Original Article

A study of standard values of two-dimensional, M-mode and doppler echocardiography in conscious small domestic rabbits

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Abstract

To determine normal cardiac values for two-dimensional, M-mode and Doppler echocardiography because standard echocardiographic values have not been adequately studied in small-breed rabbits. Data was collected from 40 healthy small-breed rabbits. The results of complete physical examination, thoracic radiography, electrocardiography and echocardiography were collected and analysed as descriptive statistics. The correlation between weight and age and echocardiographic parameters was evaluated. A t-test was performed to compare the parameters between males and females. Most parameters were normally distributed and could be reliably used as standard values of echocardiographic parameters. Weight and age correlated positively with several echocardiographic parameters. However, there was no statistically significant difference between male and female rabbits. The normal value of echocardiographic parameters was established for small-breed rabbits.

Keywords: Cardiology, Echocardiography, Exotic pet, Heart, Rabbit

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Introduction

Nowadays, small-breed rabbits (such as Lion Heads, Mini Rex, Netherland Dwarf and Holland Lop) are becoming more popular as pets in Thailand. In addition, rabbits are living longer because of improved care and diagnostic technology has evolved so that diseases and abnormalities are increasingly better detected than in the past (Orcutt and Malakoff, 2020). Consequently, the number of hospitalisations from various causes, including cardiovascular disease, is increasing (Weerakhun, 2015).

Although there are few epidemiological studies of cardiovascular disease in rabbits in Thailand, an epidemiological study in the United States (Ozawa *et al.*, 2021) has shown that the most common cardiovascular diseases diagnosed by echocardiography are degenerative valve disease and dilated cardiomyopathy. Other factors, such as malnutrition, obesity and stress, can also cause cardiovascular problems (Praag, 2020). The prevalence of cardiovascular disease is 2.6% and is likely to increase. Although the incidence of cardiovascular disease in rabbits generally increases with age, especially in rabbits older than 4 years, the disease can also occur in rabbits at a young-age (Orcutt and Malakoff, 2020).

The diagnosis of cardiovascular disease is based on history, clinical signs, physical examination and appropriate diagnostic tests, including thoracic radiography, electrocardiography and echocardiography (Orcutt and Malakoff, 2020). However, thoracic radiography cannot determine cardiac function and can only assess the silhouette of the heart and the thoracic cavity. In addition, the interpretation of radiographic results can be difficult in small rabbits due to the small size of the thorax (Pariaut, 2009). On the other hand, electrocardiography can reveal the electrical cardiac function but not the cardiac structure and mechanical function. Therefore, echocardiography is considered as the diagnostic test of choice for detecting heart disease because it can assess both cardiac structure and function with high sensitivity and accuracy (Orcutt and Malakoff, 2020).

Studies on the standard values of echocardiography in rabbits, especially in small rabbits, are insufficient as they only include New Zealand white (Giannico *et al.*, 2015) or French Lop and Dwarf Lop rabbits (Casamian-Sorrosal *et al.*, 2014), which are medium to large-breed rabbits. To the authors' knowledge, the echocardiographic reference range for small-breed rabbits has not been created yet.

In this context, the aim of this study was to determine the normal value for two-dimensional, M-mode and Doppler echocardiography in small-breed rabbits. This data will be useful in improving cardiovascular disease diagnosis and treatment planning in small-breed rabbits.

Materials and Methods

Ethics approval and consent to participate: The study was prospective. The protocol of this study was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (protocol no. 2031075).

Study location and period: This study was conducted at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand. Informed consent forms were signed by all rabbit owners. General history, physical examination, radiography, electrocardiography and echocardiography data of 40 healthy small-breed rabbits was collected from 9 June 2020 to 20 October 2021.

Animals: Forty healthy small-breed rabbits, 27 males and 13 females, were recruited from pet owners. The criteria used for recruiting the rabbits were an age of 8 months to 4 years, a weight of less than 2 kilograms, various breeds and a healthy status (without signs or history of cardiovascular or other systemic diseases). Data from each rabbit was recorded, including age, breed, sex, neutering status, weight and body condition score. All rabbits were examined by a veterinarian specialising (TL) in exotic pets. Physical examination was performed to determine the clinically normal health status. Radiography, electrocardiography and echocardiography were performed without sedation or general anaesthesia.

