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Assessment of colistin resistance in *Enterobacteriaceae*: Comparison of the rapid polymyxin NP test, MicroScan system, and broth microdilution in Thailand

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ABSTRACT

Background: Antimicrobial resistance is a global health crisis, with colistin serving as a last-resort antibiotic for the treatment of multidrug-resistant *Enterobacteriaceae*. However, colistin resistance is becoming increasingly prevalent, necessitating rapid and accurate susceptibility testing.

Objective: This study evaluated the rapid polymyxin NP (RPNP) test and the MicroScan antimicrobial susceptibility system compared to the reference broth microdilution (rBMD) method for detecting colistin resistance in colistin-resistant *Enterobacteriaceae* (CRE) isolates from clinical setting in Thailand.

Materials and methods: A total of 158 non-repetitive *Enterobacteriaceae* isolates (85 *Escherichia coli* and 73 *Klebsiella pneumoniae*) were collected from a tertiary hospital in Bangkok between August 2021 and January 2023. Colistin susceptibility was assessed using the RPNP, MicroScan system, and rBMD method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. The categorical agreement (CA), very major errors (VME), and major errors (ME) of colistin resistance test results from the RPNP and MicroScan methods were determined by comparison with the rBMD method.

Results: Among the 158 *Enterobacteriaceae* isolates, 46.2% were intermediate and 38.6% were colistin-resistant by rBMD. The sensitivity of RPNP was 98.4% and the specificity was 95.9%, compared to 95.1% and 97.9% for MicroScan. Both RPNP and MicroScan achieved high CA (96.8%) with rBMD. Discrepancies, primarily in isolates with borderline MICs (1-4 mg/L), accounted for 4 of the 8 discordant strains. In addition, *mobilized colistin resistance-1* (*mcr-1*) gene were detected in 5 colistin-resistant *E. coli* isolates with MIC of 8 mg/L.

Conclusion: Both the RPNP and MicroScan systems demonstrated high categorical agreement with the rBMD method, underscoring their reliability as practical tools for routine colistin susceptibility testing in clinical microbiology laboratories where rBMD may not be feasible.

Introduction

Antimicrobial resistance in bacteria is a critical and continuously increasing global health concern. Regarding many of the available antimicrobial agents are no longer effective in treating infections, leading to over 700,000 deaths annually from drug-resistant infections worldwide, a number that is projected to rise to 10 million deaths per year by 2050.[1] In Thailand, antimicrobial-resistant infections cause approximately 20,000-38,000 deaths

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per year, resulting in an economic loss of up to 1.3 billion dollars annually.[2]

The resurgence of colistin as a therapeutic option is driven by rising carbapenem resistance, particularly in infections caused by multidrug-resistant (MDR) gram-negative bacteria.[3] Colistin, or polymyxin E, is a cationic cyclic decapeptide that binds to the negatively charged lipid A of the lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria, disrupting the membrane and leading to bacterial cell lysis.[4] Despite its renewed clinical relevance, colistin resistance is increasingly reported. Chromosomal mutations in genes such as *pmrA/pmrB* and *mgrB* can mediate colistin resistance by modifying lipid A, thereby reducing colistin's binding affinity.[5] Moreover, efflux pumps contribute by expelling the drug from bacterial cells, lowering its intracellular concentration and effectiveness. Importantly, the 2015 discovery of the *mcr-1*, which enables plasmid-mediated horizontal transfer of colistin resistance, has heightened concerns regarding its global dissemination.[5] This increasing resistance complicates treatment, and it is critical for clinical microbiology laboratories to accurately and promptly report colistin susceptibility to ensure appropriate patient management.

Antimicrobial susceptibility testing for colistin poses challenges, as colistin's large molecular size limits its diffusion in solid media, making simple methods like disk diffusion unsuitable for testing.[6] Gradient diffusion strips are convenient for determining colistin MIC. However, their reliability is still questioned.[7,8] The rBMD method recommended by the CLSI is considered the gold standard for colistin susceptibility testing.[9] Still, it is labor-intensive and impractical for routine laboratory use. To address these limitations, faster colistin susceptibility tests have been developed, such as the RPNP test, which detects pH changes from glucose metabolization in bacteria resistant to colistin. This method provides results within 2-4 hrs and demonstrates high sensitivity and specificity.[10] Therefore, the RPNP test is well-suited as a screening method, particularly in regions experiencing endemic resistance, such as those with widespread carbapenemase producers.[10-14] However, there are some limitations to this method. It requires careful interpretation, as false positives can occur due to minor pH changes, and it may not perform as well with certain bacterial species or strains.

