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การประเมินประสิทธิภาพชุดตรวจสารพันธุกรรมต่อเชื้อโควิด-19 3 ยี่ห้อโดยใช้ค่า Cycle threshold

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Verification of Three Commercial Quantitative RT-PCR Kits for COVID-19 Using Cycle Threshold Values

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บทคัดย่อ

หลักการและวัตถุประสงค์: ในช่วงการระบาดของโรคติดเชื้อโควิด-19 ทั่วโลกนั้นมีชุดน้ำยาตรวจสารพันธุกรรมต่อเชื้อโควิด-19 ที่มีประสิทธิภาพหลากหลายยี่ห้อที่ห้องปฏิบัติการแต่ละแห่งสามารถเลือกใช้เพื่อเพิ่มขีดความสามารถในการตรวจคัดกรองโรคติดเชื้อโควิด-19 เพื่อให้การควบคุมการระบาด การป้องกัน การรักษาได้อย่างถูกต้องและรวดเร็วเพื่อลดอัตราการเกิดอาการรุนแรงและอัตราการเสียชีวิต โดยผู้วิจัยได้เลือกใช้ชุดตรวจหาสารพันธุกรรมต่อเชื้อโควิด-19 โดยใช้หลักการ quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) ทั้งหมด 3 ชุดตรวจที่ใช้งานในโรงพยาบาล ได้แก่ ชุดตรวจด้วยเครื่องอัตโนมัติ Cobas 6800, ชุดตรวจยี่ห้อ Allplex SARS-CoV2 Master Assay และชุดตรวจยี่ห้อ Sansure Assay โดยแต่ละชุดน้ำยาใช้กับเครื่องของแต่ละบริษัท ได้แก่ Cobas 6800, Biorad CFX96 และ MA6000 ตามลำดับ เนื่องจากมีการใช้ชุดน้ำยาตรวจ 3 ชนิด แต่ละชนิดมีค่า limit of detection (LOD) ที่ต่างกันเพื่อให้มีค่าตัดสินร่วมกันไม่ว่าจะตรวจด้วยน้ำยายี่ห้อใดก็ตาม ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้เพื่อประเมินประสิทธิภาพของชุดตรวจทั้ง 3 ชนิด และหาค่า cycle threshold (Ct) เพื่อใช้เป็นค่าที่ใช้ตัดสินใจในการแปลผลและการทดสอบซ้ำที่เหมาะสมและกำหนดเป็นแนวทางในการแปลผลและรายงานผลในห้องปฏิบัติการโดยใช้ตัวอย่างผู้ป่วยในการทดสอบภายใต้ห้องปฏิบัติการที่ผ่านการรับรองมาตรฐานสากล (ISO 15189)

วิธีการศึกษา: ทำการเจือจางตัวอย่าง nasopharyngeal swab ด้วยอาหารเลี้ยงเชื้อ (viral transport medium) ที่ทราบผลการทดสอบเป็นพบเชื้อโควิด-19 จำนวน 10 ตัวอย่างโดยเจือจางตัวอย่างดังนี้ undilute, 1:10, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, และ $1:10^{-6}$ แล้วทำการตรวจ qRT-PCR ตามวิธีปฏิบัติของชุดตรวจแต่ละชนิด โดยทำการทดสอบซ้ำ 3 ครั้งในทุกตัวอย่างแล้วพิจารณาค่าความไวจากความสามารถในการตรวจพบเชื้อในตัวอย่างที่ค่าเจือจางสูงสุดและบันทึกค่า Ct ที่ได้ทุกตัวอย่างจากนั้นนำผลที่ได้คำนวณค่าเฉลี่ยเพื่อกำหนดเป็นค่าตัดสินร่วมของชุดตรวจทั้ง 3 ชนิด