Thoracic Radiography: Two standard projections (right lateral view and ventrodorsal view) of radiographs were performed. Field of view of thoracic radiographs included all ribs, the diaphragm and the cranial abdomen. For the lateral projection, the rabbits were placed in the right lateral position with the thoracic limbs extended cranially. In addition, for ventrodorsal projection, the rabbits were also placed in the supine position with the forelimbs extended cranially to prevent superimposition of the muscles over the thoracic cavity (Giannico *et al.*, 2015). The vertebral heart score was performed according to the method previously described by Moarabi *et al.* (2015).

Electrocardiography: The rabbits were restrained in the right lateral position, and small clip electrodes were attached to the skin folds of both elbows and stifles. An electrocardiography consisting of six leads (I, II, III, aVR, aVL, and aVF) was recorded. Only rabbits with normal sinus rhythm were included in the study.

Echocardiography: Echocardiography was performed by an experienced ultrasonographer (SS), using an ultrasound (machine M9, Mindray, China) with a 4-12-MHz phased-array transducer to examine the structure and function of the heart. The rabbits were placed in the lateral position and shaved on both sides of the thoracic area on which the ultrasound probe was placed for examination.

Two-dimensional echocardiography measured the diameter of the left atrium (LA) and aorta (Ao) during the first diastole of aortic valve closure at the level of the aorta and aortic valves in the right parasternal short-axis view. The ratio of left atrial to aorta diameter (LA/Ao) was calculated automatically by the software of the ultrasound system.

M-mode echocardiography was performed to measure chamber size and wall thickness in the right parasternal long-axis four-chamber view. The M-mode cursor was placed at a right angle to the interventricular septum and the left ventricular wall below the edge of the mitral valves when the ventricular chamber size was largest. Echocardiographic parameters including the interventricular septal thickness at end diastole (IVSd) and systole (IVSs), left ventricular internal dimension at end diastole (LVIDd) and systole (LVIDs), and left ventricular posterior wall thickness at end diastole (LVPWd) and systole (LVPWs) were measured. Fractional shortening (FS) was measured and calculated by the internal ultrasound system software. Left atrial (LA) and aorta diameters (Ao) were measured at the level of the aortic valve. The ratio of left atrial to aorta diameter (LA/Ao) was calculated.

During Doppler echocardiography, transmitral flow velocity was measured in the left apical four-chamber view. Transmitral flow velocity was measured when the mitral valve was wide open, and the gate was placed at the tips of the valve leaflets. The following parameters included early diastolic transmitral flow peak velocity (E), late transmitral flow peak velocity (A) and the ratio between peak early diastolic and late diastolic transmitral flow velocities (E/A) was reported. Isovolumetric relaxation time (IVRT) was measured by placing the gate in the left ventricular outflow tract near the mitral valve leaflet in the left apical five-chamber view. Aortic flow velocity was also measured in the left apical five-chamber view and expressed as the maximum velocity of aortic outflow (AV). Assessment of the pulmonary vein and the pulmonary artery flow was performed in the right parasternal short-axis view. The maximum velocity of pulmonary outflow (PV), peak velocity of systolic and diastolic pulmonary vein flow (PVs, PVd), peak velocity and duration of reversal flow during atrial contraction (PVa, PVadur) were recorded. The ratio of systolic to diastolic pulmonary vein flow velocity (PVs/d) was calculated. Tissue Doppler measurements were performed and assessed along the longitudinal axis of the heart and assessed by placing the gate on the subendocardial regions of the lateral corner of the mitral annulus. The peak velocity of systolic mitral annular motion (S′), the peak velocity of early and late diastolic tissue motion (E′, A′), the ratio of the peak velocity of early to late diastolic tissue motion (E′/A′) and the peak velocity of early diastolic transmitral flow to peak velocity of early diastolic tissue motion (E/E′) were recorded.

Statistical analysis: Statistical analyses were performed with the statistical software program SPSS IBM SPSS (Statistics ver.28.0.0., Inc, Chicago, IL, USA). The Kolmogorov-Smirnov test was applied to demonstrate normality of the data. Descriptive statistical analyses were calculated for each parameter as minimum, maximum, mean and standard deviation (SD). In addition, the echocardiographic parameters were converted to normalised values, which were obtained by analysing the mean and SD of each echocardiographic value divided by the body weight of each rabbit. Differences in echocardiographic parameters between males and females were assessed with the independent sample t-test. In addition, Pearson's correlation between weight and age with each echocardiographic variable was reported. All statistical analyses were considered significant when $p < 0.05$.