Many automated drug susceptibility testing systems have been implemented, such as MicroScan, VITEK, and Phoenix systems. These platforms provide efficient and standardized methods for determining bacterial susceptibility to antimicrobial agents, enabling rapid and reliable results to support clinical decision-making. In our country, the MicroScan system is widely used in hospital settings for antimicrobial susceptibility testing. Testing was conducted in a 96-well microplate, with two wells specifically designated for colistin testing at concentrations of 2 and 4 mg/L, while in the remaining wells were allocated for testing other antibiotics, enabling comprehensive susceptibility profiling in a single run. However, few studies have investigated the performance of the MicroScan system in colistin susceptibility testing.[15,16] In this study, we evaluated the efficacy of the RPNP test and MicroScan system in *Enterobacteriaceae* clinical isolates from Thailand by comparing them with the rBMD method.

Material and methods

Sample collection

A total of 158 non-repetitive *Enterobacteriaceae* isolates were included in this study, comprising 85 and 73 isolates of *E. coli* and *K. pneumoniae*. These isolates were collected from various clinical specimens at a tertiary hospital located at Bangkok Metropolitan between August 2021 and January 2023. The bacterial isolates were initially identified through standard microbiological and conventional biochemical methods, with further confirmation performed using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) system (Bruker, Germany). The obtained *Enterobacteriaceae* isolates were stored at -80 °C in tryptic soy broth with 30% glycerol for further study.

Reference broth microdilution (rBMD)

Broth microdilution (BMD) was performed according to the CLSI guidelines for antimicrobial susceptibility testing of aerobic bacteria.[17] Cation-adjusted Mueller Hinton broth (CAMHB) was prepared by adding the appropriate salts to achieve final concentrations of 20-25 mg/L of calcium and 10-12.5 mg/L of magnesium. The powdered colistin sulfate (Sigma-Aldrich, USA) was used for this study. Bacterial suspensions in sterile 0.85% saline were adjusted to a McFarland standard No. 0.5 and then diluted 100-fold in CAMHB to achieve a bacterial concentration of 1-2×10^6 CFU/mL. Subsequently, 50 µL of the bacterial suspension was added to each well of a 96-well plate containing 50 µL of CAMHB with colistin, prepared at concentrations ranging from 32 to 1 mg/L through 2-fold serial dilutions. This resulted in a final bacterial concentration of 5×10^5 CFU/mL in each well, with colistin concentrations ranging from 16 to 0.5 mg/L. The plates were incubated at 37 °C for 18-20 hrs. The MIC was determined as the lowest concentration of colistin at which the medium remained clear, indicating bacterial inhibition. Growth control (0 mg/L colistin) and sterile control (0.85% sterile saline) were included in the testing. The antimicrobial susceptibility was interpreted according to the CLSI guidelines, which define colistin breakpoints as resistant at MIC ≥4 mg/L and intermediate at MIC ≤2 mg/L.[9]

Rapid Polymyxin NP (RPNP)

RPNP was performed in accordance with the protocol described by Nordmann *et al.*[10] The rapid polymyxin NP solution was prepared with 0.005% phenol red and 1% D(+)-glucose in CAMHB. For testing, the solution was supplemented with colistin sulfate at a 5 mg/L concentration. Bacterial suspensions were adjusted to McFarland standard No. 3, and 50 µL of the suspension was transferred into wells of a 96-well plate containing

150 µL of the rapid polymyxin NP solution, both with and without colistin (5 mg/L). The final bacterial concentration was 10^8 CFU/mL per well, and the colistin concentration in the test wells was 3.75 mg/L. Sterile 0.85% normal saline was used as a control, whereas *E. coli* ATCC 25922 and *E. coli* NCTC 13846 served as negative and positive controls, respectively. The plates were incubated at 37 °C and monitored for color changes every hour for up to 4 hrs. A shift in color from orange to yellow within 4 hrs indicated resistance to colistin, while an unchanged orange color after 4 hrs indicated susceptibility.