ผลการศึกษา: พบว่าชุดตรวจด้วยเครื่องอัตโนมัติ Cobas 6800, ชุดตรวจยี่ห้อ Allplex SARS-CoV2 Master Assay และชุดตรวจยี่ห้อ Sansure Assay โดยให้ผลการตรวจพบเชื้อโควิด-19 ในระดับค่าเจือจางที่ดีที่สุดคือ $1:10^{-5}$, $1:10^{-3}$และ $1:10^{-3}$ ตามลำดับ นอกจากนั้นได้ทำการทดสอบเพื่อหาค่า Ct ที่เหมาะสมสำหรับการแปลผลของชุดน้ำยาทั้ง 3 ชนิด พบว่าค่า Ct ที่สามารถใช้เป็นค่าตัดสินเพื่อการพิจารณาการรายงานและการทดสอบซ้ำเพื่อความถูกต้องได้ผลดังนี้ *E* gene = 36.67, *ORF1ab* gene = 35.59 และ *N* gene = 36.09

สรุป: ชุดตรวจที่มีในห้องปฏิบัติการ โรงพยาบาลขอนแก่น ที่มีความไวสูงสุดคือชุดตรวจด้วยเครื่องอัตโนมัติ Cobas 6800 ตามด้วยชุดตรวจยี่ห้อ Allplex SARS-CoV2 Master Assay และชุดตรวจยี่ห้อ Sansure Assay ที่มีความไวเทียบเท่ากัน และ ค่า Ct ที่ได้นำไปใช้ในการกำหนดแนวทางการรายงานผลการตรวจหาเชื้อโควิด-19 ด้วยวิธี qRT-PCR ในห้องปฏิบัติการเพื่อให้ผู้ปฏิบัติใช้เป็นแนวทางเดียวกันในการรายงานผลต่อไป

คำสำคัญ: qRT-PCR, Cycle threshold, ค่าตัดสิน, โควิด-19

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Abstract

Background and Objectives: During the SARS-CoV-2 pandemic, several effective and reliable qRT-PCR kits were accessible worldwide. To increase the capacity of COVID-19 detection the three commercial kits were chosen in our laboratory. Herein, we performed the verification of the three commercial qRT-PCR kits including a fully automation Cobas 6800 system, Allplex SARS-CoV2 Master Assay and Sansure Assay and finding the Ct values for interpretation and reporting. This study utilized the clinical samples and performed under our ISO 15189 certified laboratory.

Methods: Ten positive qRT-PCR for COVID-19 samples were 10-folds serially diluted with viral transport media as undilute, $1:10^{-1}$, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, and $1:10^{-6}$ dilution. Viral nucleic acid was extracted. The qRT-PCR was triplicate performed by using three commercial kits. The performance of the kits were considerated and the Ct values were recorded and calculated.

Results: We found that the performance of a fully automation Cobas 6800 system, Allplex SARS-CoV2 Master Assay and Sansure Assay with the maximum detectable dilution at $1:10^{-5}$, $1:10^{-3}$ and $1:10^{-3}$ respectively. In addition, we found the Ct values for precise interpretation for COVID-19 qRT-PCR testing. The grey zone Ct value of *E* gene is 36.67, *ORF1ab* gene is 35.59 and *N* gene is 36.09 which these values could be considered to repeat for accurate detection.

Conclusions: The result demonstrated that the best performance commercial kit was a fully automation Cobas 6800 system following by Allplex SARS-CoV2 Master Assay was equal to Sansure Assay. The Ct values of target genes (*E* = 36.67, *ORF1ab* = 35.59 and *N*= 36.09) were implemented for the qRT-PCR for COVID-19 guideline in our laboratory.