Results

Forty rabbits recruited to the study were 16 Holland Lop (40%), 12 Netherland Dwarf (30%), 4 Mini Rex (10%), 1 Teddy Bear (2.5%) and 7 mixed breed (17.5%). 67.5% of the rabbits were males [24 intact males (60%) and 3 castrated males (7.5%)]. The 13 females (32.5%) [11 intact females (27.5%) and 2 neutered females (5%)]. The age and weight of the rabbits was recorded and expressed as minimum, maximum, mean values and standard deviation (SD). The minimum and maximum values for age were 8 and 48 months, respectively. The mean age ± SD was 19.8 ± 10.6 months. The minimum and maximum weight were 0.8 and 2.0 kilograms and the mean weight ± SD was 1.5 ± 0.3 kilograms. The BCS was measured with a score of 5. The rabbits in this study had scores ranging from 2/5 (21 rabbits; 52.5%) to 3/5 (19 rabbits; 47.5%). All 40 rabbits underwent physical examination, thoracic radiography, electrocardiography and echocardiography. No rabbits had abnormal findings on physical examination. Electrocardiography revealed no cardiac arrhythmia in any rabbits.

Thoracic radiography: Thoracic radiography was measured by 3 parameters such as vertebral heart score, short axis and long axis of the heart, as shown in the Table 1. There were no remarkable abnormalities in both ventrodorsal and lateral views. However, the cranial border of the heart was difficult to measure because of the opacity of the cranial mediastinum from the fat tissue. In addition, the carina was hard to identify in lateral projection.

Table 1 Values for cardiac variables measured on a lateral view of thoracic radiography in conscious 40 rabbits

Parameter	Minimum	Maximum	Mean ± SD
RL-SA (v)	2.8	3.8	3.22 ± 0.28
RL-LA (v)	3.9	5.0	4.31 ± 0.28
VHS (v)	6.8	8.7	7.55 ± 0.49

RL-LA, long axis of right lateral view; RL-SA, short axis of right lateral view; VHS, Vertebral Heart Scale

Echocardiography: Prior to echocardiography, all rabbits were in good health. As shown by the results of echocardiographic measurements and normalised values in Table 2 and Table 3, respectively, the influences of sex, age and weight on echocardiographic parameters were investigated. There was a significant correlation between the independent variables and the echocardiographic parameters, as shown in Table 4 and 5. In addition, there were no strong correlations between each variable. The independent sample t-test was performed to compare the echocardiographic values between males and females; the values were not significantly different.

Table 2 Values for echocardiographic 2-dimensional, M-mode and Doppler variables measured in 40 conscious rabbits

Parameter	Minimum	Maximum	Mean ± SD
2D measurements			
LA (mm)	5.0	11.6	7.48 ± 1.50
Ao (mm)	3.6	7.8	5.46 ± 0.93
LA/Ao	0.95	1.73	1.38 ± 0.19
M-mode measurements			
IVSd (mm)	1.4	3.4	2.10 ± 0.48
IVSs (mm)	1.4	5.0	2.94 ± 0.81
LVIDd (mm)	6.6	12.8	10.43 ± 1.47
LVIDs (mm)	3.3	9.0	6.69 ± 1.31
LVPWd (mm)	1.0	3.7	1.95 ± 0.56
LVPWs (mm)	1.6	4.8	2.95 ± 0.72
FS (%)	20.79	54.29	36.23 ± 7.29
LA (mm)	5.4	11.9	8.27 ± 1.41
Ao (mm)	4.3	7.8	5.81 ± 0.74
LA/Ao	0.98	1.93	1.43 ± 0.22
Doppler measurements			
E (cm/s)	37.84	110.39	66.10 ± 17.58
A (cm/s)	34.27	87.94	57.21 ± 15.08
E/A	0.72	29.00	2.17 ± 4.70
IVRT (ms)	23	61	37.24 ± 7.51
AV (cm/s)	11.03	107.64	67.92 ± 16.81
PV (cm/s)	42.84	104.24	63.92 ± 12.24
PVs (cm/s)	24.99	52.83	37.31 ± 7.41
PVd (cm/s)	23.20	47.84	34.84 ± 6.79
PVa (cm/s)	7.85	25.13	14.01 ± 4.14
PVadur (ms)	23	79	44.08 ± 13.74
PVs/d	0.74	1.47	1.09 ± 0.21
TDI measurements			
S′ (cm/s)	4.77	10.53	7.23 ± 1.61
E′ (cm/s)	4.47	14.41	9.16 ± 2.40
A′ (cm/s)	3.88	12.52	7.29 ± 2.14
E′/A′	0.69	2.02	1.30 ± 0.30
E/E′	4.15	15.89	7.52 ± 2.29

