 25°C, air-dried and suspended in 40 µl of distilled water. DNA quality was determined by detecting GAPDH gene by SYBR green real-time PCR using specific primers (Table 1).

Detection of HPV, HSV and EBV by SYBR green real-time PCR

Real-time PCR was performed in Applied Biosystems 7500Fast real-time PCR Instrument (ABi). DNA was investigated for HPV, HSV and EBV DNA using

specific primers (Table 1). Reaction mixture was performed in a final volume of 10 µl containing 5 µl SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 0.2 µl of forward primer (0.2 µM), 0.2 µl of reverse primer (0.2 µM), 2.4 µl of distilled water and 2 µl of DNA template. Thermal cycling conditions for HPV detection included initial 5 min at 95°C for enzyme activation followed by 45 cycles of 95°C, 10 sec for denaturation and 42°C, 30 sec for annealing. Thermal cycling conditions for HSV and EBV detection included initial 3 min at 95°C for enzyme activation followed by 40 cycles of 95°C, 10 sec for denaturation and 49°C, 10 sec for annealing and 72°C 30 sec for extension. The melting temperature (Tm) of PCR products was analyzed. To confirm real-time PCR result, the products were electrophoresed in 2% agarose gel.

HPV genotyping

HPV L1 gene fragments in HPV positive samples were amplified usingGP5/ GP6+ primers labeled with biotin and genotyped by reverse line blot hybridization (RLBH)[18]. Biodyne C blotting membrane (Pall Life Science) was activated in 16% (w/v) 1-ethy-3-(3-dimethylaminopropyl) carbodimide (EDAC) solution (Sigma-Aldrich) at room temperature for 10 min, rinsed with distilled water and placed on mini blotter. Thirty-seven HPV type-specific 5'-amino linked oligonucleotides probes-including 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68); 12 low-risk HPV types (6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 72 and 73); and, other HPV types (34, 55, 57, 66, 70, 82MM4, 83MM7, 84MM8, 82IS39, CP6108, 71CP8061 and 81CP8304)-were dropped on Biodyne C membrane through the wells of the mini blotter in parallel lines. Subsequently, biotin-labeled PCR products were added into the channels of the mini blotter perpendicular to the oligonucleotides probe lines, then hybridized and incubated with streptavidin-peroxidase-conjugate. The HPV types were detected using chemiluminescence.

Table 1 Nucleotide sequence of specific primers.

Targets	Sequences (5'-3')	Product size
GAPDH[15]	F: TCATCAGCAATGCCTCCTGCA R: TGGGTGGCAGTGATGGCA	117 bp
HPV L1[16]	F: TTTGTTACTGTGGTAGATACTAC R: GAAAAATAAACTGTAAATCATATTC	150 bp
HSV DNA polymerase[17]	F: GTGTTGTGCCGCGGTCTCAC R: GGTGAACGTCTTTTCGAACTC	120 bp
EBV DNA polymerase[17]	F: ACC CGG AGC CTG TTT GTA GC R: GGA GAA GGT CTT CTC GGC CTC	54 bp

Results

Total of 200 MSM were enrolled in this study. DNA was extracted and checked quality by detecting GAPDH gene using SYBR green real-time PCR and used for HPV, HSV and EBV DNA detection by SYBR green real-time PCR using specific primers. HPV DNA was found in 51% of cases according to specific melting temperature (Tm) in the range of 75–80°C. For HSV and EBV DNA, Tm showed melting peak at 89.7°C and at 80.7°C, respectively. HSV and EBV infection was found in 4% and 39%, respectively (Table 2).

For HPV genotyping, almost of them was high-risk HPV (83.3%), whereas low-risk HPV was 23.5% (Table 3). HPV 16 was the most common type (31 cases) followed by HPV 58 (30 cases) and HPV 18 (29 cases). HPV infection with only one type HPV was 37.3% and multiple infections with two types and more than two types were 39.2% and 12.7%, respectively (Table 3).

The occurrence of viral co-infection in anus was detected in 38 of 200 cases (19%) and 52 cases of MSM were not infected with HPV, HSV and EBV. Thirty-four cases (23%) presented co-infection of HPV with EBV; 2 cases (1.4%) presented with a triple co-infection of HPV, HSV and EBV; 1 case (0.7%) was co-infected with HPV and HSV as well as co-infection of HSV and EBV were 1 case (0.7%). Single infection was as follows: HPV 43.9% (65 cases); HSV 2.7% (4 cases) and EBV 27.7% (41 cases) (Figure 1).

