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Growth Inhibition of *Syzygium campanulatum* Korth. for Container Planting by the Application of Uniconazole

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ABSTRACT

This study was carried out to determine the optimal dosage of a plant growth regulator, uniconazole, for controlling the growth of *Syzygium campanulatum* for container planting purposes. Uniconazole at ascending rates of 0, 10, 20, and 30 mgl^{-1} was applied as soil drench to plants grown in polyethylene bags (33 x 27 cm). The application of uniconazole significantly inhibited vegetative growth in terms of height and leaf area. Meanwhile, the most effective application rate of uniconazole for height suppression was 10 mgl^{-1}. The transpiration rate and stomatal conductance of the plants, treated with 30 mgl^{-1}, were slightly lower as compared to the control plants, while the photosynthetic rate was not affected. However, the chlorophyll fluorescence measurement indicated that the application of uniconazole did not affect the photosynthetic performance of this particular species. Uniconazole was able to extend the trimming cycle and would be very helpful in controlling the height of *S. campanulatum* without affecting the physiological processes in the plant.

Keywords: Plant growth regulator, plant physiology, container plant, chlorophyll fluorescence

INTRODUCTION

A local species, *Syzygium campanulatum* with attractive scarlet young foliage, is widely planted in urban landscapes. This species is well adapted in the harsh urban environment. However, it needs frequent trimming due to its vigorous growth. Pruning of landscape trees and shrubs to control excessive vegetative growth and improve plant form is a major expense in landscape maintenance (Keever and Foster, 1990). Meanwhile, disposal of great quantity of trimmed biomass is also a concern in certain countries (Bowles, 1985). Therefore, an alternative maintenance approach is needed to reduce time and operational cost.

Plant growth regulators have been widely used in reducing vegetative growth and increasing aesthetic value of many ornamental species (Ahmad Nazarudin *et al*., 2003; Bruner *et al*., 2001; Mike *et al*., 1999; Criley, 1997). Among the various triazoles, paclobutrazol, and uniconazole have been found to be the most effective in retarding growth in many plant species (Gilley and Fletcher, 1997). Sponsel (1995) reported that these plant growth regulators inhibited gibberellin (GA) biosynthesis by disturbing the oxidation of *ent-kaurene*. Furthermore, it

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reduces cell elongation and hence retards the plant growth (Barrett, 2001).

The growth of several woody ornamental species was consistently controlled (Wang, 1991; Bruner *et al*., 2001; Kim *et al.,* 1999) without injury after the application of uniconazole (Norcini and Knox, 1990; Warren, 1990; Keever and West, 1992). Triazole was also found to limit the rates of leaf production and leaf size (Le Cain *et al.,* 1986; Nie *et al.,* 2001). Fuller and Zajicek (1995) found that water use of plants, treated with uniconazole, was reduced by 35 % due to reduction in the leaf area and lower stomatal conductance.

The objective of the experiment was to evaluate the effects of uniconazole on the growth of *S. campanulatum.* The chlorophyll fluorescence study was carried out to confirm that uniconazole did not restrain the species to perform its physiological processes at optimal level.

MATERIALS AND METHODS

The Study Site

This study was conducted at the Forest Research Institute Malaysia (FRIM) in Kepong, Selangor. The seedlings of *S. campanulatum*, with an average height of 105 cm, were obtained from a local nursery in Yong Peng, Johor. They were one year-after planting in the polyethylene bags sized 33 x 27 cm, filled with a mixture of top soil, organic matter and sand, at a ratio of 3:2:1. A total of 16 seedlings were arranged in an open area. The plants were first trimmed to columnar shape with an approximate height of 100 cm. Uniconazole (0, 10, 20, and 30 mgl^{-1}) was applied as soil drenches after the plants had produced new shoots and recovered from the trimming effects (30 days after the trimming). Each rate of uniconazole was replicated four times in a randomised complete block design. The application volume was 1 litre per seedling. At the same time, control plants were applied with 1 litre of plain water. The plants were watered twice daily, or when necessary, depending on the weather. Nitrophoska Green, 15:15:15 (NPK Green), was applied monthly at a rate of 5 g per plant. Weeds in the polyethylene bags were controlled manually.

Data Collection and Analysis

Plant height (cm) was measured monthly, from the soil surface in the polyethylene bag to the highest shoot tip, using a telescopic height stick. Every month, the first three fully developed leaves from each plant were measured for the leaf area using the leaf area meter (Li-3100 Nebraska, USA). The average of the leaf area was recorded in square centimetres (cm^2).

Portable photosynthetic system (Li-6400, Nebraska, USA) was used to measure the photosynthetic rate, transpiration rate, and stomatal conductance. Prior to the measurements, the internal sample carbon dioxide, CO_2 concentration, was adjusted to 400 μmol m^{-2} s^{-1} and the temperature of the leaf chamber was maintained at 28 °C, while the internal radiance provided by a red LED was adjusted to 1500 μmol photon m^{-2} s^{-1} under light-saturated photosynthesis environment. Measurements were recorded at 9.00 am to 11.30 am under full sunlight. The photosynthesis and stomatal conductance were measured in mol m^{-2} s^{-1}. Meanwhile, transpiration was measured in mmol m^{-2} s^{-1}. Three fully developed leaves from each plant were selected for these measurements.

Chlorophyll fluorescence was also measured in the field at light saturation (I=100%) using a plant efficient analyzer (Hansatech Instruments Ltd., Kings Lynn, UK). The measurement was carried out at five month, after the application of uniconazole. Three fully developed leaves, from each plant, were dark adapted for 20 minutes in a leaf-exclusion clip to the central region of the leaf surface. Dark incubation of pre-illuminated leaves for 20 minutes was sufficient for the chloroplasts to return to the arrangement they had in low light. In this way, the chloroplast movement would not affect the chlorophyll fluorescence parameters. The excitation light for fluorescence was then given to the leaf disc at about 1500 μmol m^{-2} s^{-1} for 5 seconds. The measurements of F_0 (initial fluorescence), F_m (maximum fluorescence), and F_v (variable

fluorescence) were obtained from this procedure. F_v is derived as the difference between F_m and F_0 while the maximum quantum yield of PSII is obtained as F_v/F_m (Owens, 1994).

Statistical Analysis Software (SAS) was used to analyse the data. In addition, Analysis of Variance (ANOVA) was also conducted and the treatment means were then compared using the Tukey's Studentized Range (HSD) test to detect significant difference among the treatments.

RESULTS AND DISCUSSION

The treated plants were found to be significantly shorter ($p<0.01$) than the control plants (Table 1). However, there was no significant difference among the plants which were treated with different dosages of uniconazole. After five months, the height of the control plant was found to increase about 19.75%, whereas the plant which was treated with 30 mgl^{-1} uniconazole only had an increase of 1.26%. At this stage, the control plants need to be trimmed to maintain its crown form and landscape function. These results suggested that the plant height gain of *S. campanulatum* was depressed with the application of uniconazole.

Furthermore the treated plants were also found to not producing any curly leaves which would decline the aesthetic value of the plants. The leaves were, however, greener, shinier and smaller as compared to the untreated ones. Visually, the leaves of the treated plants were closely arranged and the crown was more compacted. The leaf area of the treated plants was significantly smaller ($p<0.01$) compared to the control plants (Table 2). However, no significant difference was found in leaf area among all the treated plants. At five month after the application, the leaf area of the plants treated with 30 mgl^{-1} uniconazole was reduced by 48.39%, whereas the control plants were only reduced by 13.83%. These results described the inhibition effects of uniconazole on cell elongation in the leaf. Tonkinson *et al*. (1995) reported that triazoles decreased the size of wheat leaves by the reduction of cell length.

On the contrary, no significant difference was found among all the plants in terms of photosynthetic rate after the application of uniconazole. However, a significant difference ($p<0.01$) in the transpiration rate and stomatal conductance were demonstrated between the controls and the plants which were treated with 20 and 30 mgl^{-1} uniconazole (Table 3). It showed that the transpiration rate and stomatal conductance were reduced as the uniconazole rate increased. The results also suggested that the amount of water released from the stomata was more for the control plants as compared to the treated plants, except for those treated at a rate of 10 mgl^{-1}. Wang and Lin (1992) indicated that the transpiration rate might vary following the treatment with triazoles, depending on the species. In this study, the smaller leaf area developed in the treated plants might have reduced the transpiration rate. The reduction in the transpiration rate would protect the plant against abiotic stress due to water restriction or drought period (Olsen and Andersen, 1995).

TABLE 1
The plant height of *S. campanulatum* after treatment with uniconazole

Uniconazole (mgl^{-1})	Plant height (cm)					
	Month after application					
	0	1	2	3	4	5
0	100.00a	108.25a	112.25a	114.25a	116.50a	119.75a
10	99.25a	101.50b	101.75b	102.00b	102.00b	102.50b
20	99.25a	101.25b	101.75b	102.00b	102.00b	102.50b
30	99.50a	100.50b	100.50b	100.75b	100.75b	100.75b

Means followed by the same letter(s) within the column do not differ by Tukey's Studentized Range Test at $p<0.01$

TABLE 2
The leaf area of *S. campanulatum* after treatment with uniconazole

Uniconazole (mgl^{-1})	Leaf area (cm^2)					
	Month after application					
	0	1	2	3	4	5
0	11.21a	11.06a	10.17a	9.78a	9.46a	9.66a
10	11.11a	8.42b	7.36b	6.84b	6.22b	6.32b
20	11.31a	7.92b	6.98b	6.74b	6.35b	6.06b
30	11.18a	7.25b	6.79b	6.43b	6.06b	5.77b

Means followed by the same letter(s) within the column do not differ by Tukey's Studentized Range Test at $p<0.01$

TABLE 3
The influence of uniconazole on photosynthetic rate, transpiration rate and stomatal conductance of *S. campanulatum* at five month-after the treatments

Uniconazole (mgl^{-1})	Photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$)	Transpiration rate ($mmol\ m^{-2}\ s^{-1}$)	Stomatal conductance ($mol\ m^{-2}\ s^{-1}$)
0	6.76a	2.45a	0.22a
10	5.02ab	2.15ab	0.20a
20	5.36ab	1.48bc	0.11b
30	4.29ab	1.26c	0.10b

Means followed by the same letter(s) within the column do not differ ($p<0.01$) by the Tukey's Studentized Range Test

In this study, no significant difference in F_0, F_m, F_v, F_v/F_m, and F_m/F_0 values in *S. campanulatum* were found (Table 4). The average F_v/F_m values were 0.83, showing that the plants were able to perform its physiological processes at the optimum level. According to Bjorkman and Deming (1987) and Johnson *et al.* (1993), the optimum value of 0.83 was measured for most plant species. This result showed that *S. campanulatum* was able to adapt with the application rates of uniconazole. Govindjee *et al*. (1981) reported that water is not a limiting factor for the plant's physiological processes if the F_m/F_0 ratio was above 3.0. This observation has a strong relationship with the previous results, where the treated plants had lower transpiration rate which reduced water lost through the stomata.

CONCLUSIONS

Uniconazole was found to be capable of extending the trimming cycle and would be very helpful in controlling the height and shape of *S. campanulatum*. In this study, different dosages of uniconazole did not show any differences in the plant height, suggesting that the lowest dosage of uniconazole, i.e. 10 mgl^{-1}, was more practical to be used in managing the growth of this species. This compound reduced the leaf area but no curly leaf formation was observed. Uniconazole caused variations in the transpiration rate and stomatal conductance, which were possibly due to the smaller leaves developed. However, the application of uniconazole did not affect this species to perform its physiological processes at the optimal level.

TABLE 4
Chlorophyll fluorescence values of *S. campanulatum* at five month-after treatment with uniconazole

Uniconazole (mgl^{-1})	F_0	F_m	F_v	F_v/F_m	F_m/F_0
0	72.50	437.00	364.10	0.83	6.03
10	110.75	674.50	560.75	0.83	6.09
20	84.00	505.75	417.95	0.83	6.02
30	82.25	495.5	413.00	0.83	6.02

ANOVA showed no significant difference at $p<0.01$

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Effect of Ground Basalt on Chemical Properties of an Ultisol and Oxisol in Malaysia

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ABSTRACT

Highly weathered soils in Malaysia need to be amended to rejuvenate their chemical fertility. In particular, soil pH should be increased sufficiently, while exchangeable Al eliminated in order to make them productive. A glasshouse study was conducted in Malaysia to determine changes in the chemical properties of Ultisol and Oxisol (highly weathered, infertile soils) treated with ground basalt under moist condition. The results showed that soil pH increased and exchangeable Al decreased significantly due to basalt application within 6 months, whereas the value registered was determined by the rate of application. At the application of 10 t basalt/ha, it was observed that available P and exchangeable K, Ca, and Mg were increased to the level sufficient for crop growth. Meanwhile, chemical reactions (increase in pH and decrease in pHo) in the soils had resulted in an increase of the cation exchange capacity (CEC). This means that the soils are now able to reduce the loss of basic cations via leaching under high rainfall. Thus, basalt is a good soil ameliorant with the efficacy comparable to that of limestone, which is commonly applied to eliminate acid soil infertility in the tropics.

Keywords: Basalt, cation exchange capacity, exchangeable cation, Oxisols, Ultisols

ABBREVIATIONS

CEC : cation exchange capacity
RCBD : randomized complete block design

INTRODUCTION

About 70% of Peninsular Malaysia is covered by soils which have deep and highly leached profiles. These highly weathered soils, which are taxonomically classified as Ultisols and Oxisols (Soil Survey Staff, 1999), are dominated by kaolinite, gibbsite, and goethite in the clay fraction (Tessens and Shamshuddin, 1983; Anda *et al*., 2008). Ultisols are defined by the presence of an argillic horizon in the B-horizon, depicting the accumulation of clay in that zone. On the other hand, Oxisols are defined by the presence of oxic horizon in the subsoil. By definition, an oxic horizon contains predominantly oxides of Fe and Al and it has low CEC (as $cmol_c$/kg clay). These tropical soils are infertile due to low pH and low basic exchangeable cations, but high in exchangeable aluminium. Crop production on these soils is limited by these soil constraints infertilities.

For sustainable crop production on these soils, the soil chemical fertility needs to be substantially improved by applying suitable amendments. Research shows that soil fertility is

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significantly improved after limestone is applied onto the soils at appropriate rate due to the increase in soil pH, Ca, and Mg (Shamshuddin *et al*., 1991; Shamshuddin *et al*., 1998). Studies in Queensland, Australia indicated that ground basalt is a good alternative to limestone as a soil ameliorant (Gillman *et al*., 2001; Gillman *et al*., 2002). Other than Ca and Mg, basalt contains K and P in adequate amounts. Thus, using basalt as a soil ameliorant, P- and K-fertilizers application can be reduced so as to lower the cost of crop production.

Basalt outcrops are found sporadically throughout the Malay Peninsula (Gobbett, 1972). The best basalt outcrop is located on the beach at Beserah, Pahang (in the east coast state of Peninsular Malaysia). This basalt can be mined and ground to pass through a 2-mm sieve, and used for rejuvenating highly weathered tropical soils such as the Ultisols and Oxisols. When ground basalt is applied onto the soils under tropical conditions, it disintegrates and consequently weathers under prevailing high temperature and rainfall, releasing substantial amounts of Ca, Mg, K, P, and S into the soils. Its ameliorative effects are known to last for more than 2 years (Anda, 2006). The effects of ground basalt, on the chemical properties of soils in Queensland, Australia, have been studied (Gillman *et al*., 2001; Gillman *et al*., 2002). However, there is still a dearth of information on the chemical reactions of ground basalt in soils dominated by kaolinite, gibbsite, and goethite under tropical environment. Furthermore, the mechanism by which basalt ameliorates infertile tropical soils needs to be investigated and explained.

The objective of this study was to determine the changes in the chemical properties of Ultisol and Oxisol which were treated with ground basalt in Malaysia.

MATERIALS AND METHODS

A pot experiment was carried out at Universiti Putra Malaysia (UPM) in 2007/2008 to evaluate the effects of ground basalt in Ultisol and Oxisol found in Malaysia.

Soil Used

The soils used for this pot experiment were the Bungor (Typic Paleudult) and Munchong (Haplic Hapludox) Series, which are respectively classified as Ultisols and Oxisols. The topsoil (0-15 cm depth) was sampled from the University Research Park, UPM Serdang. The samples were air-dried, ground to pass through a 2-mm sieve and kept in the glasshouse before they were treated with ground basalt in the pot experiment.

Basalt Composition

Ground basalt used in this experiment was obtained from a commercial mineral company based in Australia. According to Gillman *et al*. (2002), this particular basalt contained 216,000 ppm Si, 65,400 ppm Ca, 64,400 ppm Mg, 12,500 ppm K, 3,030 ppm P, and 2,150 ppm S. Out of the three macronutrients (N, P, K) and secondary nutrients (Ca, Mg, S) needed for crop growth, only N is not supplied by basalt.

Experimental Design

A randomized completely block design (RCBD) experiment was set up in the glasshouse. There were two separate experiments in this study, and these were done using Ultisol and Oxisol (Bungor and Munchong series), respectively. Ground basalt was mixed thoroughly with the soil samples in the pots. The rates of the basalt application were 0, 5, 10, and 20 t/ha. In addition, water was also added into the soil mixture regularly in order to maintain the soil moisture content at the field capacity (equivalent to the matric suction of 10 kPa) throughout the experiment, and this was conducted for a period of 6 months. The sub-samples were taken from each pot (of the 2 experiments) every 2 month for the soil chemical analyses.

Analytical Methods

Soil pH was measured in water at the soil to water ratio of 1:2.5 using pH meter. Exchangeable bases (Ca, Mg, K, Na) were extracted by I M NH_4OAc

buffered at pH 7 (Sumner and Miller, 1996) and the cations in the solution were determined by atomic absorption spectrophotometry (AAS). Exchangeable aluminium was extracted by 1 M KCl (Bertsch and Bloom, 1996) and the aluminium in the solution was also determined by AAS. In order to determine the CEC of the soils at the soil pH, a method of CEC determination, using non-buffered 1 M NH_4Cl proposed by Sumner and Miller (1996) and tested earlier by Tessens and Shamshuddin (1983) for a wide range of Malaysian soils, was adopted. Available P was determined using the method proposed by Pixen and Grove (1990).

Statistical Analysis

The data obtained from this study were subjected to statistical analysis (using SAS), using the Tukey's test for comparison.

RESULTS AND DISCUSSION

Initial Soil Properties

The Bungor and Munchong soils used in this experiment are classified as Ultisols and Oxisols, respectively according to the soil taxonomy (Soil Survey Staff, 1999). Both these soils were developed from shale; they only differ in their degree of chemical weathering. The Munchong soil is an Oxisol (clayey, kaolinitic, isohyperthermic family of Haplic Hapludox) and therefore by definition, it is more weathered than the Bungor soil which is an Ultisol (clayey, kaolinitic, isohyperthemic family of Typic Paleudult). These two soil types are very common in Peninsular Malaysia and often used for rubber and oil palm cultivations. According to Tessens and Shamshuddin (1983), the soils of Bungor and Munchong series are dominated by kaolinite, gibbsite, and goethite minerals in the clay fraction. Hematite can also be present in the Munchong soil as it is reddish in colour (Anda *et al*., 2008). Soil pH is low, but exchangeable aluminium is high (Paramananthan, 2000). Compounded these infertilities with low exchangeable Ca, Mg and K, the two soils need to be properly amended for sustainable crop production. In the present study, basalt was used to amend the soils.

Basalt Dissolution and Reactions

In order to determine how fast ground basalt reacts with moist soils under glasshouse conditions, the changes in both soil pH and exchangeable aluminium within 6 months for the soils added with 20 t basalt/ha were studied and discussed in detail. Table 1 shows the relevant data for discussion. Based on the data presented in Table 1, soil pH had changed from 4.33 in month 2 to 5.13 in month 6 for the Bungor soil. Meanwhile, the soil pH of Munchong soil had also been reduced accordingly.

Aluminium with a pKa value of 5.0 hydrolyzes in water. Therefore, when the soil pH goes up beyond 5, Al in the soil solution starts to precipitate as gibbsite which is inert.

TABLE 1
Effect of applying 20 t basalt/ha on the soil pH and exchangeable Al with time

Month	pH	Exch. Al $cmol_c/kg$	pH	Exch. Al $cmol_c/kg$
	Bungor Series		Munchong Series	
0	4.45	1.47	4.55	1.89
2	4.33	1.52	4.91	1.44
4	4.89	0.93	4.44	1.43
6	5.13	0.68	4.98	0.33
$HSD_{0.05}$	0.32	0.28	0.28	0.30

In the beginning (untreated soil), the exchangeable Al in the Bungor soil was 1.47 $cmol_c$/kg soil. After 6 months of incubation, the exchangeable Al was found to significantly reduce to 0.68 $cmol_c$/kg soil. Within the same period, exchangeable Al in the Munchong soil was reduced from 1.89 to 0.33 $cmol_c$/kg soil.