A, peak velocity of late transmitral flow; A′, peak velocity of diastolic mitral annular motion as determined by pulsed wave Doppler; Ao, Aorta; AV, the maximum velocity of aortic outflow; E, peak velocity of early diastolic transmitral flow; E′, peak velocity of early diastolic mitral annular motion as determined by pulsed wave Doppler; E/A, ratio of E to A dimensionless; E′/A′, ratio of E′ to A′ dimensionless; E/E′, ratio of E to E′ dimensionless; FS, fractional shortening; HR, heart rate; IVRT, isovolumic relaxation time; IVSd, interventricular septum thickness at end diastole; IVSs, interventricular septum thickness at end-systole; LA, left atrium; LA/Ao, Ratio of the left atrial dimension to the aortic annulus dimension dimensionless; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at end systole; LVPWd, left ventricular posterior wall thickness at end diastole; LVPWs, left ventricular posterior wall thickness at end-systole; PV, the maximum velocity of pulmonary outflow; PVa, pulmonary vein flow reversal at atrial contraction; PVadur, pulmonary vein flow reversal at atrial contraction duration; PVd, peak velocity of diastolic pulmonary vein flow; PVs, peak velocity of systolic pulmonary vein flow; PVs/d, the ratio of peak velocity of systolic to diastolic pulmonary vein flow; S′, Peak velocity of systolic mitral annular motion as determined by pulsed wave Doppler; SD, standard deviation; TDI, tissue Doppler imaging

Table 3 Normalized echocardiographic parameters by weight

Parameter	Mean ± SD
2D measurements	
LA (mm)	5.55 ± 1.41
Ao (mm)	4.04 ± 0.96
LA/Ao	1.04 ± 0.32
M-mode measurements	
IVSd (mm)	1.54 ± 0.41
IVSs (mm)	2.13 ± 0.59
LVIDd (mm)	7.83 ± 2.00
LVIDs (mm)	5.06 ± 1.62
LVPWd (mm)	1.41 ± 0.36
LVPWs (mm)	2.15 ± 0.54
FS (%)	26.74 ± 7.63
LA (mm)	6.17 ± 1.59
Ao (mm)	4.29 ± 0.90
LA/Ao	1.09 ± 0.38
Doppler measurements	
E (cm/s)	48.79 ± 13.01
A (cm/s)	41.56 ± 11.12
E/A	1.50 ± 2.82
IVRT (ms)	28.49 ± 11.07
AV (cm/s)	50.53 ± 13.68
PV (cm/s)	48.04 ± 13.94
PVs (cm/s)	28.04 ± 9.04
PVd (cm/s)	25.75 ± 6.87
PVa (cm/s)	10.45 ± 3.99
PVadur (ms)	34.43 ± 17.09
PVs/d	0.83 ± 0.32
TDI measurements	
S′ (cm/s)	5.34 ± 1.41
E′ (cm/s)	6.77 ± 2.14
A′ (cm/s)	5.30 ± 1.60
E′/A′	0.99 ± 0.40
E/E′	5.52 ± 1.95

