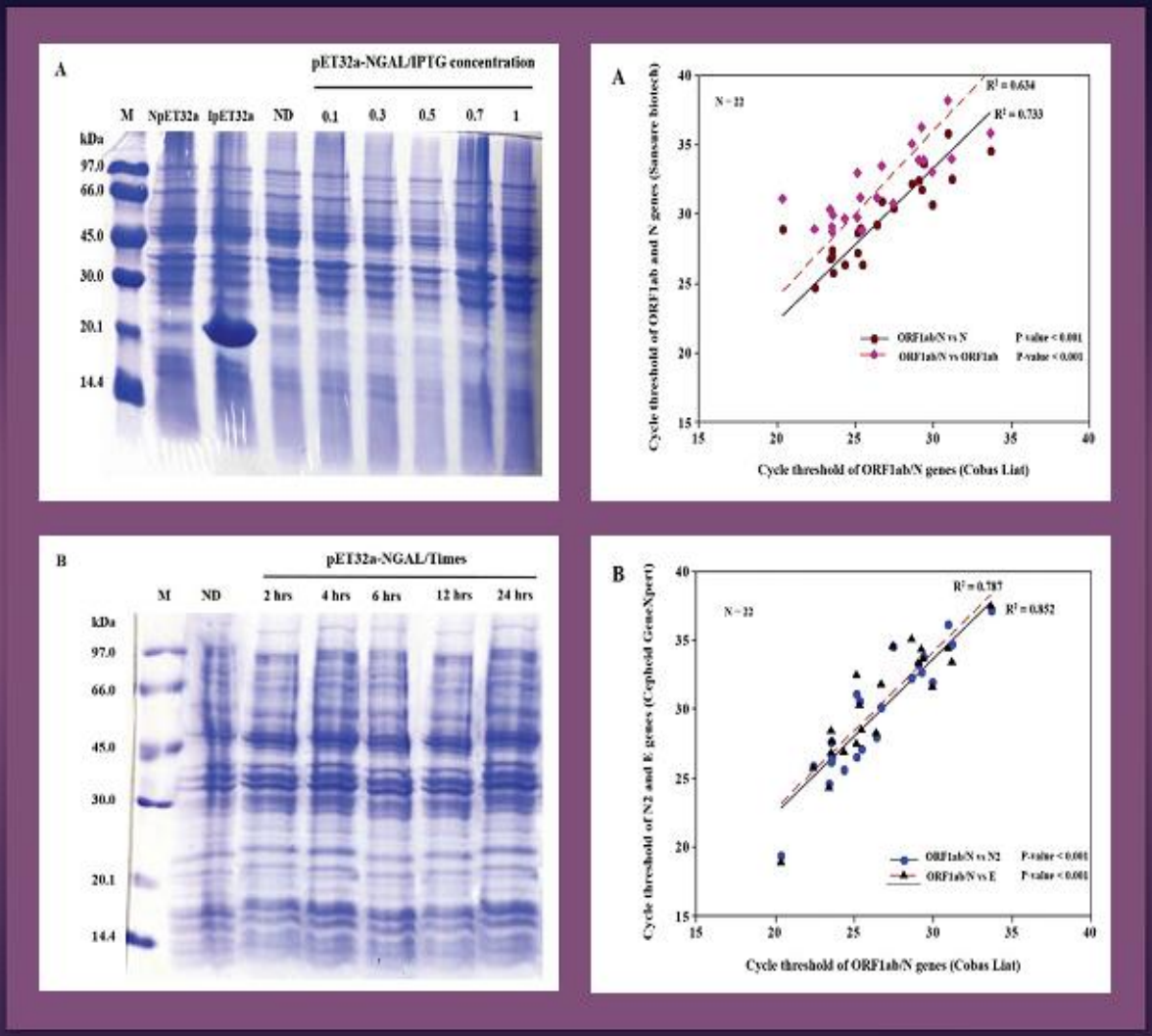
ARCHIVES OF ALLIED HEALTH SCIENCES

Archives of ALLIED HEALTH SCIENCES

Arch AHS
Volume 35
Issue 2
2023



Arch AHS
ARCHIVES OF ALLIED HEALTH SCIENCES

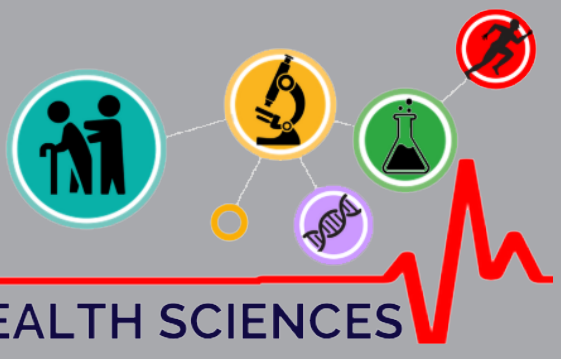
ISSN 2730-2008 (Online)

https://www.tci-thaijo.org/index.php/ams

Volume 35, Issue 2, 2023

Arch AHS

ARCHIVES OF ALLIED HEALTH SCIENCES



The **Archives of Allied Health Sciences (Arch AHS)** is an international peer-review multi-disciplinary journal published in English. It is owned by the Faculty of Associated Medical Sciences, Khon Kaen University, Thailand. The Arch AHS was formally known as *Journal of Medical Technology and Physical Therapy (JMTPT)*, which was founded in 1989. The title of the journal was changed to ***the Archives of Allied Health Sciences (Arch AHS)*** from 2020 (volume 32 issue 2: May - August) onward.

The Arch AHS aims to be a leading forum for research and knowledge in evidence-based practice relating to Allied Health Sciences. Contributions from all parts of the world and from different professionals in Allied Health Sciences are encouraged. Original articles, reviews, special reports, short communications, and letters to the editor are published 3 regular issues per year, online and in print.

Editor-in-Chief:

Sugalya Amatachaya, School of Physical Therapy, Khon Kaen University, TH.

Associate Editors:

Anchalee Techasen, School of Medical Technology, Khon Kaen University, TH.
Thiwabhorn Thaweewannakij, School of Physical Therapy, Khon Kaen University, TH.

Editorial Board Members:

Neil Robert, Clinical Research and Imaging Centre, University of Edinburgh, UK.
Marco Pang, Department of Rehabilitation Sciences, Hong Kong Polytechnic University, HK.
Michael Hamlin, Department of Sport and Exercise Sciences, Lincoln University, NZ.
Wang Xingze, Research Center in Sport and Exercise Sciences, Gannan Normal University, CN.
Prawit Janwantanakul, Faculty of Allied Health Sciences, Chulalongkorn University, TH.
Eiji Sugihara, Research and Development Center for Precision Medicine, University of Tsukuba, JP.
Charoonsak Somboonporn, Faculty of Medicine, Khon Kaen University, TH.
Wichai Eungpinichpong, Faculty of Associated Medical Sciences, Khon Kaen University, TH.
Cornelis L. Harteveld, Department of Clinical Genetics, Leiden University Medical Center, NL.
Bayden Wood, School of Chemistry, Monash University, AU.

Production and editorial assistance:
Arpassanan Wiyanad
Roongnapa Intaruk

All correspondence concerning manuscripts, editorial issues and subscription should be addressed to:
Editorial Officer: ArchAHS.TH@gmail.com
Faculty of Associated Medical Sciences,
Khon Kaen University, Thailand.

Publication information:
Arch AHS (ISSN: 2730-1990; eISSN: 2730-2008)
appears 3 issues a year.
Issue 1 January – April;
Issue 2 May – August;
Issue 3 September – December.

Indexing:
Arch AHS is indexed in Thai Citation Index (TCI tier 1) and ASEAN Citation Index (ACI) databases.

Manuscript preparation:
Please review author guideline for manuscript preparation: https://drive.google.com/drive/folders/1kO5FijEnuLSYwzgKcnQ9mxO74ZZ0wGRA

Link to website

Arch AHS
ARCHIVES OF ALLIED HEALTH SCIENCES

Contents

The potential of rapid molecular RT-PCR for SARS-CoV-2 detection: implementation of the Cobas Liat system with urgent patients during a high-incidence season in Thailand 1
Pongwut Suwannarat, Rawiporn Tiyasirichokchai, Pornparn Rojanasang, Wittaya Jomoui

Nuclear proteins of hela cells: potential autoantigenic substrate for antinuclear antibodies screening 12
Thiha Thway, Sucheewa Wongwai, Prinya Prasongdee, Chanvit Leelayuwat, Amonrat Jumnainsong

Detection of KRAS mutation at codon-12 and codon-13 associate with poor prognosis in colorectal cancer patients. 19
Namphon Kaewkla, Sarawut Chantra, Chanwit Maneenin, Parichat Pinyosri, Surasak Wanram

Isolation, cloning and optimization for expression of neutrophil gelatinase associated lipocalin (NGAL) in prokaryote 25
Tasneem Pechnur, Nisachon Jangpromma, Sakda Daduang, Patcharaporn Tippayawat, Patcharee Boonsiri, Nipaporn Ngernyuang, Jureerut Daduang

Stress and physical fitness among female university students who regularly exercise during the previous two months 35
Boonsita Suwannakul, Noppharath Sangkarit, Chula Intapunya, Narueporn Sompet, Panatda Chantakhat, Weerasak Tapanya

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2023; 35(2): 1-11.

The potential of rapid molecular RT-PCR for SARS-CoV-2 detection: implementation of the Cobas Liat system with urgent patients during a high-incidence season in Thailand

Pongwut Suwannarat, Rawiporn Tiyasirichokchai, Pornparn Rojanasang, Wittaya Jomoui*

Department of Pathology, Maha Chakri Sirindhorn Medical Center, Faculty of Medicine, Srinakharinwirot University, Ongkharak, Nakhon Nayok, Thailand.

KEYWORDS
Cepheid GeneXpert;
Cobas Liat;
COVID-19;
SARS-COV-2;
Urgency.

ABSTRACT

There had been a high incidence of COVID-19 in Thailand between May 2021 and February 2022. Urgent cases in the emergency department (ED) or labor room (LR) should be immediately tested. We aim to implement a rapid molecular point-of-care tests (POCTs) using Cobas Liat for SARS-CoV-2 identification. A total of 32 random leftover-samples represented 100% overall concordance among the 2 POCTs and standard RT-PCR assays. A total of 3,188 nasopharyngeal swabs from an ED and LR were investigated with Cobas Liat system. The incidence of SARS-CoV-2 infection was observed to be 7.53% (164/2,179) in ED and 2.87% (29/1,009) in LR. Furthermore, symptomatic cases were noted in 69.51% (114/164) of ED patients, whereas only one case was recorded from the LR. Finally, this potential strategy could reduce turnaround time and the risk of infection in medical staff during the management of patients or medical procedures in the ED and LR.

***Corresponding author:** Wittaya Jomoui, MT, PhD. Department of Pathology, Faculty of Medicine, Maha Chakri Sirindhorn Medical Center, Srinakharinwirot University, 62 Moo 7 Rungsit-Nakhon Nayok Rd., Ongkharak, Nakhon Nayok 26120, Thailand. Email address: wittayaj@g.swu.ac.th
Received: 5 February 2023/ Revised: 5 April 2023/ Accepted: 11 April 2023

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), coronavirus disease 2019 (COVID-19) was first reported in Wuhan, in the Hubei province of China, in December 2019. The infected case by the novel coronavirus was presented with an atypical pneumonia[1-3]. The phylogenetic analysis represented SARS-CoV-2 was classified as a new strain with enveloped, positive sense, and single-stranded RNA virus. The bioinformatics analysis showed that the genetic sequence of SARS-CoV-2 is 80% identical to SARS-CoV and 50% to MERS-CoV[4]. Patients with a SARS-CoV-2 infection mostly presented with fever, cough, sputum production, upper airway congestion, and shortness of breath whereas headache, diarrhea, or hemoptysis was rarely found[1]. The SARS-CoV-2 infection could transmit between person to another person via respiratory droplets by coughing or sneezing. Furthermore, environmental contamination is another way to spread the SARS-CoV-2, through contact objects that have been contaminated with droplets[1,5]. The median incubation period of COVID-19 was estimated to be 5.1 days and the symptoms developed within 11.5 days of infection[6]. In 2021, COVID-19 was a pandemic disease worldwide. The numbers of confirmed and fatal cases were increasing during 2021 and 2022. A novel variants of SARS-CoV-2 has been found recently[1].

Thailand has had a high-incidence season of COVID-19 since April 2021. The number of confirmed cases in Thailand promptly doubled to one million infected persons in August 2021[7]. During the high-season period, all urgent cases at the emergency department or pregnant women in the labor room should be examined for SARS-CoV-2 before any medical procedure. However, these patients may come to the hospital at any time. Furthermore, preventing nosocomial COVID-19 infection and maintaining the efficiency and quality of the health care service during the pandemic are essential[8]. Confirmation testing for SARS-CoV-2 infection was recommended and performed using the reverse transcription polymerase chain reaction (RT-PCR) method[9]. In laboratory practice, RT-PCR methods could be accomplished with analysis of a batch conducted at the same time. Issues arisen with patients under investigation for SARS-CoV-2 infection in the emergency department (ED) or labor room (LR) that could not be tested as soon as possible. Several management methods for resolving the problem have been suggested, such as a screening test, i.e., Ag screening or IgM antibody detection. These assays mostly represented a lower sensitivity and specificity which was not suitable for the situation and increased the risk of nosocomial infection in the medical staff[10-11]. However, Chaimayo et al[12] reported the rapid assay for SARS-CoV-2 antigen detection and showed comparable sensitivity and specificity with the real-time RT-PCR assay. Recently, rapid molecular point-of-care tests (POCTs) were developed for SARS-CoV-2 identification based on RT-PCR, including at least two platforms: the Cobas Liat and Cepheid GeneXpert systems[13-17]. It seems that these assays could resolve the problem more effectively than a screening test because the rapid molecular testing could be performed within a short time (20-50 minutes), in an individual run without batch analysis, and based on the standard RT-PCR method. The high sensitivity and specificity of the two systems have been reported in a previous work[13-17]. In this study, we aim to demonstrate the success of the implementation of rapid molecular testing for SARS-CoV-2 using the Cobas Liat system in patients from the ED and LR.

Materials and methods

Study design and clinical samples

Ethical approval for this study was obtained and got exceptions for informed consent from the Institutional Review Board of Srinakharinwirot University, Thailand (SWUEC/E/M-024/2564). A total of 32 leftover-samples were selected and validated with three methods, including the Cobas Liat and Cepheid GeneXpert systems, as well as RT-PCR (Sansure Biotech Inc., P.R. China). Sample size was selected based on method validation in guidebook of Department of Medical Sciences Ministry of Public Health, Thailand. Furthermore, a retrospective study was investigated by routine

MSMC protocol. A total of 3,188 nasopharyngeal swab specimens were recorded from urgent patients at the ED and LR, Maha Chakri Sirindhorn Medical Center (MSMC), Faculty of Medicine, Srinakharinwirot University, who were suspected of having a SARS-CoV-2 infection, and were investigated with rapid molecular RT-PCR (Cobas Liat system) between May 2021 and February 2022 by routine MSMC protocol.

Validation of RT-PCR for SARS-CoV-2 detection with three methods (Pilot study)

A total of 32 leftover specimens were recruited form routine laboratory for SARS-CoV-2 detection based on RT-PCR (Sansure Biotech Inc., P.R. China) including 10 undetected samples and 22 detected samples. All the samples were also validated for SARS-CoV-2 infection with two POCTs using the Cobas Liat and Cepheid GeneXpert systems.

Routine RT-PCR for SARS-CoV-2 was performed on a real-time PCR machine ABI7500 (Thermo Fisher, Waltham, USA) using a commercial kit (Sansure Biotech Inc., P.R. China). Two specific regions of SARS-CoV-2, including the ORF1ab and N genes, are targeted in the assay. Furthermore, an endogenous human gene (RNase P gene) was monitored as an internal control (IC) for the appropriate specimen collection in the RT-PCR. The LOD assay is 200 copies/μL. The assay was executed in 96 minutes on a real-time PCR machine. However, this assay included the pre-PCR process, i.e., RNA extraction, and the preparation of the reagent, which consumed two to four hours per batch.

The Cobas Liat system is a multiplex RT-PCR for the rapid in vitro detection and discrimination of RNA targets for three viruses: SARS-CoV-2, and the influenza A and B viruses. Two regions (ORF1a/b and nucleocapsid (N)) of the SARS-CoV-2 genome are targeted for the assay. The lower limit of detection (LOD) of the Cobas Liat SARS-CoV-2 and influenza A/B nucleic acid test is 12 copies/μL. For specimen collection, nasopharyngeal swabs were collected and preserved in viral transport media (VTM). The assay was performed according to the manufacturer's instructions. Briefly, 200 μL of specimen was added to the assay tube and inserted into the tube slot of the Cobas Liat system. Internal control checks were also analyzed. The total processing time is about 20 minutes per specimen.

The Xpert Xpress SARS-CoV-2 assay of the Cepheid GeneXpert system is a rapid RT-PCR for SARS-CoV-2 detection. The two region gene sequences of the envelope (E) and nucleocapsid (N2) proteins of SARS-CoV-2 are targeted. The LOD of the Xpert Xpress SARS-CoV-2 is 250 copies/μL. Each cartridge includes a sample processing control and a probe check control to ensure that the sample was processed correctly. For specimen collection, nasopharyngeal swabs were assembled and preserved in VTM. The test was performed on the GeneXpert instrument using the Xpert Xpress SARS-CoV-2 cartridge. Briefly, 300 μL of each specimen was added to a separate Xpert Xpress SARS-CoV-2 cartridge and loaded into an instrument. The results were interpreted automatically by the GeneXpert system with a RT-PCR curve. The total processing time is about 50 minutes per sample.

The implementation of the Cobas Liat system for SARS-CoV-2 detection in urgency

According to recommendation, the Cobas Liat system has been launched for rapid molecular testing for SARS-CoV-2 infection, which is 20 minutes, whereas the Xpert Xpress SARS-CoV-2 takes 50 minutes. Thus, in this study, we aim to assess only the Cobas Liat system. Laboratory molecular testing for SARS-CoV-2 was undertaken in our routine setting at the Department of Pathology, Faculty of Medicine, Maha Chakri Sirindhorn Medical Center (MSMC), Srinakharinwirot University. Urgent cases at the ED and pregnant women at the LR under investigation for infection who had to be examined before an operation or medical procedure were enrolled in rapid RT-PCR for SARS-CoV-2 detection between May 2021 and February 2022 using Cobas Liat system. The positive cases were recorded, along with basic patient characteristics, including age, sex, vital signs (systolic blood pressure (SBP), diastolic blood pressure (DBP), body temperature (BT), pulse rate (PR), respiratory rate (RR)), and clinical symptoms.