Important minerals in basalt are olivine and pyroxene. When they come into contact with water at low pH under high temperature of the tropics, these minerals disintegrate and dissolve, resulting in pH increase. Olivine dissolves slowly in water as follows:

$$Mg_2SiO_4 \rightarrow 2Mg^{2+} + SiO_4^{4-}$$

The SiO_4^{4-} then hydrolyzes immediately to produce a large amount of hydroxyl:

$$SiO_4^{4-} + 4H_2O \rightarrow Si(OH)_4 + 4OH^-$$

The overall reaction of olivine in moist soils can be depicted as follows (De Coninck, 1978):

$$4Mg^{2+} + 4SiO_4^{4-} + 4H_2O \rightarrow 4Mg^{2+} + Si(OH)_4 + 4OH^-$$

According to De Coninck (1978), pyroxene can hydrolyze, but less amount of hydroxyl is produced than that of the olivine. This study shows that due to the application of basalt, at 20 t/ha, the soil pH was found to increase to a value above 5. When this happens, the effect of Al toxicity to growing crop is brought to a minimal as most of the Al becomes gibbsite.

Table 2 and Table 3 presents the chemical properties of the Bungor and Munchong series, respectively as affected by the different rates of basalt applications after 6 months of incubation in the glasshouse. For the Bungor soil (Table 2), the soil pH was increased to about 5 due to the application of 10 basalt/ha. At this rate of basalt application, the exchangeable Al was concomitantly decreased to less than 1 $cmol_c$/kg soil, while the available P, exchangeable Mg and exchangeable Ca in the soil were found to be 20.84 ppm, 0.96 and 2.01 $cmol_c$/kg soil, respectively. The nutrients (P, Mg, Ca) present in the soils now are probably sufficient for a good growth of normal Malaysian crops such as rubber, oil palm, and cocoa. However, the exchangeable K was below the sufficient level for the crops to grow. The same trend of improvement in the chemical fertility was observed for the Munchong soil (Table 3). It seems that the suitable rate of ground basalt application to alleviate the infertility of Ultisol/Oxisol is 10 t/ha, based on practicality and economic viability. This is consistent with the finding of an earlier study by Anda (2006). Unlike limestone, ground basalt takes longer time to disintegrate and dissolves. According to Boniao *et al*. (2002), it took more than 9 months for basaltic rock to completely dissolve in pot under moist condition. This means that if the present experiment was extended for a longer period of time (> 12 months), the researchers would have seen a better picture of its ameliorative effects.

TABLE 2
Effect of ground basalt application on the chemical properties of Bungor soil after 6 months

Treatment t/ha	pH	Avail. P ppm	Exch. cations					CEC
			Al	Ca	Mg	K	Na	
			– – – – – – – – – – – – $cmol_c$/kg – – – – – – – – – – – –					
0	3.83	6.40	1.66	0.76	0.10	0.11	0.05	3.66
5	4.73	14.72	1.04	1.83	0.73	0.43	0.08	5.93
10	4.96	20.84	0.82	2.01	0.96	0.74	0.10	6.22
20	5.12	26.78	0.68	2.09	1.05	0.83	0.15	7.08
$HSD_{0.05}$	0.20	2.28	0.03	0.09	0.07	0.03	0.01	0.19

TABLE 3
Effect of ground basalt application on the chemical properties of Munchong soil after 6 months

Treatment t/ha	pH	Avail. P ppm	Exch. cations					CEC
			Al	Ca	Mg	K	Na	
			– – – – – – – – – – – – cmol$_c$/kg – – – – – – – – – – – –					
0	4.21	7.21	1.00	0.22	0.08	0.12	0.05	4.44
5	4.27	16.22	0.96	1.24	0.64	0.52	0.06	6.14
10	4.88	22.78	0.66	1.54	0.81	0.68	0.07	7.25
20	4.98	24.95	0.33	1.84	1.04	0.78	0.07	7.96
$HSD_{0.05}$	0.09	5.14	0.11	0.05	0.11	0.02	0.01	0.11

Changes in Soil pH According to the Rate of Basalt Application

Initially, the pH of the Bungor soil was lower than that of Munchong soil. This is consistent with the finding of Tessens and Shamshuddin (1983) who claimed that the pH of Malaysian Ultisol was usually lower than that of the Oxisol due to the presence of more exchangeable Al in the former. This finding is also proven in the present study. Data presented in Table 2 clearly show that exchangeable Al in the Bungor soil (Ultisol) is 1.66 cmol$_c$/kg soil for the untreated soil, while the corresponding value in the Munchong soil (Oxisol) is 1.00 cmol$_c$/kg soil (Table 3).

Nonetheless, applying ground basalt into the soils, at the rate of 5, 10 and 20 t/ha, did not result in the same increase in the soil pH. The pH of the Bungor soil was consistently higher than that of Munchong soil. However, this is not consistent with the higher exchangeable Al in the Bungor soil as compared to that of the Munchong soil. Higher pH is usually reflected by lower Al. Therefore, the difference in pH between the two soils was probably attributed to the differences in chemical reactions which took place because of the differing mineralogy in the clay fraction of the soils. Obviously, Oxisol contains more oxides of Fe and/or Al than that of the Ultisol because the former is more weathered than the latter. Furthermore, Munchong soil is redder in colour than Bungor soil, indicating the presence of more oxides of Fe (hematite) in the former. Actually, the reason for the phenomenon of lower pH for the Munchong soil as compared to that of the Bungor cannot be confirmed at this stage. Thus, more studies are needed to determine the plausible mechanism.

Changes in Exchangeable Al with the Rate of Basalt Application

The changes in exchangeable Al in the Bungor and Munchong soils with rate are shown in Tables 2 and 3, respectively. First and foremost, the exchangeable Al was lower in Munchong as compared to that of the Bungor soil. This is similar to the finding of Tessens and Shamshuddin (1983) who argued that because of weathering, Al in Oxisols (such as Munchong soil) became gibbsite and there would be less Al existed in its exchangeable form. Nevertheless, the opposite is true for the Ultisols, such as Bungor soil.

In this study, the application of basalt was found to reduce exchangeable Al in both soils; the decrease was more in the Munchong than that of the Bungor soils. This is consistent with the more exchangeable Al in the latter initially. Applying basalt at 10 t/ha had reduced exchangeable Al to less than 1 mol$_c$/kg soil, a level considered to be of no problem for the growth of crops sensitive to Al toxicity. Thus, it is believed that the recommended rate of basalt application to eliminate Al toxicity in Ultisols and Oxisols is 10 t/ha.

Changes in the Available P with the Rate of Basalt Application

Prior to the treatment with ground basalt, the available P in the two soils was below 10 ppm, which is below the sufficient level for crop growth. As a result of applying 10 t basalt/ha, the available P in the Bungor and Munchong soils was increased to a value above 20 ppm (Tables 2 and 3, respectively), which is well above the crop's requirement. There was less P available in the Munchong as compared to that of Bungor soil at the basalt rate of 20 t/ha. The reason can be explained as follows. Being an Oxisol, the Munchong soil contains more oxides of Fe (as reflected by its red coloration) than that of the Bungor soil. Oxides of Fe are known to fix high amount of P. Hence, more P is fixed in the Munchong than that of the Bungor soil. As such, less available P was present in Munchong soil. Nevertheless, the available P was still enough in the Munchong soil for crop consumption at the basalt application rate of 20 t/ha.

Changes in CEC with the Rate of Basalt Application

The CEC of soil is a measure of its negative charge. In this study, the CEC of both the Bungor and Munchong soils increased significantly after 6 months of basalt application (Tables 2 and 3, respectively). The mechanisms of the CEC increase are as follows:

1. As the soil pH increased, due hydrolysis of the silicate present in basalt, the broken edges of mineral silicate (e.g. kaolinite) began to react. In this case, the proton at the broken edges of the kaolinite started to detach itself, the amount detached depends on the pH increase. As a result, the surface of the kaolinite became negatively-charged. This contributed partially to the increase in the CEC.
2. The other mechanism is related to the reaction of silicate with variable charge minerals, like goethite and hematite. When silicate (basalt) is chemically reacted with variable charge minerals in the soil, its pHo is lowered (Uehara and Gillman, 1981). It is important to note that pHo is a fundamental property of individual mineral in soil, defined as the pH, at which the net charge of the variable charge mineral is zero. Past research in Malaysia showed that the pHo of Oxisol decreased due to ground basalt application (Shamshuddin and Anda, 2008). When the ionic strength of the soil solution is kept constant, the amount of negative charge (CEC or σ) in the soil is proportional to the difference between pHo and pH, which can be depicted as $\sigma = k(pHo-pH)$, where k is an overall constant. In this study, pHo was lowered and pH was concomitantly increased as a result of basalt application. These phenomena widened the gap between pHo and pH, and consequently, the negative charge (σ) in the soil was also increased.

The increase in CEC means that now the soils are able to hold more basic cations, such as K, Ca and Mg, on their newly increased negatively-charged surfaces. As a result of the CEC increase, the loss of these macronutrients via leaching under tropical environment is minimized. The same mechanism has been attributed to the accumulation of Ca and Mg in the topsoil treated with ground magnesium limestone (Shamshuddin and Ismail, 1995). This ameliorative effect is translated as a cost-saving in crop production.

Changes in Basic Exchangeable Cations with Rate of Basalt Application

The exchangeable Na, K, Mg, and Ca in the soils of Bungor and Munchong Series 6 months after ground basalt application are given in Tables 2 and 3, respectively. The lack of Na in basalt is obviously reflected by the insignificant change in the exchangeable Na in the Bungor and Munchong soils after ground basalt was applied at the rates of 5, 10, and 20 t/ha. In response to basalt application, the exchangeable Ca, Mg, and K was found to significantly increase in both soils, and that the increasing rate of

these exchangeable cations are dependent on the amount applied. The exchangeable basic cations in the two soils in the order of decreasing amount are: Ca>Mg>K>Na. This is consistent with the elemental composition of basalt used in the experiment as determined by Gillman *et al*. (2002).

CONCLUSIONS

This study clearly indicates that the application of ground basalt can rejuvenate highly weathered and/or infertile Malaysian soils by improving their chemical fertility. In the presence of water under prevailing high temperature in the tropics, basalt disintegrates and dissolves slowly and this leads to an increase of soil pH and available P as well as exchangeable K, Ca, and Mg. The pH increase would precipitate Al in the soil solution as inert gibbsite. In doing so, Al toxicity is reduced considerably. Basalt is, therefore, a good soil ameliorant, comparable to that of limestone, except that it needs more time to dissolve under moist soil condition. The recommended rate of the ground basalt application for alleviating the infertility of Bungor and Munchong soils, respectively classified as Ultisol and Oxisol, is 10 t/ha.

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Species Distribution and Resistance Phenotypes of Vancomycin-Resistant *Enterococcus* Isolated from Pigs in Pulau Pinang, Malaysia

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ABSTRACT

Vancomycin-resistant enterococci (VRE) are important nosocomial pathogens. The extensive use of avoparcin as a growth promoter in poultry and pigs is the hypothesized factor for the emergence of vancomycin resistance in enterocci in animals. As pork is one of the major protein sources for 30% of Malaysians, the present study was conducted to elucidate the role of pigs in the epidemiology of VRE. In this study, 220 rectal swabs were collected from pigs at 12 pig farms in Pulau Pinang. The study found 10 of 12 farms (83.3%) and 92 (41.8%) of the sampled pigs were positive for VRE. Of the 92 isolates examined by PCR, *E. faecium* (14%), *E. casseliflavus* (21.7%), *E. gallinarum* (1.1%) and other *Enterococcus* species (63.0%) were identified. *VanA* was detected in *E. faecium* and *E. gallinarum*. Questionnaire survey indicated that none of the sampled farms had used glycopeptides, either for growth promotion or for therapy. Tylosin, which has also been associated with vancomycin cross-resistance, was used in 41.8% of the sampled farms; however, there was no significant difference (P>0.05) between the proportion of VRE detected in the farms which used tylosin to those farms which did not. E-test on selected 49 isolates showed 16.0% of the isolates had MIC≤8 and 22.0% had MIC≥32. Single isolates of *E. faecium* and *E. gallinarum,* both possessed the resistance gene *van*A, showed very high resistance (MIC>256). About 10.0% of the isolates, in which *van* genes was not detected, had MIC>32. In conclusion *E. faecium* and *E. faecalis* were found to be present at a low rate in the pigs sampled in this study. However, detection of *van*A with high level of vancomycin resistance (MIC>256) highlights the potential public health threat associated with the pigs.

Keywords: *Enterococcus*, Malaysia, vancomycin-resistant, pigs, resistance phenotypes

INTRODUCTION

Vancomycin-resistant *Enterococcus* (VRE) is one of the major organisms causing nosocomial infections in humans (Simjee *et al*., 2006). Vancomycin is a glycopeptide antibiotic, and is the drug of choice for *Enterococcus* infections. In the recent years, an increasing resistance towards the antibiotic has been seen; therefore, treatment of the infection can be difficult. Molecular evidence suggests animals as the likely reservoir for VRE (Depardieu and Courvalin, 2005). Avoparcin, which is also of the glycopeptide antibiotic group, was used extensively in feed as a growth promoter for pigs and poultry. Such use has resulted in the ensuing cross-resistance which may be transmitted to enterococcal strains infecting humans (Centinkaya *et al*., 2000).

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The use of vancomycin in the local hospitals in Malaysia was common prior to 1994 (Cheong *et al*., 1994; Cheong *et al*., 1996). Nevertheless, limited reports are available about VRE in humans. Only one report by Raja *et al*. (2005) was encountered on the community-acquired high vancomycin-resistant *Enterococcus faecium* infection which was presented at the University Malaya Medical Centre, Selangor. This could signify that the infection is rare, under reported or under diagnosed. In contrast, detection of VRE in animal and animal products has been reported by a few authors. Among other, Ong *et al*. (2002) detected low rates (2%) of VRE in the Malaysian poultry wet markets, while Dahlia *et al*. (2005) reported five VRE isolates from a total of 172 *Enterococcus* from ducks. An epidemiological study by Hassan *et al*. (2006) documented that 43.8 % of the sampled broilers, including day-old-chicks of six poultry farms, were colonised by VRE. Radu *et al*. (2001) recorded the occurrence of *van*A and *van*C2/C3 genes in *Enterococcus* species isolated from poultry source. In addition, Radu *et al*. (1999) also molecularly characterised vancomycin-resistant *E. faecium* from the imported beef samples in Malaysia.

Pork serves as a major protein source for 7.5 million (30%) of Malaysians (DVS Perak, 2007). In other countries, the role of pigs in VRE dissemination, reservoir and human infection was suggested as VRE has been isolated from pork, pigs, pig farms and the environment (Klein *et al*., 1998; Kariyama *et al*., 2001; Manero *et al*., 2006). In addition, Lu *et al*. (2002) reported cases of VRE infection in humans which were linked to an outbreak of VRE infection in pigs (Manero *et al*., 2006). Yet, no studies have been conducted on VRE in pigs in Malaysia to elucidate its role in the epidemiology of VRE and the risk of transmission to humans. The present study was conducted to detect the occurrence of VRE at selected pig populations in Pulau Pinang, Malaysia, as well as to describe the distribution of *Enterococcus* species and their resistance phenotypes. The study employed a multiplex polymerase chain reaction (M-PCR) to simultaneously detect the species of VRE and resistance determining genes.

MATERIALS AND METHODS

Study Design

A cross-sectional study was conducted between October and November 2006 at selected pig farms in Pulau Pinang. Farms were chosen based on the farm owners' willingness to participate in the study. The State Department of Veterinary Services (DVS) was contacted via a formal letter and was informed about the study. The farmers were first contacted via the regional public health officer. They were briefed about the project and were invited to participate in the study. Once they agreed, an appointment was arranged and the researchers proceeded with the study.

Study Population

The study population was the finishing pigs from 12 farms, and for this purpose, 15 to 20 pigs were sampled from each of the 12 pig farms.

Sample Size

At the time of the study, there were a total of 219 farms and a standing pig population of 296, 232 in Pulau Pinang (DVS, 2006). Using the assumption of an overall animal-level prevalence of 18% (Butaye *et al*., 1999) and a farm-level prevalence of 30% at a confidence level of 95%, the sample size calculation was performed as suggested by Thrusfield (2005) and Dohoo *et al*. (2006).

DATA AND SAMPLE COLLECTION

Questionnaire Survey

Information related to pig herd and the management was obtained by interviewing the farm managers using a structured pre-tested questionnaire. The information included size of the farm, number of finishing pigs, antibiotics used for therapy and supplements, use of growth promoters, type of feed, source of piglets,

possibility of contact with other animals and farms' bio-security measures.

Sample Collection

Using a sterile swab, a rectal swab was performed on each pig, following the procedure described by Garcia-Migura *et al*. (2005).

MICROBIOLOGICAL ANALYSIS

Isolation of VRE and Biochemical Characterization

Each rectal swab was streaked onto a plate containing Slanetz and Bartley agar (Merck Inc., Germany), supplemented with 32µg ml^{-1} vancomycin (Sigma, USA). From each plate, all red-maroon colonies were purified. Confirmation of suspected enterococci colonies was done using biochemical tests described for the genus *Enterococcus* (Simjee *et al*., 2006). Those confirmed as *Enterococcus* were stored in 20% glycerol (Ameresco) Brain Heart Infusion (BHI; Pronadisa, Spain) broth at -20 °C until further use.

Identification of VRE: Molecular Characterization

DNA extraction

Briefly, overnight culture was made to turbidity level of McFarland 0.5 standard and the cells were digested using enzymatic lysis buffer (20mM Tris.Cl, pH 8.0; 2mM Sodium EDTA, 1.2% Triton X-100% and 20mg/ml lysozyme). DNease® Blood and Tissue DNA extraction kit (Qiagen®, Germany) was used to extract genomic DNA according to the protocol described for Gram-positive bacteria by the manufacturer.

Species and *van* gene determination

Multiplex polymerase chain reaction (M-PCR) assay developed by Kariyama *et al*. (2000) was used with a few modifications (Table 1). Briefly, seven pairs of *E. faecalis*, *E. faecium*, *E. gallinarum* (*van*C1) and *E. casseliflavus* (*van*C2/C3), *van*A, *van*B, and *rrs* 16S rRNA specific primers were used in a single reaction for a simultaneous determination of the common and clinically important VRE species and resistance genes (Dutka-Malen *et al*., 1995; Kariyama *et*

TABLE 1
Multiplex PCR primers used for species identification and vancomycin- resistance gene detection

Primer specificity	Primer	Primer pair sequences	Product size (bp)
vanA gene	*van*A	5'-CATGAATAGAATAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1,030
vanB gene	*van*B	5'- AAG CTA TGC AAG AAG CCA TG -3' 5'- CCG ACA ATC AAA TCA TCC TC-3'	536
E. gallinarum	*van*C1	5'-GGTATCAAGGAAACCTC-3' 5'-CTTCCGCCATCATAGCT-3'	822
E. casseliflavus / E. flavescens	*van*C2/C3	5'-CGGGGAAGATGGCAGTAT-3' 5'-CGCAGGGACGGTGATTTT-3'	484
E. faecalis	ddlE. faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941
E. faecium	ddlE. facium	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658
PCR internal control	*rrs* (16S rRNA)	5'-GGA TTA GAT ACC CTG GTA GTC C-3' 5'- TCG TTG CGG GAC TTA ACC CAA C-3'	320

al., 2000). Primer for *van*B was adopted from Elsayed *et al*. (2001). All the primers were synthesised by Research Biolabs, Singapore.

Species identification and *van* gene determination by multiplex PCR were repeatedly checked against ATCC *Enterococcus faecalis van*B strain (ATCC 51299), *E. faecium van*A strain (ATCC 51559), *E. gallinarum* (ATCC 49573), and *E. casseliflavus* (ATCC 25788) to evaluate and optimize the PCR conditions in reference to the expected PCR product band sizes (*Fig. 1*).

Agarose Gel-Electrophoresis Analysis

The PCR product was analysed using gel electrophoresis on 1.5% agarose gel at 75 volts for 1 hr. The gel was stained in ethidium bromide (5µg/ml) and gel photo was taken under UV using the AlphaImager®. Meanwhile, the analysis was made through visual inspection for the presence of expected band size and by comparing with standard controls and DNA markers.

E-test

The level of resistance to vancomycin was determined using E-test kit for vancomycin (AB Biodisk, Sweden) according to the manufacturer's guideline. Brief, overnight culture was used to make a suspension of McFarland 2 standard. A suspension of 150µl was evenly streaked on a plate containing BHI agar. Vancomycin strip was placed at the centre of the plate. After 48 hr of incubation at 37 °C, the MIC value was determined by reading the edge of the inhibition zone ellipse intersecting the side of the strip. The E-test was done on 49 isolates consisting most of the isolates identified to the species level, and 21 isolates which were not identified to the species level (Table 3).

