

The development of simultaneous measurement of viral load and physical status for human papillomavirus 16 and 18 co-infection using multiplex quantitative polymerase chain reaction

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Abstract. Persistent infection with human papillomavirus (HPV) type 16 and 18 is known to be a major risk factor for cervical cancer. Increased prevalence of co-infection with these high-risk types has been observed in pre-cancerous and cancerous tissues. The determination of physical status and copy numbers of viruses is therefore useful in clinical settings. A simple multiplex quantitative polymerase chain reaction (qPCR) for HPV16/HPV18 co-infection in one tube reaction was established in the present study using TaqMan[®]-based PCR for E2 and E6 viral DNA. The detection range was up to 10⁶ copies with 100% specificity and high precision (CV of cycle time <0.5%). The analytical accuracy and robustness were verified by competitive assay using an unequal mixture of HPV16/HPV18 DNA. No significant effect was demonstrated when compared with the simplex qPCR. The detection of physical status was evaluated in cervical samples, including 5 pre-cancerous and 15 cancerous samples. No significant difference was observed between simplex and multiplex qPCR (P=0.372). In conclusion, the developed multiplex qPCR method successfully demonstrated the viral status of the common HPV types in one tube. This assay will facilitate viral assessment and monitoring of cervical cancer associated with HPV16 and HPV18 co-infection.

Introduction

Cervical cancer (CXCA) is the fourth leading cause of cancer-associated mortality among females worldwide (1). The majority of CXCA cases are associated with high risk human papillomavirus (HR-HPV) infection. Among HR-HPV, HPV16 and HPV18 account for 70% of all CXCA cases worldwide (1), including in Asian populations (2). Epidemiological data revealed an increase in multiple HPV infection from 10% of CXCA cases in 2005 (3) to 65% in 2016 (3-5). However, these figures may reflect a higher sensitivity of detection, resulting in the co-infection of HPV16 and HPV18 being reported as the most common HR-HPV in Africa (6) and Asia (7), and specifically in Thailand (8), as 31.9, 20.8 and 27.8%, respectively. An increased odds ratio of HPV16 and 18 co-infection compared with single infection was demonstrated by Chaturvedi *et al* (9) and Trottier *et al* (10), but the opposite results were reported by Salazar *et al* (11). These controversial findings implied that other viral parameters, not only HPV genotype, may serve important roles in disease progression.

The physical state of HPV infection occurs as one of two forms: An episomal or an integrated form. The episomal state involves the complete life cycle of viral replication in the infected host cells, whereas the integrated form involves integration of HPV DNA into the host DNA, a major genetic event leading to cervical carcinogenesis (12). **Viral integration** and viral load have previously been reported to be biomarkers for cancer with high-grade cervical lesions (13). Several methods have been used for the detection of integrated HPV, including polymerase chain reaction (PCR) (14), *in situ* hybridization (15,16) and amplification of papillomavirus oncogene transcripts (APOT) (17). These are all qualitative measurements. Recently, we established a quantitative PCR of E2 and E6 genes to measure the viral load and physical status of HPV16 DNA in one tube (18). A ratio of E2/E6 gene of 1.0 is used to define the episomal form, while a decreased ratio (less than 1.0) indicates the integrated form, due to deletion of the E2 gene during viral integration. The present study demonstrated the benefit of using viral numbers and physical status as surrogate markers of cancer progression.

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Abbreviations: CXCA, cervical cancer; HR-HPV, high risk human papillomavirus; qPCR, quantitative polymerase chain reaction; CV, coefficient of variation

Key words: human papillomavirus type 16, human papillomavirus type 18, multiplex TaqMan[®] polymerase chain reaction, co-infection, pre-cancerous state, cervical cancer

To the best of our knowledge, the present study was the first to report the simultaneous measurement of 4 genes, E2 and E6 genes of HPV16 and HPV18, in a single tube. The development of multiplex qPCR in the present study provides a total coverage of 70% of HR-HPV-associated CXCA, including single and co-infection of HPV16 and HPV18. The analytical performance of the multiplex qPCR was evaluated in clinical samples, compared with simplex qPCR.

Materials and methods

Samples. A total of 20 cervical tissues harboring single or co-infection of HPV16 and HPV18 were collected from 5 pre-cancerous lesions (mean, 42.2±6.6 years) and 15 cancerous lesions (mean, 49.5±13.7 years). Samples were collected between 2002 and 2004 under written informed consents approved by the Ethical Committee of Khon Kaen University, Khon Kaen, Thailand (approval no. HE562296) and between 2013 and 2014 approved by the the Ubonratchathani Cancer Hospital, Ubon Ratchathani, Thailand (approval no. CE012/2013). DNA samples were extracted using a QIAamp Viral DNA kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Extracted DNA was used for HPV16 and HPV18 screening by Nested Multiplex PCR, as previously described (19).

Cell culture. The human papillomavirus Caski and HeLa cell lines containing the integrated form of HPV16 (600 DNA copies per cell) and HPV18 (20-50 DNA copies per cell) were used as internal standard for determination of physical status of HPV16 and 18, respectively. HeLa cell line containing HPV18 and CaSki cell line containing HPV16 were used as HPV positive controls. Cells were cultured in 25 cm² flasks at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium high glucose (DMEM-HG) media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin; all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). At 70% confluency, cells were trypsinized with 1 ml of 1X trypsin-EDTA at 37°C for 5 min. The cell pellets were collected and centrifuged at 2,000 rpm for 3 min and subsequently used for DNA extraction.