MicroScan system

The antimicrobial susceptibilities of *Enterobacteriaceae* tested were determined using the MicroScan antimicrobial susceptibility system with the NM EN47 panel (Beckman Coulter Diagnostics, USA). The wells were prepared with water containing PLURONIC, a non-ionic surfactant. Bacterial suspensions were prepared using the Prompt Inoculation System and inoculated using the RENOK inoculator system. The plates were then incubated for 16 to 20 hrs. Results were read automatically using WalkAway *plus* system. The antimicrobial susceptibility was interpreted following the CLSI guidelines.[9]

Quality control

E. coli ATCC 25922, *P. aeruginosa* ATCC 27853, and *E. coli* NCTC 13846 were utilized as quality control strains. The control strains were required to produce MIC values within the specified ranges. The expected MIC ranges were 0.25-2 mg/L for *E. coli* ATCC 25922, 0.5-4 mg/L for *P. aeruginosa* ATCC 27853,[9] and 2-8 mg/L for *E. coli* NCTC 13846.[18]

Detection of *mcr*-1 gene in colistin-resistant *E. coli* and *K. pneumoniae*

The presence of *mcr-1* gene in isolates displaying MIC ≥2 mg/L by rBMD was detected by PCR. Bacterial crude DNA was extracted by boiling methods. A pair of specific primers, CLR-F (5'CGGTCAGTCCGTTTGTTC'3) and CLR-R (5'CTTGGTCGGTCTGTAGGG'3) were used for amplification.[4] The PCR reaction was performed in a total volume of 25 µL, consisting of 1× DreamTaq Green PCR Master Mix (Thermo Scientific™, USA), each primer at a concentration of 1 µM, and 1 µL of the boiled DNA template. The PCR conditions included an initial denaturation at 95 °C for 20 sec, denaturation at 95 °C for 10 sec, annealing at 64 °C for 30 sec, extension at 72 °C for 30 sec, repeated for 30 cycles, followed by a final extension at 72 °C for 5 min. The PCR products were analyzed using agarose gel electrophoresis with a 1.0% (w/v) gel. In this assay, crude DNA derived from *E. coli* NCTC 13846 was used as the positive control, and distilled water served as the negative control.

Statistical analysis

The colistin resistance test results from the RPNP and MicroScan methods for *E. coli* and *K. pneumoniae* isolates were analyzed and compared with the rBMD method. VME were defined as the percentage of instances where the rBMD method indicated resistance, but the RPNP/MicroScan systems showed intermediate results. ME referred to the percentage of isolates tested where the rBMD method indicated intermediate resistance, but the RPNP/MicroScan system reported resistance. CA were defined as the percentage of test results that match between the rBMD method and the RPNP/MicroScan methods. A high CA indicates that the test methods produce results consistent with the reference method, demonstrating their reliability.[19]

Results

By using rBMD, 97 isolates (61.4%) were identified as intermediately susceptible to colistin, and 61 isolates (38.6%) were classified as colistin-resistant. Among these, 13 *E. coli* and 48 *K. pneumoniae* isolates were found to be colistin-resistant (Figure 1). Among the isolates with MICs ≥2 mg/L, five colistin-resistant *E. coli* isolates (MIC=8 mg/L) were positive for *mcr-1*, whereas none of the *K. pneumoniae* isolates tested positive.

