RESEARCH ARTICLE

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Real-time PCR Ct Values of Detection of Epstein-Barr Virus (EBV) *EBNA1* Gene in Nasopharyngeal Swab Samples

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Abstract

Objective: Nasopharyngeal cancer (NPC) is highly prevalent in Asia; however, no established screening program exists to identify high-risk individuals. This study evaluated the potential utility of real-time PCR cycle threshold (Ct) values of Epstein-Barr virus (EBV) DNA detection in nasopharyngeal swabs (NS) as a practical non-invasive screening approach. **Methods:** Nasopharyngeal cytobrush (CB) and swab (NS) samples were collected from 98 patients with NPC, and NS samples were collected from 174 non-NPC individuals. Real-time PCR targeted the *EBNA1* gene, with a Ct cutoff < 35 defined as positive. Beta-globin amplification served as an internal control to verify sample adequacy. **Results:** In NPC patients, 79% and 81% were *EBNA1* positive in CB and NS specimens, respectively. In non-NPC individuals, 10.3% were *EBNA1* positive. Median Ct values were 24.4 in NPC patients and 34.4 in non-NPC individuals ($p<0.001$). Using a Ct cutoff of <35, the diagnostic performance of detecting *EBNA1* in NS specimens showed 81% sensitivity, 90% specificity, and 90% accuracy. **Conclusion:** A real-time PCR Ct cutoff of< 35 for *EBNA1* detection provides a practical and effective non-invasive screening tool for NPC in endemic populations. Further validation in prospective cohorts is required.

Keywords: Nasopharyngeal cancer, *EBNA1*, real-time PCR, cycle threshold, swab, screening

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Introduction

Nasopharyngeal cancer (NPC) is a malignancy strongly linked to latent EBV (Epstein-Barr virus) infection [1]. NPC also shows a marked geographical distribution that exhibits a significantly higher incidence in Asia compared to other regions. In countries such as China and Indonesia, the age-standardised incidence rate reaches 3 and 10 per 100,000 person-years, contrasting with less than 1 per 100,000 in non-endemic areas [2]. Despite this prevalence, there is no established screening programme to detect NPC in Southeast Asian regions [3].

Epstein-Barr virus (EBV) is strongly associated with NPC, and its DNA detection in nasopharyngeal specimens is a potential screening biomarker, especially in endemic areas [4]. Several specimens such as cytobrush (CB) [5], nasopharyngeal swabs (NS) [6], saliva [7], and plasma [8] have been studied as sources to detect EBV DNA screening with various accuracy. A recent study shows a higher discriminatory power of nasopharyngeal swabs than saliva samples to detect the EBV DNA load [9]. Although cytobrush sampling has been studied [5], it is guided with endoscopy requiring anaesthetic application and specialised skills, which limits its use in widespread screening. During the COVID-19 pandemic, nasopharyngeal swabs became a common non-invasive method for SARS-CoV-2 detection, easily performed in primary care settings [10]. Moreover, real-time PCR Ct values of SARS-CoV-2 have been used as practical surrogate indicators of viral load in patient management [11]. This study explored the utility of real-time PCR Ct values of EBV DNA detection in nasopharyngeal swabs as a screening method for NPC, comparing its performance in NPC patients and non-NPC individuals in an EBV-

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endemic region.

Materials and Methods

Nasopharyngeal cancer (NPC) is a malignancy strongly linked to latent EBV (Epstein-Barr virus) infection [1]. NPC also shows a marked geographical distribution that exhibits a significantly higher incidence in Asia compared to other regions. In countries such as China and Indonesia, the age-standardised incidence rate reaches 3 and 10 per 100,000 person-years, contrasting with less than 1 per 100,000 in non-endemic areas [2]. Despite this prevalence, there is no established screening programme to detect NPC in Southeast Asian regions [3].