HPV16 and HPV18 integration status assay. Plasmids containing the whole genomes of HPV16, HPV18, HPV45 and HPV58 (PBR322 for HPV16 and HPV58, PGM4 for HPV18 and HPV45) were provided by Professor Pientong from the Department of Microbiology, Faculty of Medicine, Khon Kaen University. Purified recombinant plasmid copy numbers were estimated by a spectrophotometer concentration measurement (NanoDrop 2000; Thermo Fisher Scientific, Inc.). The DNA calculation formula was 6.02×10^{23} (copies/mol) $\times A_{260}$ (ng/ml)/(DNA length $\times 660$)=copies/ml. Plasmid DNA was then diluted with sterile water to obtain between 10⁶ and 10² copies, and was used to establish calibration curves for measuring E2 and E6 by multiplex qPCR using a TaqMan® probe assay (Bioneer Corporation, Daejeon, Korea). The oligonucleotide sequences of primers and probes were followed as previously described (20,21), with modifications to the quencher and reporter fluorescent

dyes (Table I). E2 and E6 DNA were amplified using a qPCR thermocycler (Exicycler™; Bioneer Corporation). Each 50 µl reaction mixture contained a premix (AccuPower® DualStar™; Bioneer Corporation) with 0.4 µM probes and primers. The PCR reaction was initiated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 63°C for 60 sec. Reactions lacking DNA template were used as negative controls as previously described. Quantification of E2 and E6 genes were analyzed using the calibration curve plotted between the quantification cycle (Cq) on the x-axis and the logarithm of the standard copy number on the y-axis (10²-10⁶ copies). Linear regression equations were estimated as indicated in Fig. 1. The obtained Cq from samples were used to calculate E2 and E6 copies from these equations. Amplification efficiency was determined from the slope of log-linear calibration curve ($10^{-1/\text{slope}-1}$) (22).

Optimization of temperature for multiplex qPCR. Multiplex qPCR temperature for HPV16 (E2 and E6 genes) and HPV18 (E2 and E6 genes) was optimized. A mixture of 10⁵ copies of plasmids containing the whole genomes of HPV16 and HPV18 was used to optimize the annealing temperature from 55 to 64°C. The temperature that produced the lowest Cq was selected as the optimal annealing temperature.

Evaluation of multiplex qPCR performances

Multiplex qPCR analytical range. Ten-fold dilutions of mixed HPV16 and HPV18 whole genomic DNA (from 10² to 10⁶ copies) were used as templates for determining the analytical range.

Multiplex qPCR analytical imprecision. Within-run and between-run precision were each determined using low (10³ copies) and high (10⁶ copies) concentrations of whole genome HPV16 and HPV18 DNA mixtures.

Multiplex qPCR analytical specificity. Cross-reactivity with two other HPV genotypes, HPV45 and HPV58, was tested. No fluorescent signal indicated a lack of cross-reaction, and uninfected HPV DNA was used as a negative control.

Competitive effect of HPV16 and HPV18 in multiplex qPCR. A mixture of unequal concentrations of HPV16 and HPV18 DNA (from 10³ to 10⁶ copies) was used to evaluate competitive effects in multiplex qPCR. E2 and E6 copies obtained from unequal HPV16 and HPV18 templates (test) were compared to those determined using a single template of HPV16 or HPV18 (control) by a paired t-test. No significant difference ($P > 0.05$) indicated no competitive effect.

Evaluation of physical status using multiplex qPCR in clinical samples. The cut-off value for an episomal form (complete E2 and E6 sequence) was first determined using plasmid DNA containing whole HPV16 and HPV18 genomes. The E2/E6 ratio was calculated by the 95% confidence interval (CI) and used to interpret physical status in clinical samples as previously described (18). To verify the accuracy of the multiplex qPCR of HPV16 and HPV18, 10⁵ copies of Caski and HeLa cells containing pure integrated HPV16 and HPV18, respectively, were prepared according to the DNA calculation

Table I. Oligonucleotide sequences and TaqMan® probes used for amplification of E2 and E6 genes of HPV16 and HPV18.

HPV type	Name	Sequence (5'-3')	Amplified product length (bp)	(Refs.)
HPV16	HPV16 E2 Forward primer	AACGAAGTATCCTCTCCTGAAATTATTAG	82	Peitsaro <i>et al</i> , 2002 (20)
	HPV16 E2 Reverse primer	CCAAGGCGACGGCTTTG		
	HPV16 E2 Probe	(TAMRA)-CACCCCGCCGCGACCCATA-(BHQ)		
	HPV16 E6 Forward primer	GAGAACTGCAATGTTTCAGGACG	81	
	HPV16 E6 Reverse primer	TGTATAGTTGTTTGCAGCTCTGTGC		
	HPV16 E6 gene Probe	(Texus red)-CAGGAGCGACCCAGAAAGTTACC ACAGTT-(BHQ)		
HPV18	HPV18 E2 Forward primer	AGAAGCAGCATTGTGGACCT	167	Damay <i>et al</i> , 2009 (21)
	HPV18 E2 Reverse primer	GGTCGCTATGTTTTTCGCAAT		
	HPV18 E2 Probe	(TeT)-TCAACC-CACCTTCTCGGTGCAGC-(BHQ)		
	HPV18 E6 Forward primer	TCACAACATAGCTGGGCACT	91	
	HPV18 E6 Reverse primer	CTTGTGTTTCTCTGCGTCGT		
	HPV18 E6 Probe	(FAM)-GCCATTCGTGCTGCAACCGA-(BHQ)		

HPV, human papillomavirus; bp, base pairs.