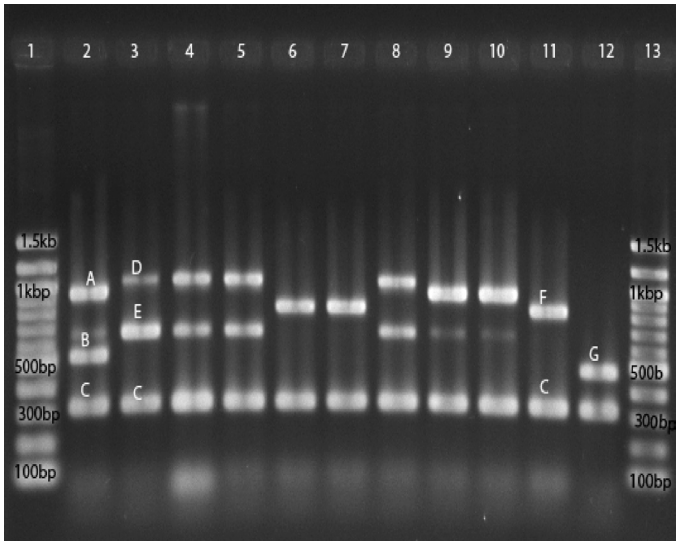

Fig. 1: Multiplex PCR products gel-electrophoresis photo Lane [L]1 & 13: 100bp DNA marker [BioLabs], L2: E.faecalis *ATCC 51299, L3:* E. faecium *ATCC51559, L11:* E. gallinarum *ATCC 49573 and L12:* E. casseliflavus *ATCC 25788 are positive controls. Lanes 4-to-10 are samples. Alphabet A indicates a band as result of amplification of* E. faecalis *specific gene [941bp], B:* van*B specific [536bp], C: rrs specific [320bp], D:* van*A specific [1,030bp], E:* E. faecium specific *[658bp], F:* E.gallinarum/van*C1 specific [822bp] and G:* E. casseliflavus/ van*C2/3 specific gene [484bp]*

Data analysis

Farm data were managed, collated, and analysed using SPSS version-15 statistical software (SPSS Inc. Chicago). A descriptive analysis was used to describe the sampled population in the study. The differences between the proportions were tested using the Chi-square (χ^2) analysis at the significance level of $\alpha = 0.05$.

Results

A total of 220 pigs were sampled throughout the period of study, from which 92 (41.8%) animals were found to be VRE positive. The organism was detected in 10 of the 12 sampled farms (83.3 %) with a detection rate ranging between 0 % and 85 % ($\chi^2=65.3$, $p<0.05$) (Table 2).

Description of the Herds

Seventy-five percent of the sampled farms supply pigs for the local, state, and nationwide markets. Meanwhile, only farms 4 and 7 supply to limited local markets in Penang. The swine population in the sampled farms ranged from 1000 to 12,000 (mean= 3205, sd = 3139.4). Each pig farm had a mean of 409 finishers with 8 to 20 pigs per pen. The age range of the farms visited was between

TABLE 2
Demography and VRE status of sampled pig farms in Pulau Pinang, Malaysia

Farm	Standing pig population	Finisher pigs	No. pigs sampled	VRE positives (%)	No. VRE isolated
1	4,369	500	20	17 (85.0)	22
2	1,400	250	15	9 (60.0)	9
3	1,200	130	15	9 (60.0)	12
4	2,500	100	20	12 (60.0)	16
5	12,000	2,000	20	0	0
6	1,000	100	15	2 (13.3)	2
7	2,000	220	20	0	0
8	2,000	200	15	7 (46.6)	9
9	2,000	110	20	11 (55.0)	13
10	6,000	700	20	10 (50.0)	15
11	3,000	300	20	10 (50.0)	13
12	1,000	300	20	5 (25.0)	6
Total (Mean)	38,469 (3205.75)	4,910 (409)	220	92 (41.8)	117

TABLE 3
The minimum inhibitory concentration (MIC) of vancomycin-resistant *Enterococcus* species isolated from pigs

VRE species	Minimum inhibition concentration [µg/ml]			
	MIC = 8	MIC 9-31	MIC 32-128	MIC>256
E. faecium (n=10)	-	60.0%	30.0%	10.0%
E. gallinarum (n=1)	-	-	-	100%
E. casseliflavus (n=17)	11.8%	64.7%	23.5%	-
Enterococcus sp.(n=21)	28.6%	71.4%	-	-
Total (N=49)	16.0%	62.0%	18.0%	4.0%

6 and 50 years (mean = 34, sd = 12.8). For ease of analysis, the farms were categorised into new (<25years) and old (≥ 25 years), respectively. Based on this categorization, three of the farms were deemed new while nine of them were categorised as old. However, the Pearson Chi-square analysis showed no significant difference (χ^2 =0.900, p>0.05) between farm age and the proportion of VRE detected in the farm.

Commercial or self-mixed pigs' meals and well water were used at all farms. None of the farmers reported using avoparcin. Glycopeptides, including vancomycin, appeared to have never been used in any of the farms for therapeutic or as a feed supplement. For therapeutic purposes, wide ranges of antibiotics obtained from the pharmaceutical company sales representative were also used. Tylosin was the commonly used antibiotic at five of the 12 farms (41.6%) which reported to use it. However, the statistical analysis did not show any significant association (χ^2 = 1.888, p>0.05) between the use of tylosin and the proportion of VRE positive pigs in the farms. All except two farms (farms 9 and 12) used disinfectant in footbath and vehicle-dip at the entrance, and restrict visitors from entering the farms. All the farms were located far from residential areas (judged by the absence of housing development at least 15 km from the farms), and they are separated from poultry and other livestock farms. All the farms are at least 500 meters away from each other.

Detection of VRE Species and Resistance Phenotypes

A total number of 117 VRE were isolated from 92 pigs (Table 2). The DNA extraction and purification were performed on 92 of the isolates and preserved for PCR.

From the 92 isolates, PCR identified three species, namely *E. faecium* (14.0%), *E. casseliflavus* (21.7%), *E. gallinarum* (1.1%), and other *Enterococcus* species (63%).

Vancomycin resistance gene *van*A was detected in *E. faecium* (1 of 13) and *E. gallinarum* (1 of 1) isolates. However, Vancomycin resistant gene *van*B was not observed in any of the isolates. *Van*C1 and *van*C2/3, which are intrinsic to *E. gallinarum* and *E. casseliflavus,* were also detected. Interestingly, the resistance gene for 70 of the 92 (76.1%) PCR tested isolates was not detected.

The E-test on 49 isolates showed that 16% of the isolates have MIC≤8, whereas 64% had MIC between 8 and 31 and 22% had MIC≥32. All *E. faecium* isolates tested had MIC>8. In fact, a single isolate of *E. faecium* and *E. gallinarum,* which both possess the resistance gene *van*A, showed very high resistance to vancomycin (MIC>256). Moreover, from 17 *E. casseliflavus* isolates tested, with natural resistance gene *van*C2/3, vancomycin resistance MIC≥32 was observed in 23% of the species, while a majority (64.7%) of them exhibited intermediate resistance of MIC ranging from 8 to 31 (Tables 3 and 4). As presented in Table 4, 10% of the isolates, where *van* genes was not detected, were resistant to MIC>32 (Table 4).

DISCUSSION

Farm owners interviewed in this study reported that they had not used avoparcin or any glycopeptide drugs, either as a growth promoter or for therapeutic purposes. Bager *et al*. (1997) were the first to establish the association between avoparcin and the presence of VRE in pig farms. The findings from the study of Bager *et al.* (1997) were followed by several others who had prompted European countries to ban the use of avoparcin in their livestock. Nevertheless, research in other countries, where avoparcin is banned, was able to detect VRE in animals and their products albeit at a lower rate (Boerlin *et al*., 2001; Manero *et al*., 2006). VRE has also been observed in farms where no antibiotics or growth promoters were used (Garcia-Migura *et al*., 2005). This led these authors to suggest that vancomycin resistance is a spontaneous defensive response by enterococci, and thus the use of avoparcin might have not been the exclusive factor for the development of vancomycin resistance in animals. Manero *et al*. (2006) proposed that the extensive use of feed additives such as tylosin could create a

TABLE 4
Resistance phenotypes and the minimum inhibitory concentrations (MIC) of vancomycin-resistant *Enterococcus* species isolated from pigs

van genes	Minimum inhibition concentration (µg/ml)			
	MIC = 8	MIC 9-31	MIC 32-128	MIC>256
*van*A (n=1)	0 %	0 %	0 %	100 %
*van*C1/A (n=1)	0 %	0 %	0 %	100 %
*van*C2/C3 (n=17)	12.5 %	62.5 %	25 %	0 %
ND (n=30)	19.4 %	71 %	9.7 %	0 %

ND: No van gene detected

co-linkage between the vancomycin resistance genes and resistance determinants to other antibiotics used as growth promoters, and thus farms using tylosin could be positive for VRE in the absence of avoparcin. In the present study, the researchers found that 41.6% of the sampled farms were using tylosin as a feed additive and/or for therapy. Nevertheless, no significant difference was observed in the VRE detection rate between the farms which were using tylosin and those which were not. However, due to the small number of farms sampled, this observation might not be conclusive. Other authors have also suggested the existence of selective pressures other than glycopeptide antibiotic usage (Bahirathan *et al*., 1998; Klein *et al*., 1998) which resulted in the development of resistance to vancomycin.

Enterococci are intrinsically resistant to a broad range of antimicrobial agents and the use of antibiotics, to which enterococci are naturally resistant, may contribute to the emergence of resistant strains (Kak and Chow, 2002). In Malaysia, farmers can purchase antibiotics without prescription from any pharmaceutical representatives. The widely accessible antibiotics for use on farms may have led to an indiscriminate usage of antibiotics which may have contributed to the observed level of resistant enterococci. However, since there is no absolute ban on avoparcin, the negative response from the farmers about the use of avoparcin may be unreliable. In many instances, the farmers may not know the exact ingredients added into the commercially-prepared or premix feed. In addition, the time of study coincided with the beta-agonist screening program by the Malaysian authorities. Therefore, this might have hampered farm owners from providing genuine information.

A total of 117 VRE were isolated from 41.8 % of the sampled pigs. There are no published data on the prevalence of VRE in pigs in Malaysia, to which a comparison of the study findings can be made. In addition, comparison with the findings of other studies from elsewhere is difficult as no standardized methods were used for isolation and identification of VRE. Among other, Butaye *et al*. (1999) used enrichment methods with 6µg ml^{-1} vancomycin and plating on Slanetz and Bartley media isolated VRE from 53% of sows, but they could not isolate VRE through direct streaking on selective agar. Meanwhile, Garcia-Migura *et al*. (2005) detected a low prevalence (2 to 5%) of *E. faecium* in pig samples using Slanetz and Bartley agar supplied with 6µg ml^{-1} vancomycin. Nevertheless, a high prevalence of VRE was found in urban sewage water (100%), pig slurry (34%) and pig faeces (16%) after enrichment with vancomycin at 8µg ml^{-1} and plating on m-*Enterococcus* agar with 20µg ml^{-1} vancomycin (Manero *et al*., 2006).

The species most broadly distributed in nature are *E. faecalis* and *E. faecium.* Manero *et al*. (2006) indicated the dominance of *E. hirae* in addition to these two species in pig and its environment. In the present study, vancomycin-resistant *E. faecium, E. casseliflavus, E. gallinarum* were isolated but *E. faecalis* was not. This finding is consistent with that of Seo

et al. (2005) in Korea who isolated neither *E. faecalis* nor *E. faecium* from 70 pigs, but instead found that *E. gallinarum* and *E. casseliflavus* were prevalent. Furthermore, the findings gathered in the present study are in agreement with those of Manero *et al*. (2006) who found vancomycin-resistant *E. casseliflavus and E. gallinarum* from pig slurry. The significance of the isolates other than *E. faecium* and *E. faecalis* was emphasised by Willey *et al*. (1999, cited in Kirschner *et al*., 2001), who noted that the incidence of less commonly found species such as *E. casseliflavus*, *E. gallinarum*, *E. durans*, and *E. hirae* had increased significantly in humans admitted to the hospital. In view of this finding, the role of these isolates in clinical cases due to *Enterococcus* must be investigated.

The multiplex PCR identified *E. casseliflavus* as the predominant species among those tested. However, a high percentage (63%) of VRE isolates, tested by PCR, were not identified to the species level since the multiplex PCR used was limited to the common and clinically important *Enterococcus* species. The results of this study indicate that there are other species to consider. These include *E. hirae* and/or *E. durans* as previously reported by Willey *et al*. (1999), Kirschner *et al*. (2001), and Manero *et al*. (2006) who indicated that these two species were isolated from pigs.

Six different gene clusters, mediating glycopeptide resistance, have been described in enterococci, including *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G (Gilmore *et al*., 2002). The gene *van*A has been the subject of much work because it encodes resistance to all glycopeptides and is associated with the plasmid mediated transposable element Tn1546. More importantly, *van*A signifies that the gene is not inherent but it is acquired, and is associated with high levels of vancomycin resistance. The Tn1546 element plays a major role in transferring resistance to *Staphylococcus aureus* (Dowling, 2006), which is another important hospital acquired nosocomial agent (Lowy, 1998). In addition, vancomycin-resistant *S. aureus* has also been reported (Tiwari and Sen, 2006; Smith *et al*., 1999). *Enterococcus faecium* with *van*A isolated in this study is consistent with the findings of Centinkaya and colleagues (2000) who stated that *van*A is primarily found in *E. faecium*. Furthermore, they also described *van*A in *E. gallinarum* which was also observed in the present study. Patel *et al*. (1997) and Devriese *et al*. (1996) described similar findings in *E. gallinarum*. In addition, *E. gallinarum* with an acquired *van*A gene has been reported to cause clinical infection in humans (Camargo *et al*., 2004). In the present study, *van*A was not detected in other isolates even though the bacteria were initially isolated on 32µg ml^{-1} vancomycin supplemented media. After isolation, the isolates were transferred into BHI vancomycin-free broth and stored at -20 ^{o}C for more than four months pending DNA extraction. The lack of stimulation due to the absence of vancomycin and long storage at low temperature could possibly explain the absence of *van*A. Manson *et al*. (2003) also reported a rapid loss of *van*A gene from *E. faecalis* when the isolates were transferred into vancomycin-free media. Khan *et al*. (2005) reported *E. gallinarum* isolates which did not possess any of the *van* genes, but they found resistant to high level of vancomycin instead.

Vancomycin resistance test using the E-test indicated different levels of susceptibility in the isolates. According to the National Committee for Clinical and Laboratory Standards (NCCLS, 2004) (presently known as Clinical and Laboratory Standards Institute-CLSI), enterococci with MIC≥32 are resistant and those showing MIC≤4 are susceptible. Using this categorisation, none of the *Enterococcus* isolates were determined as susceptible to vancomycin. Table 3 shows that the majority of isolates had MIC>8 (84%). Moreover, 40% of *E. faecium* and 23.5% of *E. casseliflavus* were completely resistant to vancomycin. In addition, a single isolate of *E. gallinarum* and *E. faecium* had a high level of vancomycin resistance.

Resistance gene *van*B, which is also acquired and encodes for intermediate level of resistance to vancomycin, was not identified from any of the VRE. A previous study conducted in Malaysia (Radu *et al*., 2001) could not detect *van*B from

70 VRE isolated from poultry. Meanwhile, Ooi (2003), who studied 81 isolates from poultry, raw vegetables and clinical sources, did not isolate any *van*B VRE. This suggests that *van*B is possibly not present in Malaysia VRE isolates.

CONCLUSIONS

This study found VRE were present in 83% of the farms and 42% of the pigs sampled. *Enterococcus casseliflavus* (21.7%), *E. faecium* (14%), *E. gallinarum* (1.1%) and other *Enterococcus* species make up 63% of the total 92 VRE isolates examined by M-PCR. Meanwhile, Vancomycin resistance gene *van*A was only detected in two of the VRE isolates (2.2%). The *van*A was observed in *E. faecium* and *E. gallinarum*. Detection of *van*A, with high level of vancomycin resistance (MIC>256), highlights potential public health threat associated with the pig industry since the resistance gene is capable of transfer within the genus *Enterococcus* and other pathogenic bacteria such as *Staphylococcus aurous*. More importantly, the multiplex PCR used in this study proved to be suitable for VRE screening. However, the incorporation of new primer sets may be required due to the high numbers of other *Enterococcus* spp. detected. In this study biochemical tests were used to identify the genus *Enterococcus*. However, using this method other bacteria with similar features (e.g. vancomycin - resistant *Pediococcus* species) may have been identified as *Enterococcus* spp. Therefore, the results should be interpreted with caution.

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Improved Anaerobic Treatment of Palm Oil Mill Effluent in a Semi-Commercial Closed Digester Tank with Sludge Recycling and Appropriate Feeding Strategy

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ABSTRACT

Anaerobic treatment of palm oil mill effluent (POME) in a semi-commercial closed digester tank with sludge recycling was studied using different feeding strategies; one fixed at every three hour and another at every six hour. The organic loading rate (OLR) was increased step-wise and stopped once inhibition on methane production occurred. The chemical oxygen demand (COD), feeding rate, hydraulic retention time (HRT), OLR, and sludge recycling ratio were measured. Performance was based on the COD removal efficiency and methane yield, while stability was assessed in terms of total volatile fatty acids (VFA) accumulation, total VFA-to-alkalinity ratio (VFA:Alk) and food-to-microorganisms ratio (F/M ratio). The feeding strategies, at every three hour and six hour, gave satisfactory COD removal efficiency of higher than 90%, but the latter feeding strategy gave a more stable process with total VFA concentration recorded below 500 mg L^{-1} and VFA:Alk ratio of less than 0.3 at maximum OLR of 6.0 kgCOD m^{-3} d^{-1}. The treatment period could also be extended up to 100 days without any obvious problems.

Keywords: Anaerobic treatment, biogas, feeding interval, methane, palm oil mill effluent, sludge recycling

INTRODUCTION

Malaysia is blessed with suitable climatic and geographical factors for the cultivation of oil palm scientifically known as *Elaeis guineensis Jacq*. The palm oil industry is very important to Malaysia and it has contributed significantly to the country's gross domestic product (GDP). The export earnings from palm oil, palm kernel oil, and its products in 1998 amounted to almost US$5.6 billion, equivalent to 5.6% of the country's GDP. Today, Malaysia is the world's largest producer and exporter of palm oil (Yusoff, 2006). However, despite the high economic returns, the generation of liquid waste or palm oil mill effluent (POME) is also huge. It was estimated that for every tonne of fresh fruit bunch processed, between 0.5 and

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0.75 tonne of POME is produced (Yacob *et al*., 2006). POME is generated from the combination of sterilization, clarification and hydrocyclone washing processes during palm oil processing (Hassan *et al*., 2004). More than 85% of the palm oil mills in Malaysia use the conventional pond systems for the treatment of POME due to its lower operating costs (Najafpour *et al*., 2006). In the future, anaerobic treatment of POME coupled with methane gas recovery will be the preferred choice for sustainable development of the palm oil industry. In addition to the production of methane gas which can be used for electricity generation, the Certified Emission Reduction (CER) mechanism could also be triggered once the project is registered as a Clean Development Mechanism (CDM) project under the patronage of the United Nations Framework Convention on Climate Change (UNFCCC). Anaerobic treatment of POME by a closed anaerobic digestion system offers several advantages in comparison with other treatment technologies such as lower energy requirements with no aeration, producing methane gas as a valuable end product and generating sludge from the process which can be used as fertilizer or for land application (Poh and Chong, 2009).

Table 1 shows a comparison of various technologies for treating POME. The technologies (except for 500 m^3 digester tank reactor studied by Yacob *et al*., 2006) exhibited high COD removal efficiency, but the experiments were performed using only laboratory scale digesters and the POME used might not represent the actually characteristics of POME produced directly from the mill. Meanwhile, the large pilot scale technology available in Malaysia is the 500 m^3 semi-commercial closed digester tank which was installed in 2005 for FELDA Palm Industries Sdn. Bhd. in Serting Hilir Palm Oil Mill. The digester was commissioned in 2005 and a series of publications have been produced since then (Yacob *et al*., 2006; Sulaiman *et al*., 2009). This study is part of the continuation of those studies on improving the anaerobic treatment of POME for higher methane gas production.