Statistical analysis

The data collection was recruited via EMR software and data analysis was prepared in Microsoft excel. Furthermore, the statistical analysis and graphic were performed by MINITAB release 14.12.0 statistical software. A *p*-value < 0.05 was considered statistically significant. The Mann-Whitney test was tested for the difference between the Ct values of the ORF1ab/N genes in the symptomatic and asymptomatic groups. The percent agreement, including the overall (OPA), positive (PPA), and negative percent agreement (NPA), were calculated in comparative assays by Microsoft excel.

Results

Validation of RT-PCR for SARS-CoV-2 detection with three methods (Pilot study)

A total of 32 random leftover-samples were recruited from two groups (10 undetected samples and 22 detected samples) in routine RT-PCR for SARS-CoV-2 detection. All the samples were also investigated for SARS-CoV-2 infection with two POCTs using the Cobas Liat and Cepheid GeneXpert systems. Based on this interpretation, a total of 22 detected samples and 10 undetected samples (32/32) represented 100% overall concordance among the three assays. Figure 1 demonstrates the comparison of the linear regression of the Ct values of the 22 detected cases in each assay. The comparison of the Ct value between RT-PCR (ORF1ab and N genes) and the rapid molecular Cobas Liat system (ORF1ab/N genes) showed the correlation in each of the Ct values of the genes (Figure 1A). Finally, the comparison of Ct values between the rapid molecular Cobas Liat and Cepheid GeneXpert systems displayed a good correlation in all samples, as depicted in figure 1B. Furthermore, the percentage of agreement, including positive (PPA), negative (NPA) and overall percentage agreement (OPA), was calculated between RT-PCR and the rapid molecular POCTs and found to be 100% in all parameters for the Cobas Liat and Cepheid GeneXpert systems, as displayed in table 1.

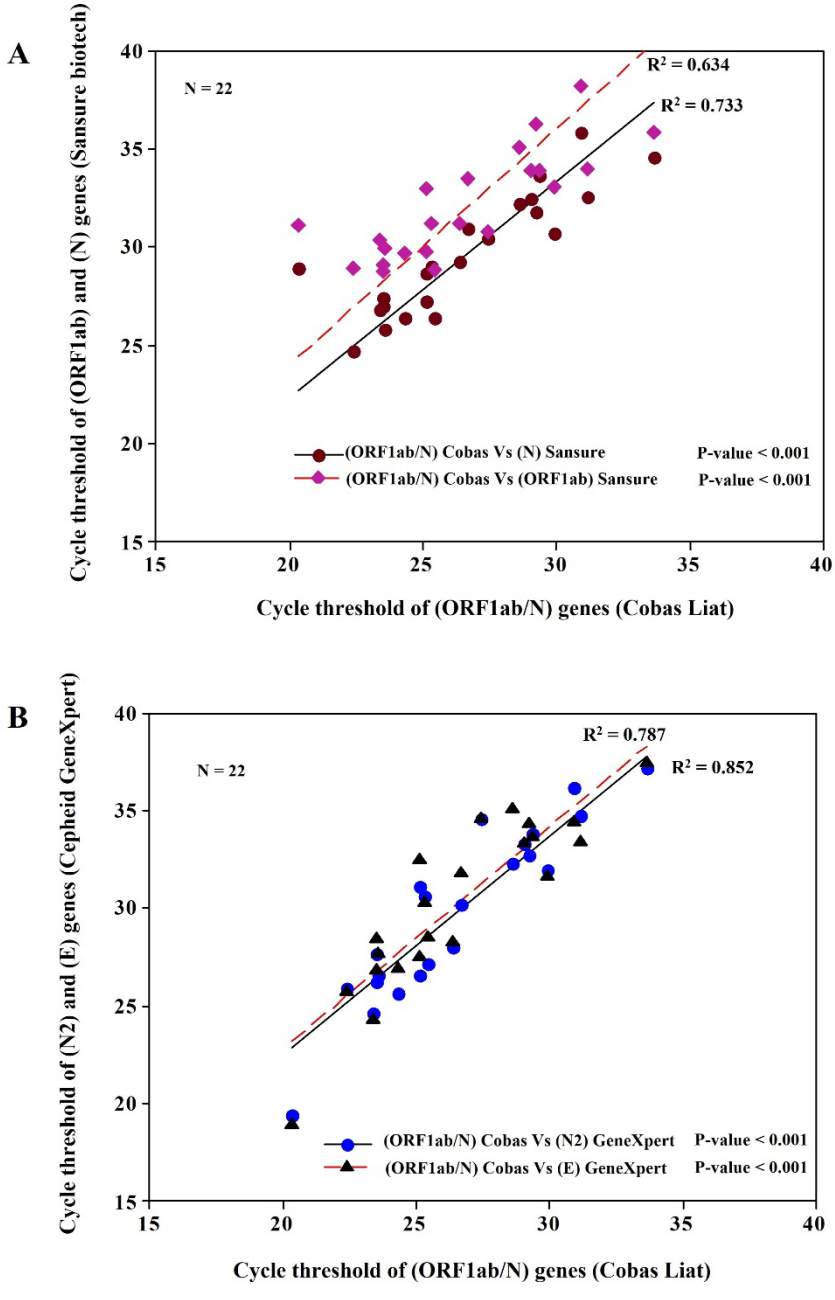


Figure 1 Ct values comparison between 3 platforms including RT-PCR (Sansure biotech), rapid molecular POCT (Cobas Liat), and rapid molecular POCT (Cepheid GeneXpert systems). Linear regression analyses (reporting the R^2 and *P*-value) between Ct values. (A) Linear regression of Ct value by RT-PCR (ORF1ab, and N genes) and Cobas Liat system (ORF1ab/N genes). (B) Linear regression of Ct value by Cobas Liat system (ORF1ab/N genes) and Cepheid GeneXpert system (N2 and E genes).

Table 1 The percentage of agreement was calculated between RT-PCR and rapid molecular POCTs including Cobas Liat system and Cepheid GeneXpert system

		RT-PCR (Sansure Biotech)	
		Positive	**Negative**
Cobas Liat system	Positive	22	0
	Negative	0	10
Cepheid GeneXpert system	Positive	22	0
	Negative	0	10
Cobas Liat system vs RT-PCR			
Positive percentage agreement = 100% (22/22; kappa = 1.0)			
Negative percentage agreement = 100% (10/10; kappa = 1.0)			
Overall percentage agreement = 100% (32/32; kappa = 1.0)			
Cepheid GeneXpert system vs RT-PCR			
Positive percentage agreement = 100% (22/22; kappa = 1.0)			
Negative percentage agreement = 100% (10/10; kappa = 1.0)			
Overall percentage agreement = 100% (32/32; kappa = 1.0)			

The potential of the Cobas Liat system for SARS-CoV-2 detection in urgency

A total of 3,188 nasopharyngeal swabs were recorded between May 2021 and February 2022 from the ED and LR according to suspected urgent cases. Figure 2 demonstrates the flowchart for SARS-CoV-2 detection in urgency. At the ED, the patients under investigation for infection accounted for 2,179 specimens, while pregnant women in the LR provided 1,009 specimens. All of these were tested with rapid molecular POCTs using the Cobas Liat system The positive cases were found in 164 samples (7.53%) and 29 samples (2.87%) from the ED and LR, respectively. Table 2 shows the patient characteristics of the detected cases in the two groups from the ED and LR. However, of the detected cases in pregnant women (LR), only one person had symptoms, which were febrile with a productive cough and sore throat.

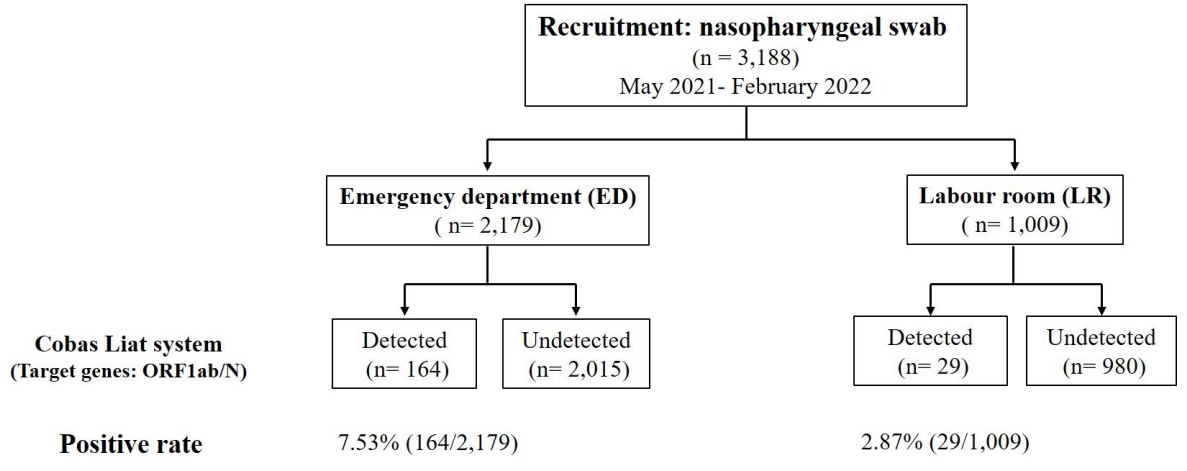


Figure 2 Flowchart of SARS-CoV-2 detection using Cobas Liat system was demonstrated in urgency at emergency department and labour room. A total of 3,188 nasopharyngeal swabs were recruited during May 2021- February 2022.

Table 2 The patient characteristics in 193 urgent cases who were positive for SARS-CoV-2 infection

	Emergency department (n = 164)	**Labor room (n = 29)**
Age: median (range): years	58 (1–102)	27 (18–40)
Sex (number)		
Female	78	29
Male	86	-
Vital sign: median (range)		
Systolic blood pressure (mmHg)	131 (117–149)	128 (120.0–132.5)
Diastolic blood pressure (mmHg)	79 (69–89)	80 (72.5–85.0)
Body temperature (°C)	36.8 (36.6–37.6)	36.7 (36.5–36.9)
Pulse rate (times)	94 (80–112)	90 (20–100)
Respiratory rate (times)	24 (20–28)	20 (20–22)
Symptomatic: n (percent)		
Febrile or fever	82 (50.0)	1 (3.5)
Cough	29 (17.7)	-
Dry cough	6 (3.7)	-
Productive cough	48 (29.3)	1 (3.5)
Anosmia	5 (3.1)	-
Sore throat	21 (12.8)	1 (3.5)
Stuffy nose or runny nose	14 (8.5)	-
Headache or dizziness	16 (9.8)	-
Myalgia	9 (5.5)	-
Fatigue	75 (45.7)	-
Nausea or vomiting	14 (8.5)	-
Diarrhea	18 (11.0)	-

Rapid molecular POCTs for SARS-CoV-2 detection were performed on the Cobas Liat in urgent cases (from the ED and LR). A total of 193 detected cases exhibited a Ct value range of 9.84 – 36.60 of ORF1ab/N genes on the Cobas Liat system. A total of 142 positive cases (73.58%) that presented with symptoms are included in table 2, but the remaining 51 (26.42%) were asymptomatic. The Ct values of the ORF1ab/N genes on the Cobas Liat system were compared between the symptomatic and asymptomatic groups, as shown on the boxplot (Figure 3). The Mann-Whitney test revealed highly significant differences (p-value < 0.001) for the Ct values of the ORF1ab/N genes in the symptomatic (median 18.28 with interquartile range (IQR) = 7.79) and asymptomatic groups (median 31.90 with IQR = 8.40).

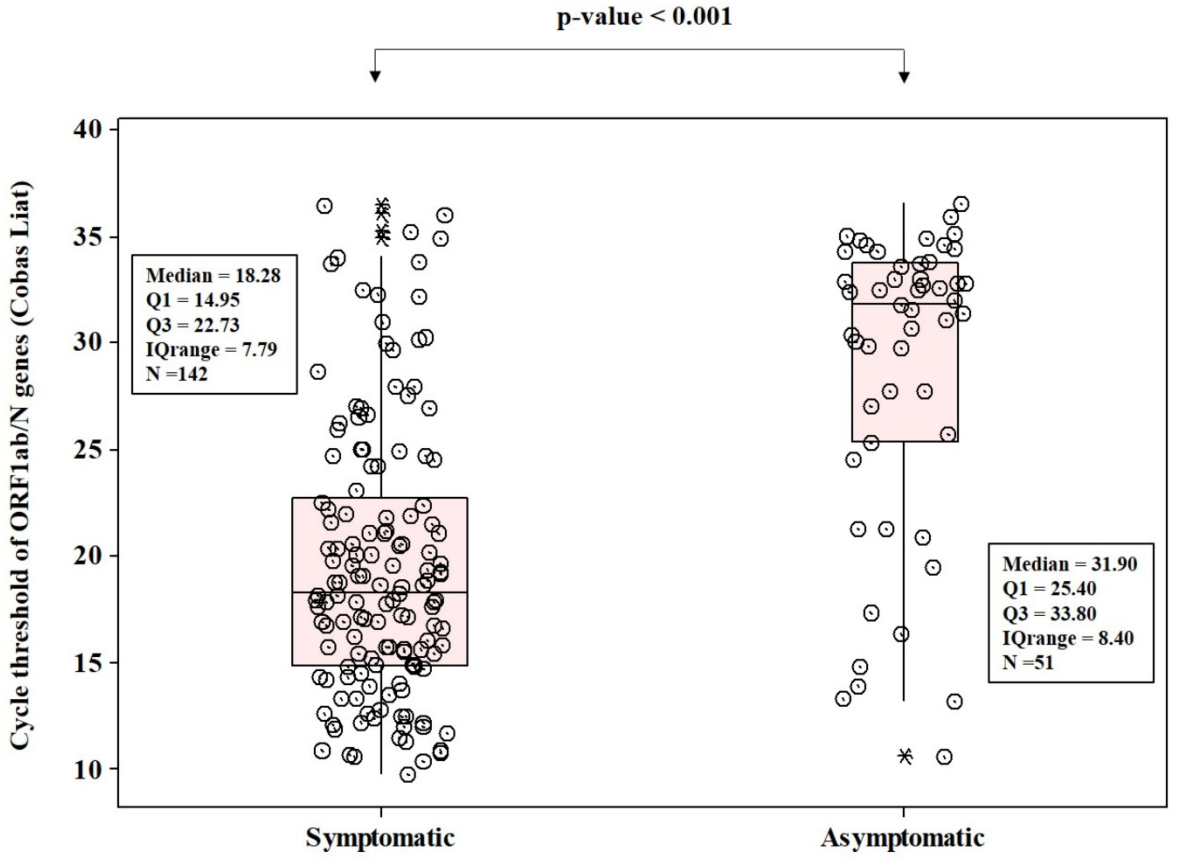


Figure 3 Distribution of Ct values of ORF1ab/N genes from positive cases grouped by symptom status and detected on the Cobas Liat system. The boxplot shows the median and interquartile range (box) of data and the box size represent sample number in each group.

Discussion

The COVID-19 pandemic has been investigated since February 2020. In Thailand, a high-incidence season has occurred since April 2021[7]. The detection of SARS-CoV-2 infection has been performed using RT-PCR as the standard method worldwide[9]. However, RT-PCR is not suitable for use in urgent cases because they should be immediately tested before management of the underlying emergency. Furthermore, identification of an infected case could lower the risk of nosocomial infection in medical staff during a procedure. This study demonstrated the rapid molecular POCTs based on the RT-PCR assay in ED and LR cases. We firstly report the proposed strategy for using rapid molecular POCTs in urgent cases.

The validation method between RT-PCR and rapid molecular POCTs demonstrated 100% overall concordance in this study. A total of 22 positive cases showed a correlation of Ct values in each assay. However, we found one sample in RT-PCR, as illustrated in figure 1A, that had a high Ct value and represents an outlier when analyzed with the two rapid molecular POCTs. This may have resulted from a random error in the sample preparation or RNA extraction process. Furthermore, the factors that led to poor analytical performance, such as the presence of inhibitors, genomic mutations, or samples with very low viral concentrations. The rapid molecular POCTs, the Cobas Liat and Cepheid GeneXpert systems, exhibit a good correlation, as depicted in the linear regression line (Figure 1C). The calculation of the percentage agreement of

results was discovered to have 100% concordance overall in three assays, including RT-PCR (Sansure Biotech), POCT 1 (Cobas Liat system) and POCT 2 (Cepheid GeneXpert system). A previous work has also noted an excellent overall 100% test concordance between the Cepheid Xpress SARS-CoV-2 assay and Cobas Liat SARS-CoV-2 and Influenza A/B assay in nasopharyngeal swabs and posterior oropharyngeal saliva[18]. Hansen et al[15] reported the overall agreement between the Cobas Liat and 68/8800 systems for SARS-CoV-2 detection and values of 100% and 97.4% for PPA and NPA, respectively. Thus, the high accuracy of rapid POCTs has been established and is suitable for applying in an emergency.

The potential of rapid molecular POCTs for SARS-CoV-2 in urgency has been evaluated in this study. The Cobas Liat system has been launched for rapid molecular testing for SARS-CoV-2 infection. This research demonstrated the successful application of the rapid molecular assays in urgent cases from the ED and LR. In the high-incidence season in Thailand that began in April 2021, SARS-CoV-2 detection has resulted in a bottleneck in laboratory investigation using RT-PCR. The proposed strategy of using rapid molecular POCT for detecting SARS-CoV-2 infection at the ED and LR started in May 2021. The patients in both departments could be immediately tested and unnecessary resources significantly reduced, i.e., wearing full personal protective equipment (PPE) in undetected cases, as this equipment is limited in the health care environment during the COVID-19 pandemic[19, 20]. This research represented the urgent cases who came to the ED or LR during the period May 2021-February 2022. As shown in figure 2, a total of 3,188 nasopharyngeal swabs were analyzed by the Cobas Liat system, which has been reported to have a short operation time of 20 minutes and the lowest LOD (12 copies/uL). However, the limitation of the Cobas Liat system is a higher cost when compared with antigen test kit. The rapid assay for SARS-CoV-2 antigen detection also showed comparable sensitivity and specificity with the real-time RT-PCR assay. Thus, there is optional use of this rapid and simple SARS-CoV-2 antigen detection test as a screening assay[12].