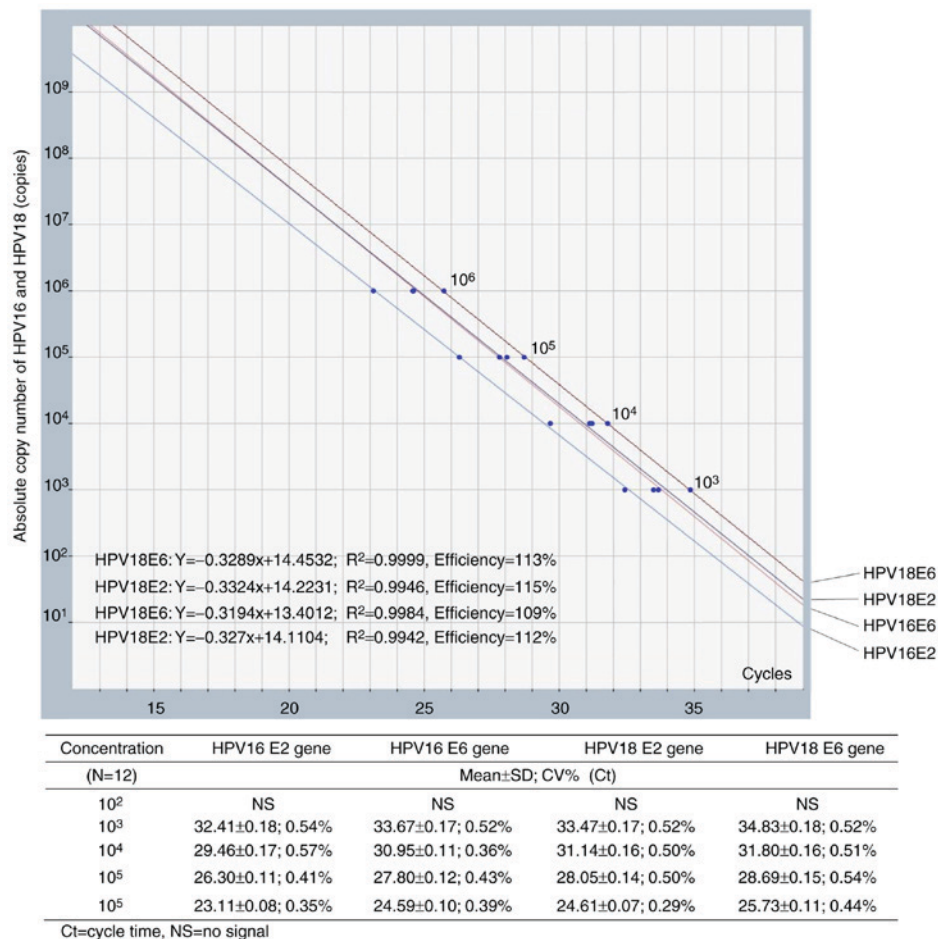


Figure 1. Standard curves of multiplex quantitative polymerase chain reaction for E2 and E6 genes of HPV16 and HPV18. The analytical range was revealed to be 10³ to 10⁶ copies. HPV, human papillomavirus; SD, standard deviation.

formula and used as internal standards for the integrated form. A total of 10⁵ copies of whole plasmid genome HPV16