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Materials and Methods

Sample collection of NPC patients

98 newly diagnosed and histopathologically confirmed undifferentiated non keratinising nasopharyngeal cancer patients (NPC) had been recruited from Dharmais Cancer Hospital and signed informed consent. For each NPC patient, NS and "blind" (non-endoscopic) CB samples were obtained. The NS specimen was collected using a 14–18 cm flexible plastic shaft (OneMed) with a breakpoint and a nylon-flocked tip (2–4 mm in diameter, 1 to 2 cm in length) for efficient absorption and comfortable insertion. In contrast, the CB was collected using a 15 cm plastic handle with a fine flocked brush tip (OneMed) designed to capture epithelial cells, a slightly flexible shaft for nasal anatomy, and a breakpoint for easy transfer to the collection tube.

NS collection was performed by clearing nasal secretions and positioning the patient's head at approximately 70 degrees of extension. A tampon soaked in lidocaine and epinephrine (4 ml : 1 ml) is inserted into the nasal cavity for 5–10 minutes prior to sampling. A sterile dacron/rayon swab is then gently inserted parallel to the palate until it reaches the nasopharynx, rotated 180 degrees for 10–15 seconds, and slowly withdrawn. The tip was broken and collected in a DNA preservative tube.

Immediately after NS sampling, 'blind' CB was performed without endoscope and was taken only on the side where the suspected tumour mass was detected. CB sampling was performed in the contralateral nostril or in the nostril corresponding to the neck mass, after the application of a lidocaine–epinephrine solution (1ml:4ml). Afterward, the CB shaft was snapped at the breakpoint, and the brush head was placed into a DNA preservative tube.

Sample Collection of Non-NPC Individuals

NS samples were also collected from 174 non-NPC individuals. The non-NPC individuals had mild respiratory symptoms and showed no clinical suspicion of any tumors. NS were collected without anaesthesia using flocked swabs inserted into both nasal openings until they reached the nasopharyngeal cavity as performed during the COVID-19 pandemic. The swab was rotated several times, quickly removed, and the tip was placed in a DNA preservative tube. The Ethics Board of YARSI University (167/KEP-UY/EA.20/VII/2024) and the Ethics Committee of the Dharmais National Cancer Centre DP04.03/11.5/211/2024 gave their approval for this study.

DNA Extraction and Quality & Quantity Assessment

Genomic DNA was isolated using the Quick-DNATM Miniprep Plus Kit (Zymo Research, USA) according to the instructions of the manual kit. The DNA concentration and purity were determined using an Infinite 200 Pro Spectrophotometer (Tecan, Switzerland). The extracted DNA samples were then stored at -20 °C for further processing.

EBNA1 gene detection

We detected the *EBNA1* gene as a surrogate marker of EBV DNA using primers and a TaqMan probe. A Ct cutoff of < 35 was used to indicate a positive result for the *EBNA1* gene. The human beta-globin (BG) gene, acting as an internal control, was used to detect intact genomic DNA. In this study, BG was assessed qualitatively via 2% agarose gel electrophoresis (yielding a 247 bp band) rather than through quantitative Ct data. As the primary goal was to confirm the presence of amplifiable human DNA to ensure sample adequacy rather than to quantify human cellularity, a qualitative PCR for the beta-globin (BG) gene was performed. This approach reliably distinguishes samples with sufficient DNA from those that are inadequate for analysis. The primer sequences are shown in Table 1.

The amplification of the QP primer and probe was performed in a total volume of 20 µl, containing a 20 ng DNA template and 1x THUNDERBIRD® Probe qPCR Mix (Toyobo, China) performed on a LC480 qPCR thermal cycler (Roche, USA). Thermal cycling conditions included pre-denaturation at 95 ° C for 1 min followed by 40 cycles of 95 ° C for 15 sec and 56 ° C for 30 sec. DNA from the RAJI cell line was used as a positive control. Beta-globin (BG) amplification was performed in a total volume of 25 µl, containing 20 ng of DNA and 1x Gotaq