TABLE 1
Different types and scales of anaerobic digester for the POME treatment and performance

Type of reactor	HRT (d)	Inlet COD concentration (kgm^{-3})	OLR ($kgCODm^{-3}d^{-1}$)	COD removal (%)	References
Up-flow anaerobic sludge fixed film	1.5	26.21	17.47	90.2	Najafpour *et al*. (2006)
Digester tank	10	56.45	5.55	>90	Yacob *et al*. (2006)
Modified anaerobic baffled	3	16	5.33	77.3	Faisal and Unno (2001)
Anaerobic hybrid	3.5	56.6	16.20	92.3	Borja *et al*. (1996)
Anaerobic filter	1.0	10.0	10.0	>90	Borja and Banks (1994a)
Anaerobic fluidized bed	0.25	2.5	10.0	>90	Borja and Banks (1994a)
Up-flow anaerobic sludge blanket	4	42.5	10.6	96	Borja and Banks (1994b)
Immobilized cell	6.2	69	10.6	96.2	Borja and Banks (1994c)
Stirred tank	5.6	70	12.60	97	Cail and Barford (1985)

The digester is of a simple and straight-forward design and hence suitable for operators with low level technical competency. The digester has recently been equipped with a sludge settling tank so that the sludge could be recycled in order to maintain a higher cell mass inside the digester and that the production of methane gas could also be improved. Many studies have reported that the operational stability of the digester could be improved in the treatment of POME and other organic wastes by recycling the sludge (Najafpour *et al*., 2006; Setiadi *et al*., 1996; Faisal and Unno, 2001). Najafpour *et al*. (2006) utilized a high recycle ratio of 11.25 to eliminate high organic over loading and supply alkalinity by blending the fresh feed with low COD and high alkalinity recycled stream. Meanwhile, the study by Setiadi *et al*. (1996) showed that a recycle ratio of more than 15 was required to maintain the pH of the anaerobic process higher than 6.8 without alkalinity supplementation. A higher recycle ratio of 30 was adopted by Faisal and Unno (2001) in stabilizing the modified anaerobic baffled reactor operation. Thus, the main objective of this study was to evaluate the performance of a large semi-commercial closed digester with sludge recycling and different feeding strategies for the anaerobic treatment of POME. In this study, the focus is on anaerobic treatment of POME, using a large 500 m^3 semi-commercial closed digester tank with sludge recycling from a newly installed settling tank and the feeding was performed at different time intervals so as to evaluate the digester performance and stability. The digester's performance parameters were measured in terms of chemical oxygen demand (COD) removal efficiency, methane yield, and digester stability in terms of the total accumulation of volatile fatty acid (VFA).

MATERIALS AND METHODS

The Set-up of the Closed Digester Tank

Fig. 1 illustrates the schematic diagram of the 500 m^3 semi-commercial closed digester tank. Raw POME was directly pumped from the mill and stored in a 50 m^3 holding tank to ensure continuous supply and consistent characteristics. A centrifugal pump was used to feed the digester. The POME feeding volume was measured online by a mass flow meter and recorded by Endress+Hauser Ecograph. During the feeding process, an equal volume of the treated effluent inside the digester was displaced and let to flow out into the settling tank, where the solids were trapped and recycled into the digester at 6 m^3 d^{-1}. After feeding, the content of the digester was mixed for 30 min using a mixing pump to ensure a good contact between substrates and micro-organisms. The generated biogas in the collection chamber was measured online by a mass flow meter before it was sent for storage.

Source of Raw POME, Seed Sludge and Recycled Sludge

Raw POME was directly obtained from the palm oil mill located beside the plant by direct pumping. The digester was seeded using the sludge from the existing 3600 m^3 open digester tank and diluted to give an initial total solid of 5% inside the digester (Yacob *et al*., 2006). The sludge from the settling tank was allowed to settle for 2-3 hours before it was recycled in the digester. After that, the recycled sludge was analyzed and characterized by a lower total COD content, high solids content (4-6% of TS), high alkalinity (2,000-4,000 mg L^{-1} $CaCO_3$), neutral pH (7.0±0.2), high nitrogen content (286-300 mg L^{-1} of TKN), low oil and grease (150-183 mg L^{-1}), high lignin content (2,280-2,350 mg L^{-1}), and high ash content (28,098-30,350 mg L^{-1}).

Experimental Procedures

In this study, two set of experiments were conducted; one with sludge recycling and the feeding interval was fixed at every three hours and another set was also with sludge recycling but the feeding interval was fixed at every six hour. The sludge recycling rate was fixed at 6 m^3 d^{-1}. The OLR was increased step-wise and the digester performance and stability were also evaluated. Table 2 shows the summary of the feeding profiles in terms of COD concentration of the raw POME, feeding rate, HRT, OLR, and

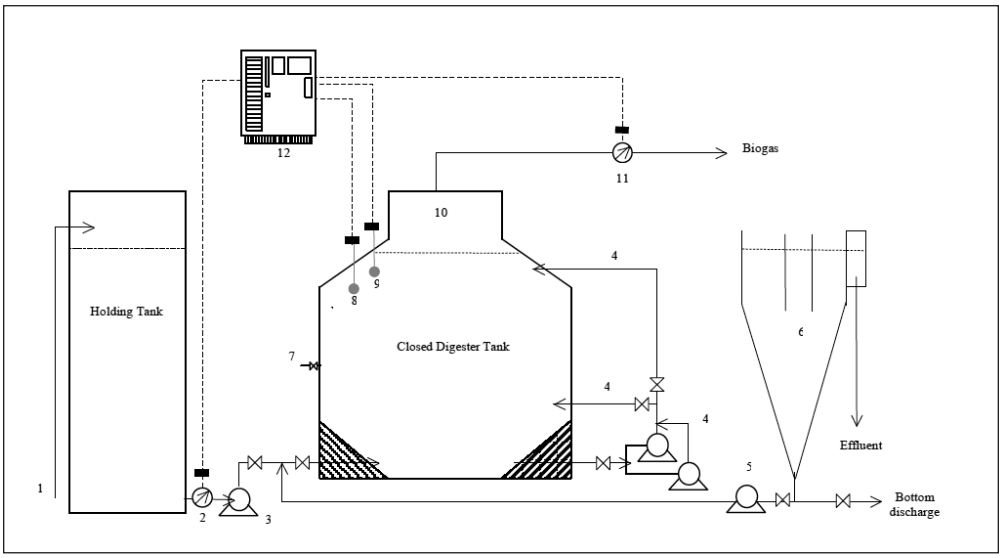

Fig. 1: Process flow diagram of the 500 m³ semi-commercial closed digester tank; 1-Raw POME inlet; 2-Mass flow meter; 3-Centrifugal pump; 4-Mixing pump system; 5-Sludge recycling pump; 6-Settling tank; 7-Sampling port; 8-pH probe; 9-Temperature probe; 10-Biogas collection chamber; 11-Biogas mass flow meter; 12-Endress+Hauser Ecograph system

sludge recycling ratio for both the experiments. For the three hours' feeding interval experiment, the operational days only lasted for 46 days, whereas for the six hours' feeding interval experiment, the operational days improved and went on up to 100 days. The COD concentrations of the raw POME utilized for both experiments were almost consistent; however, the maximum feeding rate applied was different because different maximum OLR were achieved in both the experiments. Due to different feeding rates applied, the HRT was also different. The sludge recycling rate was fixed at 6 $m^3 d^{-1}$ and the recycling ratio was varied daily.

Chemical Analyses and On-line Data

Analysis for Chemical Oxygen Demand (COD), total volatile fatty acids (VFA), total solid (TS), volatile suspended solid (VSS), pH, alkalinity, total kjeldah nitrogen (TKN), lignin, and ash were performed according to the APHA standard methods (APHA, 1985). The raw POME volume feeding rate was measured online using an electromagnetic flow measuring system (PROline promag 50, Endress+Hauser, Germany) and the biogas produced was also measured online using a thermal mass flow meter (T-Mass AT70, Endress+Hauser, Germany). The online data were temporarily stored in

TABLE 2
Chemical Oxygen Demand inlet, feeding rate, HRT, OLR and sludge recycling ratio

Experiment	Days of operation	COD inlet (mg L^{-1})	Feeding rate ($m^3 d^{-1}$)	HRT (d)	OLR (kgCOD $m^{-3}d^{-1}$)	Recycling ratio
Experiment 1[a]	46	17,100 - 82100	3.3-50.0	10.0 - 152.2	0.5 - 4.0	0.13 - 1.35
Experiment 2 [b]	100	28,900 - 79600	18.2 - 67.2	7.4 - 27.4	2.0-6.0	0.11 - 0.30

[a] Feeding of every three hours with sludge recycling, [b] Feeding of every six hours with sludge recycling

the Endress+Hauser Ecograph (Germany) and retrieved weekly for analysis. The concentration of methane was determined using a calibrated portable methane gas analyzer (XP-314A, Shin-Cosmos Electric Co. Ltd., Japan).

Scanning Electron Microscope (SEM)

Samples for the scanning electron microscope were fixed with 4% glutaraldehyde in 0.1 M of sodium cacodylate buffer for three changes of 10 min each. After post-fixation with 1% osmium tetroxide for 2 h, the samples were washed again with 0.1 M of sodium cacodylate buffer for three changes of 10 min each. The samples were then dehydrated in a graded acetone series of 35% for 10 min, 50% for 10 min, 75% for 10 min, 95% for 10 min, and finally three changes of 100% for 15 min each. After that, the samples were dehydrated using the critical point drying method (CPD) for 30 min. Finally, the CP-dried samples were sputter-coated with gold and examined using a Philips XL30 ESEM (Holland), operating at an accelerating voltage of 15 kV. The samples were viewed and the most prominent was selected for the analysis.

RESULTS AND DISCUSSION

Effects of the Three Hours' Feeding Interval on Digester Performance and Stability

The digester performance and its stability, over 46 days of operation when the digester was subjected to a feeding interval of every three hours, are shown in Tables 3 and 4, respectively. The performance was evaluated in terms of the COD removal efficiency and methane yield. The methane yield is expressed in mass of methane, produced at a standard temperature and pressure over the mass of COD removed in a day ($kgCH_4$ $kgCODremoved^{-1}$). This experiment only lasted for 46 days and was stopped due to the lower methane production. Over this period, the OLR was gradually increased from 0.5 to 4.0 kgCOD m^{-3} d^{-1} in order to eliminate shock loading to the system. The strategy was proven successful, based on the satisfactory COD removal efficiency. The results showed a good treatment performance by the digester with > 90% removal. This is consistent with some previous studies on POME treatment, as reported by several researchers listed in Table 1 (Cail and Barford, 1985; Borja and Banks, 1994a; Borja and Banks, 1994b; Borja and Banks, 1994c; Borja *et al*., 1996; Faisal and Unno, 2001; Najafpour *et al*., 2006). In general, high COD removals (higher than 90% removals) were recorded using various technologies tested at various HRT (0.25 - 10 days), inlet COD concentrations (2.5 – 70 kgm^{-3}) and OLR (5.33-17.47 $kgCODm^{-3}d^{-1}$), except for the Modified Anaerobic Baffled Reactor where only 77.3% COD removal was reported. In addition, our previous studies also observed high COD removal efficiency using the same digester (Yacob *et al*., 2006; Sulaiman *et al*., 2009). This further implies the suitability of anaerobic treatment method for POME.

The methane yield showed a reducing trend towards the end of the experiment. Initially at lower OLR, a high methane yield was recorded as can be seen in the case OLR of 0.5 kgCOD m^{-3} d^{-1}, where the methane yield was between 0.22 and 0.37 $kgCH_4$ $kgCODremoved^{-1}$. In this case, the methane yield was higher than the theoretical methane yield of 0.25 $kgCH_4$ $kgCODremoved^{-1}$. This is because when the OLR applied is low, adequate retention time is available for the micro-organisms to utilize the substrate in the digester and eventually result in higher methane production. Moreover at this stage, a higher recycling ratio was also applied to the system at 1.35 m^3 sludge m^{-3} POME, which brought about the benefit of higher cell accumulation in the digester. The methane yield of higher than theoretical value of 0.25 $kgCH_4$ $kgCODremoved^{-1}$ or 0.35 L CH_4 $kgCODremoved^{-1}$ was also reported by Faisal and Unno (2001), where methane yield ranging from 0.355 – 0.420 were reported when the digester was operated at 5 – 8 days HRT, using a Modified Anaerobic Baffled Reactor and high recycling ratio. The trend of high COD removal efficiency and satisfactory methane yield continued up to OLR application of 1.5 kgCOD m^{-3} d^{-1}. However, once the OLR was further increased to 2.0 kgCOD m^{-3} d^{-1}, the methane yield was drastically reduced to only

TABLE 3
Digester performance parameter for the three hours feeding interval experiment and the sludge recycling ratio

Days	Organic loading rate[a]	COD removal efficiency (%)		Methane yield [b]		Recycling ratio [c]
		Range	Mean±SD	Range	Mean±SD	Mean±SD
1-5	0.5	95-98	97±1	0.22-0.37	0.27±0.1	1.35±0.43
6-8	1.0	95-98	96±2	0.17-0.22	0.19±0.02	0.56±0.20
9-11	1.5	97	97	0.19-0.20	0.19±0.01	0.43±0.02
12-16	2.0	95-98	97±1	0.09-0.14	0.11±0.02	0.34±0.14
17-21	2.5	95-98	96±1	0.06-0.10	0.08±0.02	0.28±0.12
22-29	3.0	90-95	94±2	0.10-0.13	0.11±0.01	0.18±0.03
30-37	3.5	83-96	92±4	0.06-0.13	0.09±0.02	0.13±0.02
38-46	4.0	90-97	94±2	0.06-0.13	0.09±0.02	0.15±0.02

[a] unit is in kgCOD m^{-3} d^{-1}, [b] unit in is $kgCH_4$ kgCODremoved $^{-1}$, [c] is defined as the sludge recycling rate over the volumetric feeding rate of the POME

TABLE 4
Digester stability for the three hours' feeding interval experiment

Operation days	Organic loading rate[a]	Total VFA (mg L^{-1})		VFA:Alk
		Range	Mean±SD	Average
1-5	0.5	164-202	182±16	0.05
6-8	1.0	170-299	219±70	0.07
9-11	1.5	209-304	253±48	0.07
12-16	2.0	104-167	147±25	0.05
17-21	2.5	150-688	370±287	0.07
22-29	3.0	389-922	627±229	0.12
30-37	3.5	194-621	417±167	0.20
38-46	4.0	321-1291	742±373	0.28

[a] unit is in kgCOD m^{-3} d^{-1}

0.11 $kgCH_4$ kgCODremoved^{-1}. At even higher OLR application (2.5 to 4.0 kgCOD m^{-3} d^{-1}), the trend of low methane yield continued, i.e. at only between 0.08 and 0.11 $kgCH_4$ kgCODremoved^{-1} which is only 32-44% of the theoretical yield. At this stage, the methanogenesis was inhibited and the sludge recycling rate at 6 m^3 d^{-1} was inadequate to cater for the higher accumulation of VFA (742 mg L^{-1}) in the system. The recycling ratio was also lower, i.e. at only 21-25% of the initial recycling ratio. At the end of the experiment, the process was stopped to recover.

The digester's stability in terms of the total VFA accumulation and VFA:Alk ratio in the system is shown in Table 4. At a lower OLR range of 0.5 to 2.0 kgCOD m^{-3} d^{-1}, the total VFA accumulation inside the system was low, i.e. ranging from 147 to 253 mg L^{-1}. This particular phenomenon is a common indication of a good VFA utilization by the methanogens for methane production. In addition, the available alkalinity is also adequate to buffer the total VFA accumulation in the system, as indicated by low VFA:Alk ratio of between 0.05 and 0.07. At this stage, the results of COD removal efficiency

and methane yield were satisfactory. However, once the OLR was increased to 2.5 kgCOD m^{-3} d^{-1} and the maximum of 4.0 kgCOD m^{-3} d^{-1}, the process became unstable, as indicated by the drastic increase of the total VFA inside the system. The maximum total VFA accumulation recorded in the system was 742 mg L^{-1} at the end of the process before it was stopped for recovery. At this stage, the VFA:Alk ratio was increased to 0.28, which was almost the critical level of 0.3. Due to this non-conducive environment, the methane yield was lower at 0.09 $kgCH_4$ kg COD $removed^{-1}$. This phenomenon of higher total VFA accumulation, when the OLR was increased, was also observed in the study by Yacob *et al*. (2006) and Sulaiman *et al*. (2009).

The Effects of Six Hours' Feeding Interval on Digester Performance and Stability

In this experiment, the digester was subjected to a feeding interval of six hours which resulted in four times of feeding per day. The digester performance was evaluated in terms of the COD removal efficiency and methane yield achieved, whereas the stability was determined in terms of the total VFA accumulation and VFA:Alk ratio. These results are shown in Tables 5 and 6, respectively. The feeding interval of every six hour was found to produce a more stable anaerobic treatment process, where the total VFA accumulation was below 500 mg L^{-1} even when the OLR was increased to 6.0 kgCOD m^{-3} d^{-1}. Initially at low OLR ranging from 2.0 to 2.5 kgCOD m^{-3} d^{-1}, the system maintained a remarkably high COD removal efficiency of 96-97% and the methane yield was also high at 0.17 $kgCH_4$ $kgCODremoved^{-1}$. The total VFA concentration in the system was also low (i.e. below 300 mgL^{-1}) and this phenomenon indicates a good VFA utilization by the methanogens. At this stage, it is believed that a balanced microbial population existed between acidogens and methanogens. As OLR was gradually increased to 3.0 and eventually to 4.0 kgCOD m^{-3} d^{-1}, the methane yield was slightly reduced to 0.14 $kgCH_4$ $kgCODremoved^{-1}$ but the COD removal efficiency was maintained (i.e. higher than 90%). As discussed earlier, the reduction of the methane yield might be caused by the inhibition of methanogenesis process. At this stage, the recycling ratio was only 50% of the initial recycling ratio which might have resulted in lower cell retention in the digester to counter the VFA accumulation in the system at 224 mg L^{-1}. Therefore, in order to understand the effect of higher OLR on the system, the OLR was gradually increased to 4.5, 5.0, 5.5, and finally to

TABLE 5
Digester performance parameter for the six hours' feeding interval experiment and the sludge recycling ratio

Days	Organic loading rate[a]	COD removal efficiency (%)		Methane yield [b]		Recycling ratio [c]
		Range	Mean±SD	Range	Mean±SD	Mean±SD
1-10	2.0	94-99	97±1.6	0.08-0.21	0.17±0.04	0.30±0.13
11-18	2.5	95-97	96±0.7	0.16-0.18	0.17±0.01	0.23±0.04
19-27	3.0	90-98	95±2.1	0.12-0.18	0.14±0.01	0.16±0.04
28-37	3.5	95-97	96±0.8	0.13-0.18	0.15±0.01	0.16±0.03
38-47	4.0	86-97	94±3.5	0.12-0.17	0.14±0.01	0.15±0.02
48-57	4.5	88-98	96±2.8	0.14-0.16	0.14±0.01	0.17±0.03
58-77	5.0	91-97	95±1.6	0.11-0.17	0.13±0.01	0.12±0.02
78-89	5.5	91-95	94±1.2	0.11-0.14	0.12±0.01	0.11±0.01
90-100	6.0	92-99	96±2.0	0.09-0.11	0.10±0.01	0.11±0.01

[a] unit is in kgCOD m^{-3} d^{-1}, [b] unit is in $kgCH_4$ $kgCODremoved^{-1}$, [c] is defined as the sludge recycling rate over the volumetric feeding rate of the POME

TABLE 6
Digester stability for the six hours feeding interval experiment

Operation days	Organic loading rate[a]	Total VFA (mgL^{-1})		VFA:Alk
		Range	Mean±SD	Average
1-10	2.0	163-506	284±122	0.08
11-18	2.5	211-308	268±32	0.08
19-27	3.0	195-375	293±67	0.08
28-37	3.5	171-414	255±76	0.10
38-47	4.0	105-386	224±89	0.10
48-57	4.5	211-517	343±88	0.15
58-77	5.0	183-515	336±85	0.15
78-89	5.5	318-583	432±83	0.20
90-100	6.0	327-726	500±109	0.28

[a] unit is in kgCOD m^{-3} d^{-1}

6.0 kgCOD m^{-3} d^{-1}. Remarkably, the system was found to maintain a satisfactory COD removal efficiency of higher than 90%. In spite of the good COD removal, the methanogensis process was inhibited due to the accumulation of VFA in the system. The methane yield was decreased to 0.09 $kgCH_4$ $kgCOD_{removed}^{-1}$ at the end of the process, before it was stopped for recovery. This is only 36% of the theoretical yield. At this stage, the total VFA accumulation was only around 500 mg L^{-1}, but the VFA:Alk ratio was high, i.e. almost similar to its critical level of 0.3. At this stage, the available alkalinity was limited and inadequate to sustain a good buffering capacity to the system and therefore created non-conducive environment for methanogenesis. The sludge recycling ratio was only 0.11, which was only 37% of the initial recycling ratio. This was inadequate to provide a higher buffering capacity to the digester which suggests that a higher sludge recycling ratio is required, as adopted by Setiadi *et al*. (1996), Faisal and Unno (2001), Najafpour *et al*. (2006) and Sulaiman *et al*. (2009).