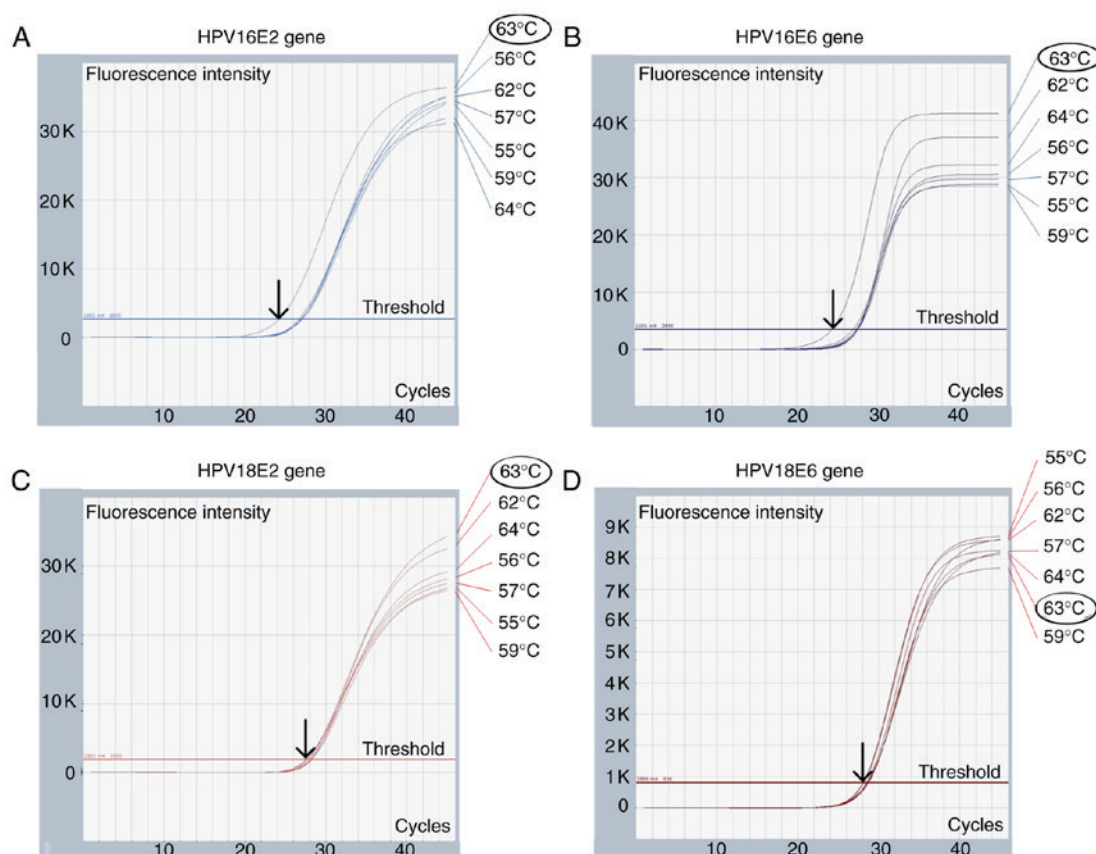


Figure 2. Optimization for (A) E2 and (B) E6 genes of HPV16, and (C) E2 and (D) E6 genes of HPV18. The optimal Cq for HPV16 and HPV18 was selected at 63°C. HPV, human papillomavirus; Cq, quantification cycle.

and HPV18 were used as internal standards for the episomal form. The two cell lines were provided by Professor Pientong. A total of 20 cervical samples with single and mixed infection were used to compare the copy number and physical status between the simplex and multiplex qPCR using paired t-tests and χ^2 tests.

Statistical analysis. The linear regression equation was estimated from standard curves between viral copy number (y-axis) and cycle time (x-axis). The percentage of efficiency between 80-120% and correlation (R^2) >0.98 were used to determine the standard curve. The comparison of viral copy number between multiplex and simplex was performed using paired t-tests, while physical status was compared by χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS 19 software (IBM Corp., Armonk, NY, USA) under a Khon Kaen University license.

Results

Multiplex qPCR performance for the detection of HPV16 and HPV18 (E2 and E6 genes). The optimization of Cq was performed under annealing temperatures from 55 to 64°C, as demonstrated in Fig. 2. The optimal Cq for the E2 and E6 genes of HPV16 was 63°C, whereas the optimal Cq for HPV18 was between 55 and 64°C. Therefore, 63°C was used to construct a standard curve for HPV16 and HPV18

multiplex qPCR, as demonstrated in Fig. 1. According to the guidelines for validation of quantitative PCR methods, linear regression and correlation (R^2) analyses for each gene revealed an acceptable efficiency of 109-115% (23). The analytical range was verified at 1,000-1,000,000 copies for HPV16 and HPV18, with average imprecision from 0.42 to 0.50% CV as demonstrated in Fig. 1. The imprecision of measured HPV copies is presented in Table II. The average CV of within-run and between-run was 10.2 and 12.1, respectively. No cross reactivity was observed with HPV58 and HPV45 (Fig. 3).

Evaluation of competitive effect of unequal HPV16 and HPV18 template concentrations in multiplex qPCR. To mimic the presence of HPV16 and HPV18 co-infection up to 1,000-fold difference in the same sample, measurement of mixed HPV16 and HPV18 DNA was compared in parallel with that of single HPV16 and HPV18. Different concentrations of HPV16 and HPV18 exhibited no effect on the quantification of E2 ($P=0.319$ and $P=0.526$, respectively) and E6 genes ($P=0.347$ and $P=0.146$, respectively), as demonstrated in Tables III and IV. Therefore, our established multiplex qPCR platform provided an accurate measurement for the presence of HPV16 and HPV18 co-infection.

Evaluation of physical status in clinical samples. Cut-off values for viral status were calculated as previously described by Wanram *et al* (18) and are shown in Table V. The E2/E6 ratio was identified as 0.78-1.10 and 0.85-1.18 for HPV16

Table II. Analytical imprecision of the HPV16 (E2 and E6 genes) and HPV18 (E2 and E6 genes) measurement using multiplex quantitative polymerase chain reaction.

Precision	HPV16 E2 gene		HPV16 E6 gene	
	Mean \pm SD (CV%) Cq	Mean \pm SD (CV%) Copy number	Mean \pm SD (CV%) Cq	Mean \pm SD (CV%) Copy number
Within-run (n=12) ^a				
Low level ^c	32.41 \pm 0.18 (0.54)	961 \pm 127.1 (13.23)	33.67 \pm 0.17 (0.52)	1,084 \pm 149.1 (13.74)
High level ^d	23.11 \pm 0.08 (0.35)	952,490 \pm 59,040.35 (6.19)	24.59 \pm 0.10 (0.39)	1,097,119 \pm 81,784.1 (7.45)
Between-run (n=15) ^b				
Low level ^c	32.61 \pm 0.20 (0.60)	973 \pm 141.36 (14.53)	34.26 \pm 0.19 (0.54)	929 \pm 137.45 (14.79)
High level ^d	23.37 \pm 0.14 (0.59)	911,140 \pm 91,626.87 (10.06)	25.04 \pm 0.14 (0.55)	1,040,597 \pm 112,028.74 (10.77)
Precision	HPV18 E2 gene		HPV18 E6 gene	
	Mean \pm SD (CV) Cq	Mean \pm SD (CV) Copy number	Mean \pm SD (CV) Cq	Mean \pm SD (CV) Copy number
Within-run (n=12) ^a				
Low level ^c	33.47 \pm 0.17 (0.52)	1,159 \pm 160.1 (13.81)	34.83 \pm 0.18 (0.52)	1,067 \pm 144.35 (13.52)
High level ^d	24.61 \pm 0.07 (0.29)	1,123,790 \pm 60,126.32 (5.4)	25.73 \pm 0.11 (0.44)	1,023,242 \pm 86,472.54 (8.45)
Between-run (n=15) ^b				
Low level ^c	34.03 \pm 0.19 (0.57)	996 \pm 147.1 (14.76)	34.77 \pm 0.19 (0.53)	958 \pm 134.18 (14.00)
High level ^d	25.00 \pm 0.11 (0.43)	992,011 \pm 81,890.14 (8.25)	25.42 \pm 0.13 (0.51)	1,054,498 \pm 101,053.01 (9.58)