Table 1. PCR Primer Sequences Used in This Study

Primer	Region Amplifikasi	Direction	Sequences 5` - 3`	Size	Reference
QP1	*EBNA-1*	Forward	GCC GGT GTG TTC GTA TAT GG	213	[12]
QP2		Reverse	CAA AAC CTC AGC AAA TAT ATG AG		
Probe			(FAM)ACCCGGCCCACAACCTG(MGB)		[8]
BG-1F	Beta-Globin TAL57	Forward	TAG CAA CCT CAA ACA GAC ACC A	247	[12]
BG-1R		Reverse	CAG CCT AAG GGT GGG AAA AT		

Green Master mix (Promega, USA) performed on the conventional T100 thermal cycler (BioRad, USA). The amplification profile was initiated at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized using UV gel documentation. A 247 bp band indicated that the BG gene was positive.

Statistical analyses

Normality was tested in each group. Mann whitney was used to test the numerical difference of independent datasets that were not normally distributed. The chi-square or Fisher's exact test was used to test the difference in proportion (EBV positivity) between independent groups. For paired groups (CB and NS from the same NPC patients), the significance proportion of EBV positivity was tested using the McNemar test. To test the concordance of EBV positivity in CB and NS, we used the Cohen Kappa test with possible values between 0 and 1. Kappa values less than 0, 0.0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8, and 0.81 to 1.0 indicate no agreement, fair agreement, moderate agreement, substantial agreement, and almost perfect agreement, respectively. Statistical analysis was performed using DATAtab (Online Statistics Calculator. University of Graz, Austria. URL https://datatab.net) and GraphPad Prism.

Results

Demography of NPC patients and non-NPC individuals

As shown in Table 2, the median age of patients with NPC and non-NPC individuals was similar, with a slightly higher median age in non-NPC individuals. Smoking history was also more frequent in patients with NPC than in non-NPC individuals. Finally, the distribution of males and females was similar in both groups. Within the NPC patients, the DNA concentration of the CB specimen (median 15.2ng/ul) was higher than that of the NS specimens (11.8 ng/ul, Wilcoxon $p < 0.001$). Moreover, the DNA concentration of the NS specimens of patients with NPC was significantly higher than that of the swab specimens of non-NPC individuals (6.6 ng/ul, Mann–Whitney $p = 0.003$).

Reliability of Nasopharyngeal Swabs (NS) to detect EBV in histopathologically confirmed NPC patients

As shown in Supplementary Table 1, the Ct values of the *EBNA1* PCR amplicon found in NS versus CB samples from NPC patients were highly correlated. Moreover, the median Ct values of *EBNA1* were also similar to NS and CB of patients with NPC (Supplementary Table 2).

To determine the concordance of positivity for *EBNA1* found in NS and CB samples from NPC patients, we calculated Cohen's kappa values. As shown in Supplementary Table 3, the concordance improved when the *EBNA1* positivity criteria were Ct cutoff <35 (from 0.31 to 0.43, or from fair to moderate agreement). In particular, the agreement improved from 0.11 to 0.33 when Ct cutoffs <35 were used as positive results.

Using a Ct cutoff of <35 as positive for *EBNA1*, the positivity of EBV *EBNA1* in different clinical variables is summarized in Supplementary Table 4.

Using a Ct cutoff of <35 to indicate positive EBV infection, we found that EBV positivity was similar in NS (80%) and CB (79%) specimens. However, positivity in males and patients with a smoking history was higher

Table 2. Demographic Data of Patients with Nasopharyngeal Cancer and Non-NPC Individuals

Clinical variables		NPC CB (N=98)	Non-NPC (N=174)	p values
Age (years)				
	Range	17-84	45-77	
	Mean	49	50	
	Median (IQR)	50 (42-56)	48 (47-52)	0.79 (Mann-Whitney)
Sex				
	Males	73 (75%)	132 (75%)	0.91 (Chi Square)
	Females	25 (25%)	42 (24.1%)	
Smoking history				
	Yes	71 (73.2%)	65 (37%)	<0.05 (Chi Square)
	No	19 (19%)	92 (53%)	
	Unknown	8 (8%)	17 (17%)	