In this study, we compared the symptomatic and asymptomatic groups by using the Ct values of the ORF1ab/N genes. The symptomatic group shows a significant difference from the asymptomatic one (p-value < 0.001). Previous studies have also reported significantly lower Ct values in the symptomatic group when compared with asymptomatic cases. Nearly 60% of specimens from asymptomatic individuals had a PCR Ct value >30 as measured using the Cobas 6800 assay E gene[21]. The active cases who demonstrated clinical symptoms may have an early stage of infection and there is a high growth of the replication of the virus in this period. During a late infection, the virus may be eliminated in the nasopharynx and a lower copy number of the virus (representing a high Ct value) might be found, meaning that asymptomatic cases are present in this group. Moreover, a previous work has reported that the viral load may be low (a high Ct value) in patients who were IgG-positive at discharge and high in those who were IgG-negative[22].

Conclusion

The implementation of rapid molecular point-of-care tests for SARS-CoV-2 detection using the Cobas Liat system in urgent patients from an emergency department and labor room could be promising and highly effective. This proposed strategy could reduce turnaround time and the risk of infection in medical staff during the management of patients or medical procedures in the ED and LR.

Clinical implication

- The validation method between RT-PCR and rapid molecular POCTs demonstrated 100% overall concordance in this study.
- During the high-season period, all urgent cases could be examined for SARS-CoV-2 infection before any medical procedure in our setting.
- The implementation of Cobas Liat system for SARS-CoV-2 detection in urgent case could be promising and highly effective.
- The preventing nosocomial COVID-19 infection and maintaining the efficiency and quality of the health care service during the pandemic are demonstrated.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by a research grant from HRH Princess Mahachakri Sirindhorn Medical Center, Faculty of Medicine, Srinakharinwirot University (Grant No. 162/2565).

References

1. Tsang HF, Chan LWC, Cho WCS, Yu ACS, Yim AKY, Chan AKC, et al. An update on COVID-19 pandemic: the epidemiology, pathogenesis, prevention and treatment strategies. Expert Rev Anti Infect Ther 2021; 19(7): 877-88.
2. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell 2020; 181(2): 271-80.
3. Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S, et al. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerg Microbes Infect 2020; 9(1): 221-36.
4. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. J Autoimmun 2020; 109: 102433.
5. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. N Engl J Med 2020; 382(16): 1564-7.
6. Lauer SA, Grantz KH, Bi Q, Jones FK, Zheng Q, Meredith HR, et al. The incubation period of coronavirus disease 2019 (COVID-19) from publicly reported confirmed cases: estimation and application. Ann Intern Med 2020; 172(9): 577-82.
7. Prasertbun R, Mori H, Mahittikorn A, Siri S, Naito T. Pneumonia, influenza, and dengue cases decreased after the COVID-19 pandemic in Thailand. Trop Med Health 2022; 50(1): 27.
8. Hsu CH, Chiu CL, Lin YT, Yu AY, Kang YT, Cherng M, et al. Triage admission protocol with a centralized quarantine unit for patients after acute care surgery during the COVID-19 pandemic: A tertiary center experience in Taiwan. PLoS One 2019; 17(3): e0263688.
9. Gdoura M, Abouda I, Mrad M, Ben Dhifallah I, Belaiba Z, Fares W, et al. SARS-CoV2 RT-PCR assays: In vitro comparison of 4 WHO approved protocols on clinical specimens and its implications for real laboratory practice through variant emergence. Virol J 2022; 19(1): 54.
10. Porte L, Legarraga P, Vollrath V, Aguilera X, Munita JM, Araos R, et al. Evaluation of a novel antigen-based rapid detection test for the diagnosis of SARS-CoV-2 in respiratory samples. Int J Infect Dis 2020; 99: 328-33.

11. Mak GC, Cheng PK, Lau SS, Wong KK, Lau CS, Lam ET, et al. Evaluation of rapid antigen test for detection of SARS-CoV-2 virus. J Clin Virol 2020; 129: 104500.
12. Chaimayo C, Kaewnaphan B, Tanlieng N, Athipanyasilp N, Sirijatuphat R, Chayakulkeeree M, et al. Rapid SARS-CoV-2 antigen detection assay in comparison with real-time RT-PCR assay for laboratory diagnosis of COVID-19 in Thailand. Virol J 2020; 17(1): 177.
13. Er TK, Chou YC, Chen SY, Huang JW. Rapid Cobas Liat SARS-CoV-2 assay in comparison with the laboratory-developed real-time RT-PCR Test. Clin Lab 2021; 67(11): e210316.
14. Blackall D, Moreno R, Jin J, Plotinsky R, Dworkin R, Oethinger M. Performance characteristics of the roche diagnostics cobas Liat PCR system as a COVID-19 screening tool for hospital admissions in a regional health care delivery system. J Clin Microbiol 2020; 59(10): e0127821.
15. Hansen G, Marino J, Wang ZX, Beavis KG, Rodrigo J, Labog K, et al. clinical performance of the point-of-care cobas liat for detection of SARS-CoV-2 in 20 minutes: a multicenter study. J Clin Microbiol 2021; 59(2): e02811-20.
16. Granato PA, Kimball SR, Alkins BR, Cross DC, Unz MM. Comparative evaluation of the Thermo fisher TaqPath™ COVID-19 combo kit with the Cepheid Xpert® Xpress SARS-CoV-2 assay for detecting SARS-CoV-2 in nasopharyngeal specimens. BMC Infect Dis 2021; 21(1): 623.
17. Graham M, Williams E, Isles N, Buadromo E, Toatu T, Druce J, et al. Sample pooling on the Cepheid Xpert® Xpress SARS-CoV-2 assay. Diagn Microbiol Infect Dis 2021; 99(2): 115238.
18. Hin FT, Wai L, Lawrence C, William C, Sze C. Performance comparison of the Cobas® Liat® and Cepheid® GeneXpert® systems on SARS-CoV-2 detection in nasopharyngeal swab and posterior oropharyngeal saliva. Expert Rev Mol Diagn 2021; 21(15): 515-8.
19. Kim H, Hegde S, LaFiura C, Raghavan M, Sun N, Cheng S, et al. Access to personal protective equipment in exposed healthcare workers and COVID-19 illness, severity, symptoms and duration: a population-based case-control study in six countries. BMJ Glob Health 2021; 6(1): e004611.
20. Park SH. Personal protective equipment for healthcare workers during the COVID-19 pandemic. Infect Chemother 2020; 52(2): 165-82.
21. Raju S, Anderson NW, Robinson E, Squires C, Wallace MA, Zhang R, et al. Comparison of 6 SARS-CoV-2 molecular methods and correlation with the cycle threshold distribution in clinical specimens. J Appl Lab Med 2021; 6(6): 1452-62.
22. Sakano T, Urashima M, Takao H, Takeshita K, Kobashi H, Fujiwara T. Differential kinetics of cycle threshold values during admission by symptoms among patients with mild COVID-19: a prospective cohort study. Int J Environ Res Public Health 2021; 18(15): 8181.

Arch AHS

ORIGINAL ARTICLE

Archives of Allied Health Sciences 2023; 35(2): 12-18.

Nuclear proteins of hela cells: potential autoantigenic substrate for antinuclear antibodies screening

Thiha Thway[1,2], Sucheewa Wongwai[3], Prinya Prasongdee[4], Chanvit Leelayuwat[2,3], Amonrat Jumnainsong[2,3*]

[1] Biomedical Sciences Program, Graduates School of Khon Kaen University, Khon Kaen, Thailand.

[2] The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[3] Department of Clinical Immunology and Transfusion Sciences, School of Medical Technology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[4] Clinical Laboratory section, Srinagarind hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

KEYWORDS
Antinuclear antibody;
Autoimmune disease;
Hela cell;
Nuclear protein;
Rapid test.

ABSTRACT

Antinuclear antibodies (ANA) are important in diagnosis and follow-up of patients with autoimmune conditions. The current increase in ANA requests is driven by broadening the use of ANA from a test for lupus to a test for diverse autoimmune diseases, but the standard method is protracted, cumbersome and prone to error. Therefore, simple and reliable testing are needed and autoantigen substrates are required for the development to capture the autoantibodies. This study evaluates the nuclear proteins of Hela cells (NP-HL) as screening marker for systemic autoimmune diseases. Reactivity of 38 ANA positive and 10 negative sera, against NP-HL was determined by western blotting. We demonstrated that NP-HL reacts with 37 ANA positive sera (97%), and without showing any reaction with negative ones. NP-HL was shown to have a diagnostic value as a screening marker for ANA and, therefore, is a suitable alternative substrate for a new antibody test. This research implies that the NP-HL provides a potential to be used as autoantigen substrate in the rapid testing to define ANA for screening of autoimmune diseases.

***Corresponding author:** Amonrat Jumnainsong, MT, PhD. Department of Clinical Immunology and Transfusion Sciences, School of Medical Technology, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand. Email address: amonrat@kku.ac.th

Received: 10 February 2023/ Revised: 18 March 2023/ Accepted: 26 March 2023

Introduction

Autoimmunity is defined as an immune response against self (autologous) antigens and is an important cause of disease. The diseases caused by autoimmunity are called autoimmune diseases[1]. When autoimmune diseases occur, it can cause inflammation, pain, diminished mobility, fatigue and other non-specific symptoms[2]. Autoimmune diseases are chronic diseases in nature and can lead to morbidity and mortality. Therefore, autoimmune diseases are medically and economically important due to high treatment costs, long hospital stays, and early retirement. In the last decade, the incidence and prevalence of autoimmune diseases rise steadily[3]. The estimated prevalence of all autoimmune diseases is about 5-7% of the general population[4].

The presence of autoantibodies in serum against to nuclear antigens such as nucleic acids and intracellular proteins is the hallmark of systemic autoimmune diseases such as systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjogren's syndrome (SjS), scleroderma (SS), systemic sclerosis (SSc), and idiopathic inflammatory myopathies (IIMs)[5]. These autoantibodies, also known as antinuclear antibodies (ANA), are predominantly reactive with nuclear antigens. Due to an excellent diagnostic sensitivity of the ANA test, it is considered as the best screening test for these diseases[6,7]. The positivity of ANA screening can make guidance to make confirmatory tests and may be applicable for clarifying an exact clinical diagnosis or prognosis.

Among the currently used ANA screening tests, the indirect immunofluorescence assay (IFA) method applying HEp-2 cells as the substrate is recommended as the gold standard test for ANA detection, with a wide range of advantages and limitations[8-13]. However, there are some challenges such as labor-intensiveness, subjectivity in result interpretation, time-consuming, inadequate reagents standardization and a workforce shortage in clinical laboratories[8-11,14,15]. Therefore, simple and reliable testing are needed for early diagnosis and effective treatment of autoimmune diseases, thereby improving productivity of health care systems by minimizing costs, time, and errors. If available, rapid testing for ANA can improve diagnostics in primary, urgent/emergency, and remote care clinics and enhance medical intervention for patients[16].

For the development of rapid testing, autoantigen substrate is required to capture the autoantibodies. Hela cell is the first continuous cancer cell line. Through application of Hela cells and the various other cell lines, lots of knowledge aspect to human cells have already been perceived. Among thousands of human cancer cell lines, Hela cell is still the most commonly used cell line in biomedical research. It is also non-fastidious nature to grow and has large nucleus which shows a wide range of nuclear antigens correlated with systemic autoimmune diseases and non-expensive. In addition, in concerned of Hep-2 cells substrate, some clinically important autoantibodies can be missed, like SS-A/Ro60 is missed by Hep-2 cells due to the low cellular abundance of this particular protein on Hep-2 cells. Thus, the aim of this study is to evaluate the diagnostic value of nuclear proteins of Hela cells (NP-HL) to be used as screening marker for total ANA.

Materials and methods

Human serum samples

Patient sera were obtained from the patients under auspices of a human subject protocol, approved by the Center for Ethics in Human Research, Khon Kaen University (HE631169). The patients were diagnosed at the Srinagarind Hospital, Khon Kaen, Thailand with ANA positive and titer was determined by indirect immunofluorescence assay (IFA-ANA). All samples were assigned number codes and used in the experiments without knowledge of clinical laboratory information. The IFA-ANA positive sera with clinically significant pattern, homogeneous, speckled, centromere and nucleolar, were chosen and the titer was equal or grater than 1:80. For the negative sera, the samples which showed IFA-ANA negative or the titer was lower than 1:80 were selected.

Cultivation of Hela cells

The Hela cells used in this study were cultured in standard plastic flasks with basic growth medium (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) Penicillin-Streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C.

Nuclear extraction of Hela cells

Nuclear and cytoplasmic protein fractions were collected using the nuclear extraction kit (ab113474; Abcam, UK) In brief, the cells were lysed with cytoplasmic extraction buffer (CEB) and centrifuged at 12,000 rpm for 1 minute to separate the supernatant containing the cytoplasmic proteins and nuclear pellet. Nuclear proteins were extracted from the nuclear pellet with the aid of nuclear extraction buffer (NEB) and centrifugation at 14,000 rpm for 10 minutes. The protein concentration of the nuclear extracts and cytoplasmic extracts were measured. Then, the nuclear extracts and cytoplasmic extracts were used immediately or stored at -80°C until use.

Purity checking of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic proteins extracted from Hela cells were prepared for purity checking by western blotting. In brief, after electrophoresis, gels were electroblotted onto a 0.45-µm pore size polyvinylidene fluoride (PVDF) membrane (10600023, GE Healthcare, UK) which was subsequently blocked in 5% skimmed milk in 0.1% tween 20-TBS (t-TBS) for 1 h at room temperature before incubating with anti-histone H3 antibody (1:1000; ab1791; Abcam, UK) and anti-beta actin antibody (1:5000; ab227387; Abcam, UK). The secondary goat anti-rabbit IgG H & L horseradish peroxidase labeled antibody (ab6721; Abcam, UK) was visualized by the Enhanced Chemiluminescence (ECL) plus reagent (GE Healthcare, UK) using an Amersham imager 600.

Western blotting to detect the reaction of human serum and extracted nuclear proteins from Hela cells (NP-HL)

NP-HL (20, 10, 5, 2 & 1µg) were heated in sample buffer and separated at room temperature by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to PVDF membrane for 1 h at 300 V and 90 mA, the membranes were blocked for 1 h with 5% skimmed milk in t-TBS and sequentially incubated for overnight at 4 °C with 100X diluted IFA-ANA sera, followed by 5,000X diluted of horseradish peroxidase conjugated goat anti-human IgG secondary antibody (Cat-A00166, GenScript, USA) for 1 h at room temperature. Blots were washed 3X for 10 min between steps with t-TBS. Bound antibodies were detected by using Enhanced Chemiluminescence (ECL) plus reagent (GE Healthcare, UK) and quantified on an Amersham imager 600. Anti-histone antibody was used as positive control in the uniform condition to assure the accuracy and quality of the results.

Results

Purity checking of extracted nuclear and cytoplasmic protein

Western blotting results from cellular extracts of Hela cells were shown in figure 1, which indicated that nuclear proteins were sufficiently separated from cytoplasmic proteins. The nuclear marker, histone H3, was highly expressed in nuclear fractions but low amount in cytoplasmic fractions. Conversely, the cytoplasmic marker, ß actin, was detected only acceptable amount in nuclear fraction while it was detected higher concentration in cytosolic portion.

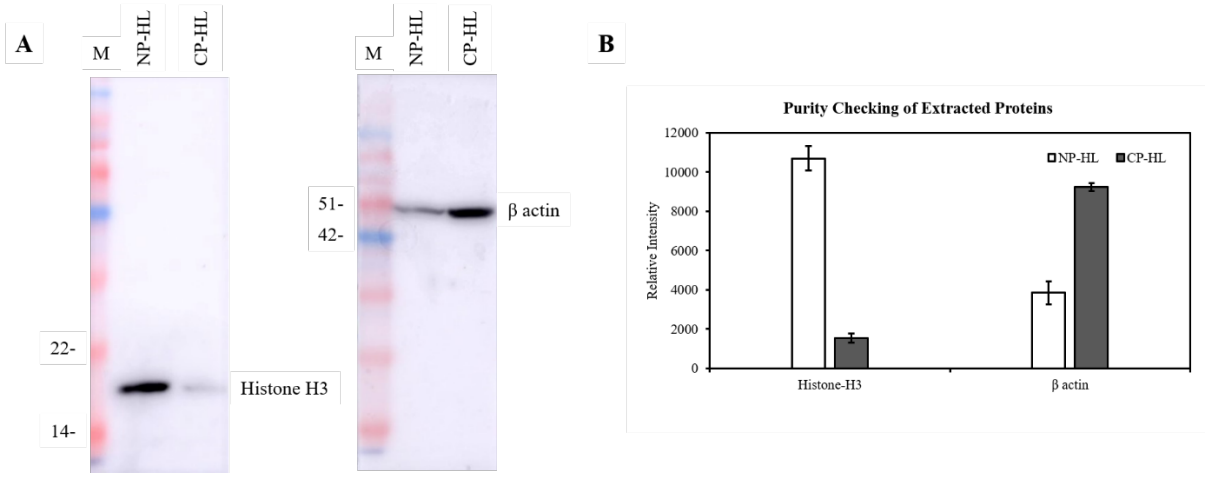


Figure 1 Purity checking of extracted nuclear and cytoplasmic protein from Hela cells (A) Western blotting of extracted nuclear and cytoplasmic protein from Hela cells (NP-HL and CP-HL, respectively) probed with anti-histone H3 antibody (nuclear protein marker) and anti-β actin antibody (cytoplasmic protein marker). (B) From the western blotting result, the in tensity of the western blotting band from NP-HL and CP-HL with anti-histone H3 and anti-β actin was determined. The experiments were performed for three times and data were expressed as mean±SD. (M = molecular weight markers)

Evaluation the reactivity of NP-HL with ANA negative and positive sera

To determine the optimal reactive concentration of NP-HL, western blot analysis was performed using 1, 2, 5, 10, 20 µg of extracted nuclear protein against positive and negative ANA sera which tested with IFA-ANA testing. All proteins of NP-HL in the SDS-PAGE were demonstrated in figure 2A. As could be seen in figure 2B, autoantibody reactivity increased proportionally to the amount of protein bound to the membrane and the result indicated that 20 µg of NP-HL provided the strongest binding ability of autoantigens and antibodies without showing cross-reactivity in negative serum. Therefore, for the rest of the study, 20 µg of NP-HL was routinely used as the optimal reactive concentration of autoantigens.