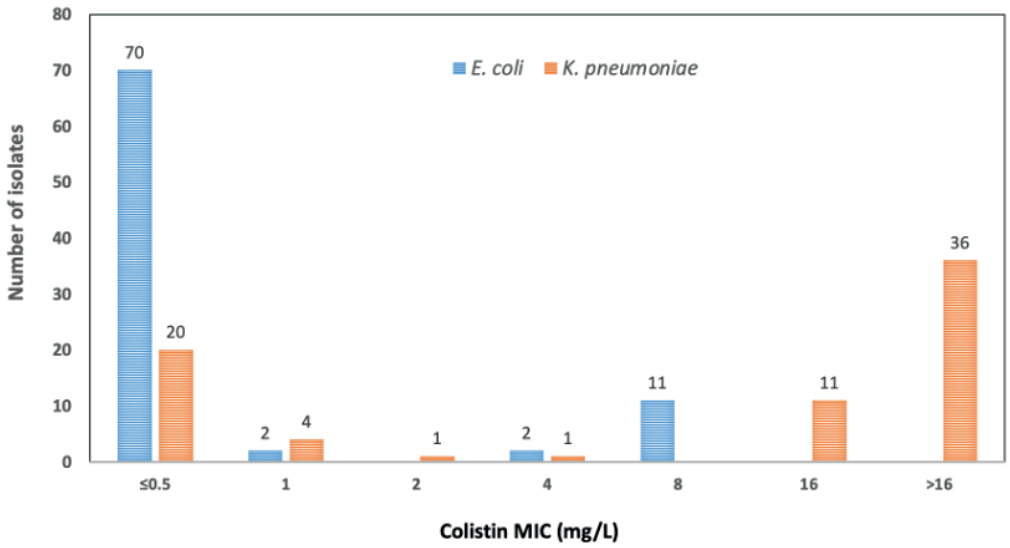


Figure 1. *Colistin MIC for 158 isolates of E. coli and K. pneumoniae determined by rBMD.*

The RPNP yielded negative results in 94 isolates (59.5%) and positive results in 64 isolates (40.5%), with 12 *E. coli* isolates and 52 *K. pneumoniae* isolates testing positive. Meanwhile, the MicroScan system detected colistin intermediate in 98 samples (62.0%) and colistin resistance in 60 samples (38.0%), including 12 *E. coli* isolates and 48 *K. pneumoniae* isolates (Table 1).

When comparing the colistin susceptibility results and MIC values between the RPNP and the rBMD, RPNP demonstrated agreement with rBMD in 153 isolates, achieving a CA of 96.8% (Tables 1 and 2). Of the tested isolates, 60 were categorized as colistin-resistant and 93 as colistin-intermediate. However, discrepancies were observed in four *K. pneumoniae* isolates, where rBMD classified them as intermediate (MIC ≤0.5-2 mg/L), but the RPNP test yielded resistant results, suggesting potential overestimation of resistance by the RPNP method (Table 3).

The MicroScan system also showed a CA of 96.8%. The concordant results included 58 isolates as colistin-resistant and 95 as colistin-intermediate by both MicroScan and rBMD. However, discrepancies were noted in four *K. pneumoniae* isolates where colistin susceptibility results from MicroScan systems were inconsistent with those from the rBMD method (Table 3). In two of these isolates, colistin resistance was detected by the MicroScan system (MIC >4 mg/L), but rBMD was intermediate (MIC ≤0.5 mg/L). Conversely, in two other *K. pneumoniae* isolates, the rBMD method identified them as resistant (MIC ≥16 mg/L), while the MicroScan system yielded intermediate results (MIC ≤2 mg/L).

Additionally, in one *E. coli* isolate, colistin resistance was detected using the rBMD method (MIC=4 mg/L), but both the RPNP and MicroScan systems returned negative results.

Table 1. Results of RPNP and MicroScan system for the detection of susceptibility to colistin among *Enterobacteriaceae*.

rBMD Colistin MIC (mg/L)	N		RPNP		MicroScan system		
			Pos	Neg	>4	4	≤2
>16	36	Resistance	36	0	35	0	1
16	11		11	0	10	0	1
8	11		11	0	9	2	0
4	3		2	1	1	1	1
2	1	Intermediate	1	0	0	0	1
1	6		2	4	0	0	6
≤0.5	90		1	89	2	0	88

***Note**: MIC: minimum inhibitory concentration, RPNP: rapid polymyxin NP test, rBMD, reference broth microdilution, Pos: positive, Neg: negative.*

Table 2. Performance of RPNP and MicroScan system to detect colistin resistance among *Enterobacteriaceae* (N=158) compared to rBMD.