Keywords: qRT-PCR, Cycle threshold, Cut off, COVID-19

Introduction

Coronavirus disease 2019 (COVID-19) is an emerging infectious disease caused by a novel coronavirus 2 (SARS-CoV-2). This virus emerged in human since the end of 2019. It was first reported as deadly pneumonia in Hubei Province, Wuhan, China. Recently, it is a tremendous burden worldwide and World Health Organization (WHO) declared to be a pandemic since March 2020[1]. SARS-CoV-2 is closely related to the previous SARS-CoV and Meddle East respiratory syndrome coronavirus (MERS-CoV). It is sharing 80% sequence identity with SARS-CoV and 50% with MERS-CoV[2]. Initially, the virus affected respiratory system caused flu-like symptoms such as a cough, fever, fatigue, dyspnea which might lead to pneumonia in some cases and cause death in the severe cases especially patient with underlying disease[3]. Moreover, it might cause a respiratory failure, shock, kidney failure, cardiovascular damage and liver failure[4,5]. COVID-19 is extremely contagious infectious disease by one patient can transmit an estimate of three surrounding people which is greater than SARS-CoV and MERS-CoV[6]. SARS-CoV-2 is an enveloped, sense-single stranded RNA virus with 30-kb genome, a member of the *Coronaviridae* family. SARS-CoV-2 genome consist of several gene that encodes the structural, non-structural, and accessory proteins. The *ORF1a* and *ORF1b* genes encode two polyproteins that are cleaved into 16 non-structural proteins, such as RNA-dependent RNA polymerase (RdRP), helicase, and various proteases. In the last third part of genome, genes for four structural proteins including spike (S), membrane (M), envelope (E), nucleocapsid (N) and several accessory proteins are located[2,7]caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2. Spike (S) is most diverse than other expression genes and it relevant to unusual epidemiological characteristics[8]. To date, WHO has classified COVID-19 variants as follows: variants of concern (VOC) such as Alpha, G ramma, Beta, Delta, and Omicron and variants of interest (VOI) for instance Eta, lota, and Kappa. Some of VOCs are evidence of a highly transmissibility and more severity. Therefore, VOCs are required more suitable public health approaches[9] coupled with the high performance of detection system. Nevertheless, COVID-19 is now dropped down as an endemic disease in partial globe included Thailand. Early detection is yet necessary for transmission control and well-organized treatment leading to decline of severity and lethality. Recently, there are serological testing such COVID-19 antigen (ATK) and COVID-19 antibodies along with nucleic acid detection, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), for COVID-19 diagnosis. The qRT-PCR is used when the starting nucleic acid is RNA. RNA was transcribed into complementary DNA (cDNA) by reverse transcriptase. The cDNA is then utilized as the template for qPCR. The qRT-PCR is a gold standard molecular detection and basically use to detect gene expression levels, also facilitating the early diagnosis of acute respiratory viral infection. Basically, conserve (*ORF1ab*, *RdRP*) and variable region (*E, N, S)* of viral genome were used as the targeted genes for molecular detection. During the pandemic, the several high-performance qRT-PCR kits were distributed by several venders. In the extremely course of pandemic, three of commercial qRT-PCR kits were utilized in our laboratory to support the high throughput workload. There are different of the limit of detection, targeted genes, thermal cycle protocol in each kit and controversial accuracy between clinical symptoms and cycle threshold values. In this study, we aimed to improve and verify three of qRT-PCR kits to obtain the appropriated coincidence cut off with in three commercial qRT-PCR kits under ISO 15189 certified laboratory. Later, the guideline for interpretation of qRT-PCR for COVID-19 testing was constructed and implemented in Khon Kaen Hospital.

Materials and Methods

1. Sample collection

This study conducted in Khon Kaen Hospital between July to August 2022. Left-over nasopharyngeal swab in viral transport media were included in this study. Inclusion criteria are (1) positive for COVID-19 by qRT-PCR either of commercial kits in our laboratory (2) Cycle threshold of target genes > 30 (3) Sample volume > 2 mL. The samples after routine test were stored at -20°C then were detected within 7 days. Ethical approval was received from Ethics Review Committee, Khon Kaen Hospital (Approval number KEXP65031).