Evaluation NP-HL with ANA negative and positive samples

NP-HL was tested with the IFA-ANA sera, negative (n = 10) and positive (n = 38) samples, by western blotting. Among the various patterns observed in ANA-positive cases, homogeneous pattern (n = 13), speckled pattern (n = 10), centromere pattern (n = 8) and nucleolar pattern (n = 7) were observed. In western blotting, all negative sera (100%) were observed no detectable band revise and 37 out of 38 positive samples (97%) were detected at least one band. One sample (Homogeneous pattern) was not detected with NP-HL. Additionally, the western blotting results showed the different band in each sample that is shown in table 1. Protein at molecular weight 60 was found to be the most prevalent antibody which is the representation of SS-A/ Ro60 antigen.

Table 1 Serological frequencies of autoantibodies in IFA-ANA patterns

MW in WB (kD)	number (percent)					Autoantibody to*
	Homogeneous (n=13)	Speckled (n=10)	Centromere (n=8)	Nucleolar (n=7)	Total (n=38)	
60	4 (30.8)	1 (10)	2 (25)	3 (42.9)	10 (26)	SS-A/Ro60
52	7 (53.8)	0	2 (25)	0	9 (23.7)	SS-A/Ro52
48	1 (7.7)	3 (30)	2 (25)	2 (28.6)	8 (21)	SS-B/La
33/70	6 (46.1)	1 (10)	0	1 (14.3)	8 (21)	U1-nRNP
60-72	1 (7.7)	1 (10)	5 (62.5)	1 (14.3)	8 (21)	
29	1 (7.69)	4 (40)	1 (12.5)	0	6 (15.8)	Sm
12-24	3 (23.1)	2 (20)	0	0	5 (13.2)	Histone
80	0	0	5 (62.5)	0	5 (13.2)	CENP-B
120	0	1 (10)	3 (37.5)	0	4 (10.5)	CENP-C
135	0	0	1 (12.5)	3 (42.9)	4 (10.5)	RNAP-III
100	3 (23.1)	0	0	0	3 (7.9)	Topoisomerase-I
80	1 (7.7)	2 (20)	0	0	3 (7.9)	Ku
75/100	0	0	0	2 (28.6)	2 (5.3)	PM/Scl
262	2 (15.4)	0	0	0	2 (5.3)	Nucleosome
140-180	0	1 (10)	1 (12.5)	0	2 (5.3)	
37	1 (7.7)	0	0	0	1 (2.6)	Rib-P

*reference number 17

Discussions

Autoantibodies are hallmarks of autoimmune diseases, and testing for total ANA has become a valuable tool at both primary care and subspecialty settings as a trigger for further clinical investigation. Standard ANA testing is performed by indirect immunofluorescence microscopy in centralized clinical laboratories by technicians trained in carrying out its multiple steps and in interpreting fluorescence microscopy images. As a screening tool, the ANA patterns can guide confirmatory testing useful in elucidating a specific clinical diagnosis or prognosis. However, routine use of IFA-ANA testing as a global screening test is hampered by its labor-intensiveness, subjectivity, and limited diagnostic specificity. Thus, the need for simple testing which will be easy to perform and reliable is still a burning issue and the autoantigen substrates with high diagnostic values are required to apply in development of the testing. In this context, we developed and evaluated the extracted nuclear protein from Hela cell with the clinical sample.

The results of western blot analysis clearly established the value of the reactivity of NP-HL for ANA detection, showing strong positive bands on ANA positive sera, versus showing no reaction on all ANA negative sera. In IFA-ANA, some clinically important autoantibodies can be missed, as SS-A/Ro60 was missed by Hep-2 cells due to the low cellular abundance of this particular protein on Hep-2 cells. In contrast, SS-A/Ro60 was highly expressed in NP-HL and was found as the most prevalent autoantibodies in our study. This may be due to the use of extracted autoantigenic nuclear proteins with less content of cytoplasmic proteins. Therefore, autoantigenic targets were highly expressed and masking effect of cytoplasmic proteins to target proteins was also inhibited.

Consequently, NP-HL was evaluated as potential autoantigenic substrate for total ANA detection in western blotting, providing important autoantigenic targets for autoantibodies from cheap source.

In comparison of the results of IFA-ANA and western blotting, high concordance data were found out in negative samples (100%) and positive samples (97%). One sample (homogeneous pattern) was not detected with NP-HL. This difference may be due to the inter-operator variability of performing the different assays and result interpretation. In addition, although our NP-HL contained the major autoantibody targets, including SS-A, SS-B, U1-nRNP, Sm, histone, CENP, RNAP, Topoisomerase-I, Ku, PM/Scl, nucleosome and ribosomal protein, some important autoantigens may not be present. Moreover, this positive sample showed the low titer with IFA-ANA. To understand the association of IFA patterns with specific autoantibodies, our finding was compared to standard reference[17], and found to be very similar with published literature. The speckled pattern showed an association with Sm and SS-B/La which was similar to other studies. Likewise, the centromere pattern is shown in association with the CENP-B & C antibodies and nucleolar pattern with RNAP and PM/Scl antibodies, in accordance with other published studies. However, there are some exceptions, like histones in speckled pattern, SS-A/Ro and U1-nRNP in the homogeneous pattern, and 60-72kD protein in centromere pattern which may be due to the use of different detection techniques. These differences may also be due to the subjectivity and the inter-operator variability of performing the assay and identification the patterns. These technical differences have been observed earlier in literature, and this is one of the major limitations of using IFA as a screening assay for the detection of autoantibodies. Although the validation studies have been performed for automated IFA[18,19], they are not routinely used in developing countries due to the expenses involved. This correlation is very helpful in predicting a specific antibody with a particular ANA pattern and also reminding of limitation in patterns identification of IFA. However, in our study, nuclear antigens were focused and cytoplasmic antigens that binds to cytoplasmic organelles such as mitochondria and the Golgi complex were limited to be identified.

The diagnostic value of the NP-HL is related for screening of total ANA and, warrants further work to set up and validate an autoantibody clinical test for systemic autoimmune diseases based on this autoantigen.

Conclusion

We described the NP-HL as a screening marker for total ANA with potential diagnostic value, strengthened by reactivity evaluation against clinically important ANA sera. This research implies that the NP-HL provides a potential to be used as alternative autoantigen substrate in defining antinuclear antibodies (ANA) for screening of systemic autoimmune diseases.

Take home messages

Our study provides the antigenic avidity of extracted nuclear proteins of Hela cells in capturing antinuclear antibodies. Further study for application of this substrate in method development of ANA screening is warranted.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The grant of this work was supported by the Centre for research and development of medical diagnostic laboratories.

References

1. Abbas AK, Lichtman AH. Basic immunology: Functions and disorders of the immune system. 2nd ed. Philadelphia, Pa: Elsevier Saunders; 2006.

2. Playfair JHL, Lydyard PM. Medical immunology made memorable. 2nd ed. London: Churchill Livingstone; 2000.
3. Lerner A, Jeremias P, Matthias T. The world incidence and prevalence of autoimmune diseases is increasing Int J Celiac Dis 2016; 3(4):151–5.
4. Mackay IR, Rose NR. The Autoimmune Diseases. 5th ed. Philadelphia, Pa : Elsevier; 2013.
5. von Mühlen CA, Tan EM. Autoantibodies in the diagnosis of systemic rheumatic diseases. Semin Arthritis Rheum 1995; 24(5): 323–58.
6. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982; 25(11): 1271–7.
7. Steiner G, Smolen J. Autoantibodies in rheumatoid arthritis and their clinical significance. Arthritis Res 2002; 4(Suppl 2): S1-5.
8. Agmon-Levin N, Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. Ann Rheum Dis 2014; 73(1): 17–23.
9. Meroni PL, Schur PH. ANA screening: an old test with new recommendations. Ann Rheum Dis 2010; 69(8): 1420–2.
10. Pisetsky DS. Antinuclear antibody testing — misunderstood or misbegotten? Nat Rev Rheumatol 2017; 13(8):495–502.
11. Tan EM, Feltkamp TE, Smolen JS, Butcher B, Dawkins R, Fritzler MJ, et al. Range of antinuclear antibodies in "healthy" individuals. Arthritis Rheum 1997; 40(9): 1601–11.
12. Emlen W, O'Neill L. Clinical significance of antinuclear antibodies: comparison of detection with immunofluorescence and enzyme-linked immunosorbent assays. Arthritis Rheum 1997; 40(9): 1612–8.
13. Homburger HA, Cahen YD, Griffiths J, Jacob GL. Detection of antinuclear antibodies: comparative evaluation of enzyme immunoassay and indirect immunofluorescence methods. Arch Pathol Lab Med 1998; 122(11): 993–9.
14. Chan EKL, Damoiseaux J, Carballo OG, Conrad K, de Melo Cruvinel W, Francescantonio PLC, et al. Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 Cell Patterns 2014-2015. Front Immunol 2015; 6: 412-9.
15. Hoffman IEA, Peene I, Veys EM, De Keyser F. Detection of specific antinuclear reactivities in patients with negative anti-nuclear antibody immunofluorescence screening tests. Clin Chem 2002; 48(12): 2171–6.
16. Konstantinov KN, Tzamaloukas A, Rubin RL. Detection of autoantibodies in a point-of-care rheumatology setting. Autoimmunity Highlights 2013; 4(2): 55-61.
17. Bizzaro N, Antico A, Platzgummer S, Tonutti E, Bassetti D, Pesente F, et al. Automated antinuclear immunofluorescence antibody screening: A comparative study of six computer-aided diagnostic systems. Autoimmunity Reviews 2014; 13: 292–8.
18. Mathiaux F, Barrot A, Elong C, Parent X. Evaluation of an automated system of immunofluorescence analysis in daily practice. Ann Biol Clin (Paris) 2018; 76: 407–15.

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2023; 35(2): 19-24.

Detection of KRAS mutation at codon-12 and codon-13 associate with poor prognosis in colorectal cancer patients

Namphon Kaewkla[1,2], Sarawut Chantra[1,2], Chanwit Maneenin[2,3], Parichat Pinyosri[2,3], Surasak Wanram[2,3*]

[1] Biomedical Sciences student, College of Medicine and Public Health, Ubon Ratchathani University, Thailand.

[2] Biomedical Research and Medical Innovation Unit, College of Medicine and Public Health, Ubon Ratchathani University, Thailand.

[3] Department of Pathology, College of Medicine and Public Health, Ubon Ratchathani University, Thailand.

KEYWORDS

Codon-12 mutation;
Codon-13 mutation;
Colorectal cancer;
KRAS mutations;
Prognosis outcomes.

ABSTRACT

Colorectal cancer (CRC) associated with KRAS mutations relevance to clinical outcome of progression especially for codon mutation remain unclear, clinically. Thus this study aimed to detect KRAS mutations on specific codon-12 and codon-13. DNA extraction was performed from 40 formalin-fixed paraffin-embedded (FFPE) tissues. The KRAS codon-12 and codon-13 mutations were detected by quantitative multiplex real-time PCR consisting of seven primers of G12A, G12D, G12R, G12C, G12S, G12V, and G13D, specifically. Our results showed mutation of codon-12 at 37.5% (15/40 cases), especially for 86.7% (13/15 cases) were found G12D, G12V and G12R mutation for 40%, 40% and 6.67%, respectively. On the other hand, the codon-13 mutation found only G13D at 20% (3/15 cases). The present findings showed a statistically significant difference between KRAS mutations with lymph node metastases (p-value = 0.004), and mucosal lymphoma with other tumor types (p-value = 0.016). We noticed that KRAS mutations are associated with codon-12 and codon-13 and relevanced to prognostic outcomes of CRC patients on lymph node metastases as well as histological types. However, increased samples, novel prognostic biomarkers and more specific clinical information could be further explored to the clinical molecular biomarkers for monitoring and treatments.

***Corresponding author:** Surasak Wanram, MT, PhD. Ubon Ratchathani University, 85 Sathonlamark Road, Warin Chamrap, Ubon Ratchathani, 34190 Thailand. Email address: Surasak.w@ubu.ac.th

Received: 22 February 2023/ Revised: 23 April 2023/ Accepted: 20 May 2023

Introduction

Nearly 2 million cases of colorectal cancer (CRC) were diagnosed in 2020, making it the third most prevalent cancer form globally. Approximately 1 million people die from it each year, making it the second most frequent cause of cancer death including Thailand[1]. Over 10,000 new CRC cases occur annually, and about 40% are rectal cancer. The International Agency for Research on Cancer (IARC) estimates that the global burden of colorectal cancer will increase by 56% between 2020 and 2040, to more than 3 million new cases per year. For several reasons, CRC continues to be a significant healthcare burden in Thailand. It is the only malignancy with an increased incidence in both sexes in Thailand. Due to the lack of CRC screening and public awareness, non-metastatic cancer accounts only for 60%–70% of overall cases. The demand for general or colorectal surgeons outmatches the supply at a ratio of 1 general surgeon to 35,000 individuals. There are about 70 board-certified colorectal surgeons serving Thailand's population of nearly 70 million. As a result, more than 25% of cancer patients wait more than a month before surgery[2]. Although surgery is the main treatment modality for early CRC, adjuvant treatment is usually given to patients with advanced disease. Therapies with epidermal growth factor receptor (EGFR) inhibitors have been shown to be effective treatments in a subset of patients with metastatic colorectal cancer. Two anti-EGFR biologics, cetuximab and panitumumab, have been approved by the Food and Drug Administration for the treatment of refractory metastatic colorectal cancer.

The oncogene family RAS consists of three members (KRAS, HRAS and NRAS) that play important roles in human cancers. All RAS genes encode 21-kDa monomeric GTPases that relay extracellular signals to intracellular signal transduction cascades. The on/off state of rat sarcoma proteins (RAS) is determined by nucleotide binding[3], with the GTP-bound form exhibiting active signaling conformation. Missense mutations in RAS proteins alter the homeostatic balance of GDP and GTP binding toward the active state by either reducing GTP hydrolysis or increasing GTP loading rate[3]. Approximately, 40% of metastatic colorectal cancers (mCRC) have KRAS mutations, typically in exon 2 and codons 12 (nearly 80% of all KRAS mutations) and 13, and less frequently in exon 3 (codons 59 and 61) and 4 (codons 117 and 146). For decades, the different KRAS mutations were considered equivalent in terms of lack of anti-EGFR response and a negative prognostic factor[4]. It is known that most mutations in RAS are missense mutations in three hotspot residues, G12, G13, and Q61. The order of frequency of G12 is G12D, G12V, G12C, G12A, G12S, and G12R, with the G12C mutation most common in lung cancer and the G12D most common in PDAC[5]. In fact, KRAS G12D (KRAS G12D) is one of the most important therapeutic targets in tumors. Up to 40% of patients with KRAS wild-type CRC show at least a partial response to anti-EGFR therapy, an improvement over the 10% prior to KRAS mutation stratification. Whereas patients with KRAS mutated tumors do not benefit from anti-EGFR therapy. Thus, the advantage of determining KRAS mutation status is to identify those who will not respond to EGFR monoclonal antibody therapy[6]. Therefore, detection of KRAS mutation on the specific codon is an essential investigation for the further molecular biomarker's association, clinically.

Materials and methods

Patients and tumor samples

A total of 40 CRC patients from the lower part of the north-eastern Thai population were collected from January 2019 to June 2020. They were assembled with the approval from the hospital ethics committee (EC003/2021). The clinical information, pathological diagnosis, and formalin-fixed paraffin-embedded (FFPE) tissue samples were collected from the CRC patient's registration.

DNA extraction

Genomic DNA was isolated from ti ssue samples using the PureLink® Genomic DNA kits following the manufacturers' instructions. All FFPE tissue sections were evaluated for tumor cell content more than 20% using hematoxylin and eosin (H&E) staining by pathologists. A total of 5-8

sections were taken from each sample followed by a thickness of each section was 10 µm. The FFPE tissue sections were placed into a 1.5 microcentrifuge tube and deparaffinized with xylene. Each sample was incubated with proteinase K at 56°C overnight until completed digestion. The lysate was subsequently incubated at 80°C for 4 hours to reverse formaldehyde crosslinks. DNA was precipitated with ethanol, fixed on the membrane by centrifugation, washed and eluted by using the elution buffer solution. The DNA concentration and purity were determined by spectrophotometer (Thermo Fisher Scientific) at 230, 260 and 280 nm using Nanodrop 2000, subsequently.

Detections of KRAS mutations

The KRAS gene mutations were detected using Therascreen RGQ PCR KRAS Kit (Qiagen, Manchester, UK). KRAS mutations in codons-12 and codon-13 were identified using a principle of multiplex real-time PCR that combines an amplification refractory mutation system. Scorpion fluorescent primers and probes system were used as directed by the manufacturer 12 GCT (Ala), 12 GAT (Asp), 12 CGT (Arg), 12 TGT (Cys), 12 AGT (Ser), 12 GTT (Val) and 13 GAC (Asp). The seven primer-specific reactions with one control reaction were produced in each sample. DNA template was used at concentration of 20-25 ng. The PCR profile consisted of an initial hold phase at 95 °C for 4 min, followed by 40 cycles of two-step amplification with denaturation at 95 °C for 30 s and annealing at 60 °C for 1 min. The DNA samples with Ct-value between 23.5 and 29.5 were considered valid and suitable for subsequent KRAS analysis. The PCR reaction and data analysis were performed on the QMDx System (Qiagen, Hilden, Germany) and Rotor-Gene Q Software, version 2.3.1.49 (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Statistical analysis

All statistical analysis was calculated in SPSS v.25 (IBM Corp., Armonk, NY, USA). The relationships between clinicopathological variables and the presence of KRAS mutation were determined using Chi-square test. The significance difference was considered at *p*-value less than 0.05, statistically.

Results

Determine KRAS mutation detection by multiplex real-time PCR

The results showed 37.5% (15/40 cases) with KRAS mutations including of 86.7% (13/15 cases) with codon-12 mutations (p.G12D, p.G12V, and p.G12R was 40%, 40%, and 6.67%, respectively), and 20% (3/15) with codon 13 mutations (p.G13D was 5.08%). The most common mutation in codon 12 was glycine to aspartate (GGT to GAT) and glycine to valine (GGT to GTT), which was detected in 6 of 15 cases (40%), whereas the other mutation observed in codon 12 mutations resulting in a replacement of glycine by arginine (GGT to CGT; 1 case, 6.677%). On the other side, the most common mutation in codon 13 was glycine to aspartate (GGC to GAC) which occurred in 3 of 15 cases (20%), whereas the other mutation was glycine to cysteine (GGC to TGC).