Ratio of Food-to-Microorganisms (F/M Ratio)

The ratio of food-to-microorganisms (F/M), expressed in mass of COD applied over the mass of mixed liquor volatile suspended solid in the digester of the entire experiments period for both the experiments, is shown in *Fig. 2*. In many studies the sludge recycling was observed to be able to provide active microorganisms to the system and maintain its pH without additional alkalinity supplementation as reported by Najafpour *et al*. (2006), Setiadi *et al*. (1996) and Faisal and Unno (2001). In addition to providing additional alkalinity and maintaining the pH of the system, the sludge also contains denser population of microorganisms responsible for biodegradation of organic substances in POME and conversion to methane gas. *Fig. 3* shows the scanning electron microscope (SEM) picture of the microorganisms in the sludge sample taken from the settling tank. It shows the existence of microorganisms in the flocs of the treated POME sludge believed to be *Methanosarcina* sp. and *Methanosaeta* sp. This is consistent with the earlier finding by Sulaiman *et al*. (2009) on a similar sample using the Fluorescent In-situ Hybridisation (FISH) technique. Both the methanogens are known to be very important for the production of methane from acetate (Robinson *et al*., 1984; Sekiguchi *et al*., 1999; Saiki *et al*., 2002; Yang *et al*., 2007). These researchers also confirmed the presence of both *Methanosarcina* sp. and *Methanosaeta* sp. in the samples of the anaerobic wastewater treatment plants.

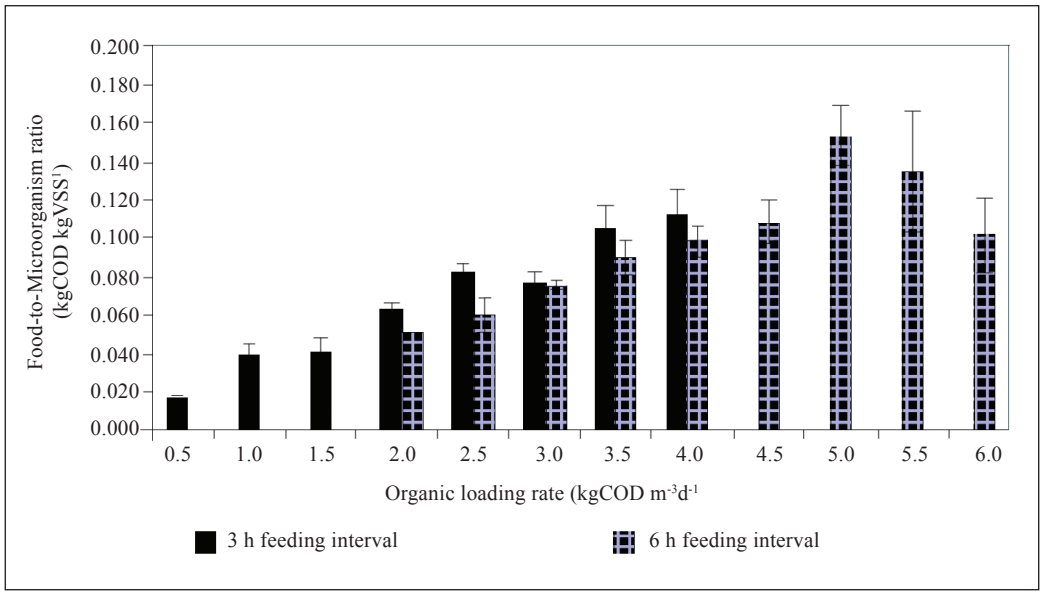

Fig. 2: The ratio of food-to-microorganisms (F/M ratio) at different organic loading rates applied for the three hours feeding interval and six hours feeding interval experiments

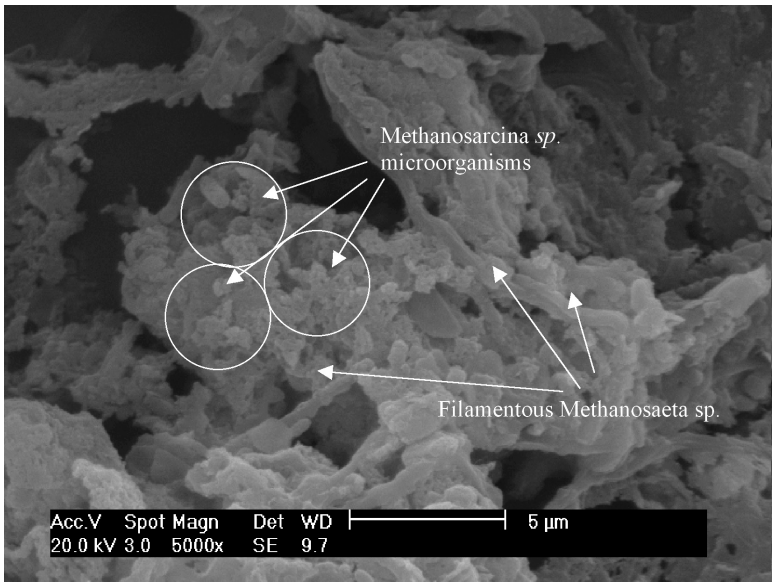

Fig. 3: Photograph of scanning electron microscope (SEM) of the recycled sludge showing the existence of microorganisms in the flocs of POME sludge - Methanosarcina *sp. and* Methanosaeta *sp. (magnification of 5000X)*

In both the experiments, the sludge was recycled at 6 m^3 in a day. The sludge recycling ratio was gradually reduced due to the higher OLR applied to the system. This resulted in higher F/M ratio at higher OLR applied, as indicated in *Fig. 2*. Generally, the F/M ratio for experiment 1 was relatively higher than experiment 2, indicating a lower concentration of microorganisms available in the system. In the three hours' feeding interval experiment, the maximum F/M ratio recorded was approximately 0.12 kgCOD $kgVSS^{-1}$ before the process was stopped for recovery due to VFA inhibition. In the six hours' feeding interval experiment, however, at the same OLR, a slightly lower F/M ratio of 0.1 kgCOD kg VSS^{-1} was recorded and the methanogenesis was not yet inhibited. Moreover, it is believed that at this stage, the continuous supply of active microorganisms from the settling tank and less disturbance, due to longer feeding interval, helped to maintain the methanogenesis rate in the digester and consequently maintaining higher VFA uptake rate by the methanogens.

In the six hours' feeding interval experiment, when the OLR was increased to 5.0 kgCOD m^{-3} d^{-1}, the F/M ratio also increased to 0.148 kgCOD $kgVSS^{-1}$. At this stage, the methane yield was further reduced, indicating a lower microorganism activity in the system. Upon further increment of the OLR (i.e. 6.0 kgCOD m^{-3} d^{-1}), the adverse effect on methanogenesis was clearly observed where the recorded methane yield was only 0.11 $kgCH_4$ kg $CODremoved^{-1}$. At this stage, although the F/M ratio was lower, it was unable to recover due to shock loading and microorganisms' washout. Furthermore the sample of the mixed liquor volatile suspended solid (MLVSS) inside the digester might have been contaminated by the VSS of the raw POME itself, and thus resulted in a lower ratio of food-to-microorganisms. At this stage, higher sludge recycling ratio should be applied in order to maintain a higher level of microorganisms in the system which are responsible for methane production, as suggested by some researchers (e.g. Najafpour *et al*., 2006; Setiadi *et al*., 1996; Faisal and Unno, 2001; Sulaiman *et al*., 2009).

CONCLUSIONS

This study has demonstrated the feasibility of anaerobic treatment of POME at the sludge recycling rate of 6 m^3 d^{-1} and feeding interval of three hours or six hours. In both the experiments, the COD removal efficiency was higher than 90%. The feeding interval of six hours showed a more stable anaerobic treatment process with the total VFA concentration recorded below 500 mg L^{-1} and the VFA:Alk of less than 0.3 at OLR of 6.0 kgCOD m^{-3} d^{-1}. At the end of the treatment period, the methane yield recorded the lowest values (0.09 and 0.10 $kgCH_4$ $kgCODremoved^{-1}$) for the three hours' feeding interval and six hours' feeding interval, respectively. Future research will focus on the methane yield improvement for renewable energy capture.

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Physical Changes to Oil Palm Empty Fruit Bunches (EFB) and EFB Mat (Ecomat) during Their Decomposition in the Field

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ABSTRACT

The main objectives of this study were to determine the physical changes in oil palm empty fruit bunches (EFB) and EFB mat (Ecomat), which were used as soil mulching materials, during their decomposition in the field, as well as to compare the soil water content under these mulches and with bare soil. A field experiment was conducted at an estate using ten-year-old oil palm trees. Experimental design was a Randomized Complete Block with two treatments (EFB and Ecomat) and three replications. EFB was applied at 1000 kg palm^{-1} as a single layer on the soil surface. Ecomat was applied as a single layer with an area of 4 m^2. Physical properties of EFB and Ecomat, measured every two month for six months, were bulk density, water content, water retention, and saturated hydraulic conductivity. Soil water content up to 750 mm depth was further measured on a daily basis. Results showed that EFB was better than Ecomat as a mulching material to conserve soil water. As compared to Ecomat, EFB had a lower bulk density (two times less dense), higher saturated hydraulic conductivity (about two times higher) and higher water content (between 20 to 57% more water). EFB was also found to hold its water more strongly than Ecomat. On average, the soil under EFB mulches had, nearly 27% more water than the soil under Ecomat mulches, and 38% more than bare soil. The soil under Ecomat mulches had only 8% more water than bare soil on average. Based on the model simulations, 5 layers of Ecomat would conserve as much soil water as 1 layer of EFB. Both mulching materials were estimated to fully decompose in the field in about 9 months.

Keywords: Empty fruit bunches, Ecomat, oil palm, soil conservation, water conservation, organic matter

INTRODUCTION

Malaysia and Indonesia are the two largest producers of palm oil in the world. In Malaysia, oil palm occupies over four million hectares, occupying about 12% of Malaysia's land area. One of the by-products of the palm oil milling process is the empty fruit bunches (EFB), and on average, every one tonne of fresh fruit bunches (FFB) produces about 220 kg of EFB as a by-product (Singh *et al*., 1999). Considering that Malaysia produces 2.8 to 3 million tonnes of EFB annually (Kamaruddin *et al*., 1997), determining ways to reuse the EFB waste is therefore vital. One of the most common methods practiced in oil palm estates is to use EFB as a mulching material to protect the soil surface and conserve soil water and nutrients.

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In terms of fertiliser use, one tonne of EFB is equivalent to 7 kg of urea, 2.8 kg of rock phosphate, 19.3 kg of muriate of potash, and 4.4 kg of kieserite (Singh *et al*., 1999). EFB is also a source of organic matter which increases soil aggregation, aggregate stability, and water infiltration, and hence, it reduces soil erosion.

Nevertheless, one well-known disadvantage of EFB is that it is rather bulky. One recent method used is to compress the EFB into a mat or carpet known as Ecomat. Being less bulky and easier to pack, transportation and handling of Ecomat is easier and cheaper than EFB. According to Yeo (2007), Ecomat is produced by shredding the EFB into its raw fibre and then combed out, after which EFB undergoes a high pressure hydraulic press to remove impurities such as water, sludge, and oil traces. EFB is then dried to about 15% gravimetric water content before it is trimmed to the required size and packed for shipping. In an unpublished study by the Beijing Forestry and Parks Department of International Cooperation conducted from 2002 to 2006, Ecomat was found to increase soil water content by 35.5% after two years, soil nitrogen by 3.5% and 6.7% in the summer and winter periods, respectively, as well as potassium content by between 20 to 128.6%.

Although much has been researched, particularly on the effects of EFB on the properties of soil, little has been studied on the physical changes of EFB and Ecomat over prolonged periods. However, this particular study focused on the changes of both mulching materials, rather than on the changes of the soil properties due to these mulching materials. Therefore, the main objectives of this study were: 1) to determine the physical changes in EFB and Ecomat used as soil mulching materials during their decomposition in the field; and 2) to compare the soil water content under EFB and Ecomat mulches, as well as with bare soil. Understanding the temporal physical changes of both these mulching materials will enable a better understanding on how they affect soil properties, particularly the ones related to conserving soil water and reducing soil erosion.

MATERIALS AND METHODS

The field experiment was conducted in an oil palm estate located at Balau Estate (2.9325 °N and 101.8822 °E) in Semenyih, Selangor. The estate had ten-year-old palms (Elaeis guineensis), and the soil was of the Rengam series (Typic Paleudult). The oil palm trees were planted in 8-by-8 m spacing on a hill slope of 6°. The total area of the experiment was 2240 m^2. The experimental design was a Randomized Complete Block (RCB) with two treatments (EFB and Ecomat) and three replications. For each replication, EFB was applied as a single layer on the soil surface at a rate of 1000 kg $palm^{-1}$. The mean weight of EFB was 3.5 kg per bunch and the mean thickness was 130 mm. For the Ecomat treatment, it was applied as a single layer of four pieces of Ecomat carpet, arranged side-by-side and without gaps between the pieces. Each piece of Ecomat carpet had an area of 1 m^2 and average weight and thickness of 3.3 kg and 20 mm, respectively. The experiment was conducted for six months, starting from February to September 2008. The EFB and Ecomat samples were collected every two months. Two samples were collected randomly from every plot.

Four physical parameters of EFB and Ecomat were measured. They were bulk density (core ring method by Blake and Hartge, 1986), gravimetric water content (Gardner, 1973), water retention (ceramic plate method by Richards, 1947), and saturated hydraulic conductivity (method adapted from Klute and Dirksen, 1986). In addition, volumetric soil water content, up to 750 mm depth, was measured daily using a soil moisture probe AquaPro-Sensor (Aquatic Sensors, Nevada). The statistical analyses were done using SPSS version 14 (SPSS Inc., Chicago). Meanwhile, the mean separation tests were carried out according to Duncan's New Multiple Range Test.

In addition to the field measurements, a soil water model was also used to simulate the effects on EFB and Ecomat on soil water content. Simulations were for a three-month's dry period (i.e. no rainfall) using information collected

about EFB, Ecomat and the soil properties. The soil water model was based on Hillel (1977), where the soil profile was divided into several layers, and the net water flux calculated for each soil layer was based on Darcy's law.

RESULTS AND DISCUSSION

The mean thickness of EFB and Ecomat, at the start of the experiment, was about 130 and 20 mm, respectively. As for the EFB, its thickness was reduced at the rate of 15.1 mm month^{-1}, and this was 1.7 mm month^{-1} for Ecomat, as shown in *Fig. 1*. Therefore, EFB and Ecomat would lose on average of 11% and 9% per month of their original thickness, respectively. Using the fitted linear regression curves (*Fig. 1*), it was estimated that both mulching materials would be fully decomposed (reduced to zero thickness) in about 9 months.

On average, Ecomat was two times more compact than EFB, as presented in *Fig. 2*. For example, at the application date (start of experiment), the bulk density of Ecomat was 0.24 Mg m^{-3} compared to only 0.11 Mg m^{-3} for the EFB. Hence, the bulk density for both the mulching materials was expected to increase with time because they would decompose into increasingly finer materials and, in turn, reduce the total pore size and increase compaction. Although the bulk density for both the mulching materials did generally increase with time, the ANOVA revealed that only the sole treatment factor had a significant effect on bulk density at 5% level of significance. The sole time factor and the interaction between the treatment and time factors were not significant at 5% level. Meanwhile, the non-significant effect of the time factor could be due to the high variability in the measurements of bulk density in this study.

As for the saturated hydraulic conductivity (K), the ANOVA revealed that the sole effects of time and treatment factors (but not the interaction between the two factors) on K were significant at 5% level. On average, K for the EFB and Ecomat was 3.8 and 2.0 mm s^{-1}, respectively (*Fig. 3*). This indicates that on average, the EFB would conduct water into the

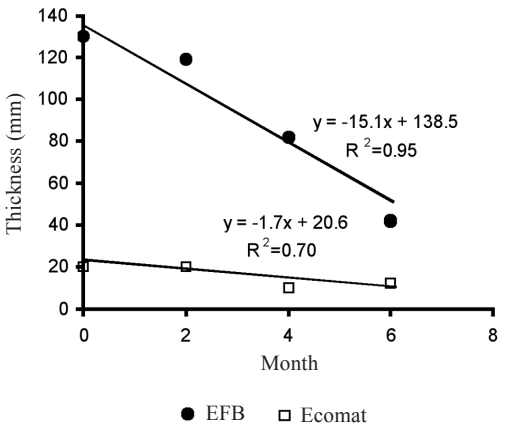

Fig. 1: Thickness of EFB and Ecomat

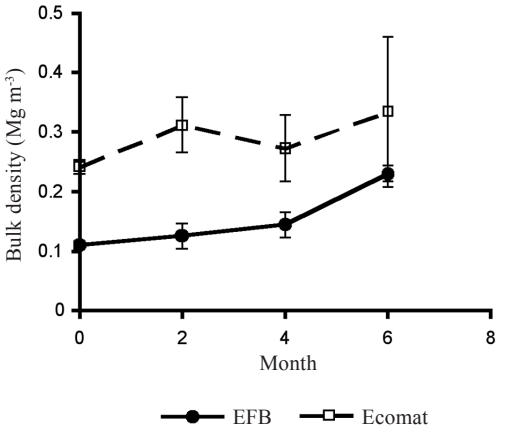

Fig. 2: Bulk density of EFB and Ecomat

soil nearly two times faster than Ecomat. For both the mulching materials, the reduction in their K over time was nearly two times. As for the EFB, its K was found to sharply decrease two months after the application (i.e. reduced from 5.0 mm s^{-1} in the second month to 2.5 mm s^{-1} in the fourth month), whereas for Ecomat, its K was shown to have reduced significantly immediately after the application (i.e. reduced from 3.5 mm s^{-1} at the start of the application to 1.8 mm s^{-1} two months later).

Similarly for the saturated hydraulic conductivity, the ANOVA revealed that the sole effects of time and treatment factors (but not the

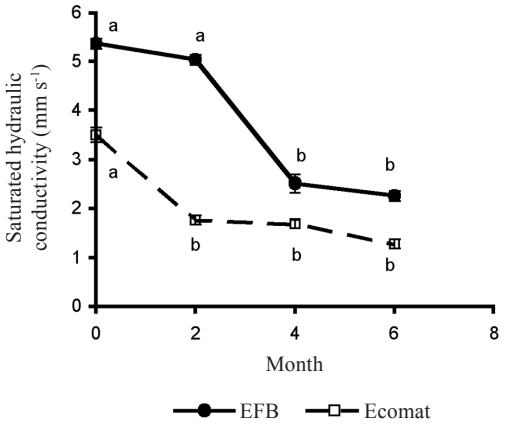

Fig. 3: Saturated hydraulic conductivity of EFB and Ecomat. For the same treatment, means with the same letter are not significantly different from each other at 5% level of significance

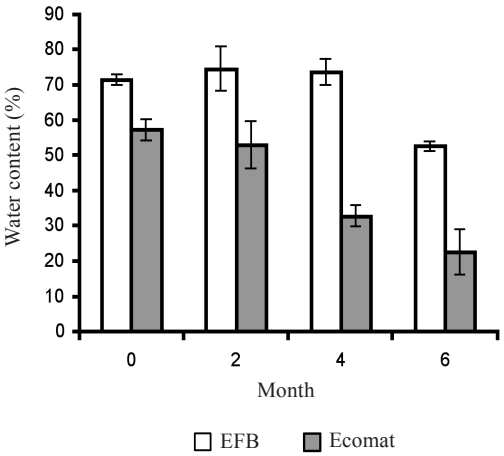

Fig. 4: Gravimetric water contents of EFB and Ecomat, at the time of sampling (mean separation test revealed that for the same month, the water content in EFB and Ecomat, were significantly different from each other at 5% level of significance)

interaction between them) on the water content of the mulching materials were significant at 5% level. On average, EFB was found to have 26.6 % more water than Ecomat at each sampling time (*Fig. 4*). At the start of the experiment, EFB had 20% more water than Ecomat, and this was increased to 57% at the sampling date on the sixth month. The mean separation test revealed that for the same month, the water content in the EFB and Ecomat was significantly different from each other at 5% level of significance.

Not only would EFB hold more water than Ecomat, the EFB was also found to hold or retain the water much stronger than Ecomat (*Fig. 5*). The mean negative slope of the water retention curve for EFB was 0.13 and this was 0.23 for Ecomat. A material with a smaller slope denotes water being held much stronger (therefore, harder to dry and more difficult to lose its water) than the material with a larger slope (which means it holds water less strongly). With the increase in time, the water retention slopes for both the mulching materials were generally increased. This meant that over time, both the mulching materials would hold their water increasingly less strongly due to the decomposition of the mulches.

However, the soil treated with EFB was found to have more water than the soil with Ecomat treatment (*Fig. 6*). On average, the total daily soil water content (up to 0.75 m depth), under EFB and Ecomat mulches, was 382 and 300 mm, respectively. In other words, the soil water content under EFB had, nearly 27% more water than the soil under Ecomat on average. The total soil water content for bare soil was the least as its surface was unprotected by any cover mulching material. On average, the soil with EFB mulches had 38% more water than the bare soil. Meanwhile, the soil with Ecomat mulches had only 8% more water than bare soil on average.