^a12 replicates per experiment; ^bTriplicate in 5 independent experiments; ^c10³ copies of HPV16 and HPV18 DNA; ^d10⁶ copies of HPV16 and HPV18 DNA. HPV, human papillomavirus; Cq, quantification cycle.

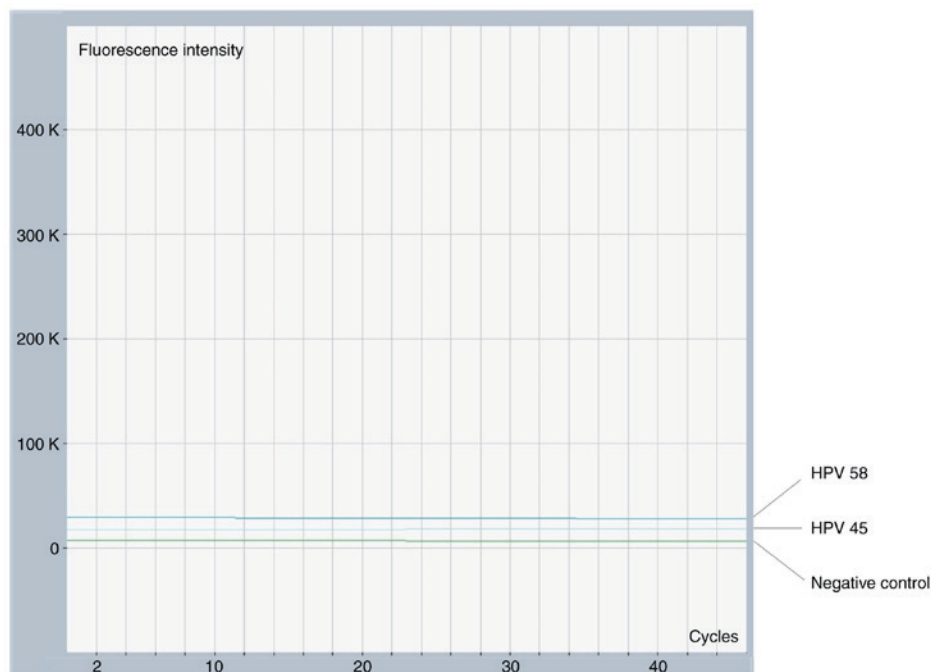


Figure 3. Analytical specificity of multiplex quantitative polymerase chain reaction. No cross reactivity was observed with 10⁴ copies of HPV45 and HPV58. Uninfected HPV DNA was used as a negative control. HPV, human papillomavirus.

and HPV18, respectively. An E2/E6 ratio of 0 was defined as the absolute integrated form, whereas E2/E6 >0 and less

than the cut-off value was interpreted as the mixed form of episomal and integrated HPV (18). Comparisons of HPV16

Table III. Competitive effect of unequal DNA copies between HPV16 and HPV18 on the measurement of HPV16 E2 and E6 genes using multiplex quantitative polymerase chain reaction.

HPV16 gene				HPV18 gene			
Unequal mixture HPV16 and HPV18		Single HPV16		Unequal mixture HPV16 and HPV18		Single HPV16	
Copy Ratio (HPV16:HPV18)	Measured copies (Test) ^a	Copy of HPV16	Measured copies (Control) ^a	Copy Ratio (HPV16:HPV18)	Measured copies (Test) ^a	Copy of HPV16	Measured copies (Control) ^a
10 ⁶ :10 ³	945,367	10 ⁶	1,026,287	10 ⁶ :10 ³	979,497	10 ⁶	1,039,326
10 ⁶ :10 ⁴	901,450	10 ⁶	915,772	10 ⁶ :10 ⁴	900,328	10 ⁶	913,776
10 ⁶ :10 ⁵	921,335	10 ⁶	897,221	10 ⁶ :10 ⁵	914,673	10 ⁶	911,439
10 ⁶ :10 ⁶	979,241	10 ⁶	980,160	10 ⁶ :10 ⁶	1,039,326	10 ⁶	964,189
10 ⁵ :10 ³	89,554	10 ⁵	90,112	10 ⁵ :10 ³	87,556	10 ⁵	89,341
10 ⁵ :10 ⁴	95,749	10 ⁵	103,356	10 ⁵ :10 ⁴	91,586	10 ⁵	98,371
10 ⁵ :10 ⁵	96,968	10 ⁵	105,065	10 ⁵ :10 ⁵	97,092	10 ⁵	103,174
10 ⁵ :10 ⁶	94,159	10 ⁵	92,332	10 ⁵ :10 ⁶	112,708	10 ⁵	100,329
10 ⁴ :10 ³	8,832	10 ⁴	9,149	10 ⁴ :10 ³	11,219	10 ⁴	10,439
10 ⁴ :10 ⁴	8,836	10 ⁴	10,626	10 ⁴ :10 ⁴	9,175	10 ⁴	9,303
10 ⁴ :10 ⁵	8,913	10 ⁴	9,319	10 ⁴ :10 ⁵	8,641	10 ⁴	10,322
10 ⁴ :10 ⁶	11,347	10 ⁴	10,861	10 ⁴ :10 ⁶	10,989	10 ⁴	9,018
10 ³ :10 ³	1,143	10 ³	1,200	10 ³ :10 ³	1,039	10 ³	1,153
10 ³ :10 ⁴	867	10 ³	912	10 ³ :10 ⁴	907	10 ³	865
10 ³ :10 ⁵	881	10 ³	1,122	10 ³ :10 ⁵	848	10 ³	973
10 ³ :10 ⁶	848	10 ³	901	10 ³ :10 ⁶	846	10 ³	908