Table 2 Prevalence of anal HPV and herpesviruses infection in MSM, KhonKaen, Thailand (n=200)

Viral infection	Cases, n (%)
HPV	102 (51)
HSV	8 (4)
EBV	78 (39)

Table 3 Distribution of HPV genotypes in MSM, KhonKaen, Thailand. (n=102)

HPV genotypes	Cases, n (%)
Low-risk types	24 (23.5)
6	9(8.8)
11	9 (8.8)
42	6 (5.9)
43	2(2)
61	1(1)
High-risk types	85 (83.3)
16	31 (30.4)
18	29 (28.4)
31	4 (3.9)
33, 35	10 (9.8)
39, 45, 52	12 (11.8)
56	9 (8.8)
58	30 (29.4)
59	2(2)
Others types	
66, 70, 83MM7	5(4.9)
Undetermined	11(10.8)
Infected with one type	38(37.3)
Multiple infections	53(52)
- Infected with high-risk and low-risk types	21 (20.6)
- Infected with 2 types	40(39.2)
- Infected with $\geq$ 3 types	13 (12.7)

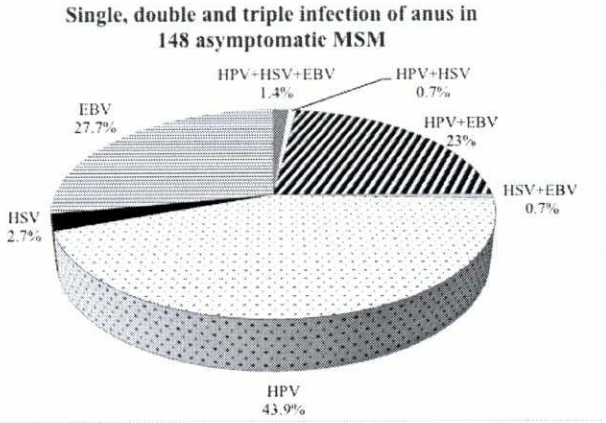

Figure 1 Frequency of anal viral co-infection

Discussion

This study investigated HPV, HSV and EBV infection at anal site of asymptomatic MSM in KhonKaen, Thailand using real-time PCR and RLBH for HPV genotyping. The HSV prevalence (4%) in anal mucosa of MSM was corresponded to previous reports that showed HSV infection of 2.3-17.6% in HIV-positive and 0-7.8% in HIV negative[13, 14, 19, 20] as well as the anal EBV infection rate (39%) of this study was corresponded to previous reports that showed EBV infection in 27.5-32% of HIV-positive and 7.7% of HIV negative[13, 14, 21]. The prevalence of HPV (51%) in anus of asymptomatic MSM in KhonKaen, Thailand was corresponded to previous reports that showed anal HPV infection in 71.4-85% of HIV-positive and 33.8-58.5% of HIV-negative MSM[22, 23]. However, our study has a limitation in data of HIV infection. Previous studies of HPV infection in MSM, showed that 37.3% of MSM were infected with at least one type of HPV and 52% of MSM were infected with multiple HPV types. HPV 16 was the most frequently detected type in the anus[22, 24-26]. Corresponding with this study, HPV 16 was the most common type and followed by HPV 58 and HPV 18 as well as high prevalence of multiple HPV infections was found (Table 2).

In the present study, HPV-HSV-EBV co-infection was 1.4% (2/148); HPV-HSV co-infection and HSV-EBV co-infection were 0.7% (1/148); HPV-EBV co-infection was 23% (34/148). These data are different from the previous report which showed that a triple co-infection, HPV-HSV and HPV-EBV were 9%, 55% and 27%, respectively in HIV patients[21]. However, our study has a limitation in data of HIV status.

Conclusion

Our study presents the prevalence of HPV, HSV and EBV as well as HPV genotypes in asymptomatic MSM in KhonKaen, northeastern Thailand. High-risk HPV, multiple HPV infections and HPV co-infected with EBV are common. These data are a crucial for seeking methods to prevent HPV, HSV and EBV transmission and anal cancer development.

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