2. Viral nucleic extraction and qRT-PCR

Samples were 10-fold serially diluted with viral transport media as undiluted, $1:10^{-1}$,$1:10^{-2}$,$1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, and $1:10^{-6}$ dilution. Viral nucleic acid was extracted from samples by using Nucleic Acid Extraction kit and EXM3000 extraction system (Zybio Inc, Germany). A sample volume of 200 µL was used for nucleic acid extraction and the elution volume was 50 µL. The qRT-PCR was triplicate performed by using Allplex SARS-CoV2 Master Assay (Seegene Inc. Republic of Korea) which amplified *E, RdRP/S, N* and *S* variants gene. The thermal cycle was performed at 50 ℃ for 20 min, 95 ℃ for 15 min, 45 cycles of 95 ℃ for 10 s, 60 ℃ for 15 s, 72 ℃ for 10 s. Following triplicate detected by Sansure SAR-CoV-2 Assay (Sansure Biotech Inc, Republic of China) which amplified *ORF1ab* and *N* gene. The protocol was performed at 37 ℃ for 1 min, 50 ℃ for 5 min, 95 ℃ for 2 min, and 45 cycles of 95 ℃ for 5 s, 60 ℃ for 30 s. In addition, undiluted and $1:10^{-1}$ to $1:10^{-6}$ diluted samples were triplicate detected COVID-19 by fully automation Cobas 6800 system (Roche Diagnostics GmbH, Germany) which amplified *ORF1ab* and *E* gene. The protocol followed by the confidential manufacturer's instruction. Cycle threshold (Ct) of target genes and housekeeping gene were recorded on Microsoft excel. Mean and standard deviation were computerized.

3. Interpretation of qRT-PCR for COVID-19

The results were interpreted by the COVID-19 interpretation's protocol of the Department of Medical Sciences, Ministry of Public Health, Thailand[10]. Housekeeping gene or internal quality control must be valid (Ct <40). Detectable result is two of target genes were detected result (Ct<40). Undetectable result is none of target genes were detected (Ct>40). Inconclusive result is one of two or one of three target genes were detected; this result must be repeated or recollect specimen. Herein, Detectable dilutions were included for verification of the three commercial qRT-PCR kits.

4. Consideration the cut off all three qRT-PCR kits.

The Ct value of each individual genes which detectable result by all three commercial kits were considered. Cut off Ct values were calculated from the mean +/- SD of Ct values of lowest detection dilution of each gene from three kits by using Microsoft Excel. These Ct values were used as the new cut off for COVID-19 qRT-PCR interpretation protocol in our laboratory.

Results

During COVID-19 pandemic, the high throughput detection tools were required to early and rapidly diagnosed for the restriction of outbreak and decline the severity and death. In our hospital, three commercial qRT-PCR were used. However, it was a novel emerging disease which confused strategies and limited of understanding the pathogenesis of the disease. Therefore, detection tools especially qRT-PCR kits were required the verification by using the clinical samples. We investigated the limit of detection of three qRT-PCR kits which used in the Department of Medical Technology, Khon Kaen Hospital. Total ten COVID positive samples were included in this study. In this context, the study demonstrated that Cobas 6800 system showed the highest sensitivity for detection with the lowest of detection at the dilution $1:10^{-5}$ 7 samples and $1:10^{-4}$ 3 samples. While Allplex SARS-CoV2 Master Assay and Sansure Assay was equal. The result was showed the lowest of detection at the dilution $1:10^{-3}$ of individual one sample C and G respectively, as showed in Table 1-3.

Table 1 The limit of detection of Allplex SARS-CoV-2 Master Assay

Sample	Mean of Ct values					Limit of Detection (dilution)
	E	RdRP/S	N	S variants	IC	
A	34.82	34.79	36.5	35.58	35.16	$1:10^{-2}$
B	34.57	34.79	37.18	37.6	36.1	$1:10^{-2}$
C	37.04	34.87	36.54	36	37.29	$1:10^{-3}$
D	36.8	36.05	36.83	36.51	35.54	$1:10^{-2}$
E	33.74	35.85	33.76	38.03	38.52	$1:10^{-1}$
F	36.35	35.11	37.44	36.63	37.48	$1:10^{-2}$
G	33.45	38.74	36.99	38.39	37.58	$1:10^{-2}$
H	36.38	34.67	37.82	38.01	33.84	$1:10^{-2}$
I	35.6	36.45	37.06	37.1	36.7	$1:10^{-2}$
J	37.34	37.83	38.36	36.27	38.19	$1:10^{-2}$
Mean	35.61	35.92	36.85	37.01	36.64	
SD	1.39	1.41	1.23	0.96	1.47	