Table 3. Ct Values of EBV *EBNA1* in NPC Patients and Non-NPC Individuals

	NPC Median (Min - Max))	Non-NPC Median (Min - Max))	p values (Dunn Bonferoni)
All	24.4 (17.9 to 36.9)	34.4 (20.9 to 37.9)	<.001
Males	24.5 (17.9 to 36.9)	34.7 (23.5 to 37.9)	<.001
Females	23.8 (17.9 to 35)	34.2 (20.9 to 37.9)	0.026
Smoking	24.5 (17.9 to 34.6)	34.7 (26.8 to 37.9)	<.001
Non-Smoking	24.5 (17.9 to 35)	35.6 (31.8 to 37.9)	0.005

in NS than in CB.

Difference in EBNA1 detection in (NS) specimens of NPC patients vs non-NPC individuals

The median Ct values of *EBNA1* in the NS of patients with NPC (24.4) were lower than those in the NS of individuals without NPC (34.4), reflecting a higher burden of EBV *EBNA1* in patients with NPC, as expected (Table 3). Low Ct values of *EBNA1* were consistently found in all clinical variables, such as sex and smoking history, in NPC patients. Lastly, as shown in Table 4, diagnostic accuracy was better when using positivity as defined by Ct values less than 35 than by any Ct values. The positivity of *EBNA1* in non-NPC individuals was 10% when positivity was defined as Ct cutoff <35. In contrast, any Ct values led to positivity rate of 18% in non-NPC individuals leading to low specificity (Table 4).

Discussion

In this study, we showed that the detection of *EBNA1* as a surrogate marker of EBV DNA in nasopharyngeal swab (NS) collection could match the diagnostic performance of *EBNA1* in cytobrush (CB) sampling in patients with histologically confirmed nasopharyngeal carcinoma (NPC). Furthermore, we found that *EBNA1* detection using real-time PCR with Ct cutoff <35, NS achieved similar *EBNA1* positivity (81%) comparable to CB (79% positivity). Ct cutoff <35 also led to higher specificity than when using any detectable Ct values as positive. These findings have important implications for NPC screening in EBV endemic Asian settings, where the logistical simplicity and patient acceptability of NS could facilitate wider adoption.

NS versus CB: Performance and Practicality

Although CB samples yielded higher DNA concentrations, EBV detection rates were marginally lower than those for NS. A plausible explanation is that CB collection in this study was unguided and performed without endoscopic visualization, which may have reduced the likelihood of directly sampling the tumor-involved mucosa. Moreover, this "blind" technique likely underestimates the sensitivity achievable with endoscopically guided CB, which remains a valuable tool for targeted sampling. In contrast, NS sampling, similar in technique to that used for SARS-CoV-2 testing, sweeps a larger mucosal surface area and can capture viral DNA from exfoliated tumor cells and associated secretions. This pattern is consistent with earlier Asian studies: Coghill et al. in Taiwan reported the detection of EBV in 88% of NPC cases using NS [13], while Li et al. in China found NS to outperform saliva in EBV load discrimination between NPC and non NPC individuals [9]. Our data extend these findings by directly comparing NS and CB in the same patients and demonstrating statistical equivalence in positivity, despite the procedural simplicity of NS.