Method	Genera	Performance (%)						
		Sensitivity	Specificity	PPV	NPV	CA	ME	VME
RPNP	Total	98.4	95.9	93.8	98.9	96.8	4.1	1.6
	E. coli	92.3	100.0	100.0	98.6	98.8	0.0	7.7
	K. pneumoniae	100.0	84.0	92.3	100.0	94.5	16.0	0.0
MicroScan	Total	95.1	97.9	96.7	96.9	96.8	2.1	4.9
	E. coli	92.3	100.0	100.0	98.6	98.8	0.0	7.7
	K. pneumoniae	95.8	92.0	95.8	92.0	94.5	8.0	4.2

***Note**: RPNP: rapid polymyxin NP test, CA: categorical agreement, PPV: positive predictive value, NPV: negative predictive value, ME: major error, VME: very major error.*

Table 3. Discrepancies observed among *Enterobacteriaceae* with RPNP and MicroScan system compared to rBMD.

Species	rBMD		RPNP	MicroScan (mg/L)	Category error
	MIC (mg/L)	Result			
K. pneumoniae K4	≤0.5	I	Neg	>4	Major error
K. pneumoniae K47	≤0.5	I	Pos	>4	
K. pneumoniae K40	1	I	Pos	≤2	
K. pneumoniae K44	1	I	Pos	≤2	
K. pneumoniae K60	2	I	Pos	≤2	
E. coli E21	4	R	Neg	≤2	Very major error
K. pneumoniae K26	16	R	Pos	≤2	
K. pneumoniae K19	>16	R	Pos	≤2	

***Note**: MIC: minimum inhibitory concentration, RPNP: rapid polymyxin NP test, rBMD: reference broth microdilution, Pos: positive, Neg: negative, I: intermediate, R: resistance.*

Discussion

Detecting colistin resistance in *Enterobacteriaceae* poses significant challenges due to the variability in resistance mechanisms, such as the presence of *mcr* genes and modifications of lipid A in the bacterial outer membrane. These factors complicate the accuracy and reliability of phenotypic testing methods, thereby impacting treatment decisions and patient outcomes. This study aimed to evaluate the performance of the RPNP test and MicroScan system for detecting colistin susceptibility in *Enterobacteriaceae* isolates, using rBMD as the gold standard. The findings revealed notable levels of CA for both methods, with RPNP and MicroScan systems achieving a CA of 96.8%. This agreement underscores the reliability of these methods for colistin susceptibility testing in clinical settings.

RPNP quickly detects colistin resistance by identifying the pH changes due to glucose metabolism. Resistant bacteria metabolize glucose in the presence of colistin, causing a color change from orange to yellow, with results available within 2-4 hrs.[10] In this study, RPNP demonstrated high sensitivity (98.4%), with a positive predictive value (PPV) of 93.8% and a negative predictive value (NPV) of 98.9%. However, it exhibited an error rate of 4.1%, particularly in strains with MIC values in the borderline range (0.5-4 mg/L). Several possible explanations for the error observed in the RPNP method include difficulty in interpreting color changes, especially when they are subtle. This can lead to the misclassification of results, particularly near MIC breakpoints. The inoculum effect is another factor, as variations in the bacterial concentration can affect the pH change and subsequent color shift, leading to false positives or negatives. Additionally, borderline MIC values near the interpretive thresholds (such as MICs of 0.5-4 mg/L, as found in our results) can cause discrepancies and contribute to the observed error. Previous studies have highlighted similar limitations of in-house and commercial RPNP tests, suggesting that these challenges are consistent across different testing settings.[20]

The MicroScan antimicrobial susceptibility testing system is an automated platform designed to identify bacteria and determine their antimicrobial susceptibility profiles. Broth microdilution technology provides MIC values by detecting bacterial growth in the tested panel. In this study, the MicroScan system exhibited VME and ME rates of 4.9% and 2.1%, respectively. Comparatively, Jayol *et al*. reported a higher accuracy for MicroScan in detecting colistin resistance in *Enterobacteriaceae*, with no VME and an ME rate of 3.1%, suggesting possible variability in performance due to differences in study populations or methodologies.[16] The differences in results between our study and previous studies could be attributed to several factors. First, variations in sample size, source, and isolate composition may have played a role, with previous studies potentially including fewer strains with borderline MIC values. Second, differences in the prevalence of resistance mechanisms between studies could impact the ability of the system to detect resistance with consistent accuracy. A notable limitation of the MicroScan panel is its restricted testing range for colistin concentrations, which is confined to 2 and 4 mg/L. This narrow range makes it difficult to accurately determine the MIC values for isolates with MICs outside this range, potentially leading to under- or overestimation of resistance. As such, the MicroScan system may be more suitable as a screening tool rather than for comprehensive MIC determination. These limitations reduce the panel's utility for monitoring resistance trends or validating results in clinical settings where precise MIC data are critical for guiding treatment decisions. To improve the accuracy of MicroScan, laboratories may consider using confirmatory testing such as rBMD and colistin broth disk elution (CBDE) with a broader concentration range. Expanding the MIC dilution range in automated systems may also strengthen the reliability of colistin susceptibility reporting.