Correlation between KRAS mutation and clinicopathological parameters

Our results showed a statistically significant difference between KRAS mutations and lymph node metastasis *p*-value = 0.004. Our results also found a statistically significant difference between KRAS mutations with mucinous adenocarcinoma and other tumor types *p*-value = 0.016. However, our findings did not show a significant difference between KRAS mutations and other pathological factors such as gender, age, clinical tumor markers, and degree of differentiation, as shown in table 1.

Table 1 Correlation between clinicopathological characteristics and KRAS mutation status (missense type) in 40 patients with CRC patients

Patient's information	n	KRAS genotypes (n)		*p*-value
		KRAS Mutation	KRAS Wild type	
Total CRC patients	40	15	25	
Sex				
Male	21	9	12	0.462
Female	19	6	13	
Age				
< 70 years	13	5	8	0.960
>70 years	27	10	17	
Primary tumor				
Right CRC	9	4	5	0.654
Left CRC	26	10	16	
Rectum	5	1	4	
Grade of differentiation				
Poor	1	0	1	0.575
Moderate	13	5	7	
Well	26	8	17	
Metastasis at diagnosis				
Lymph node	27	14	13	0.004
Peritoneum	1	1	0	
none	12	0	12	
Histology				
Mucinous	3	1	2	0.016
None	37	14	23	

Note: *p*-value by using chi-square test. KRAS, kirsten rat sarcoma; CRC, colorectal cancer.

Discussion

CRC is the third most common cancer and the leading cause of cancer-related deaths worldwide [7]. In Thailand, the incidence of CRC has increased in recent years. In CRC patients, KRAS mutations are present in 45% of metastatic tumors and in nearly 15-37% of early-stage tumors [8,9]. Recently, KRAS gene mutation in CRC has been intensively studied to clarify whether it is the reason for the tumors' expansive growth. Several researchers have reported that the KRAS gene mutation plays an important role in the protrusive growth of CRC [10].

In previous study from Chinese population that represent KRAS mutation rate for codon 12 was 72.7%, of which G12D was the highest (47.5%) followed by G12V (30.6%), and the mutation rate for codon 13 was 22.0%, of which G13D was reported[11]. Like our study of KRAS mutations in codon-12 and codon-13 from the lower part of the north-eastern Thai population, we notice that a mutation in KRAS

codon-12 has much greater oncogenic potential than a mutation in codon-13. The CRC patients with KRAS codon-12 mutations are commonly present at G12V and G12D, while mutations in G12R are much less commonly found. Among the patients who carry on the KRAS codon-13 mutation, G13D is the most frequently observed mutation. From previous study of KRAS mutation detection on FFPE had been evaluated with a multiplex QPCR assay for the rapid detection of common KRAS mutations. Similar to our study that using multiplex real-time PCR has been shown to be more sensitive and cost effective than sanger sequencing in paraffin-embedded archival tissue [12]. It is a sensitive sequencing assay and can reliably detect mutant alleles at low frequencies (1% mutated) among wild-type alleles, which is often the case in solid tumors [12, 13].

To date, clinical information and biomarkers for drug responses are necessary for CRC managements. Treatments of mCRC with crucial prognostic biomarkers are one of the improvement and reduction risk of disease progression. Recently, Cetuximab and Panitumumab, as the highly effective antibodies targeting epidermal growth factor receptor (EGFR), have clinical activity in the mCRC patients' treatments[14-16]. So, we suggest that study of the molecular KRAS codon-12 and codon-13 mutations with chemotherapeutic drugs response for anti-EGFR could be further explored.

Conclusion

The association between KRAS codon-12 and codon-13 mutations and prognostic outcome of CRC patients should be used for the prognostic biomarkers, clinically. The clinical outcome of progression in the patients related to lymph node metastases as well as a mucinous colorectal adenocarcinoma histology type.

Take home messages

The mCRC patients including of both the sporadic and hereditary CRC patients with the specific clinical outcome of progression as well as the novel molecular biomarkers for diagnosis, prognosis, monitoring, and treatments could be further investigated, clinically.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank the supporting fund from College of Medicine and Public Health (grant number: CMP2021), Ubon Ratchathani University, Thailand. Thanks to the staff of the Office of International Relations (UBU) for assistance with English.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71(3): 209-49.
2. Lohsiriwat V, Chaisomboon N, Pattana-Arun J. Current colorectal cancer in Thailand. Ann Coloproctol 2020; 36(2): 78-82.
3. Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. Cell 2017; 170(1): 17-33.
4. Ciardiello D, Maiorano BA, Martinelli E. Targeting KRASG12C in colorectal cancer: the beginning of a new era. ESMO Open 2023; 8(1): 100745.
5. Plangger A, Rath B, Stickler S, Hochmair M, Lang C, Weigl L, et al. Cytotoxicity of combinations of the pan-KRAS SOS1 inhibitor BAY-293 against pancreatic cancer cell lines. Discover Oncology 2022; 13(1): 84.

6. Plesec TP, Hunt JL. KRAS mutation testing in colorectal cancer. Adv Anat Pathol 2009; 16(4): 196-203.
7. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin 2020; 70(3): 145-64.
8. Vaughn CP, ZoBell SD, Furtado LV, Baker CL, Samowitz WS. Frequency of KRAS, BRAF, and NRAS mutations in colorectal cancer. Genes Chromosomes Cancer 2011; 50(5): 307-12.
9. Pang XL, Li QX, Ma ZP, Shi Y, Ma YQ, Li XX, Cui WL, Zhang W. Association between clinicopathological features and survival in patients with primary and paired metastatic colorectal cancer and KRAS mutation. Onco Targets Ther. 2017 May 19;10:2645-54.
10. Margonis GA, Kim Y, Spolverato G, Ejaz A, Gupta R, Cosgrove D, et al. Association between specific mutations in KRAS codon 12 and colorectal liver metastasis. JAMA Surgery 2015; 150(8): 722-9.
11. Li W, Liu Y, Cai S, Yang C, Lin Z, Zhou L, et al. Not all mutations of KRAS predict poor prognosis in patients with colorectal cancer. Int J Clin Exp Pathol 2019;12(3): 957-67.
12. Wolf WL, Ye F, Tran V, Yang Z, White R, Bloom K, et al. Sensitive multiplex detection of KRAS codons 12 and 13 mutations in paraffin-embedded tissue specimens. J Clin Pathol. 2011; 64(1): 30-6.
13. Tol J, Dijkstra JR, Vink-Börger ME, Nagtegaal ID, Punt CJ, Van Krieken JH, et al. High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. J Cell Mol Med 2010; 14(8): 2122-31.
14. Heinemann V, Stintzing S, Kirchner T, Boeck S, Jung A. Clinical relevance of EGFR- and KRAS-status in colorectal cancer patients treated with monoclonal antibodies directed against the EGFR. Cancer Treatment Reviews 2009; 35(3): 262-71.
15. Jones RP, Sutton PA, Evans JP, Clifford R, McAvoy A, Lewis J, et al. Specific mutations in KRAS codon 12 are associated with worse overall survival in patients with advanced and recurrent colorectal cancer. Br J Cancer 2017; 116(7): 923-9.
16. Passot G, Denbo JW, Yamashita S, Kopetz SE, Chun YS, Maru D, et al. Is hepatectomy justified for patients with mutant colorectal liver metastases? An analysis of 524 patients undergoing curative liver resection. Surgery 2017; 161(2): 332-40.

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2023; 35(2): 25-34.

Isolation, cloning and optimization for expression of neutrophil gelatinase associated lipocalin (NGAL) in prokaryote

Tasneem Pechnur[1,2,3], Nisachon Jangpromma[4,5], Sakda Daduang[4,6], Patcharaporn Tippayawat[2], Patcharee Boonsiri[7], Nipaporn Ngernyuang[8], Jureerut Daduang[2*]

[1] Master's degree student, Medical Technology Program, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[2] The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[3] Department of Pathology, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand.

[4] Protein and Proteomic Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Thailand.

[5] Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

[6] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand.

[7] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

[8] Chulabhorn International College of Medicine, Thammasat University, Pathum Thani, Thailand.

KEYWORDS

Acute kidney injury; Neutrophil gelatinase associated lipocalin; Prokaryote expression.

ABSTRACT

Acute kidney injury (AKI) is a sudden loss of kidney function followed by a failure to maintain fluid, which is associated with increased mortality. Recently, neutrophil gelatinase associated lipocalin (NGAL) has been recommended as the first biomarker for prediction of kidney damage in the early phase. For medical applications of the NGAL protein, a large amount and expression system of this protein is required. Currently, the assay to determine NGAL levels is enzyme-linked immunosorbent assay (ELISA), which is accurate but inconvenient for clinical applications, because its long-time process takes about 4 hours and the antibody against human NGAL is expensive. Therefore, high-level expression of NGAL is required in order to develop a commercially NGAL strip test. The present study aimed to construct the recombinant plasmid, to optimize the expression of NGAL in Escherichia coli BL21(DE3) and Origami™B(DE3) strains. The host strain, IPTG concentration and induction time were also evaluated. NGAL gene was cloned in pET32a and transformed to E. coli BL21(DE3) and Origami™B(DE3). The construct of NGAL and the expression vector was successful. BL21(DE3) showed a high growth rate indicated by reaching the mid-log phase faster than Origami™B(DE3). However, both E. coli BL21(DE3) and Origami™B (DE3) showed no expression of NGAL. In conclusion, this study demonstrates the efficiency of the expression host to produce protein for development as a commercial strip test. The suitable system to express NGAL protein should be further investigated.

***Corresponding author:** Jureerut Daduang, MT, PhD. Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand. Email address: jurpoo@kku.ac.th

Received: 29 March 2023/ Revised: 29 April 2023/ Accepted: 9 May 2023

Introduction

Acute kidney injury (AKI) is an abrupt reduction in kidney function followed by failure to maintain fluid, acid balance and electrolyte homeostasis. AKI is a serious condition globally and it is associated with high mortality in patients in an intensive care unit (ICU). Early diagnosis and treatment of AKI are important. Nowadays, AKI is typically diagnosed based on an elevation of serum creatinine (SCr). However, rapid change of kidney function cannot be indicated in real time by the level of SCr because the level of SCr can only be detected after kidney function has been destroyed beyond 50%[1]. SCr can also be affected by several factors such as age, muscle mass, diet, drugs and exercise[2]. The level of SCr has low sensitivity to detect kidney damage in an early diagnosis. Therefore, it is necessary to explore for highly sensitive biomarkers for early detection of AKI. Gaspari et al[3] reported that a protein called neutrophil gelatinase associated lipocalin (NGAL) was an alternative choice for the prediction of kidney injury in the early phase, instead of SCr. NGAL has potential for use in clinical applications because its level is rapidly increased into the blood and urine[4,5]. Taken together, NGAL is considered as an appropriate biomarker for diagnosis of AKI and also for prediction of clinical outcomes, including the need for initiation of dialysis and for the onset of mortality[6].

At present, measuring NGAL level is performed by enzyme-linked immunosorbent assay (ELISA), which has a high accuracy but is not suitable for clinical applications. The ELISA process for AKI diagnosis takes a long time, about 4 hours[7], with a high cost for the antibodies against human NGAL. Therefore, the development of a reliable, rapid, easy-to-use and cost-effective method for NGAL detection is needed. For medical applications of the NGAL protein, a large amount of NGAL protein is necessary. In the present study, the bacterial expression system is the method of choice for the expression of the recombinant NGAL protein, due to its simplicity, cost effectiveness, and capacity to produce high yield protein[8].

The aim of this study was to construct and produce recombinant NGAL protein by cloning in *Escherichia coli*. The optimal conditions for the expression host, *E. coli* BL21(DE3) and Origami™ B(DE3) strains, were compared. The IPTG concentrations and induction time were also evaluated.

Materials and methods

Neutrophil gelatinase associated lipocalin gene

Total RNA was isolated from colon carcinoma and extracted by reagent TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Then, total RNA was used to generate the first-strand Complementary DNA (cDNA) using the cDNA synthesis kit (Invitrogen, USA), which was provided by the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand and preserved at -80°C before use.

Escherichia coli and vectors

E. coli DH5α strain was used as a host for cloning plasmid. *E. coli* strains, BL21(DE3) and Origami™ B(DE3) were used as the expression host. The pGEM®-T easy vector (Promega, USA) was used as a vector for cloning and the pET-32a vector (Invitrogen, USA) was used as an expression vector.

Primers design

Two primers were designed for amplification of the NGAL gene fragment (GenBank accession number: EU644752.1) by polymerase chain reaction (PCR). The forward and reverse primers were extended with tails that contain *EcoR*I and *Xho*I-cutting sites respectively for subsequent digestion and cloning into a pET32a vector. In addition, special attention to primer design parameters such as length of 18-24 bases, 40-60% GC content, melting temperature (Tm) of 50-60°C have been considered. To reduce the likelihood of primer-dimer and secondary structure formation for all the primers, the oligonucleotide calculator was used (http://biotools.nubic.northwestern.edu/OligoCalc.html), and target specificity was also evaluated using BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). This primer set was also optimized for annealing temperature.

Amplification of NGAL gene

Complementary DNA (cDNA) was used as the template and the NGAL gene was amplified with specific primers using the polymerase chain reaction (PCR) system. The PCR parameters were set using a thermal cycler as follows: pre-denaturation at 94°C for 5 min; 35 cycles of heat denaturation at 94°C for 30 secs, primer annealing at 55°C for 30 secs, DNA extension at 72°C for 30 secs; and a final extension at 72°C for 7 min. The expected PCR product size was eluted from electrophoresis gel before being ligated into pGEM®-T easy vector. The identity of NGAL was verified by sequencing.

Construction of a plasmid expressing recombinant NGAL

Initially, the NGAL gene was amplified by PCR using the primer set F-*EcoR*I: 5'-GAATTCGTGC-CACTAGGTCTCCT-3' and R-*Xho*I: 5'-CTCGAGGC-CGTCGATACAC-3'. The resulting PCR product, with the predicted size and expression vector, was digested by the same restriction enzyme *EcoR*I/ *Xho*I using the double digestion method at 37°C for 3 hours and cloned into pET32a.

Preparation of recombinant NGAL

Protein expression was carried out in *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells using the heat shock method. The following day, single colonies were selected on LB agar containing ampicillin and screened by colony PCR. The bacterial colonies were transferred onto an LB broth and cultured at 37°C with shaking at 180 rpm for 18 hours. Then, the recombinant plasmid was extracted from the bacteria using the Presto™ Mini Plasmid kit according to the manufacturer's instructions.

Optimization of expression condition

The positive colonies were inoculated in LB broth containing ampicillin and incubated at 37°C with shaking at 180 rpm until the cell density reached the mid log-phase of growth. Then, protein expression was induced with isopropyl ß-D-1-thiogalactopyranoside (IPTG). The concentration of IPTG was varied in each tube with final concentrations of 0.1, 0.3, 0.5, 0.7, and 1 mM. The time of induction was examined at different time intervals of 2, 4, 6, 12 and 24 hours. Then, the cells were harvested by centrifugation at 10,000g for 5 minutes and the expressed protein was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of expressed recombinant NGAL was analyzed by SDS-PAGE. The cell pellets were prepared by mixing with 2X solubilizing buffer (0.5 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.5% (w/v) bromphenol blue, and 10% ß-mercaptoethanol), and then heating in boiling water for 5 minutes. The SDS-PAGE system contained two gels, including a 13% polyacrylamide gel as a separating gel and a 4% polyacrylamide gel as a stacking gel. Twenty µg Samples were loaded into the wells and separated at 150 volts until the tracking dye reached the bottom of the gel, continued by staining with Coomassie Brilliant Blue R-250 solution for 30 min. Then the gel was destained with destaining solution until the background cleared and protein bands appeared.

Results

Specific primer design

The sequence of the NGAL gene from the NCBI database (GenBank accession number: EU644752.1) as a template for designing new primers with cloning enzyme restriction sites for pET32a vector construction is shown in Figure 1A. The cloning enzyme at the restriction sites of these primers did not affect the template primer binding.

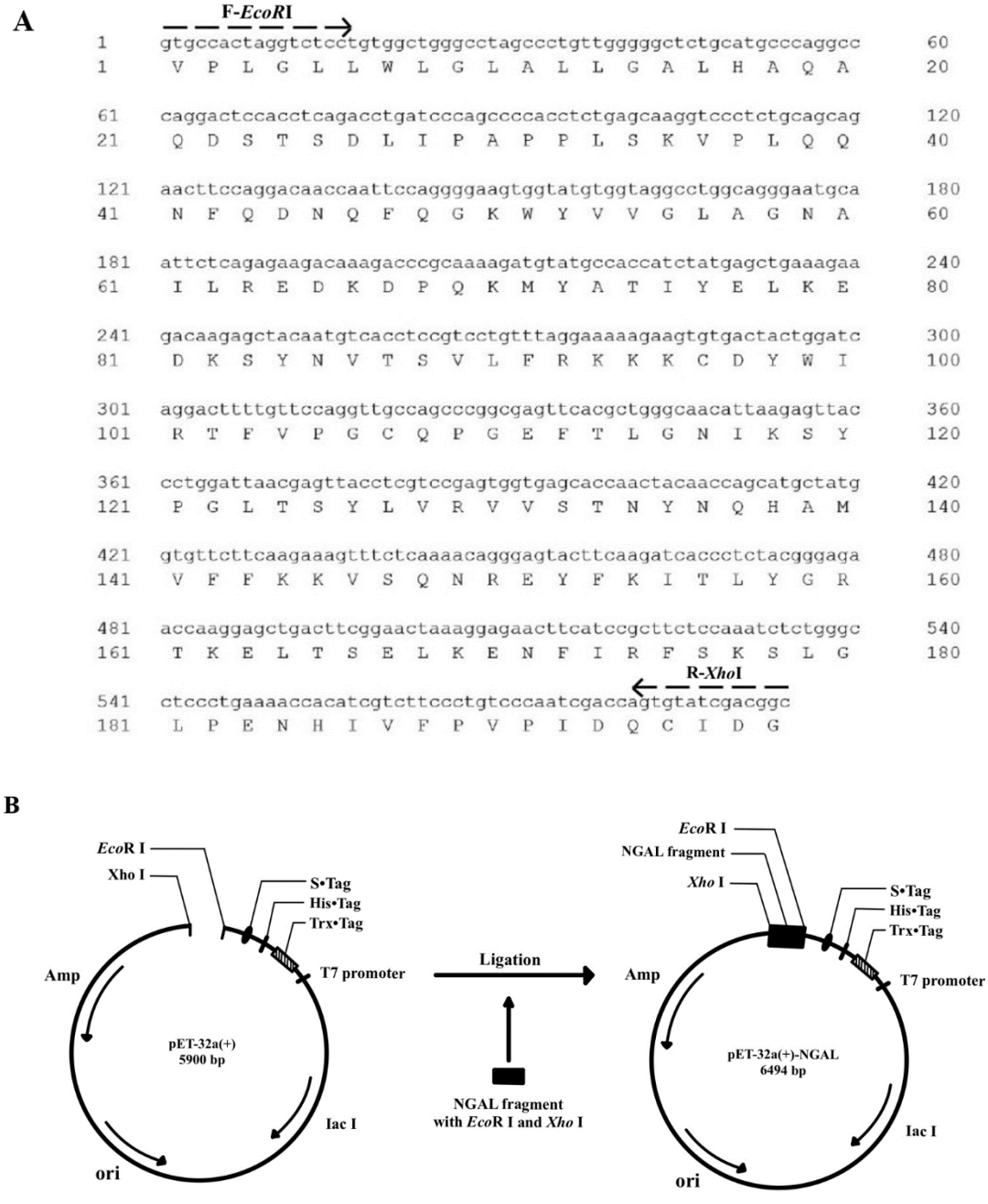


Figure 1 Characteristics of *E. coli* expression plasmid pET32a-NGAL.
(A) Sequence of the NGAL gene and the amino acids it encodes. Primer binding sites are indicated by a dotted arrow.
(B) Schematic of the recombinant plasmid construction containing the NGAL gene insert and key features required for subsequent protein expression and purification.