Ct Values as a Surrogate for Viral Burden

The adoption of a Ct < 35 positivity threshold increased the specificity of~81% to ~90% by filtering out low level EBV detection in non NPC individuals in our study. This mirrors the widespread use of Ct thresholds during the COVID 19 pandemic to approximate viral load and infer clinical relevance [11]. In our cohort, NPC patients consistently exhibited lower median Ct values (~24) than non NPC individuals (~34), supporting the biological plausibility of Ct as a proxy for tumor

Table 4. Any Ct Values vs Ct Cutoff <35 for *EBNA1* Positivity in NS of NPC vs Non-NPC

Variables	Any Ct				Ct values <35			
	EBNA1 Positivity NPC	*EBNA1* Positivity non-NPC	Sensitivity (95%CI)	Specificity (95% CI)	*EBNA1* Positivity NPC	*EBNA1* Positivity non-NPC	Sensitivity (95%CI)	Specificity (95% CI)
All	82.6%	18.9%	82.6% (73.6 to 89.5)	81% (74 to 86)	80%	10%	80.6% (71.3 to 87.9)	89.6 % (84.1 to 93.7)
Males	89%	15%	89% (79 to 95)	84% (76.8 to 89.9)	87%	8%	87.6% (77.8 to 94.2)	91.7% (85.6 to 95.8)
Females	64%	29%	64% (42.5 to 82)	70.7% (54.4 to 83.8)	60%	17%	60% (36.6 to 78.8)	82.9% (67.9 to 92.8)
Smoking	88%	20%	88.7% (79 to 95)	80% (68.2 to 88)	88%	10%	88.7% (79 to 95)	89.2% (82.7 to 93.8)
No Smoking	65%	11%	63.1% (36.3 to 83.7)	88% (79 to 93)	57%	4%	57.8% (33.5 to 79.7)	95.6% (89.2 to 98.8)

associated EBV burden [14]. This approach could enable semi quantitative risk stratification in screening contexts and guide thresholds for referral to endoscopy.

Comparison with Other Sampling Modalities

Plasma EBV DNA testing, as established in large Hong Kong and Guangdong screening programmes, achieves sensitivities above 95% but requires venipuncture, cold chain transport, and centralized qPCR infrastructure [4]. In lower resource environments, such requirements may be prohibitive. NS sampling, in contrast, is low cost, non invasive, and compatible with decentralised collection in community clinics. Previous studies from Vietnam [15] and China [16] have demonstrated EBV positivity rates in NPC ranging from 45–95% using NS, similar to our findings. Saliva testing, although even less invasive, tends to show lower viral loads and higher variability [9], limiting its standalone use for NPC screening.

Public Health and Screening Implications

NPC remains a leading malignancy in several Asian populations, with a peak incidence in Southern China, Southeast Asia. Organised screening is rare, leading to frequent late-stage diagnosis and poorer survival. Our findings suggest that NS-based EBV DNA testing, interpreted using an evidence-based Ct cutoff, could be deployed as part of tiered screening in endemic communities. For example, individuals with *EBNA1* Ct < 35 could be prioritised for confirmatory naso-endoscopy and biopsy, optimising the use of specialist resources.

Strengths and Limitations

The strengths of this study include the head-to-head evaluation of NS and CB in the same patients, robust statistical analysis of paired samples, and comparison with a large non-NPC control group. Limitations include the absence of endoscopic guidance for CB, which may underestimate its maximal sensitivity, and the cross-sectional design, which precludes the evaluation of the predictive value for incident NPC in asymptomatic carriers. Future studies employing endoscope-guided CB would provide a more definitive assessment of CB's maximal diagnostic sensitivity of CB. Furthermore, the use of qualitative, rather than quantitative, beta-globin PCR provided binary confirmation of sample adequacy but precluded a more nuanced analysis of how sample cellularity might influence EBV DNA detection and Ct values. Finally, this study lacked an external validation cohort, and the clinical outcomes of false-positive individuals (non-NPC with EBV-positive swabs) are unknown, as no longitudinal follow-up was performed.

Future Directions

Further research should focus on refining Ct thresholds for an optimal balance between sensitivity and specificity, integrating NS testing with serology (e.g., antiEBV VCA/EA IgA), and validating longitudinal NS sampling for early recurrence detection. Regional implementation trials can assess the cost-effectiveness and feasibility of NS-based screening in primary care or community outreach settings.

Author Contribution Statement

All authors contributed equally in this study.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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