A, peak velocity of late transmitral flow; A′, peak velocity of diastolic mitral annular motion as determined by pulsed wave Doppler; Ao, Aorta; AV, the maximum velocity of aortic outflow; E, peak velocity of early diastolic transmitral flow; E′, peak velocity of early diastolic mitral annular motion as determined by pulsed wave Doppler; E/A, ratio of E to A dimensionless; E′/A′, ratio of E′ to A′ dimensionless; E/E′, ratio of E to E′ dimensionless; FS, fractional shortening; HR, heart rate; IVRT, isovolumic relaxation time; IVSd, interventricular septum thickness at end diastole; IVSs, interventricular septum thickness at end-systole; LA, left atrium; LA/Ao, Ratio of the left atrial dimension to the aortic annulus dimension dimensionless; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at end systole; LVPWd, left ventricular posterior wall thickness at end diastole; LVPWs, left ventricular posterior wall thickness at end-systole; PV, the maximum velocity of pulmonary outflow; PVa, pulmonary vein flow reversal at atrial contraction; PVadur, pulmonary vein flow reversal at atrial contraction duration; PVd, peak velocity of diastolic pulmonary vein flow; PVs, peak velocity of systolic pulmonary vein flow; PVs/d, the ratio of peak velocity of systolic to diastolic pulmonary vein flow; S′, Peak velocity of systolic mitral annular motion as determined by pulsed wave Doppler; SD, standard deviation; TDI, tissue Doppler imaging

Table 4 Correlation analysis for age with the echocardiographic parameters in 2-dimensional, M-mode and Doppler echocardiography in 40 rabbits

Parameter	*r*	*p*-value
HR	0.075	0.644
LA (2D)	0.052	0.752
Ao (2D)	0.146	0.370
LA/Ao (2D)	-0.129	0.427
IVSd	0.367*	0.020
IVSs	0.456**	0.003
LVIDd	-0.199	0.217
LVIDs	-0.415**	0.008
LVPWd	0.299	0.061
LVPWs	0.347*	0.028
FS	0.524**	<0.001
LA (M-mode)	-0.007	0.966
Ao (M-mode)	0.272	0.090
LA/Ao (M-mode)	-0.206	0.203
E	-0.073	0.657
A	0.177	0.274
E/A	0.073	0.653
IVRT	-0.174	0.283
AV	0.101	0.534
PV	0.111	0.495
PVs	0.077	0.635
PVd	-0.066	0.685
PVa	0.073	0.656
PVa dur	-0.335*	0.034
PVs/d	0.142	0.381
S′	0.094	0.563
E′	-0.199	0.219
A′	-0.011	0.949
E′/A′	-0.206	0.202
E/E′	0.230	0.153

A, peak velocity of late transmitral flow; A′, peak velocity of diastolic mitral annular motion as determined by pulsed wave Doppler; Ao, Aorta; AV, the maximum velocity of aortic outflow; E, peak velocity of early diastolic transmitral flow; E′, peak velocity of early diastolic mitral annular motion as determined by pulsed wave Doppler; E/A, ratio of E to A dimensionless; E′/A′, ratio of E′ to A′ dimensionless; E/E′, ratio of E to E′ dimensionless; FS, fractional shortening; HR, heart rate; IVRT, isovolumic relaxation time; IVSd, interventricular septum thickness at end diastole; IVSs, interventricular septum thickness at end-systole; LA, left atrium; LA/Ao, Ratio of the left atrial dimension to the aortic annulus dimension dimensionless; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at end systole; LVPWd, left ventricular posterior wall thickness at end diastole; LVPWs, left ventricular posterior wall thickness at end-systole; PV, the maximum velocity of pulmonary outflow; PVa, pulmonary vein flow reversal at atrial contraction; PVadur, pulmonary vein flow reversal at atrial contraction duration; PVd, peak velocity of diastolic pulmonary vein flow; PVs, peak velocity of systolic pulmonary vein flow; PVs/d, the ratio of peak velocity of systolic to diastolic pulmonary vein flow; S′, Peak velocity of systolic mitral annular motion as determined by pulsed wave Doppler; SD, standard deviation; TDI, tissue Doppler imaging

*Correlation values are significantly ($p < 0.05$) related to the echocardiographic parameter

**Correlation values are highly significantly ($p < 0.01$) related to the echocardiographic parameter

Discussion

The normal echocardiographic values in 40 healthy small-breed rabbits were determined in this study. Rabbits aged of 8 months to 4 years, all of which were adults, were recruited for the study. A rabbit usually reaches puberty between the age of 3 to 8 months depending on its genetic make up, sex and environment (McClure, 2020). The 27 male and 13 female rabbits had no signs or history of cardiovascular or systemic disease and belonged to popular breeds in Thailand, such as Holland Lop, Netherland Dwarf, Mini Rex and Teddy Bear.