Therefore, the results gathered from the field experiment have indicated that a single layer of EFB is a better mulching material than a single layer of Ecomat in conserving more water in the soil. In order to determine the number of Ecomat layers which is required to equal the efficacy of a single layer of EFB to conserve soil water, this study has used a soil water model based on Hillel (1977), in which the soil profile was divided into seven layers (with

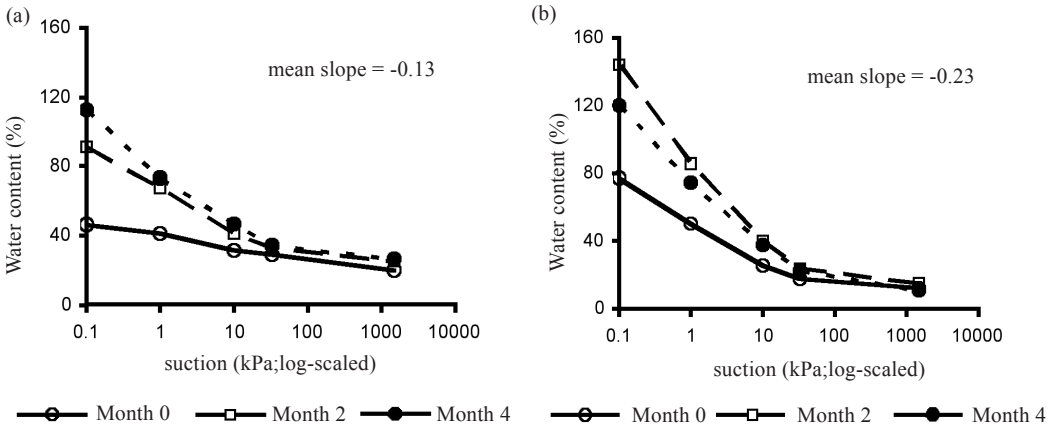

Fig. 5: Volumetric water retention curve of: a) EFB and b) Ecomat

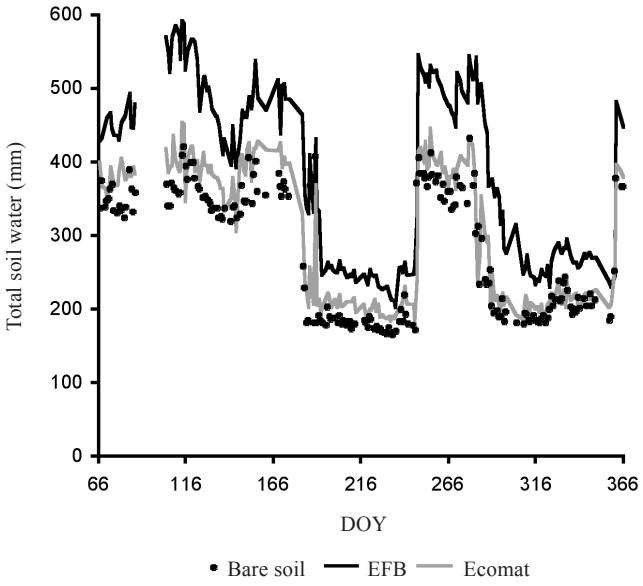

Fig. 6: Total daily soil water content (up to 750 mm depth) under EFB and Ecomat mulches and bare soil

the first layer the given mulching material), and the net water flux calculated for each soil layer was based on Darcy's law. Simulations were for a three-month dry period of no rain and the model parameters were based on those properties collected from EFB, Ecomat and soil in the experimental site. The simulations revealed that between 4 to 6 layers of Ecomat were required to give comparable soil water content as the soil under 1 layer of EFB (Table 1). Thus, based on the findings of the present study, it is concluded that 5 layers of Ecomat is equivalent to 1 layer of EFB so as to conserve the same amount of soil water.

TABLE 1
Simulated total soil water content under EFB (1 layer) and under different numbers of Ecomat layers for a three-month's dry period

Days	EFB (1 layer)	Ecomat (1 layer)	Ecomat (2 layers)	Ecomat (4 layers)	Ecomat (6 layers)
30	253.2	239.0	242.7	248.6	253.8
60	215.8	207.8	208.8	212.1	215.9
90	210.2	202.3	203.3	206.6	210.1

CONCLUSIONS

This study has showed that EFB is better than Ecomat as a mulching material to conserve water in soil. As compared to Ecomat, EFB has a lower bulk density (two times less dense), higher saturated hydraulic conductivity (about two times higher) and higher water content (between 20 to 57% more water). In addition, EFB can also hold its water much stronger than Ecomat. All these properties have helped the soil treated with EFB to have more water than the soil treated with Ecomat. The soil under EFB mulches had, on average, nearly 27% more water than the soil under Ecomat mulches, and 38% more than the bare soil. Meanwhile, the soil under Ecomat mulches had an average of only 8% more water than the bare soil. Based on model simulations, this study determined that 5 layers of Ecomat were required to conserve as much soil water as that equivalent to 1 layer of EFB. Finally, both the mulching materials were estimated to fully decompose in the field at nearly the same time, i.e. about 9 months.

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Concentrations of Heavy Metal in Different Parts of the Gastropod, *Faunus ater* (Linnaeus), Collected from Intertidal Areas of Peninsular Malaysia

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ABSTRACT

Marine gastropods, *Faunus ater* (Linnaeus), were collected from Pantai Sri Tujoh (Kelantan), Pantai Bisikan Bayu (Kelantan), Kg. Telaga Nenas (Perak) and Kesang Laut (Johor). Soft tissues of gastropods were dissected into digestive caecum (DC), foot, remainder, muscle, and operculum. The shell and dissected parts were analyzed for Cd, Cu, Ni, and Pb. It was found that the DC and the remainder accumulated high concentrations of Cu ranging between 159.1 and 290.2 μg/g dw. The shell was shown to highly accumulate non-essential Pb, Ni, and Cd compared to the soft tissues. Meanwhile, higher bioavailabilities of Cd and Cu were found in Pantai Sri Tujoh, whereas higher bioavailabilities of Ni and Pb were found in Pantai Bisikan Bayu compared to other sampling sites. The present results suggested that *F. ater* could be used as a potential biomonitor of heavy metal contamination. However, further studies are still needed in order to validate the use of *F. ater* as a good biomonitor of heavy metal pollution.

Keywords: Different parts, *Faunus ater*, heavy metals, Peninsular Malaysia

INTRODUCTION

Peninsular Malaysia is known to have a high diversity of marine molluscs. This advantage allows the advancement of biomonitoring studies, especially for heavy metal contamination in coastal areas. In its 2005-06 Malaysia Fisheries Directory, the Department of Fisheries Malaysia (2005) indicated that there are about 18 species of marine gastropods in Malaysian coastal areas. Apart from that, it is crucial to maintain the marine environment at pristine levels since extensive industrialization and urbanization have led to a strong risk of heavy metal contamination in many coastal environments around the world (Tam and Yao, 1998), including Peninsular Malaysia. Malaysia's economic growth is rapidly increasing and this leads to the increment in the production and usage of toxic chemicals such as trace elements (Yap *et al.*, 2002) to the marine system of Malaysia. The west coast of Peninsular Malaysia is a principle repository for agricultural, industrial, and domestic wastes originating from land-based and sea-based activities (Shazili *et al.*, 2006). In order to obtain sustainable resources from coastal areas, the ecological distributions and the background densities of intertidal molluscs should first be determined. Besides, the well-established green-lipped mussel as a biomonitor of heavy

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metal pollution (Yap *et al*., 2002: 2003: 2004: 2006), other intertidal gastropods should also be utilized for biomonitoring studies since a suite of intertidal biomonitors could reflect metal bioavailabilities and contaminations better (Rainbow *et al*., 2002). In Malaysia, there is a line of different gastropods species which have been attempted to be used as good biomonitoring agents such as *Nerita lineata* (Amin, 2006b; Yap *et al.*, 2009a), *Telescopium telescopium* (Yap *et al.*, 2008a) and *Pomacea insularum* (Yap *et al.*, 2008b).

Locally known "Siput belitung", *Faunus ater* (Potamididae), is a brackish-water snail inhabiting mangrove areas. Studies on the distributions of *F. ater* in this region have only been reported in Thailand (Sri-aroon *et al*., 2005; Sri-aroon *et al*., 2006). The likely habitats of this mangrove snail may include water plants, leaf-filled surface depressions, log-mud interfaces, log and stone crevices, soil, sand or mud around roots and on leaves, stones and trunks of mangrove trees (Sri-aroon *et al*., 2006). They are filter-feeders which use their gills to extract organic matter from the water in which they live (Yap *et al*., 2009b).

The use of gastropods as biomonitor organisms offer several advantages (Goldberg, 1975). Firstly, gastropods have reasonable sizes for analysis and repeatable samplings. Secondly, they are sedentary or less mobile than any other organisms such as fishes, and thus accumulate contaminants more efficiently than that of the surrounding waters. Thirdly, gastropods exhibit low or undetectable enzyme activities which metabolize pollutants. Finally, some gastropods are important seafood or source of protein; thus, studying them has significant human health implications.

It is known that the ability of aquatic molluscs to accumulate heavy metals, in their different parts to elevated levels reaching concentrations which are much higher than those of the ambient water concentrations, makes these molluscs useful for heavy metal biomonitoring purposes (Phillips and Rainbow, 1994; Rainbow, 2002; Saha *et al*., 2006). Besides, Rainbow (1995) stated that the use of particular organisms as biomonitors of heavy metal bioavailabilities in coastal waters allows comparisons to be made over space and time for biomonitors provide integrated measures of the ecotoxicologically significant fraction of ambient metal in those waters. On the other hand, Rainbow *et al*. (2002) mentioned that a biomonitor could provide information on heavy metal bioavailabilities specific to that particular biomonitor. However, it is usually considered valid to extrapolate from that biomonitor to draw conclusions about heavy metal bioavailabilities of the site in general. *F. ater* could probably act as a potential biomonitor of heavy metal bioavailabilities in the sites undertaken in this study.

The reliability of gastropods as a biomonitor of heavy metal contaminations has been revealed by a number of researchers. Among other, Liang *et al*. (2004), who conducted a study in the Chinese Bohai Sea, found that *Rapana venosa* accumulated a high level of Cd. The ability of *Patella caerulea* to accumulate heavy metals was revealed in the study conducted by Hamed and Emara (2006) in the Gulf of Suez, Red Sea. The studies of heavy metals in *Patella caerulea* and *Mullus barbatus* in the Ionian Sea, Italy were done by Storelli and Marcotrigiano (2005). These studies strongly supported the use of gastropods as biomonitors of heavy metal pollution in the marine environments. Meanwhile, suggestions on the monitoring of heavy metal contaminations and bioavailabilities, using the different parts of gastropods, were also very interesting. The strategy may overcome the inaccuracy caused by determining the heavy metal level in the total soft tissues. In addition, the spawning season of the gastropods and environmental factors may contribute to the wide variability of heavy metal concentrations in the total soft tissues of molluscs (Yap *et al*., 2006).

Since there have been no reported studies on heavy metals in *F. ater* from Malaysia, the present study focused on the work done using four geographical populations of *F. ater* in Peninsular Malaysia and the heavy metal distributions in the different parts of *F. ater*.

MATERIALS AND METHODS

Samplings were conducted from June to September 2007 in Pantai Seri Tujoh and Pantai Bisikan Bayu in the eastern part of Peninsular Malaysia and in Kg. Telaga Nenas and Kesang Laut in the western coast (*Fig. 1*). Further details of the sampling sites are given in Table 1. The samples of *F. ater* (Linnaeus) were identified for their family, genus, and species with the aid of the identification keys proposed by Uptham *et al*. (1983), Brandt (1974) and Van Benthem Jutting (1956).

Approximately 20 snails, with sizes ranging from 3.82 - 6.79 cm, were collected from each sampling site. The samples were brought back to the laboratory and were stored at -10 °C until further analysis. Prior to the analyses, the soft tissues of the snails (besides the shells) were carefully dissected and pooled into five different parts, namely remainder, operculum, muscle, foot, and digestive caecum (DC). The samples were then dried in an oven for 72 hours at 105 °C to constant dry weights (Mo and Neilson, 1994).

The dried shell and soft tissue parts were weighed and placed in acid-washed digestion tubes. 10 ml of concentrated nitric acid (AnalaR grade, BHD 69%) was added into the digestion tube for digestion. The samples were placed in a digestion block at 40 °C for 1 hour and they were fully digested at 140 °C for 3 hours after that (Yap *et al*., 2006). The cooled samples were diluted to 40 ml with double deionised water (DDW). The digested samples were then filtered through Whatman No. 1 (filter speed: medium) filter paper into acid-washed pill boxes. The samples were analyzed for Cd, Cu, Ni, and Pb using an air-acetylene flame Atomic Absorption Spectrophotometer (AAS) Perkim-Elmer™ Model 800. Standard solutions were prepared from 1000 ppm stock solutions provided by MERCK Titrisol for Cd, Cu, Ni, and Pb.

In order to avoid possible contamination, all the glassware and equipment used were acid-washed. Procedural blanks and quality control samples made from standard solutions for Cd, Cu, Ni, and Pb were analyzed after every 5 - 10 sample in order to check for the sample accuracy. The percentages of recoveries for the heavy metal analyses were acceptable at 80 - 110 %. The analytical procedures for the gastropod were checked with the Certified Reference Material (CRM) for dogfish liver (DOLT-3, National Research Council Canada). The recoveries of all the metals were satisfactory (Table 2).

One-way ANOVA-Student-Newman-Keuls (S-N-K) was applied to detect significant differences among the mean values using the statistical software, SPSS version 12.

TABLE 1
Locations, sampling dates, number of samples analyzed (*N*), longitude, latitude and descriptions of sampling sites of *Faunus ater* collected from Peninsular Malaysia

No	Location	Sampling date	N	Longitude	Latitude	Description of sampling site	Individual size, (cm)
1	Pantai Sri Tujoh	29 July 2007	20	5°52'N	102°30'E	Aquacultural area and recreational beach	4.83-6.79
2	Pantai Bisikan Bayu	03 July 2007	20	06°13'N	102°07'E	Aquaculture area	3.82-5.20
3	Kg. Telaga Nenas	25 August 2007	20	4°27'N	100°37'E	A fishing village	3.98-6.78
4	Kesang Laut	15 September 2007	20	2°10'N	102°34'E	A fishing village	4.33-6.71

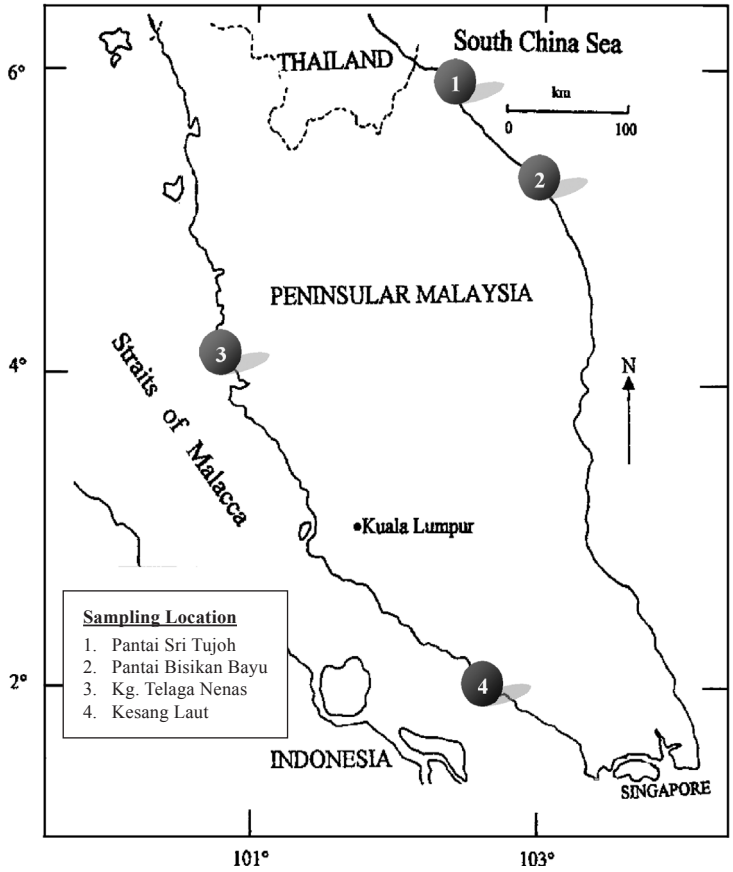

Fig. 1: Map showing sampling sites of Faunus ater *in the East and West Coast of Peninsular Malaysia*

TABLE 2
Analytical results for the Certified Reference Material (CRM) and the certified values for each metal (All values are presented µg/g dry weight)

Metal	Sample	CRM values	Measured values	Percentage of recovery
Cd	DOLT-3 Dogfish-liver	19.4 ± 0.600	20.5 ± 0.439	106 ± 2.26
Cu	DOLT-3 Dogfish-liver	31.2 ± 1.00	26.5 ± 2.58	85.0 ± 8.28
Ni	DOLT-3 Dogfish-liver	2.72 ± 0.350	2.77 ± 0.741	102 ± 27.2

NA: Pb value is not available

RESULTS AND DISCUSSION

Heavy Metals Distribution in the Tissues of F. ater

The heavy metal concentrations in the different parts of *F. ater* from the four sampling sites are shown in Table 3. It was found that the concentrations of Cu in DC and the remainder are in higher range (159.1-290.2 μg/g dw) than the shell of *F. ater*, (9.28-18.5 μg/g dw). On the other hand, the shell was the main tissue for the accumulation of non-essential heavy metals such as Pb, Ni, and Cd. The heavy metal concentrations found in the shell were in the range of 54.2-65.7, 24.5-31.5, and 4.97-5.52 μg/g dw for Pb, Ni and Cd, respectively.

The differences in the affinities of the metals to the binding sites of the metallothioneins in the different tissues (Roesijadi, 1980; Viarengo *et al*., 1985; Rainbow, 2002; Yap *et al*., 2009b) could cause the different heavy metal levels found in the gastropods. Besides, the function or the location of a specific organ in the gastropod could also be associated with the heavy metal accumulations in the different tissues (Rainbow, 2002). The heavy metals found in the shell could be explained on the basis of calcification in molluscs occurring within the extrapallial fluid, which is secreted by the mantle. The composition of the extrapallial fluid might be significantly altered with respect to seawater due to the influence of mantle metabolic activity on the transport of metals through the mantle (Klein *et al*., 1996), to the contributions of metals and carbon from metabolic source (Tanaka *et al*., 1986; Klein *et al*., 1996) or to organic complexation (Crenshaw, 1972). Additionally, heavy metals are not necessarily incorporated into the calcite crystal structure but they can also be adsorbed onto the skeletal organic matrix (Lingard *et al*., 1992) or entrapped as a separate mineral phase (Fritz *et al*., 1990). Consequently, shells may provide a more realistic indication of the degree of contamination for shell to exhibit less variability (unlike soft tissues due to seasonal changes), integrate elemental concentrations over the life of the animal (Foster and Cravo, 2003; Cravo *et al.*, 2002) and the shells also act as a biodeposition site of unwanted chemical species such as heavy metals (Yap *et al.*, 2003).

Heavy Metal Bioavailability in the Sampling Sites

Heavy metal bioavailability of the sampling locations was estimated using the concentrations in the different parts of *F. ater* (*Figs. 2-7*). It was assumed that high bioavailabilities of Cd and Cu were found in Pantai Sri Tujoh based on the elevated concentrations of these metals exhibited in the different parts such as the foot, muscle, remainder, DC, and shell. High bioavailabilities of Ni and Pb were found in Pantai Bisikan Bayu as exhibited in the similar tissues. According to Rainbow *et al*. (2002), the accumulated concentrations in a biomonitor are a direct reflection of the total integrated bioavailability and contamination of the sampling sites. This is because biomonitors such as molluscs bioaccumulate metals in their tissues in proportion to the degree of environmental contamination from seawater, suspended particles, and sediments and through food chains (Louma, 1983; Blackmore, 2001). Therefore, the comparisons of such accumulated concentrations in a biomonitor among sites are measurements of the bioavailabilities and contaminations of heavy metals of the sampling sites (Phillips and Rainbow, 1994).