The optimal condition for NGAL gene amplification

The annealing temperature of the PCR reaction for primer was optimized for NGAL fragment amplification (Figure 2A). The results suggested that the optimal annealing temperature is 55°C, because the NGAL product from PCR showed an intense band at this temperature. The specific band of 594 bp appeared as expected (Figure 2B).

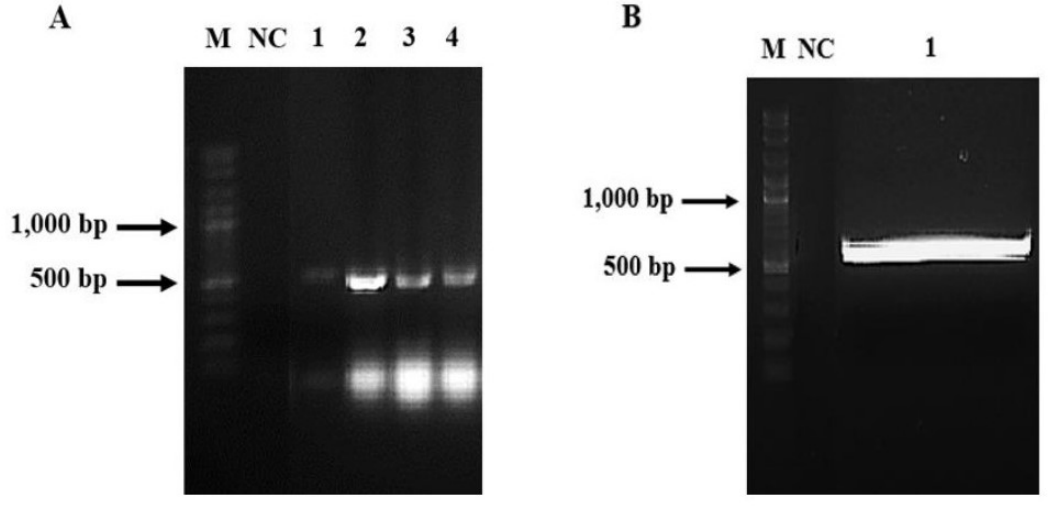


Figure 2 Agarose gel electrophoresis of NGAL product from PCR amplification
(A) Annealing temperature.
Lane 1 at 50°C.
Lane 2 at 55°C.
Lane 3 at 60°C.
Lane 4 at 65°C.
(B) Purified NGAL.
Note: Lane 1, NGAL purified from agarose gel electrophoresis amplification at 55°C; lane M, marker 100 bp DNA ladder; lane NC, negative control (PCR buffer without DNA template).

Construction of recombinant expression plasmid

The NGAL fragment, which was inserted in the pGEM®-T easy vector, was transformed into an *E. coli* DH5α competent cell. Then the pET32a vector and recombinant plasmid were prepared by digestion with a restriction enzyme. After that, the NGAL fragment and the pET32a vector were ligated between the *EcoRI*/*XhoI* cloning sites and transformed to *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells (Figure 1B). The transformant colonies were selected for identification by colony PCR to ensure that the NGAL fragment carried the vector. The results showed that 10 positive clones of recombinant plasmid were transformed into *E. coli* BL21(DE3) competent cells, as shown in Figure 3A in lanes 1 to 10. This demonstrated that NGAL fragments at approximately 594 bp were obtained as expected. Ten transformant colonies were selected and screened for positive clones in *E. coli* Origami™ B(DE3) competent cells, only 7 clones contained the NGAL fragments as shown in Figure 3B (lanes 11 to 20). These results revealed that the recombinant processes were successful, because the NGAL fragments at approximately 594 bp were observed in lanes 11, 12, 14, 16, 17, 18 and 19 of agarose gel electrophoresis (Figure 3B).

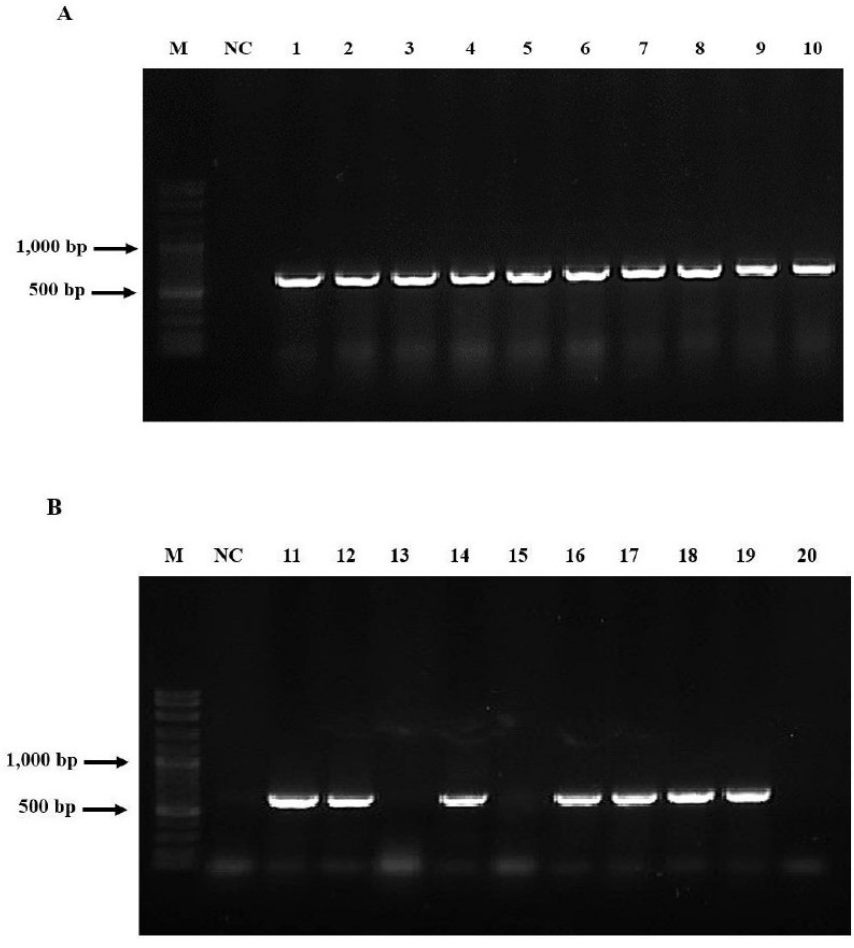


Figure 3 Agarose gel electrophoresis of positive clones for confirmation of the correct insertion of NGAL into competent cells.
(A) *E. coli* BL21(DE3) competent cell. *Note:* Lanes 1 to 10, expected clones of recombinant plasmid pET32a-NGAL.
(B) *E. coli* Origami™ B(DE3) competent cell. *Note:* Lanes 11 to 20, expected clones of recombinant plasmid pET32a-NGAL; lane M, marker 100 bp DNA ladder; lane NC, negative control (PCR buffer without DNA template).

SDS-PAGE analysis

The molecular weight of pET32a-NGAL, by SDS-PAGE analysis, was approximately 42.96 kDa, while the molecular weight of pET32a alone and NGAL were approximately 20.4 and 22.56 kDa, respectively.

To select the optimized protein expression condition, three parameters were studied for the expression of the proteins including the bacterial host, IPTG concentrations and induction times. The lowest IPTG concentration and lowest induction time were investigated. IPTG concentrations in ranges of 0.1, 0.3, 0.5, 0.7 and 1 mM, at a fixed incubation time of 24 hours, were performed. SDS-PAGE reveals that the bands of both *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells (Figures 4A and 5A) were similar, with no over expression at all concentrations of IPTG. In Figures 4B and 5B, 1 mM IPTG was fixed at different induction times, ranging through 2, 4, 6, 12 and 24 hours. The results showed no over expression and no difference was observed in *E. coli* BL21(DE3) and Origami™ B(DE3) competent cells for all induction times when compared with the non-induced lane.

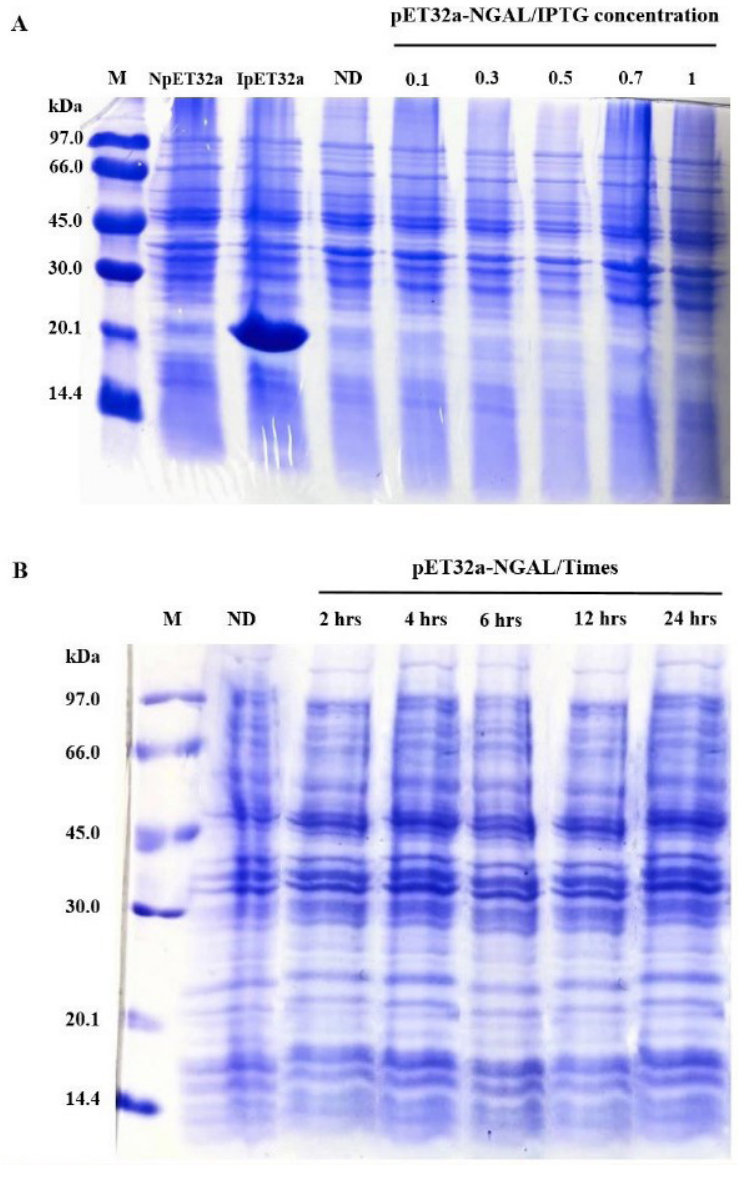


Figure 4 Optimization of IPTG concentration and induction time of recombinant NGAL in *E. coli* BL21(DE3) competent cell by SDS-PAGE.

(A) Optimization of IPTG concentration (0.1 - 1 mM), fixed incubation time of 24 hours.

(B) Optimization of induction time (2 - 24 hours), fixed IPTG 1 mM.

Note: Lane M, low MW protein marker; lane NpET32a, pET32a uninduced with IPTG; lane IpET32a, pET32a induced with 1 mM IPTG; lane ND, uninduced.

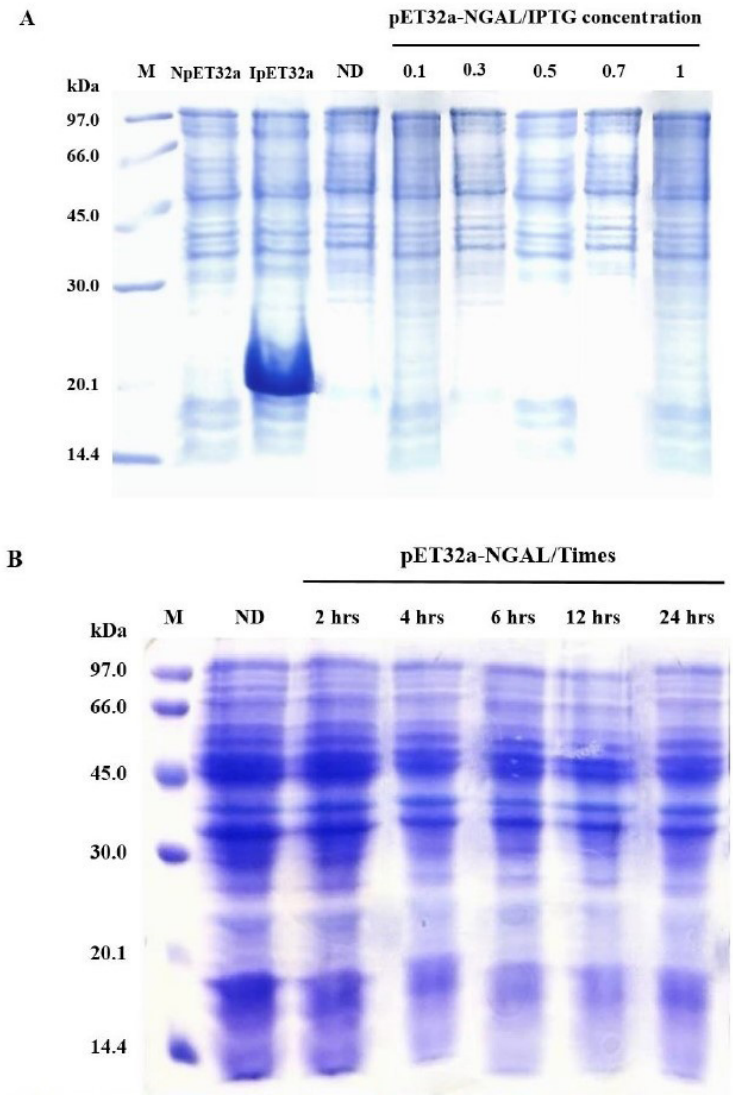


Figure 5 Optimization of IPTG concentration and induction time of recombinant NGAL in *E. coli* Origami™ B(DE3) competent cell by SDS-PAGE.
(A) Optimization of IPTG concentration (0.1 - 1 mM), fixed incubation time of 24 hours.
(B) Optimization of induction time (2 - 24 hours), fixed IPTG 1 mM.
Note: Lane M, Low MW protein marker; lane NpET32a, pET32a uninduced with IPTG; lane IpET32a, pET32a induced with 1 mM IPTG; lane ND, uninduced.

Discussion

NGAL plays an important role in several pathological conditions such as kidney injury, burn injury, bacterial infection and also in some types of human cancers such as colon cancer, rectal cancer, and pancreatic cancer[9]. It has been recommended as the first biomarker for the prediction of AKI[10]. NGAL has potential for use in clinical applications because the level of NGAL rapidly increases in the blood and urine during AKI[4]. ELISA is a standard method for assaying NGAL, but it is not convenient for clinical applications due to its high cost and time consuming. Developing a reliable and cost-effective detection for NGAL may help in the prediction of AKI.

To produce an NGAL detection kit, a large amount of NGAL is required. The expression system of NGAL is also necessary for developing commercial NGAL antibodies. Cloning is one of the suitable strategies for NGAL production. The bacterial expression system is a preferred choice for the expression of recombinant proteins due to its capacity to produce high yield protein at a low cost[8].

In the present study, three parameters were investigated, including *E. coli* host strain, IPTG concentration and induction time. The purification of NGAL carried out in the *E. coli* system was successful (Figures 3A and 3B). One of the popular prokaryotic expression systems is *E. coli* strain BL21. The NGAL was cloned to pET32a vector that contained a histidine tag (His-tag) and a thioredoxin tag (Trx-tag). Upon expression, these tags are integrated into the NGAL protein which facilitates protein purification (His-tag) and reduces protein aggregation (Trx-tag) in the bacterial system [11]. Therefore, we used pET32a as the expression vector system. In an attempt to express recombinant NGAL in *E. coli*, the recombinant plasmid was constructed. *E. coli* BL21(DE3) and Origami™ B(DE3) were cultivated and optical density (OD) at logarithmic growth phase was measured. The concentration of bacterial growth at OD_{600} was 0.6. pET32a-NGAL/ BL21(DE3) was induced at 3 hours and pET32a-NGAL/Origami™ B(DE3) was induced at 5 hours after bacterial inoculation, respectively. BL21(DE3) was considered to have higher efficiency to produce recombinant protein because the growth rate of BL21(DE3) was faster than the Origami™ B(DE3) strain. The possible explanation is that it may enhance amino acid synthesis rate because these strain recombinant protein productions can be driven by the very strong T7 promoter system[12]. Due to the mutations of trxB and gor genes in Origami™ B(DE3), it can increase the disulfide bond formation in the cytosolic fraction. However, these strains may increase protein production but sometimes decreases the solubility of protein[13]. After cultivations of both *E. coli* BL21(DE3) and Origami™ B(DE3) in the optimization of expression conditions, they were compared with negative control (pET32a without insertion) from which no over expression bands were observed (Figures 4A-B and 5A-B). In general, low temperature cultivation of *E. coli* enhances soluble protein expression[14]. Moreover, the high concentration of inducer can reduce the protein expression due to its toxic effects on cells. In an attempt to express NGAL in *E. coli*, using the specific epitopes of NGAL instead of cloning whole NGAL protein should be further investigated and codon optimization is recommended. It is important to note that all the factors including host, vector, concentration of IPTG, time and temperature should be considered and optimized in order to determine the appropriate conditions for the expression of the protein.