^aNon-significant differences between test and control for HPV16 E2 (P=0.319; paired Student's t-test) and HPV16 E6 (P=0.347; paired Student's t-test). HPV, human papillomavirus.

HPV E2 gene				HPV E6 gene			
Unequal mixture HPV18 and HPV16		Single HPV18		Unequal mixture HPV18 and HPV16		Single HPV18	
Copy ratio (HPV18:HPV16)	Measured copies of HPV18E2 (Test) ^a	Copy of HPV18	Measured copies of HPV18E2 (Control) ^a	Copy ratio (HPV18:HPV16)	Measured copies of HPV18E6 (Test) ^a	Copy of HPV18	Measured copies of HPV18E6 (Control) ^a
10 ⁶ :10 ³	1,135,661	10 ⁶	1,075,620	10 ⁶ :10 ³	1,015,464	10 ⁶	1,107,241
10 ⁶ :10 ⁴	1,097,976	10 ⁶	1,181,450	10 ⁶ :10 ⁴	1,107,170	10 ⁶	1,125,579
10 ⁶ :10 ⁵	877,385	10 ⁶	1,135,265	10 ⁶ :10 ⁵	911,742	10 ⁶	893,768
10 ⁶ :10 ⁶	1,092,086	10 ⁶	993,922	10 ⁶ :10 ⁶	965,678	10 ⁶	1,040,114
10 ⁵ :10 ³	104,611	10 ⁵	96,998	10 ⁵ :10 ³	90,546	10 ⁵	94,778
10 ⁵ :10 ⁴	101,164	10 ⁵	113,785	10 ⁵ :10 ⁴	97,952	10 ⁵	113,933
10 ⁵ :10 ⁵	105,464	10 ⁵	101,469	10 ⁵ :10 ⁵	110,175	10 ⁵	87,996
10 ⁵ :10 ⁶	102,760	10 ⁵	113,715	10 ⁵ :10 ⁶	87,492	10 ⁵	107,587
10 ⁴ :10 ³	9,129	10 ⁴	8,789	10 ⁴ :10 ³	10,201	10 ⁴	9,746
10 ⁴ :10 ⁴	9,550	10 ⁴	9,443	10 ⁴ :10 ⁴	9,732	10 ⁴	10,395
10 ⁴ :10 ⁵	8,639	10 ⁴	9,988	10 ⁴ :10 ⁵	10,403	10 ⁴	8,665
10 ⁴ :10 ⁶	10,700	10 ⁴	9,812	10 ⁴ :10 ⁶	8,910	10 ⁴	10,550
10 ³ :10 ³	912	10 ³	1,120	10 ³ :10 ³	937	10 ³	1,056
10 ³ :10 ⁴	936	10 ³	1,078	10 ³ :10 ⁴	910	10 ³	938
10 ³ :10 ⁵	849	10 ³	831	10 ³ :10 ⁵	901	10 ³	833
10 ³ :10 ⁶	896	10 ³	1,168	10 ³ :10 ⁶	869	10 ³	1,063

Non-significant difference between test and control for HPV18E2 ($P=0.526$; paired t-test) and HPV18E6 ($P=0.146$; paired t-test). HPV, human papillomavirus.

Table V. Estimation of cut-off values (E2 and E6 ratios) for the interpretation of physical status using various concentrations of whole genome plasmid DNA from 10^4 to 10^6 copies.