In addition, the characteristics (anthropogenic activities) of the sampling locations could also contribute to the heavy metal bioavailabilities and contaminations of these areas. Based on the data presented in Table 1, it is known that all the four places are well-known as aquacultural areas and fishing villages. Therefore, it is suggested that the heavy metal bioavailabilities of these four sampling locations could be due to the anthropogenic activities found in these areas besides natural sources. There was a study reported in the literature which mentioned that heavy metal such as Pb contaminations originated from aquacultural activities and fishing villages (Yap *et al*., 2002). Moreover, the organic wastes discharged from fish/or mussel

TABLE 3
Decreasing order of heavy metal (μg/g) concentrations in the different parts of *Faunus ater*

Metal	Site	Metal distribution in the different parts					
Cu	Pantai Sri Tujoh	226.43 ± 4.38 DC	177.20 ± 10.37 Foot	153.08 ± 5. 47 Remainder	122.97 ± 1.18 Muscle	37.83 ± 9.74 Operculum	18.47 ± 3.18 Shell
	Pantai Bisikan Bayu	193.86 ± 10.52 Remainder	138.14 ± 9.35 Foot	127.01 ± 2.58 DC	104.85 ± 6.16 Muscle	94.64 ± 15.09 Operculum	9.28 ± 1.54 Shell
	Kg. Telaga Nenas	159.09 ± 3.16 DC	144.38 ± 4.88 Remainder	139.03 ± 0.87 Foot	115.48 ± 10.67 Muscle	51.84 ± 3.54 Operculum	11.66 ± 1.80 Shell
	Kesang Laut	290.16 ± 9.17 Remainder	211.79 ± 3.18 DC	163.98 ± 2.41 Foot	146.91 ± 13.68 Muscle	32.61 ± 0.68 Operculum	9.65 ± 0.82 Shell
Pb	Pantai Sri Tujoh	61.31 ± 1.57 Shell	27.30 ± 8.55 Operculum	24.78 ± 1.23 Remainder	15.50 ± 2.07 DC	7.59 ± 1.34 Foot	8.88 ± 1.23 Muscle
	Pantai Bisikan Bayu	65.66 ± 3.68 Shell	26.60 ± 4.75 DC	15.62 ± 4.95 Remainder	11.52 ± 2.62 Muscle	5.36 ± 0.45 Foot	4.80 ± 1.79 Operculum
	Kg. Telaga Nenas	54.22 ± 3.37 Shell	18.08 ± 0.23 Operculum	14.69 ± 2.20 Remainder	3.57 ± 1.46 DC	3.39 ± 0.48 Muscle	1.04 ± 0.02 Foot
	Kesang Laut	57.80 ± 1.50 Shell	31.64 ± 0.97 Operculum	18.87 ± 2.80 Remainder	6.68 ± 0.48 DC	2.89 ± 0.84 Foot	2.75 ± 1.35 Muscle
Ni	Pantai Sri Tujoh	26.42 ± 1.42 Shell	7.19 ± 1.25 DC	7.01 ± 0.82 Remainder	3.41 ± 0.45 Operculum	2.26 ± 0.73 Muscle	0.34 ± 2.00 Foot
	Pantai Bisikan Bayu	31.49 ± 2.21 Shell	10.29 ± 1.31 DC	9.77 ± 1.10 Remainder	6.77 ± 0.37 Foot	5.35 ± 0.29 Muscle	1.62 ± 0.55 Operculum
	Kg. Telaga Nenas	26.53 ± 1.94 Shell	11.05 ± 2.85 Operculum	9.86 ± 1.64 Remainder	8.59 ± 0.33 DC	1.70 ± 0.66 Muscle	0.63 ± 0.20 Foot
	Kesang Laut	24.53 ± 1.23 Shell	4.82 ± 1.75 Remainder	2.62 ± 0.13 Operculum	1.81 ± 0.62 Muscle	0.64 ± 1.69 DC	0.11 ± 0.05 Foot

Cd	Pantai Sri Tujoh	5.52 ± 0.13	2.58 ± 0.25	1.57 ± 0.11	1.40 ± 0.14	0.86 ± 0.11	0.74 ± 0.24
		Shell	Remainder	DC	Muscle	Foot	Operculum
	Pantai Bisikan Bayu	5.11 ± 0.08	2.09 ± 0.05	1.20 ± 0.16	0.77 ± 0.11	0.72 ± 0.23	0.11 ± 0.03
		Shell	Remainder	DC	Muscle	Foot	Operculum
	Kg. Telaga Nenas	4.99 ± 0.19	2.26 ± 0.15	1.40 ± 0.12	1.29 ± .19	0.69 ± 0.09	0.55 ± 0.10
		Shell	Remainder	DC	Operculum	Muscle	Foot
	Kesang Laut	4.97 ± 0.19	1.53 ± 0.14	1.11 ± 0.06	0.77 ± 0.23	0.57 ± 0.06	0.32 ± 0.06
		Shell	Remainder	Operculum	DC	Muscle	Foot

Note: DC = Digestive caecum

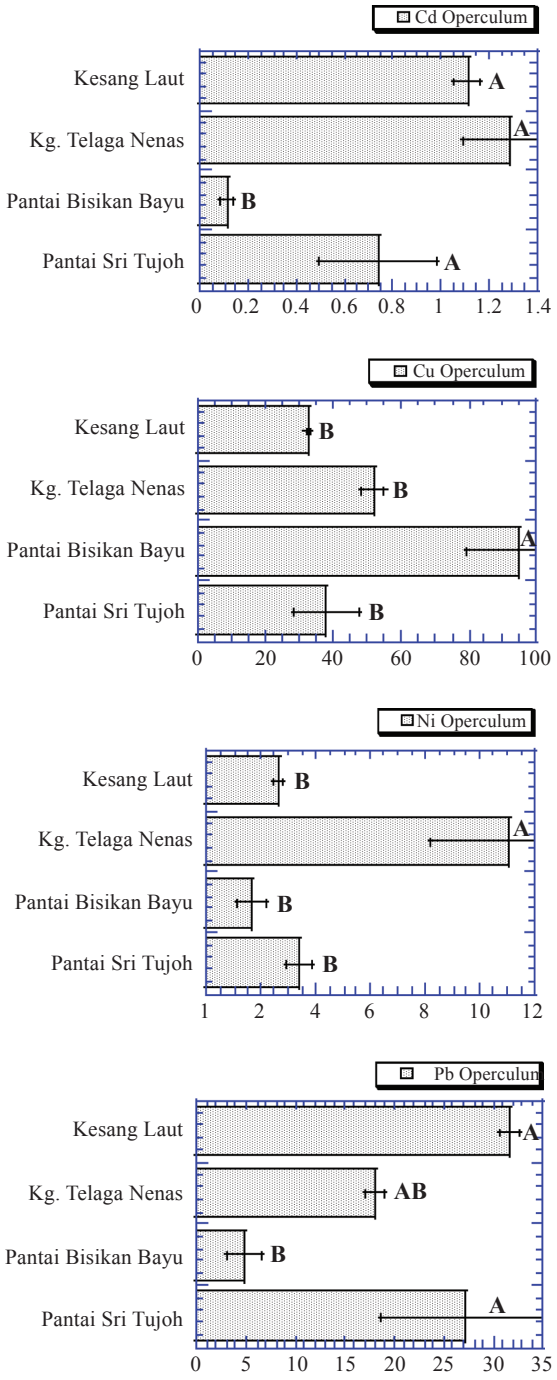

Fig. 2: Heavy metal concentration (mean µg/g dw ± S.E. (n=3)) in the operculum of Faunus ater *collected from the East and West Coasts of Peninsular Malaysia*

Note: Students-Newman-Keuls (SNK) comparisons of metal levels in different soft tissues and shell of *F. ater*; Means with different letters are significantly different, P≤0.05

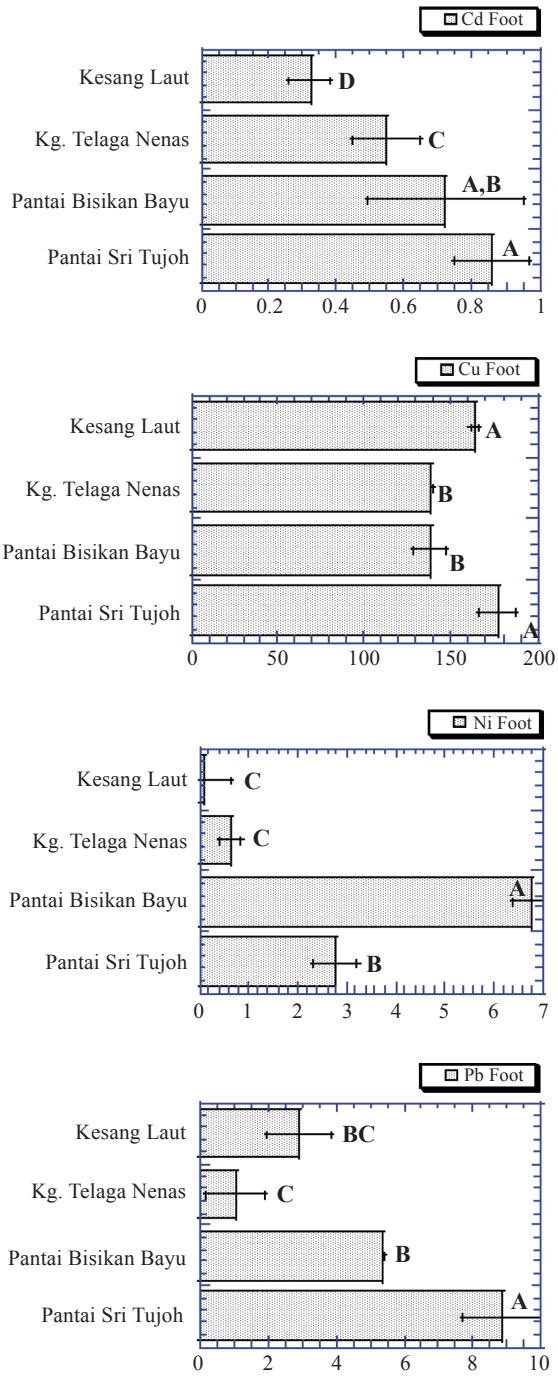

Fig. 3: Heavy metal concentration (mean µg/g dw ± S.E. (n=3)) in the foot of Faunus ater *collected from the East and West Coasts of Peninsular Malaysia*

Note: Students-Newman-Keuls (SNK) comparisons of metal levels in different soft tissues and shell of *F. ater*; Means with different letters are significantly different, P≤0.05

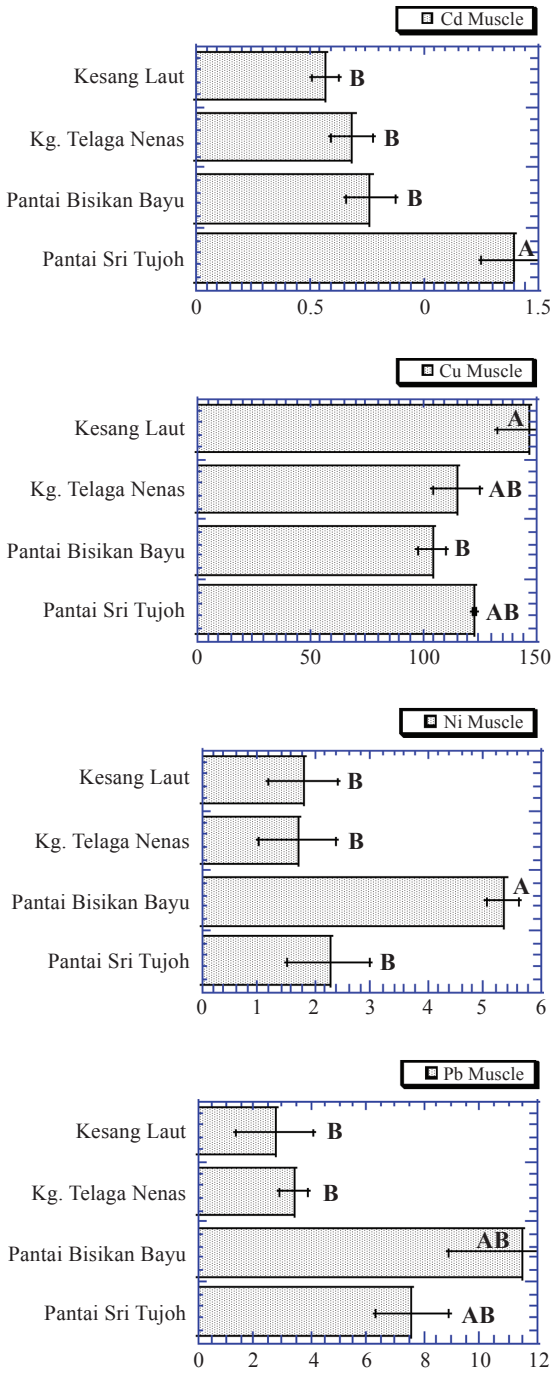

Fig. 4: Heavy metal concentration (mean µg/g dw ± S.E. (n=3)) in the muscle of Faunus ater *collected from the East and West Coasts of Peninsular Malaysia*

Note: Students-Newman-Keuls (SNK) comparisons of metal levels in different soft tissues and shell of *F. ater*; Means with different letters are significantly different, P≤0.05

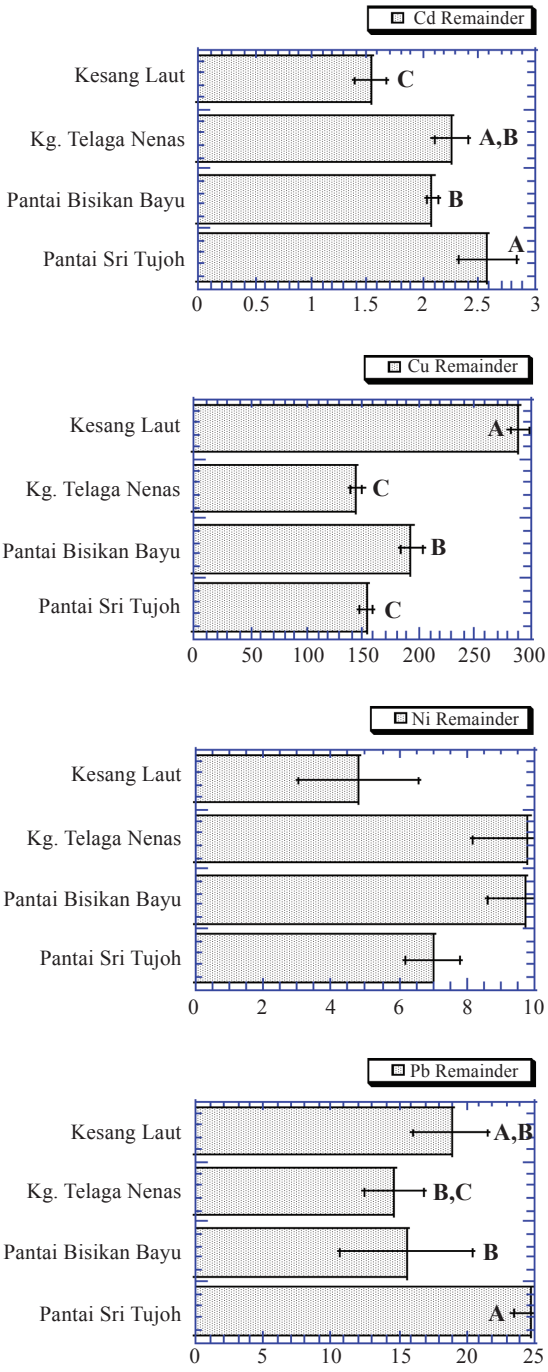

Fig. 5: Heavy metal concentration (mean µg/g dw ± S.E. (n=3)) in the remainder of Faunus ater *collected from the East and West Coasts of Peninsular Malaysia*

Note: Students-Newman-Keuls (SNK) comparisons of metal levels in different soft tissues and shell of *F. ater*; Means with different letters are significantly different, P≤0.05

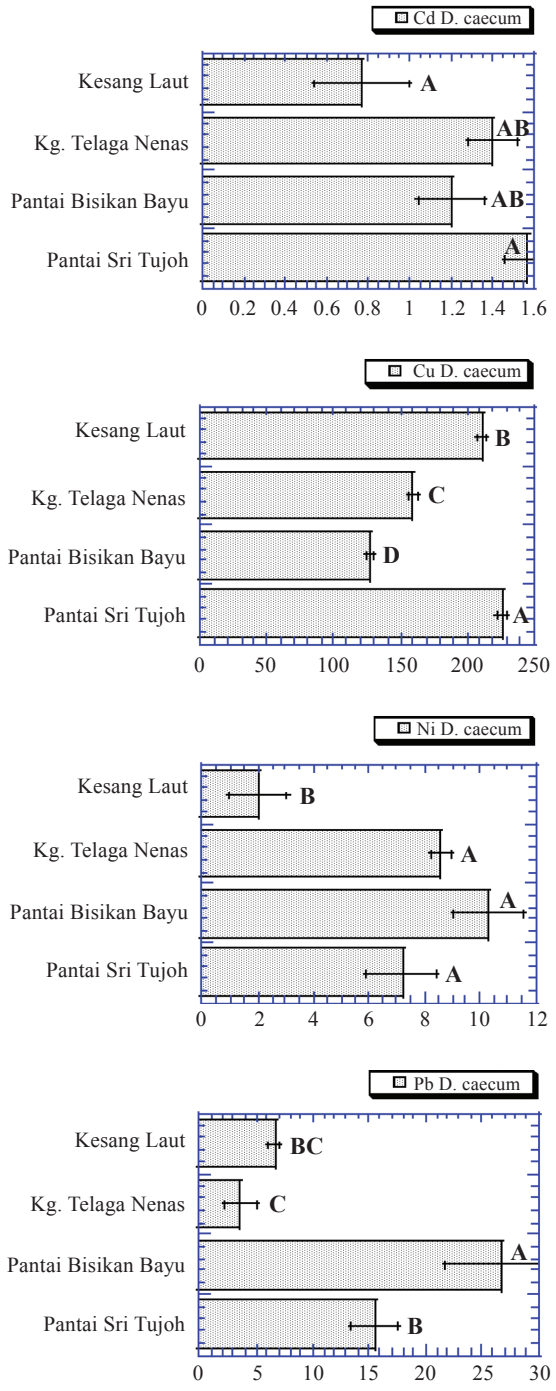

Fig. 6: Heavy metal concentration (mean µg/g dw ± S.E. (n=3)) in the digestive caecum of Faunus ater *collected from the East and West Coasts of Peninsular Malaysia*

Note: Students-Newman-Keuls (SNK) comparisons of metal levels in different soft tissues and shell of *F. ater*; Means with different letters are significantly different, P≤0.05

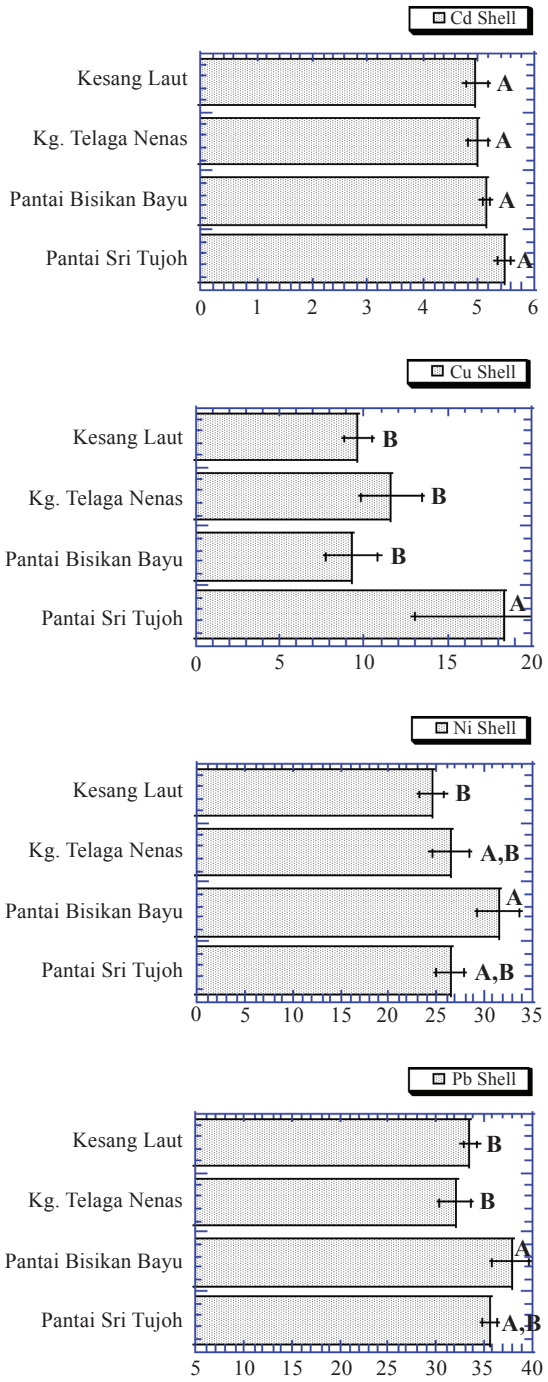

Fig. 7: Heavy metal concentration (mean µg/g dw ± S.E. (n=3)) in the shell of Faunus ater *collected from the East and West Coasts of Peninsular Malaysia*

Note: Students-Newman-Keuls (SNK) comparisons of metal levels in different soft tissues and shell of *F. ater*; Means with same letters are not significantly different, P≥0.05

farms were found to have impacts on the water quality around fish culture zones (Wu *et al*., 1994; Yap *et al*., 2003) which could contribute to the bioavailabilities and contaminations of the metals. The heavy metal concentrations in *F. ater* were slightly higher for Cd, Cu, and Ni and within the range for Pb as compared to other species in Malaysia (Amin, 2006b; Yap *et al.*, 2008a; Yap *et al.*, 2008b; Yap *et al*., 2009a).