Conclusion

This study has successfully constructed the recombinant plasmid pET32a-NGAL. *E. coli* BL21(DE3) is considered to be more efficient to produce recombinant protein because its growth rate is faster than the Origami™ B(DE3) strain. Expression of recombinant NGAL in *E. coli* with this construct plasmid, expressing protein with optimal solubility and appropriately, should be further investigated. Other expression hosts, expression systems, and methods for the production of recombinant protein should also be studied.

Take home messages

The construction of the recombinant plasmid and the evaluation of the production of recombinant protein was compared between two expression hosts. This study demonstrates the efficiency of the expression host to produce protein for development as a commercial strip test in the future.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

T. Pechnur has been holding a scholarship from the Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), and Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. This research was supported by Innovation and Enterprise Affairs, Khon Kaen University in the year 2019.

References

1. Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P. Acute renal failure-definition, outcome measures, animal models, fluid therapy and information technology needs: the second international Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. Crit Care 2004; 8: 204-12.
2. Khawaja S, Jafri L, Siddiqui I, Hasmi M, Ghani F. The utility of neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney injury (AKI) in critically ill patients. Biomark Res 2019; 7: 4.
3. Gaspari F, Cravedi P, Mandala M, Perico N, Deleon FR, Stucchi N, et al. Predicting cisplatin-induced acute kidney injury by urinary neutrophil gelatinase-associated lipocalin excretion: a pilot prospective case-control study. Nephron Clin Pract 2010; 2: 154-60.
4. Srisawat N, Kulvichit W, Mahamitra N, Hurt C, Praditipornsilpa K, Lumlertgul N, et al. The epidemiology and characteristics of acute kidney injury in the Southeast Asia intensive care unit: a prospective multicentre study. Nephrol Dial Transplant 2019; 1-10.
5. Bennett M, Catherine L, Ma Q, Dastrala S, Grenier F, Workman R, et al. Urine NGAL predicts severity of acute kidney injury after cardiac surgery: A prospective study. CJASN 2008; 3: 665-73.
6. Roy JP, Devarajan P. Acute kidney injury: diagnosis and management. Indian J Pediatr 2020; 8: 600-7.
7. Lei L, Zhu J, Xia G, Feng H, Zhang H, Han Y. A rapid and user-friendly assay to detect the neutrophil gelatinase-associated lipocalin (NGAL) using up-converting nanoparticles. Talanta 2017; 1: 339-44.
8. Cowland JB, Borregaard N. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics 1997; 45: 17-23.
9. Roudkenar MH, Kuwahara Y, Baba T, Roushandeh AM, Ebishima S, Ade S, et al. Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions. J Radiat Res 2007; 1: 39-44.
10. Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systemic review and meta-analysis. Am J Kidney Dis 2009; 6: 1012-24.
11. Stewart EJ, Aslund F, Beckwith J. Disulfide bond formation in *Escherichia coli* cytoplasm: an in vivo role reversal for the thioredoxins. EMBO J 1998; 17: 5543-50.
12. Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF. Understanding the differences between genome sequence of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the *E. coli* B and K-12 genomes. J Mol Biol 2009; 394: 653-80.
13. Fathi-Roudsari M, Akhavian-Tehrani A, Maghsoudi N. Comparison of three *Escherichia coli* strains in recombinant production of reteplase. Avicenna J Med Biotechnol 2016; 8(1): 16.
14. Xu SY, Carlson M, Engstrom A, Garcia R, Peterson CG, Venge P. Purification and characterization of human neutrophil lipocalin (HNL) from the secondary granules of human neutrophil. Scand J Clin Lab Invest 1994; 5: 365-76.

ORIGINAL ARTICLE

Arch AHS

Archives of Allied Health Sciences 2023; 35(2): 35-45.

Stress and physical fitness among female university students who regularly exercise during the previous two months

Boonsita Suwannakul, Noppharath Sangkarit, Chula Intapunya, Narueporn Sompet, Panatda Chantakhat, Weerasak Tapanya*

Department of Physical Therapy, Faculty of Allied Health Science, University of Phayao, Phayao, Thailand.

KEYWORDS
Mental health;
Physical health;
Physical activity;
Health promotion.

ABSTRACT

University students, particularly females, are susceptible to stress affecting their health. Previous evidence suggested that regular exercise promotes good mental and physical health. Female physical therapy students of University of Phayao frequently contend with a challenging academic environment and a lack of daily physical activity. Thus, this study aims to compare stress levels, anthropometric indices, and physical fitness between the groups of female university students who regularly exercise (PE), and those who did not i.e., a non-physical exercise (non-PE) group. One hundred and one female university students, aged between 18-23 years were recruited. The participants were assigned into two groups regarding exercise history over the last two months before participation in this study, including a regular PE group (exercise > 3 days/week, n = 55) and a non-PE group (exercise < 3 days/week, or no exercise, n = 46). All participants were measured for stress levels via the Suan Prung Stress Test-60, including susceptibility to stress, sources of stress, and symptoms of stress. Then, they were assessed for body mass index, waist-to-hip circumference ratio (WHR), and body fat percentage. The grip and leg muscle strength and muscle flexibility were determined. Finally, the participants performed the Queens College step test to measure maximal oxygen consumption (VO_{2max}). Independent t-test or Mann-Whitney U test were used to compare all parameters between the two groups. A *p*-value < 0.05 was considered statistically significant. The results showed that the susceptibility to stress scores and WHR in PE group were significantly lower than non-PE group (*p*-value = 0.01 and 0.03, respectively). In addition, VO_{2max} in PE group were significantly higher than non-PE group (*p*-value = 0.02). The present findings suggested benefit of regular exercises among female physical therapy students who are vulnerable to stress, while they will be health professionals who are directly responsible to health promotion using exercises. The university or faculty may use strategies facilitating the students to engage in regular exercise programs that may promote their health, study outcomes, and awareness to positive exercise effects to be applied for their future patients.

***Corresponding author:** Weerasak Tapanya, PT, PhD. Department of Physical Therapy, Faculty of Allied Health Science, University of Phayao, Phayao 56000, Thailand, Email address : weerasak.ta@up.ac.th
Received: 3 March 2023/ Revised: 1 June 2023/ Accepted: 20 June 2023

Introduction

University students, especially health sciences students, are vulnerable to stress from a number of factors such as difficult academic assignments, demanding responsibilities, and a competitive academic environment. Additionally, female university students reported greater stress than males[1]. Stress is defined as an imbalance between a person's expectations and their ability to cope[2] . Stress negatively affects mental health, including psychological functioning, happiness, and life satisfaction[3]. The previous study shows that academic stress leads to depression among students which negatively affecting their academic performance and learning outcomes[4]. Physical inactivity and sedentary behavior are also reported in the student population. These unhealthy lifestyles could lead to decreased physical fitness. Previous study reported that physical activity was positively associated with cardiorespiratory fitness, vital capacity, muscular strength, and flexibility among participants in the fit group of university students[5]. In addition, sedentary lifestyles or a deficiency in physical activity are risk factors for non-communicable diseases, such as hypertension, diabetes mellitus, and cardiovascular disease[6-8].

Physical activity (PA) is any activity which allows the body to expend more energy as a consequence of skeletal muscle contraction[8]. PA also refers to all movements performed during leisure time such as housework, and physical exercise. Regular moderate- to vigorous-intensity PA or physical exercise promotes both good mental and physical health[9,10]. Stress levels and PA are inversely associated. The previous studies revealed that stress was lower in the group with regular PA, particularly for women[11,12]. There is evidence that a decrease in PA leads a decrease in the oxygen transport capacity[13]. Exercise is a stressor on the body, and stress hormones may be reduced while endorphin levels are increased. These suppress pain perception and improve mood[14]. In other research, the impact of PA and exercise on lowering depression and anxiety levels was revealed[15,16].

The physical therapy students of the University of Phayao were the health science students who vulnerable to stress. In addition, most of them are female and had generally sedentary lifestyle. These factors might cause adverse effects on both physical and mental health, while they will be health professionals who are directly responsible to health promotion using exercises. The university or faculty may use strategies facilitating the students to engage in regular exercise programs that may promote their health, study outcomes, and awareness to positive exercise effects to be applied for their future patients. Therefore, the aim of this study was to compare the stress levels, anthropometric indices, and physical fitness between the female university students who reported regular physical exercise in the past 2 months and non-physical exercise group.

Materials and methods

Study design and participants

This retrospective cohort study was carried out during July to August 2022. The population in this study were 193 female physical therapy students from the Department of Physical Therapy, School of Allied Health Science, University of University of Phayao, Northern Thailand. We recruited the number of physical therapy students as much as we can. The inclusion criteria were healthy females aged 18 to 22 years, with no major adverse health conditions such as heart disease, asthma, hypertension, or myopathy. The participation flowchart is shown in Figure 1. After selection, the participants were subdivided according to the exercise history in the past 2 months into two groups, including the regular physical exercise (PE), and a non-physical exercise (non-PE) group. The PE group was fifty-five physical therapy students who performed exercise regularly such as walking, jogging, running, cycling, dancing, yoga, and weight training, for at least 30 minutes/session, 3 days per week over the previous two months prior to participation in this study. The non-PE group was forty-six physical therapy students who performed physical exercise less than 3 days per week or

did not perform any exercise. The participants received an explanation of the objectives and study protocols. All participants volunteered to participate and provided written informed consent before data collection began. The study was reviewed and approved by the Human Ethical Committee at the University of Phayao (protocol code UP-HEC 1.2/036/65 and date of approval, 1 July 2022), prior to data collection.

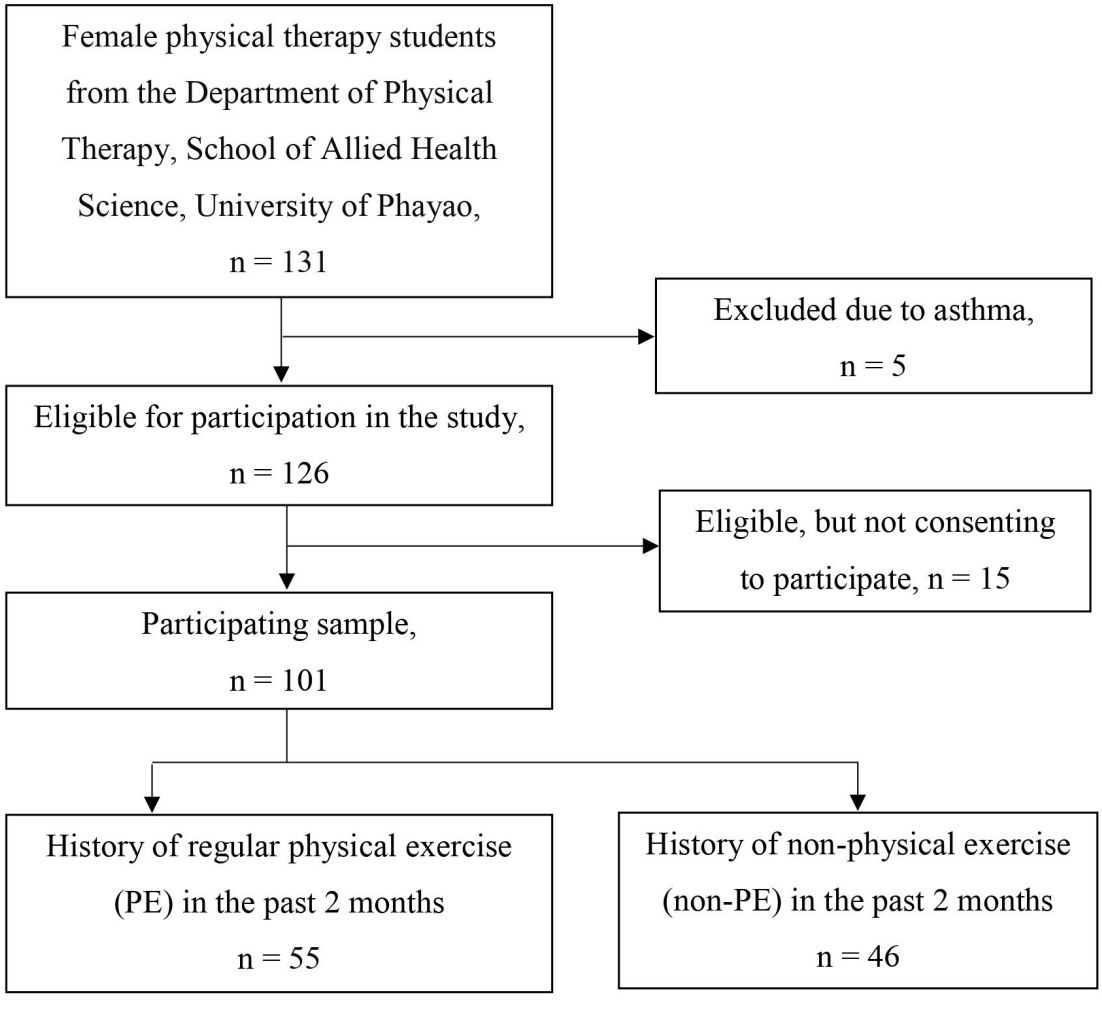


Figure 1 The participation flowchart.

Research protocols

The participants were completed two self-report questionnaires, one for their demographics (including age, underlying diseases, alcohol use, and cigarette smoking) and the participation in any exercise programs (i.e., frequency, duration, and types of exercise), and the other one for their stress levels using the Suan Prung Stress Test-60 (SPST-60). Then, anthropometric indices including body mass index (BMI), waist-to-hip circumference ratio (WHR), and body fat percentage, were measured. The participants were assessed for their muscular strength using grip, and back and leg dynamometers. For assessing their trunk flexibility, the participants were performed modified sit-and-reach test. Finally, the participants performed the Queens College step test for assessing their cardiorespiratory fitness.

Anthropometric indices, muscular fitness, and cardiorespiratory fitness were measured with two blinded assessors. The assessors were two physical therapy students who had received administration training from an experienced physical therapist. The first assessors evaluated BMI, WHR, body fat percentage, and trunk flexibility. The second assessors evaluated grip and leg muscle strength, and Queens College step test. Details of the measurements were explained below.

Suan Prung Stress Test-60

The SPST-60 is a widely accepted measure to indicate stress levels among Thai people[17]. The questionnaire comprises 60 items with the scores ranging from 1 to 5. This questionnaire presents Cronbach's alpha reliability > 0.7 and concurrent validity > 0.27 (statistically significant based on electromyography values at a 95% CI)[18]. The SPST-60 was designed to assess participants' stress levels from the prior six months. The scores of each item were separated into five categories based on the frequency of the event or symptom. The participants were given only one category in each item. In the susceptibility to stress aspect, the scales ranged from 1 = always occurs to 5 = never occurs (scoring rates were reversed for items 4-6). In the sources of stress and the symptoms of stress aspects, the scales ranged from 1 = no stress or never to 5 = very high stress or always occur. SPST-60 has three aspects as following.

Susceptibility to stress

The first aspect of SPST-60 consisted of 12 items. The questionnaire accessed daily activities which could affect stress levels in participated subjects. This section provided power of detecting the probability of susceptibility to stress. In this part, the stress level was quantified by numerical system where 1 means always occur (scored 1), 2 means often (scored 2), 3 means sometimes (scored 3), 4 means rarely (scored 4), and 5 means never (scored 5). The reverse rating systems were applied for items 4-6; smoking, alcohol consumption and taking sleeping pills or antidepressants. Total score was used for interpreting the stress levels ranging from mild (score 0-20), moderate (score 21-26), high (score 27-33), to severe (score > 33).

Sources of stress

This aspect provided data about sources of stress namely work or study, personal issues, family, social problems, environment, and financial issues (24 items in total). Conversely, for the sources of stress aspect, the participants scored 5 for the answer of very high stress, 4 for high, 3 for average, 2 for low, and 1 for no stress. A higher total score means higher stress. Total score was used for interpreting the stress levels from mild (0-36 scores), moderate (27-57 scores), high (58-79 scores), to severe (> 79 scores).

Symptoms of stress

The last aspect illustrated the symptoms of stress in terms of skeletal muscles, parasympathetics and sympathetics nervous systems, emotion, cognition, and the endocrine and immune systems (24 items in total). The participants scored 5 for the answer of always occur, 4 for often, 3 for sometimes, 2 for rarely, and 1 for never. Total score was used for interpreting the stress levels from mild (score 0-17), moderate (score 18-36), high (score 37-57), to severe (score > 57).

Anthropometric indices

The anthropometric indices including BMI, WHR, and body fat percentage were determined. Each participant's weight and height were measured to calculate BMI. Waist circumference was measured midway between the costal margin and the iliac crest at the end of inspiration. Hip circumference was measured as the largest value above the buttocks. Both waist and hip circumference were recorded in centimeters. Then, WHR was calculated by dividing waist circumference by hip circumference[19]. Body fat percentage was measured via the skinfold thickness technique (Lange skinfold caliper, Beta Technology, Santa Cruz, CA, USA) at four sites: the triceps, biceps, subscapular, and suprailiac crest[20].

Grip, and back and leg strength

The hand grip dynamometer was applied to evaluate grip strength (kilograms). Each participant performed the hand grip dynamometers (T.K.K 5001 Grip-A, Takei Scientific Instruments, Niigata, Japan) three times with a 1-minute rest

interval in between. Then relative strength was calculated by dividing the best grip-strength score by the participant's body weight (kilograms)[21]. To assess leg muscle strength, a back and leg dynamometer (T.K.K 5402 Back-D, Takei Scientific Instruments, Niigata, Japan) were used. The participants performed two trials of the back and leg dynamometer with a 1-minute rest interval. The best score of the leg muscle strength test (kilograms) was divided by the participant's body weight (kilograms) to determine relative strength.

Trunk flexibility

Trunk flexibility was assessed using a modified sit-and-reach with a standing trunk flexion meter (T.K.K. 5403 Flexion-D, Takei Scientific Instruments, Niigata, Japan). In this test, a yard stick was placed on top of a 12-inch sit-and-reach box. The most distant point of the fingertips on the yard stick was measured in inches[22].