No.	Mixture of equal concentration of HPV16 and HPV18	HPV16			HPV18		
		E2 gene (copy)	E6 gene (copy)	E2/E6	E2 gene (copy)	E6 gene (copy)	E2/E6
1	10^3	1,115	1,103	1.01	1,153	1,233	0.94
2	10^3	1,115	1,185	0.94	1,019	1,210	0.84
3	10^3	908	1,044	0.87	1,193	1,211	0.99
4	10^3	1,168	1,169	1	1,155	1,134	1.02
5	10^3	1,038	1,047	0.99	1,146	1,179	0.97
6	10^4	8,882	10,814	0.82	10,760	9,858	1.09
7	10^4	8,789	10,438	0.84	9,187	9,408	0.98
8	10^4	9,019	10,379	0.87	8,703	9,062	0.96
9	10^4	9,233	9,268	1	8,513	8,220	1.04
10	10^4	9,117	9,337	0.98	9,687	11,622	0.83
11	10^5	94,603	105,819	0.89	110,462	107,970	1.02
12	10^5	108,055	108,928	0.99	107,699	102,882	1.05
13	10^5	88,246	100,449	0.88	106,389	98,269	1.08
14	10^5	87,938	105,370	0.83	114,166	112,276	1.02
15	10^5	106,246	100,978	1.05	109,971	113,442	0.97
16	10^6	1,098,531	1,059,641	1.04	1,086,195	991,320	1.1
17	10^6	926,410	982,153	0.94	1,097,976	996,524	1.1
18	10^6	912,783	1,076,185	0.85	1,085,161	927,645	1.17
19	10^6	1,020,080	931,143	1.1	1,086,195	1,005,826	1.08
20	10^6	940,240	997,234	0.94	1,146,435	1,142,588	1
		Mean		0.94	Mean		1.01
		SD		0.08	SD		0.08
		Mean \pm 2SD		0.78-1.10	Mean \pm 2SD		0.85-1.18

HPV, human papillomavirus; SD, standard deviation.

and HPV18 copy numbers between the simplex and multiplex qPCR in CXCA samples are summarized in Table VI. No significant difference between simplex and multiplex qPCR was observed for HPV16 E2 and E6 ($P=0.307$ and $P=0.288$; paired t-test) and HPV18 E2 and E6 genes ($P=0.396$ and $P=0.252$; paired t-test). The physical status obtained from multiplex qPCR was also compared with that from the simplex qPCR. The cut-off value for the episomal form of HPV16 (0.79-1.10) and HPV18 (0.85-1.18) was calculated as previously described for multiplex qPCR. Interpretation of physical status was similar in 95% (19/20) of cases between multiplex and simplex qPCR assays, and differed in one case, CX-1 ($P=0.372$; χ^2 test; Table VI).

Discussion

Multiple HR-HPV infection, particularly HPV16 and HPV18 co-infection, is now a concern due to its effects on cervical neoplasia development. The failure rate of treatment was previously reported to be increased by 5-fold in multiple infection (57%), compared with single infection (12%) (24). Therefore, a suitable risk assessment among patients with persistent multiple HR-HPV infection is required. To assess the

risk, viral load and viral physical status may be used for cancer prognosis. In the present study, a one tube qPCR assay for HPV16 and HPV18 co-infection was successfully established with acceptable performance in terms of specificity, accuracy and precision.

Upon performing a literature search for multiplex qPCR of 4 genes in one tube, one study by Zhao *et al* (25) was identified. The authors reported detection of 4 viral DNAs: HPV16 (E6 gene), HPV18 (E6 gene), HSV1 and HSV2, with an improved detection limit at 10 copies compared with the present study. The difference in the detection limit may result from the different size of viral DNA standard. Small fragments of viral DNA (66-139 bp) were used as standard in the study undertaken by Zhao *et al*, whereas the whole HPV genome (10728-12267 bp) was used in the present study. Accordingly, the small size template has an advantage for amplification when compared with the whole genome. Therefore, our established technique better represents the real viral infection in a clinical setting. To resolve this limitation, more DNA template may be adjusted. The accuracy of interpretation, including possible cross reactivity, was verified using DNA of known viral status from cervical Caski and HeLa cell lines. HPV58 and HPV45 were selected for cross reactivity according to