During electrocardiography, there were problems with restraining; as a result, the electrocardiographic signals of the rabbits were distorted by noise, and parameters such as amplitude and interval could not be interpreted clearly.

Thoracic radiography is a widely used diagnostic tool to visualise the structure of the thoracic region, including the cardiovascular system. In general, cardiac size can be assessed by thoracic radiography via measuring the size of the heart compared with the long axis of the thoracic vertebrae, which is also known as the vertebral heart score (VHS). In this study, the mean ± SD of the VHS was 7.55±0.49, which is consistent with a previous report in the New Zealand white rabbit (mean ± SD, 7.60±0.39) (Giannico *et al*., 2015). However, the measurement of the heart size by VHS in rabbits was difficult to interpret. In lateral projection, the rabbit heart likely shifted cranially and contacted the intrathoracic fat, making the cranial border unclear and difficult to define (Giannico *et al*., 2015). As mentioned previously, the interpretation of VHS in rabbit thoracic radiography could be inaccurate, and it has therefore been suggested that other diagnostic tools for cardiovascular disease, such as echocardiography, should be used in conjunction with thoracic radiography.

Echocardiography parameters could adequately be properly evaluated because the rabbits could be sufficiently restrained. A total of 40 rabbits were examined by only one specialised ultrasonographer (SS) to avoid variation and bias. Differences in heart rate and echocardiographic parameters have been found between studies using anesthetised and non-anesthetised rabbits (Giannico *et al.*, 2015) which suggests that the use of sedative or anaesthetic agents can alter cardiovascular parameters (Giannico *et al.*, 2015). Therefore, echocardiographic examination in this study was performed without anaesthetic agents to mimic the real procedure in the small animal hospital. Electrocardiography was not performed during the echocardiographic examination because the rabbits were uncomfortable with the electrocardiographic electrodes applied during the procedure without anesthesia.

Statistical analysis of echocardiographic parameters showed that there were no differences in any echocardiographic values between male and female rabbits. However, there were weak to moderate correlations between weight and age with echocardiographic parameters. Precisely, there were positive correlations between weight and HR, LA, Ao, IVSd, IVSs, LVIDd, LVPWd, LVPWs, E, A, AV, PVd, S′ and A′. However, weight was negatively correlated with PVadur. On the other hand, age was positively correlated with IVSd, IVSs, LVPWs and FS, but showed a negative correlation with LVIDs and PVadur. There was no strong correlation between age and weight and any variables. Due to the correlation between weight and some echocardiographic variables, the normal values normalised with body weight were created.

We are confident that our results and measurements represents the healthy population of small-breed rabbits in Thailand. The number of 40 rabbits recruited for the study was based on a previous study which reported normal echocardiographic values in domestic pet rabbits (Cassamian-Sorrosal *et al.*, 2014). The different breeds of rabbits may affect the echocardiographic values and it is therefore recommended to create breed-specific reference values for a more accurate evaluation.

This study has some limitations. Only adult rabbits with no history of disease and no evidence of cardiovascular disease were studied and the echocardiographic parameters of rabbits with diseases were not examined to compare their results with those of the normal population. Therefore, it is recommended that diseased rabbits should be examined in future studies. Another limitation is that the age and breed of the samples were varied. However, the analysed data was normally distributed according to the Kolmogorov-Smirnov test.

The echocardiographic values in this study were obtained from 40 conscious small-breed rabbits to determine the normal range for echocardiographic parameters in small-breed rabbits. The influences of weight and age on echocardiographic parameters were also investigated. In contrast, echocardiographic parameters were not influenced by sex. The data from this study can be used as normal values for echocardiographic evaluation in small-breed rabbits.

Author Contribution: Nattanicha Ngosurachet, Tipparat Tangtraitha, Preeyanit Kaewyarat: Performing experiments, collecting data, analysing data and writing first draft. Pattharat Asvaesoontorn: collecting samples. Thawat Lekdumrongsak, Sirilak Disatian Surachetpong: Supervising, performing experiments, validating data, reviewing and editing. All authors read and approved the final manuscript.

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