E= Envelope, RdRp/S =RNA dependent RNA polymerase/Spike, N = Nucleocapsid, S variants = Spike variants, IC =Internal control

Table 2 The limit of detection of Sansure Assay

Sample	Mean of Ct values			Limit of Detection (dilution)
	ORF1ab	N	IC	
A	36.97	36.6	36.46	$1:10^{-2}$
B	36.95	35.67	37.28	$1:10^{-1}$
C	38.77	37.23	33.78	$1:10^{-2}$
D	37.03	38.87	35.77	$1:10^{-2}$
E	38.15	37.12	37.84	$1:10^{-1}$
F	36.4	35.46	38.42	$1:10^{-1}$
G	35.75	38.7	36.42	$1:10^{-3}$
H	38.56	36.97	35.27	$1:10^{-2}$
I	36.55	36.16	39.03	$1:10^{-1}$
J	37.12	34.63	35.64	$1:10^{-1}$
Mean	37.23	36.74	36.59	
SD	0.97	1.35	1.58	

ORF1ab=Open Reading Frame 1ab, N=Nucleocapsid, IC =Internal control

Table 3 The limit of detection of Cobas 6800 system

Sample	Mean of Ct values			Limit of Detection (dilution)
	ORF1ab	E	IC	
A	36.08	36.67	33.35	$1:10^{-5}$
B	35.99	36.47	33.99	$1:10^{-5}$
C	36.46	37.72	33.94	$1:10^{-5}$
D	36.22	36.58	33.31	$1:10^{-4}$
E	35.97	38.72	33.76	$1:10^{-4}$
F	35.52	35.85	34.19	$1:10^{-4}$
G	35.98	38.28	33.98	$1:10^{-5}$
H	35.89	37.87	33.34	$1:10^{-5}$
I	35.35	36.96	33.55	$1:10^{-5}$
J	36.87	37.28	33.75	$1:10^{-5}$
Mean	36.03	37.24	33.72	
SD	0.43	0.90	0.31	

ORF1ab=Open Reading Frame 1ab, E= Envelope, IC =Internal control

Table 4 The Ct cut off of three commercial qRT-PCR kits

Ct values of the limit of detection of all three kits					
	E	ORF1ab	N	RdRp/S	S variants
	37.04	35.75	36.54	34.87	36.00
	36.67	36.08	38.7	-	-
	36.47	35.99	-	-	-
	37.72	36.46	-	-	-
	38.28	35.98	-	-	-
	37.87	35.89	-	-	-
	36.96	35.35	-	-	-
	37.28	36.87	-	-	-
Mean	37.29	36.05	37.62	34.87	36.00
SD	0.62	0.46	1.53	-	-

E= Envelope, ORF1ab=Open Reading Frame 1ab, N=Nucleocapsid, IC =Internal control

Recommend cut off value for each kit was Ct <40 followed by the manufacturer's protocol of all three kits. In this study the mean cycle thresholds ±SD were considered as the grey zone for cut off value of all three qRT-PCR kits which represent Ct value for detection which may need to confirm the result for true detection. From the results, grey zone Ct value of *E* gene, *ORF1ab* gene and *N* gene are 37.29 ±0.62, 36.05 ± 0.46 and 37.62 ±1.53 respectively. Additionally, by using Allplex SARS-CoV2 Master Assay the cycle threshold value of *RdRP/S* gene and *S* variants were 34.87 and 36.00 respectively, as showed in Table 4. The Ct values lower than grey zone Ct – SD was considered detectable in our laboratory.

Following Ct value of *E* gene, *ORF1ab* gene and *N* gene were 36.67, 35.59 and 36.09 respectively.