Queens College step test

For cardiorespiratory fitness, the Queens College step test was used to measure maximal oxygen consumption (VO_2max). After three minutes of the step test, heart rates (beats per minute; bpm) were recorded. Then, the predicted VO_2max (milliliters/kilogram/minute; ml/kg/min) was calculated[23].

Two physical therapy students who had received administration training from an experienced physical therapist administered the tests. The first evaluator assessed BMI, WHR, and step test. Grip and leg muscle strength, and muscle flexibility were tested by the second assessor.

Statistical analysis

Descriptive statistics were used to explain demographic data and findings of the study. All data were tested for normally distributed pattern by the Kolmogorov–Smirnov test. Independent t-test or Mann-Whitney U test were used to compare the differences in SPST-60 scores, anthropometric indices, and physical fitness parameters between the two groups. *Chi* square test was used to examine the differences between categorical variables between both groups. A p-value < 0.05 was considered statistically significant. Following a statistical examination of the differences between the groups, G*Power was used to compute the power of the test of the findings in order to demonstrate the strength of the findings. We assessed the power of the tests of 0.80 as a standard for adequacy.

Results

Demographic characteristics of the PE group and the non-PE group

Table 1 shows demographic characteristics of the participants. The results showed that aerobic exercise was the most popular exercise for both groups (PE group = 76.4%, non-PE group = 30.4%).

Table 1 Demographic characteristics of participants in the regular physical exercise (PE) and non-physical exercise (non-PE) groups

Parameters	Non-PE group (n = 46)	PE group (n = 55)	*p*-value
Age[a]: years[#]	20.04 ± 1.33	19.98 ± 1.39	0.88
Alcohol use[c]: n (%)	5 (10.9)	9 (16.4)	0.43
Cigarette smoking: n (%)	0 (0)	0 (0)	
Underlying disease: n (%)	0 (0)	0 (0)	
Resting heart rate[a]: beat/minute[#]	80.41 ± 17.25	79.64 ± 9.51	0.51
Systolic blood pressure[b]: mm Hg[#]	111.78 ± 15.97	108.65 ± 13.96	0.30
Diastolic blood pressure[b]: mm Hg[#]	72.74 ± 11.93	70.24 ± 11.27	0.28
Type of Exercise			
- Aerobic (jogging, running, cycling)[c]: n (%)	14 (30.4)	42 (76.4)	0.00[*]
- Strengthening (gym weight)[c]: n (%)	1 (2.2)	2 (3.6)	0.67
- Flexible (yoga)[c]: n (%)	2 (4.3)	11 (20.0)	0.02[*]
- No exercise[c]: n (%)	29 (63.0)	0 (0)	<0.01[*]
Duration of exercise[b]: minutes[#]	14.34 ± 20.26	38.27 ± 10.54	<0.01[*]
Frequency of exercise[b]: days/week[#]	0.65 ± 0.89	3.76 ± 0.76	<0.01[*]

Note: [#] The data are presented as mean ± SD (95% confidence interval), and the data between regular physical exercise (PE) and non-physical exercise (non-PE) groups were compared using [a] the independent samples *t* test for data with normal distribution, [b] the Mann-Whitney U test for variables with non-normal distribution, and [c] the data are compared using the *Chi* square test. [*] significant difference between group, *p*-value < 0.05.

Stress levels among PE group and non-PE group

The participants' stress levels were assessed via the SPST-60. Table 2 shows the results of the comparison of stress scores between the PE group and the non-PE group. The results showed that the susceptibility to stress scores was significantly lower than non-PE group (*p*-value = 0.01).

Physical fitness tests

Table 2 shows the results of the comparisons of the anthropometric indices and physical fitness tests. The results showed that WHR in PE group were significantly lower than non-PE group (*p*-value = 0.03). In addition, VO_2max in PE group were significantly higher than non-PE group (*p*-value = 0.02).

Table 2 Comparison of SPST-60 scores, anthropometric indices, and physical fitness between the regular physical exercise (PE) and non-physical exercise (non-PE) groups

Parameters	Non-PE group (n = 46)		PE group (n = 55)		*p*-value
	Mean	SD	Mean	SD	
Susceptibility to stress (total 60 scores)[a]	27.72	5.37	25.04	5.14	0.01*
Sources of stress (total 120 scores)[a]	61.57	14.91	57.87	17.37	0.26
Symptom of stress (total 120 scores)[b]	49.33	15.65	46.58	14.69	0.41
BMI[b]: kg/m^2	21.77	5.44	21.70	3.77	0.46
WHR[b]	0.79	0.11	0.76	0.04	0.03*
Body fat[a]: percent	30.11	4.99	29.75	3.68	0.69
Forward back flexibility[b]: inch	7.60	4.74	7.95	4.45	0.45
Relative hand grip strength[a]	0.46	0.10	0.47	0.07	0.56
Relative leg strength[a]	1.10	0.41	1.12	0.28	0.71
VO_2max[b] (ml/kg/min)	39.09	3.29	41.05	4.78	0.02*

Note: The data between regular physical exercise (PE) and non-physical exercise (non-PE) groups were compared using [a] the independent samples *t* test for data with normal distribution and [b] the Mann-Whitney U test for variables with non-normal distribution. * significant difference between group, *p*-value < 0.05.

Discussion

In this study, we compared the stress levels, anthropometric indices, and physical fitness among female university students between the PE group, who reported the exercise history for at least 3 days/week before participating in this study, and the non-PE group, who had the exercised for less than 3 days/week, or not at all. Stress levels were assessed using the SPST-60, a questionnaire for measurement of stress in the Thai population. The results of this study demonstrated that the PE group had a significantly lower stress scores, as well as greater cardiorespiratory fitness and anthropometric indices than the non-PE group. In the present study, the average exercise duration and frequency of the PE group were 38.27 minutes and 3.76 days a week, respectively. These tended to be effective at lowering stress and promoting physical fitness in the PE group. The World Health Organization (WHO) recommends that all adults engage in moderate intensity exercise for 30 minutes a day of physical activity, 4 to 5 days a week, or at least 150 minutes a week to obtain health benefits. The exercise duration and frequency that the participants reported in this study are considerably less than this minimum recommendation[24]. However, the exercise duration and frequency of the PE group in the present study were consistent with the American College of Sports Medicine (ACSM) guidelines, which recommended that a minimal exercise program should consist of at least three 20- to 60-minute exercise sessions each week to develop and maintain cardiovascular fitness and reduce body fat[25].

Our results showed that the PE group had significantly lower outcomes in susceptibility to stress scores than the non-PE group (*p*-value = 0.01, actual power of test = 0.81). These findings tended to show that frequent physical activity was one of the most efficient ways to reduce stress in female university students. Our results corroborated those of a previous study, which found that an aerobic exercise program lasting eight weeks (1 hour/ session, 3 sessions/ week) led to a lower stress score in the experimental group compared to the control group[26]. In addition, de Camargo et al[11] reported that the lower the weekly

frequency of physical activity was, the greater the chances of experiencing stress were, especially for women[11]. Our results were also confirmed the findings in the previous studies that stated the relationships between low physical activity or sedentary lifestyles and high stress levels[12,27]. Nonetheless, stress level is affected by a number of factors which should be considered, including the amount of stress, the duration of its influence, genetic components, and patient history[28].

The results showed that the PE group had a lower mean WHR compared to the non-PE group (*p*-value = 0.03, actual power of test = 0.80). Moreover, the mean VO_2max was higher in the PE group than in the non-PE group (*p*-value = 0.02, actual power of test = 0.80). However, there were no significant differences in BMI or body fat percentage. As previously mentioned, the PE group exercised according to the ACSM recommendations (20–60 minutes per session, three days per week), which can improve and maintain cardiovascular fitness and reduce body fat[25]. As a result, the PE group improved their cardiovascular fitness and reduced their body fat in comparison to the non-PE group. According to our survey data, we found that aerobic exercise, such as jogging, running, and cycling, were the most popular activities in the PE group (76.4%). Regular aerobic exercise has been proven to help maintain the cardiovascular, locomotor, and nervous systems' working capacities[29,30]. Adequate cardiovascular fitness levels can lower obesity rates and total and central body fat[31,32]. Our results were consistent with a study of Milovan et al[37] who found a significant difference in VO_2max between the aerobic exercise training group and the control group among female college students. WHR is an anthropometric predictor of visceral or abdominal obesity while BMI and body fat percentage indicate overall obesity[33]. Additionally, it was discovered that WHR was linked to a higher chance of cardiovascular conditions like myocardial infarction[34]. According to reports, aerobic exercise with a moderate to high intensity has the best potential to decrease visceral fatty tissue[35]. The current research found a significant difference in WHR between the PE and non-PE groups, though not in BMI or body fat percentage. Our study was inconsistent with the previous study which discovered a significant decrease in body fat percentage, fat mass and visceral fat compared to the control group[36]. The PE group in the present study may have exercised at a lower intensity than those in the previous studies (57-90% HRmax). This might not significantly affect the PE group's BMI or body fat percentage compared to the non-PE group. Accordingly, we did not measure individual exercise intensity, which could have impacted the study findings. The present findings did not reveal any significant differences in muscle strength or flexibility. These may be due to the fact that the majority of participants in both groups engaged in aerobic activity, which appears to have had little impact on muscle characteristics. Nonetheless, some data suggests that aerobic exercise of moderate to high intensity led to mild declines in muscle strength[37]. Although the results of this study revealed the significant differences of WHR and VO_2max between the both groups, the results could be confounded by many factors, such as lifestyle, eating, health and personal behaviors of participants.

Our study had some limitations. First, aside from demographic traits, no known variables that influence stress levels have been identified. Second, the group arrangement was done according to the interview data which could affect the findings of the study. Finally, the SPST-60 was used for assessed the stress levels during the past six months, but the specific duration and length of exposure to chronic stress for each participant were not identified. All limitations should be considered when developing further studies. In addition, the different levels found in this study should be compared with clinical significance and standard measurement errors (SEM) of the raters and clinical significance (MDC).

Conclusion

Female university students who engaged in regular physical exercise had better control over their stress levels, WHR, and cardiorespiratory fitness than the non-physical exercise group. Therefore, it is important to encourage female

university students to engage in regular physical exercise. However, a number of factors, including individuals' personal behaviors, eating habits, and lifestyles, should be taken into consideration.

Take home messages

Regular physical exercise lasting at least 30 minutes, three days a week, for two months could control over stress levels, waist to hip circumference, and cardiorespiratory fitness.

References

1. Graves BS, Hall ME, Dias-Karch C, Haischer MH, Apter C. Gender differences in perceived stress and coping among college students. PLoS One 2021; 16(8): e0255634.
2. Danielsson M, Heimerson I, Lundberg U, Perski A, Stefansson C, Akerstedt T. Psychosocial stress and health problems: health in Sweden: the national public health report 2012. chapter 6. Scand J Public Health 2012; 40(9): 121-34.
3. Galderisi S, Heinz A, Kastrup M, Beezhold J, Sartorius N. Toward a new definition of mental health. World Psychiatry 2015; 14: 231-3.
4. Deng Y, Cherian J, Khan NUN, Kumari K, Sial MS, Comite U, et al. Family and academic stress and their impact on students' depression level and academic performance. Front Psychiatry 2022; 13: 1-13.
5. Zhang C, Liu Y, Xu S, Sum RK, Ma R, Zhong P, Liu S, Li M. Exploring the Level of Physical Fitness on Physical Activity and Physical Literacy Among Chinese University Students: A Cross-Sectional Study. Front Psychol 2022; 13: 1-9.
6. Harris ML, Oldmeadow C, Hure A, Luu J, Loxton D, Attia J. Stress increases the risk of type 2 diabetes onset in women: A 12-year longitudinal study using causal modelling. PLoS One 2017; 12(2): e0172126.
7. Vrijkotte TG, van Doornen LJ, de Geus EJ. Effects of work stress on ambulatory blood pressure, heart rate, and heart rate variability. Hypertension 2000; 35(4): 880-6.
8. Bull FC, Al-Ansari SS, Biddle S, Borodulin K, Willumsen JF. World Health Organization 2020 guidelines on physical activity and sedentary behaviour. Br J Sports Med 2020; 54(24): 1451-62.
9. Rantalainen T, Ridgers ND, Gao Y, Belavý DL, Haapala EA, Finni T. Physical activity accumulation along the intensity spectrum differs between children and adults. Eur J Appl Physiol 2021; 121: 2563-71.
10. Verswijveren SJJM, Lamb KE, Martín-Fernández JA, Winkler E, Leech RM, Timperio A, et al. Using compositional data analysis to explore accumulation of sedentary behavior, physical activity and youth health. J Sport Health Sci 2021; 11(2): 234-43.
11. de Camargo EM, Piola TS, Dos Santos LP, de Borba EF, de Campos W, da Silva SG. Frequency of physical activity and stress levels among Brazilian adults during social distancing due to the coronavirus (COVID-19): cross-sectional study. Sao Paulo Med J 2021; 139(4): 325-30.
12. Kong S, Koo J, Lim SK. Associations between stress and physical activity in Korean adolescents with atopic dermatitis based on the 2018-2019 Korea youth risk behavior web-based survey. Int J Environ Res Public Health 2020; 17(21): 8175.
13. Aksović N, Bjelica B, Joksimović M, Skrypchenko I, Filipović S, Milanović F, et al. Effects of aerobic physical activity to cardio-respiratory fitness of the elderly population: systematic overview. Pedagogy Phys Cult Sports 2020; 24(5): 208-18.
14. Caplin A, Chen FS, Beauchamp MR, Puterman E. The effects of exercise intensity on the cortisol response to a subsequent acute psychosocial stressor. Psychoneuroendocrinology 2021; 131: 105336.
15. Goodwin RD. Association between physical activity and mental disorders among adults in the United States. Am J Prev Med 2003; 36(6)): 698-703.

16. Martinsen EW. Physical activity in the prevention and treatment of anxiety and depression. Nord J Psychiatry 2008; 62(47): S25-9.
17. Pannawee T, Luweela V. Stress among public health worker in primary care units, Nonbualumpoo Province. Community Health Development Quarterly Khon Kaen University 2018; 4(2): 185-97.
18. Mahatnirunkul S, Pumpisalchai W, Thapanya P. The construction of suan prung stress test for Thai population. Bulletin of Suan Prung 1997; 13(3): 1-20.
19. World Health Organization. Waist circumference and waist–hip ratio: report of a WHO expert consultation, Geneva, 8–11 December 2008. Geneva: WHO; 2011.
20. Alvero-Cruz JR, Marfell-Jones M, Alacid F, Orta PA, Correas-Gómez L, Medina FS, et al. Comparison of two field methods for estimating body fat in different Spanish dance disciplines. Nutr Hosp 2014; 30(3): 614-21.
21. Massy-Westropp N, Rankin W, Ahern M, Krishnan J, Hearn TC. Measuring grip strength in normal adults: reference ranges and a comparison of electronic and hydraulic instruments. J Hand Surg Am 2004; 29(3): 514-9.
22. Hoeger WW, Hopkins DR. A comparison of the sit and reach and the modified sit and reach in the measurement of flexibility in women. Res Q Exerc Sport 1992; 63(2): 191-5.
23. Das B, Ghosh T, Gangopadhyay S. A comparative study of physical fitness index (PFI) and predicted maximum aerobic capacity (VO_2max) among the different groups of female students in west Bengal, India. Int J Appl Sports Sci 2010; 22(1): 13-23.
24. World Health Organization. Global recommendations on physical activity for health. Geneva: WHO; 2010.
25. American College of Sports Medicine Position Stand. The recommended quantity and quality of exercise for developing and maintaining cardiorespiratory and muscular fitness, and flexibility in healthy adults. Med Sci Sports Exerc 1998; 30(6): 975-91.
26. Mohebbi Z, Dehkordi SF, Sharif F, Banitalebi E. The effect of aerobic exercise on occupational stress of female nurses: a controlled clinical Trial. Invest Educ Enferm 2019; 37(2): e05.
27. Chust-Hernández P, Fernández-García D, López-Martínez L, García-Montañés C, Pérez-Ros P. Female gender and low physical activity are risk factors for academic stress in incoming nursing students. Perspect Psychiatr Care 2022; 58(4): 1281-90.
29. Esch T, Stefano GB, Fricchione GL, Benson H. Stress in cardiovascular diseases. Med Sci Monit 2002; 8(5): RA93-101.
30. Bernardi M, Romano S, Squeo MR, Guerra E, Adami PE, Alviti F, et al. Aerobic fitness is a potential crucial factor in protecting paralympic athletes with locomotor impairments from atherosclerotic cardiovascular risk. Sport Sci Health 2021; 17: 363-74.
32. Craighead DH, Heinbockel TC, Hamilton MN, Bailey EF, MacDonald MJ, Gibala MJ, et al. Time-efficient physical training for enhancing cardiovascular function in midlife and older adults: promise and current research gaps. J Appl Physiol 2019; 127(5): 1427-40.
33. Gonçalves ECA, Alves Junior CAS, Silva VS, Pelegrini A, Silva DAS. Anthropometric indicators of body fat as discriminators of low levels of cardiorespiratory fitness in adolescents. J Pediatr Nurs 2022; 62: 43-50.
34. Gonçalves ECA, Nunes HEG, Silva DAS. Clusters of anthropometric indicators of body fat associated with maximum oxygen uptake in adolescents. PLoS ONE 2018; 13(3): e0193965.
35. Milovan B, Pantelic S, Milanovic Z, Mustadanagic J. The effect of aerobic exercise program on the cardiorespiratory fitness and body composition of female college students. Facta Univ Ser Phys Educ Sport 2016; 14(2): 145-58.
36. Murray S. Is waist-to-hip ratio a better marker of cardiovascular risk than body mass index?. Can Med Assoc J 2006; 174(3): 308.

37. Vissers D, Hens W, Taeymans J, Baeyens JP, Poortmans J, Van GL. The effect of exercise on visceral adipose tissue in overweight adults: a systematic review and meta-analysis. PLoS One 2013; 8(2): e56415.
38. Lan C, Liu Y, Wang Y. Effects of different exercise programs on cardiorespiratory fitness and body composition in college students. J Exerc Sci Fit 2022; 20(1): 62-9.
39. Markov A, Chaabene H, Hauser L, Behm S, Bloch W, Puta C, et al. Acute effects of aerobic exercise on muscle strength and power in trained male individuals: a systematic review with meta-analysis. Sports Med 2022; 52(6): 1385-98.