CXCA samples (HPV)	Simplex qPCR (Control)					Multiplex qPCR (Test)										
	Copies/ng DNA		Physical status	Copies/ng DNA		Physical status	Copies/ng DNA		Physical status							
	HPV16 E2	HPV16 E6	E2/E6 ratio (0.79) ^a	Interpretation	HPV18 E2	HPV18 E6	E2/E6 ratio (0.81) ^b	Interpretation	HPV16 E2	HPV16 E6	E2/E6 ratio (0.78) ^c	Interpretation	HPV18 E2	HPV18 E6	E2/E6 ratio (0.85) ^d	Interpretation
PCX 1 (16)	5,139	498,762	0.01	Mixed	-	-	-	-	4,196	470,492	0.01	Mixed	-	-	-	-
PCX 2 (16)	312	32,190	0.01	Mixed	-	-	-	-	203	30,613	0.01	Mixed	-	-	-	-
PCX 3 (16)	5,815	6,087	0.96	Episomal	-	-	-	-	5644.39	6196.23	0.91	Episomal	-	-	-	-
PCX 4 (18)	-	-	-	-	-	191,065	0	Integrated	-	-	-	-	-	153,540	0	Integrated
PCX 5 (18)	-	-	-	-	-	9,380	0	Integrated	-	-	-	-	-	11,897	0	Integrated
CX 1 (16)	118,584	157,522	0.75	Mixed	-	-	-	-	108,968	133,776	0.81	Episomal	-	-	-	-
CX 2 (16)	30,353	26,353	1.15	Episomal	-	-	-	-	32,588	24,118	1.35	Episomal	-	-	-	-
CX 3 (16)	-	24,237	0	Integrated	-	-	-	-	-	26,814	0	Integrated	-	-	-	-
CX 4 (16)	38,041	44,122	0.86	Episomal	-	-	-	-	38,788	48,108	0.81	Episomal	-	-	-	-
CX 5 (16)	6,209	6,721	0.92	Episomal	-	-	-	-	6,879	7,395	0.93	Episomal	-	-	-	-
CX 6 (18)	-	-	-	-	15,649	45,455	0.34	Mixed	-	-	-	-	16,857	42,240	0.4	Mixed
CX 7 (18)	-	-	-	-	-	73,929	0	Integrated	-	-	-	-	-	70,912	0	Integrated
CX 8 (18)	-	-	-	-	74,237	56,271	1.32	Episomal	-	-	-	-	71,243	54,039	1.32	Episomal
CX 9 (16+18)	855	3,468	0.25	Mixed	326	521	0.63	Mixed	1,014	3,919	0.26	Mixed	205	420	0.49	Mixed
CX 10 (16+18)	39,350	123,420	0.32	Mixed	266	410	0.65	Mixed	36,000	155,429	0.23	Mixed	113	237	0.48	Mixed
CX 11 (16+18)	2,834,887	6,008,765	0.47	Mixed	4,090	3,087	1.32	Episomal	2,592,857	5,571,429	0.47	Mixed	3,064	2,414	1.27	Episomal
CX 12 (16+18)	4,122	14,979	0.28	Mixed	403	478	0.84	Episomal	3,305	17,360	0.19	Mixed	371	356	1.04	Episomal
CX 13 (16+18)	120	277	0.43	Mixed	219	415	0.53	Mixed	92	227	0.41	Mixed	197	339	0.58	Mixed
CX 14 (16+18)	9,124	24,510	0.37	Mixed	-	1,034	0	Integrated	8,733	20,600	0.42	Mixed	-	853	0	Integrated
CX 15 (16+18)	-	512,980	0	Integrated	-	981	0	Integrated	-	488,500	0	Integrated	-	741	0	Integrated
Internal control																
HPV16; Caski	0	95,859	0	Integrated	0	0	0	-	0	91,791	0	Integrated	0	0	0	-
HPV18; HeLa	0	0	0	-	0	102,825	0	Integrated	0	0	0	-	0	110,275	0	Integrated
Whole plasmid HPV16	109,334	102,675	1.06	Episomal	-	-	-	-	106,445	105,988	1.00	Episomal	-	-	-	-
Whole plasmid HPV18	-	-	-	-	99,545	98,9655	1.01	Episomal	-	-	-	-	100,599	99,453	1.01	Episomal

The cut-off value of physical status interpretation for ^aHPV16 and ^bHPV18 using simplex qPCR; for ^aHPV16 and ^bHPV18 using multiplex qPCR. There was no significant difference in copy numbers between simplex and multiplex qPCR for HPV16 E2 gene (P=0.307; paired t-test); HPV16 E6 gene (P=0.288; paired t-test). There was no significant difference in copy numbers between simplex and multiplex qPCR for HPV18 E2 gene (P=0.396; paired t-test); HPV18 E6 gene (P=0.252; paired t-test). There was no significant difference in physical status from multiplex qPCR compared with simplex qPCR (P=0.372; χ^2 test). Caski and HeLa cell lines containing 100,000 copies of E6 gene were used as internal standard for integrated HPV16 and HPV18/Whole plasmid HPV16 and HPV18 100,000 copies of E2 and E6 genes were used as internal standard for episomal HPV16 and HPV18. qPCR, quantitative polymerase chain reaction; HPV, human papillomavirus; PCX, pre-cancerous cervical tissues; CX, cervical cancer tissues.

the top 4 common HR-HPV types (HPV 16, 18, 45 and 58), covering 90% of cases among Thai women (26). Accurate quantification was not only demonstrated via comparison with simplex qPCR, but it was also revealed that there was no competitive effect of an unequal mixed HPV DNA template from 10- to 1,000-fold (Tables III and IV).

Furthermore, the comparison of viral physical status between simplex and multiplex qPCR in clinical samples achieved 95% (19/20 samples) agreement in the results of viral physical status. The different physical status of CX1 was caused by the variation of E2/E6 ratio between simplex (0.75) and multiplex (0.81) which closed to the cut-off value for episomal form (0.79 and 0.78) resulting in discrepancy results as mixed form and episomal form, respectively. This limitation of accuracy occurred at values close to the cut-off values.

In conclusion, the increased incidence of HPV16 and HPV18 co-infection is a high-risk factor for CXCA progression in patients with persistent HR-HPV infection. Therefore, the successful development of multiplex qPCR for detecting HPV16 and HPV18 viral load and physical status in a single tube would provide a significant benefit in terms of cost effectiveness and shorter assay time in the clinic. To assess the potential of using this assay as a risk assessment for cancer progression in patients with single and co-infection with HPV16 and HPV18, a larger sample size with clinical outcome data should be included in future studies. In particular, pre-cancerous and early cancerous cases harboring high risk factors should be followed up frequently with monitoring of risk factors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PJ supervised, designed and assisted in the coordination of the present study. PP performed the research, the analysis and prepared data for the first draft of manuscript. TL and CL made substantial contributions in the conception and experimental design. MW was involved in data analysis, interpretation of data and drafting the final manuscript. PT assisted with data

interpretation and revising the final manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Ubonratchathani Cancer Hospital (approval no. CE012/2013; Ubonratchathani, Thailand) and Khon Kaen University (approval no. HE562296; Kho Kaen, Thailand).

Patient consent for publication

All patients provided written informed consent for the publication of the present study.

Competing interests

The authors declare that they have no competing interests.

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