Discussion

During the COVID-19 pandemic, the WHO has widely emphasized the urgent increase the molecular diagnosis for COVID-19 to limit the outbreak as well as classification of the patients. The number of suspected cases were increased. Accuracy and timely results were required. Several commercial qRT-PCR kits for COVID-19 were chosen for the high throughput workloads including our laboratory, Khon Kaen Hospital. Herein, we verified of three in used commercial qRT-PCR kits comprised of Allplex SARS-CoV2 Master Assay, Sansure Assay and a fully automation Cobas 6,800 system. In this context, the best performance of detection of COVID-19 nucleic acid in this study was Cobas 6,800 system followed by Allplex SARS-CoV2 Master Assay was equal to Sansure Assay which the limit of detection at dilution $1:10^{-5}$, $1:10^{-3}$, and $1:10^{-3}$ respectively. However, the performance of three qRT-PCR kits from previous studies indicated the high performance following, Allplex SARS-CoV-2 Master Assay was claimed 98.2% and 100% (91.6-100%) of sensitivity, 100% and 100% of specificity in single sample and pooled samples respectively[11,12] Sansure Assay was better performance (sensitivity 99.5%, specificity 91.3%) when compared with GeneFider, TaqPath and 2019-nCoV CDC EUA (IDT)[13,14]. A fully automation Cobas 6,800 system have been compared to the semi-automated platform, resulting in the satisfactory of agreement between two platform (Cohen's κ coefficient was found to be 0.76 (95% CI, 2.5897–13.4103)[15–17]. Therefore, all of three qRT-PCR kits were effective and reliable assay for the detection of SARS-CoV-2 infection. Furthermore, the cycle threshold value of lowest detection in dilution manner of *E* gene, *ORF1ab* gene and *N* gene are 37.29±0.62, 36.05±0.46 and 37.62 ±1.53 respectively. The Ct values lower than grey zone (Ct - SD) were considered as detectable in our laboratory and were implement in routine to construct the appropriated guideline to diagnose COVID-19 infection. These Ct values were consistency with the previous studies which were correlated to the clinical symptoms[18,19]. Thus, the Ct values of 36.67, 35.59 and 36.09 for *E* gene, *ORF1ab* gene and *N* gene respectively, can be used as the cut off for the decision of treatment strategies or disease control. Herein, our study was claimed as the first report to verify three of commercial qRT-PCR kits with clinical samples and consideration of the coincidence Ct values of all three commercial kits. However, there were several affecting the Ct values including collection technique such as nasopharyngeal swab was recommended[20], patient should be appropriately prepared for the best specimen collection. The samples having Ct > 36 were recommended for repetition to rule out the possibility of contamination [18,21] many scientists disagree, and it is essential to understand that several factors and variables can cause a false-negative test. In this context, cycle threshold, sampling time and viral kinetic were also affected the Ct values, viral loads were increased on the fifth day and decline on the seventh day onset. On the 13th and 14th days during the course of infection, qRT-PCR was positive opposite viral load was undetectable[22]. Some studies indicated Ct value was relevant to the severity and stages of disease[23,24]. The Ct values were significantly lower in severe condition[25,26].

Liu et al reported an average Ct value of 34.92 from asymptomatic individuals[25]. The high Ct value of 35.00 can be detected in recovery stage[27]as reflected by the Ct However, to increase accuracy and precision the more sample size was recommended in this study. Due to the limitation of manufacturer's data let to the lower of detection as the copies number could not be provided. There were different primers, PCR conditions, reagents, and other components of all three commercial kits which might cause the different process and result. Nevertheless, the Ct values must be considered with the clinical symptoms, sample collection technique, sampling timing and epidemiological data for the corrective diagnosis. This study provided the coincidence Ct values among three qRT-PCR kits to beware the decision of lab report whether performing by any three kits. Verification of the selected commercial qRT-PCR kits are recommended individual laboratory.

Conclusion

The best performance of three commercial qRT-PCR kits was a fully automation Cobas 6800 system. Allplex SARS-CoV2 Master Assay and Sansure Assay was equal. The gray zone Ct value of 36.67, 35.59 and 36.09 for E gene, *ORF1ab* gene and *N* gene respectively were recommended for the repetition of qRT-PCR for COVID-19 detection. We used all of three commercial kits to detect COVID-19 depended on the amount of the suspected cases. In this context we used these Ct values as a gray zone for consideration of repetition whether detection by any of three kits and construction of reporting COVID-19 guideline.

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Conflict of interest

The authors declare no conflict of interest

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