We performed PCR to detect the *mcr-1* in isolates with MIC ≥2 mg/L, focusing on those with phenotypic evidence of colistin resistance or reduced susceptibility. In this study, *mcr-1* gene was detected in five *E. coli* isolates but was absent in *K. pneumoniae*. This finding aligns with previous studies in Thailand, which reported a higher prevalence of *mcr-1* in *E. coli* (29.7 %) compared to *K. pneumoniae* (1.4 %), suggesting that plasmid-mediated colistin resistance may be more common in *E. coli* in Thailand.[21] All five *mcr-1*-harboring

E. coli isolates in our study exhibited colistin MIC values of 8 mg/L as determined by rBMD and were identified as colistin-resistant by both the RPNP and MicroScan methods. This finding aligns with most studies reporting MIC values for colistin in *mcr-1*-positive *E. coli* typically ranging from 4 or 8 mg/L.[5,22,23] Moreover, *mcr-1*-positive *E. coli* can also display low or no phenotypic resistance.[24] While the clinical relevance of polymyxin therapy for *mcr*-positive isolates remains uncertain, it is important to recognize that *mcr-1* may facilitate the selection of chromosomal mutations, potentially leading to high-level colistin resistance (HLCR).[25] This study focused on *mcr-1* due to its predominant prevalence in *Enterobacteriaceae.* However, other *mcr* variants (*e.g*., *mcr-2, 3, 8* and *9*) are acknowledged in Thailand.[26,27] These variants may confer low- to moderate-level colistin resistance, often with MIC values ranging from 2 to 8 mg/L. However, the phenotypic expression associated with these genes can be variable, resulting in minimal or no detectable resistance. Thus, reliance on phenotypic detection alone may be insufficient. Enhancing laboratory protocols to include targeted testing of isolates with borderline MIC values (e.g., 2 mg/L) using complementary methods or molecular detection of *mcr* genes is crucial for accurate and comprehensive resistance monitoring.

The limitations of this study include the relatively small sample size and collection of isolates from only one hospital. Moreover, the molecular mechanisms underlying colistin resistance have not yet been determined. This might limit the application of our findings to other geographical areas because of the differences in the mechanism of antimicrobial resistance. Another notable limitation of this study was the limited number of isolates with MIC values at borderline interpretive thresholds (e.g., 1-2 mg/L). This could affect the robustness of the data in evaluating the performance of the RPNP and MicroScan systems in accurately detecting resistance within these critical ranges. Future studies with a greater representation of borderline MIC values would help to provide a more comprehensive understanding of the reliability of each method under these conditions.

Conclusion

This study provides critical insights into the performance of different methods for detecting colistin resistance in *Enterobacteriaceae* isolates, including RPNP test, MicroScan, and rBMD methods. The RPNP and MicroScan systems both demonstrated high CA (96.8%) with the rBMD method, underscoring their reliability as practical tools for routine colistin susceptibility testing in clinical microbiology laboratories. However, with 4.12% ME for RPNP and 4.92% VME for MicroScan, this study highlights the challenges of relying solely on these methods. Discrepancies frequently arise when MIC values are near the interpretive threshold, and the limited testing range of the MicroScan system further complicates accurate resistance detection. Therefore, while these methods are suitable for initial screening, confirmation with rBMD or expanded testing protocols is crucial for borderline isolates (2-4 mg/L). Nevertheless, the small sample size, particularly with borderline MIC values, and the lack of a detailed investigation into the resistance mechanisms are key limitations of this study, highlighting the need for further study.

Conflict of interest

The authors declare that there is no conflict.

Ethical approval

This study was conducted in accordance with the ethical guidelines approved by The Human Research Ethics Committee of Thammasat University No.3 Sciences (COE 017/2565).

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