CONCLUSIONS

From the present findings, it was concluded that the DC and remainder of *F. ater* accumulated high concentrations of Cu. The shell was accumulative of the non-essential metals Pb, Ni and Cd. On the other hand, bioavailabilities of Cd and Cu were found to be high in Pantai Sri Tujoh, whereas high bioavailabilities of Ni and Pb were found in Pantai Bisikan Bayu. The results gathered in the present study suggested that *F. ater* could be used as a potential biomonitor of heavy metal contamination in the intertidal area of Peninsular Malaysia and as an alternative biomonitor mollusc to the well-established green-lipped mussel *P. viridis*.

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Physiochemical Traits as Potential Indicators for Determining Drought Tolerance during Active Tillering Stage in Rice (*Oryza sativa* L.)

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ABSTRACT

It is well known that water scarcity limits crop production and further expansion of agriculture. In order to combat the adverse effect, plants have developed various morphological, physiological, and biochemical responses. Several studies were carried out separately, but there have been limited reports which explored on the combined effects of stress factors. Therefore, the present study was attempted to relate physiochemical traits under water deficit condition and explore the possibility of utilizing it for further crop improvement in rice. Five commercial rice varieties, namely MR-37, MR-84, MR-219, MR-220 and MR-232, were grown in randomized block design replicated thrice. At active tillering stage (i.e. 45 days after sowing), drought was induced by withholding water for a period of 7-10 days. Drought symptom was noticed by wilting of leaves. At the time of wilting, physiological parameters like relative water content (RWC), total chlorophyll content, chlorophyll stability Index (CSI), proline accumulation and protein content were estimated for both stress induced and control plants. Statistical analyses were performed to determine the effect of drought stress on the rice varieties. Significant differences were noticed for the physicochemical traits studied under drought condition. Mean performance of the genotypes also showed a sharp decline in RWC (%), total chlorophyll content, CSI (%), and protein. Meanwhile, free proline accumulation was found to be more in water deficit condition. Correlation coefficient (r) revealed a significant negative association of RWC (%) with total chlorophyll content, chlorophyll stability index (CSI %), proline and protein with, while total chlorophyll content itself had significant positive relation with CSI (%) under water deficit condition. Correlation coefficient clearly demonstrated that reduction in RWC would affect the osmotic adjustment, cell membrane stability, and photosynthetic machinery in crop plants. Estimates of genotypic variance, board sense heritability, and genetic advance revealed moderate to high estimates for RWC (%), CSI (%), and proline. These high estimates indicated that the rice varieties had inherent potential for drought improvement program. Moreover, the physiochemical parameters involved in the study are fair enough to distinguish between tolerance and susceptible genotypes. Therefore, these parameters could be utilized as key indicators for laboratory screening in determining drought tolerance in rice plants.

Keywords: **Drought, rice, physiochemical parameters, correlation coefficient, heritability, genetic advance**

INTRODUCTION

Water scarcity limits crop production and further expansion of agriculture in the world. As water resources for agronomic use are becoming scarce, development of drought tolerant lines has been considered as a valid breeding target to mitigate yield loss. Several efforts have been taken to improve rice production under water limiting conditions through conventional

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breeding techniques. However, progress in traditional breeding approach has been slow due to limited knowledge on genetics of drought tolerance and involvement of several complex tolerance mechanisms (Yeo and Flower, 1986). Moreover, inadequate screening techniques and low screening efficiency (Gregorio *et al.*, 1997; Fukai *et al*., 1999) have also limited the progress in breeding drought tolerance plants.

It is recognized that plants under stress have developed various physiological and biochemical adaptive responses like accumulation of sugars, amino acids, organic acids, and inorganic compounds (Morgan, 1984; Sairam *et al.,* 2002). These biochemical compounds play a key role in preventing membrane disintegration and provide tolerance against drought and cellular dehydration (Hanson and Hitz, 1982; Bohnert and Jensen, 1996; Mahajan and Tuteja, 2005). Although many studies were carried out separately on physiological and biochemical basis of drought tolerance, there have been limited studies exploring the combined effects of the stress factors (Tal, 1985). Therefore, physiochemical parameters associated with drought tolerance should be evaluated extensively for screening drought tolerance in rice. As mechanism of responses to drought stress varies with genotypes and growth stages of its life cycle (Ashraf and Harris, 2004), it would be much more valuable if biochemical indicators are specified for individual crop species. Knowledge on heritability, genetic advance and inter-relationship, among various physiochemical dehydration responses, will offer an insight for developing practicable strategies to improve drought stress tolerance in rice. In the present study, drought induced physiochemical responses were monitored in five commercial rice varieties using parameters like total chlorophyll content, chlorophyll stability index (CSI), relative water content (RWC), proline accumulation and total soluble protein, and responses of individual varieties are correlated using statistical tools.

MATERIALS AND METHODS

Seeds of five commercial rice varieties (MR 232, MR 37, MR 87, MR 219, and MR 220) were grown in 30cm x 30 cm plastic pots filled with clay loam soil. Thinning was done on the 15th day after sowing (DAS) and only five plants were retained per pot. A slow release commercial fertilizer (15% N, 15% P, and 15% K) of 7g was added to each pot to maintain healthy crop stand. Randomized block design was followed with three replications, while two sets of experimental materials were maintained - one was kept as a control and the others for stress induction. A gradual dehydration condition was applied on 45 DAS by withholding water for a period of 7-10 days. The response was noticed by wilting. At the time of wilting, the leaf samples were collected from both the control and stress plants and analyzed for their physiochemical parameters.

Determination of Relative Water Content

Leaf relative water content (RWC) was measured according to the method of Weatherly (1950); four leaf samples were collected and weighed to determine their fresh weight (FW). The leaves were rehydrated by placing them in distilled water for 12 h at 25 °C in order to obtain turgid weight (TW), followed by oven drying at 80 °C for 48 h and reweighed the leaf samples to get their dry weight (DW). Meanwhile, relative water content was calculated using the following formula:

$$\text{RWC (\%)} = \frac{\text{Fresh weight (FW)} - \text{Dry weight (DW)}}{\text{Turgid weight (TW)} - \text{Dry weight (DW)}} \times 100$$

Determination of the Total Chlorophyll Content

Chlorophyll content was determined using the methods proposed by Harbone (1984). 500 mg of the leaf tissues was homogenized in 80 % acetone at 4 °C. The supernatant was taken for

determination of photosynthetic pigments using the following formula:

$$\text{Total chlorophyll content } (mgL^{-1}) = 17.3\ A_{646} + 7.18\ A_{663}$$

$$\text{Chlorophyll } a\ (mgL^{-1}) = 12.21\ A_{663} - 2.81\ A_{646}$$

$$\text{Chlorophyll } b\ (mgL^{-1}) = 20.13\ A_{646} - 5.03\ A_{663}$$

Estimation of Chlorophyll Stability Index (CSI)

CSI in the leaves was estimated using a UV spectrometer following the method of Koleyoreas (1958). Two leaf samples of 250 mg each were put in two test tubes containing 10 ml of distilled water. One of the test tubes was placed in a water bath and heated to 65 °C for 30 minutes, while the other was kept as a control. Then total chlorophyll content was estimated using a spectrophotometer at 652 nm. CSI was calculated using the following formula:

$$\text{CSI (\%)} = \frac{\text{Total chlorophyll content (heated)}}{\text{Total chlorophyll content (control)}} \times 100$$

Estimation of Free Proline Content

Free proline content in the leaves was determined using the method described by Bates *et al*. (1973). The leaf tissue of 0.5g was homogenized in 3% of aqueous sulphosalicylic acid using mortar and pestle. The homogenate was centrifuged at 9000 rpm. The reaction mixture consisted of 2 ml of supernatant, 2 ml of ninhydrin, and 2 ml of glacial acetic acid. The reaction mixture was incubated in a water bath at 100 °C for 1 h, and then allowed to cool to room temperature. After cooling the reaction mixture, about 2 ml of toluene was added and mixed on a vortex mixture for 20s in a fume hood. The test tube was allowed to stand for 5 min to allow the separation of toluene and aqueous phases. The absorbance of toluene phase was read at 520 nm in the spectrophotometer against toluene blank. Meanwhile, the concentration of proline was estimated by referring to a standard curve of proline and expressed as mg/g fresh weight.

Protein Extraction and SDS-PAGE Analysis

About 1.0 g of the leaf tissues was ground in a cold mortar. The grinding medium (4-6 ml/g fresh mass) consists of 50mM Tris-HCL buffer (pH 8.0), 1mM PMSF, 10% (v/v) glycerol and homogenizing beads. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12000 rpm for 20 minutes at 4 °C, and the supernatant was taken after that. An aliquot of the extract was used for protein concentration following the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

SDS-PAGE was performed according to Laemmli (1970) with 12.5% acrylamide gels. Prior to electrophoresis, an equal volume of loading buffer [100 mM Tris-HCL, pH – 8.0, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.025% (w/v) bromophenol blue] was added to the protein sample and boiled at 100 °C for 2 minutes. Electrophoresis was carried out at a constant current of 30mA per plate towards the cathode for 2 h. Gels were stained with 0.03% Coomassie Brilliant Blue R-250.

STATISTICAL ANALYSIS

The experiment was performed using a randomized block design with three replications. Differences among the stress induced, as well as control, were tested using the SPSS software (Version 11.) Statistical variance analysis of the data was performed using ANOVA. From the ANOVA table, genotypic variance, broadsense heritability and genetic gain were estimated. The correlation coefficient analysis was performed and compared with the least significant differences at 5% level.

RESULTS AND DISCUSSION

Effect of Drought on RWC (%)

Plant water stress was measured in terms of leaf water potential or leaf relative water content. *Fig. 1(a)* depicts the performance of genotypes for RWC (%), under water deficit condition, in

which a sharp decline in RWC was recorded among the genotypes. From *Fig. 1*, it is evident that the genotypes MR 220 and MR 219 showed a decrease in RWC (%). This genotypic variation might be attributed to the variations in stomatal control of transpiration (Dingkuhn *et al.,* 1989), water extraction ability (Lilley and Fukai, 1994), and variation in the canopy size at the onset of stress (Mitchell *et al*., 1998). Progressive decline in RWC was reported by Silva *et al*. (2007) in sugarcane. Some previous studies (e.g. Jamauex *et al*., 1997; Altinkut *et al*., 2001; Colom and Vazzana, 2003) have shown that maintenance of a relatively high RWC during mild drought is an indicative of drought tolerance.

Effect of Drought on Total Chlorophyll Content

Total chlorophyll was found to decrease with the severity of stress. Under controlled condition, the amount of total chlorophyll ranged from 3.72 ± 0.05 to 20.201 ± 1.034 mg/g FW. On the other hand, stressed plants revealed total chlorophyll between 0.58 ± 0.02 and 17.78 ± 0.29 mg/g FW, and the extent of this decrease was strongly cultivar-dependent. In both the conditions, the highest mean for this particular trait was obtained in MR219 and the lowest in MR37 (*Fig. 1(b)*). Chlorophyll degradation is considered as one of the consequences of drought stress which has resulted from sustained photo-inhibition and photo breeding (Long *et al*., 1994). Meanwhile, the decrease in chlorophyll is associated with a reduction in the flux of nitrogen into the tissue, as well as alteration in activity of enzyme systems such as nitrate reductase (Begaum and Paul, 1993).

Effect of Drought on CSI (%)

Wider variation was noticed between the control and drought stress plants for chlorophyll stability index (*Fig. 1(c)*). Under a normal growth condition, a slight variation for CSI (%) was seen among the genotypes. In more specific, significant differences were noticed when the same genotypes were exposed to water deficit condition. CSI (%) was found to range from 47.79 ± 4.67 to 91.62 ± 3.13. Based on the result obtained for CSI (%), MR232 had a high mean value and it also tended to be stable, while MR37 was found to be sensitive to water deficit stress. Meanwhile, higher CSI indicates the level of polyunsaturated lipids stabilizes chloroplast membrane and increases adaptive response to tolerance under water stress condition.

Effect of Drought on Proline

Accumulation of proline is one of the common characteristics in many monocotyledons under stress (Wyn Jones, 1981; Asraf, 1994; Mansour, 2000). From *Fig. 1(d)*, two to three-folds of increase in the accumulation of proline were observed among the genotypes. In particular, MR220 exhibited a higher accumulation of proline (67.13 ± 0.245), whereas MR232 showed a minimal value for this trait (17.57 ± 1.02). The synthesis of proline helps the cell to maintain their hydrated state and therefore functions to provide resistance against drought and cellular hydration (Hockstra *et al*., 2001; Ramanjulu and Bartels, 2002). Similarly, Maggaio *et al.* (2002) suggested that proline might act either as a signalling or regulatory molecule which is capable of activating multiple responses which are component of the adaptation process.

Effect of Drought on Protein

Like other physiochemical traits, water stress condition has also been known to alter protein metabolism. Total soluble protein was found to respond to stress either by increasing or decreasing its amount. Four genotypes, namely MR232, MR37, MR84, and MR220, showed a decreasing trend, whereas MR219 alone revealed an increasing trend (*Fig. 1(e)*). The increase and decrease in the total soluble proteins under drought stress were found to be consistent with the findings of some other researchers (e.g. Riccardi *et al.,* 1998; Ti da *et al.,* 2006; Nayer and Reza, 2008). Proteins synthesize *de novo* in response to stress and increase when plants are exposed to stress (Pareek *et al.,* 1997; Ashraf

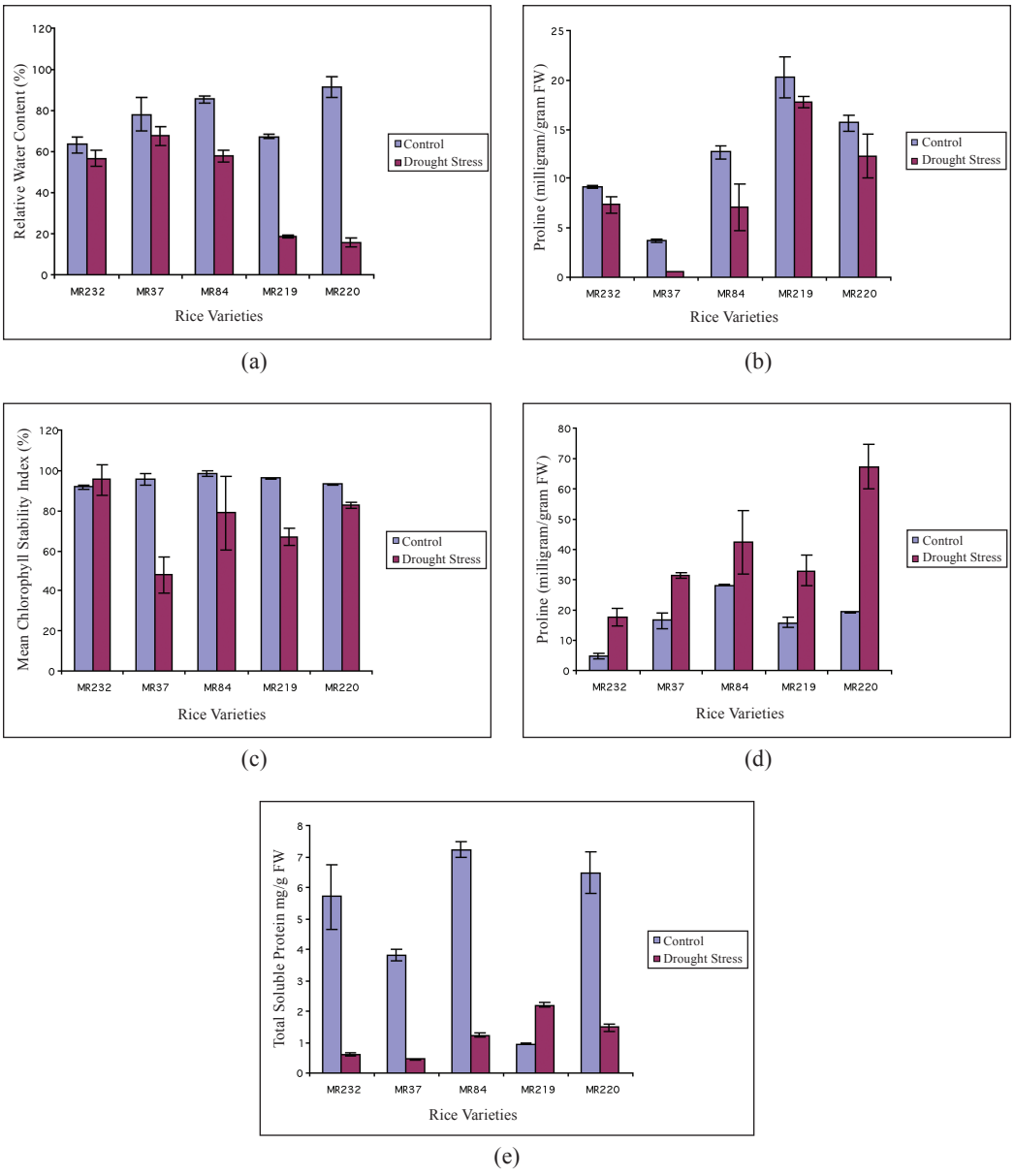

Fig. 1: Effect of drought stress on (a) RWC (%), (b) Total chlorophyll, (c) CSI (%), (d) Proline and (e) Total soluble proteins. Values shown are the average of five plants in each replication (mean ± S.E). All the treatments were significantly different from the control ($p < 0.05$)

and Harris, 2003). Proteins which accumulate in plants, which are grown under stress conditions, may provide a storage form of nitrogen that is re-utilized when the stress is over (Singh *et al*., 1987) and it may also play a role in osmotic adjustment. In general, drought induces metabolic changes related to protein turnover. This is probably due to the alteration in protein synthesis, and maintenance the level of the same proteins or protein degradation.

The leaf tissues of both the control and stress genotype of MR219 were analyzed for protein using one-dimensional SDS-PAGE. The SDS gel electrophoresis revealed a considerable difference in the banding pattern. A gel comparing control and stressed proteins from the leaf tissue is shown in *Fig. 2*. Meanwhile, low (~ 15.5 –18.0 kDa) and high molecular weights (~ 250 kDa) of protein bands were also detected. In response to drought, protein synthesis exhibits a wide range in terms of size, i.e. from 9 to 200 kDa (Close, 1996). These proteins are called dehydrins and belong to group II of the late embryogenesis abundant protein (LEA). The results obtained in the study are similar to the findings of Mohammadkhani and Heidari (2008). They reported a leaf protein accumulations of 15, 17, 20, 27, 30, 33, 37 kDa in wheat in progressive water deficit condition.

Estimates of Correlation Coefficient

Correlation coefficient (r) of physiochemical parameters was estimated for both the controlled and water deficit conditions and the results obtained are presented in Table 1. In this investigation, an interesting association was found among the various physiochemical parameters towards drought. The correlation coefficient (r) in Table 1 illustrates that the total chlorophyll has a negative significant correlation with RWC (%) ($r = –0.872$, $p<0.05$), while a strong positive significant relation was noticed with CSI (%) ($r = 0.603$, $p<0.05$) and protein ($r = 0.899$, $p<0.05$). The magnitude of association between the two traits was found to be more in stressed plants than in well-watered plants. The findings of this study are in agreement with the findings of Thornber *et al*. (1991).

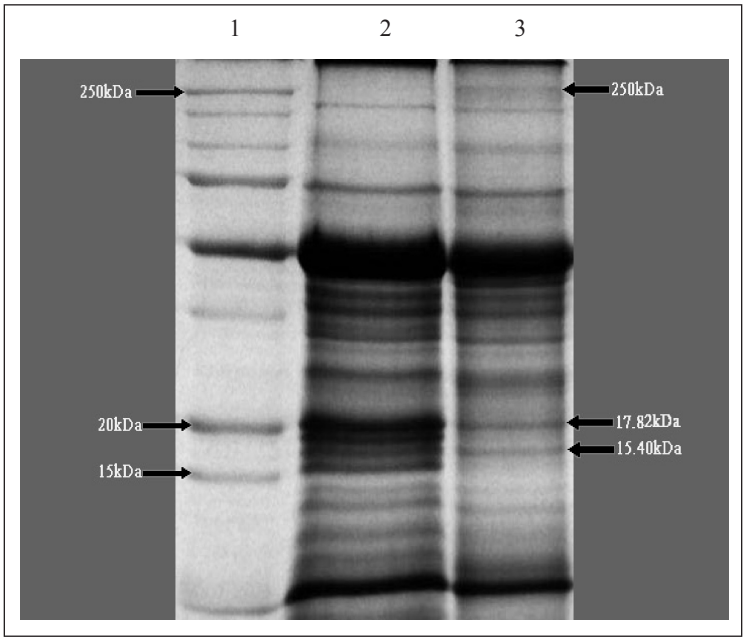

Fig. 2: SDS PAGE profile indicating the changes in protein expression in both stress-induced and control plants. The lane on the left side indicates the molecular weight of standard protein (Precision plus protein # 161-0373 was used as a molecular marker). Lane 1: molecular marker, Lane 2: Proteins obtained from control plants, and Lane 3: Proteins obtained from water deficit plant. The arrow head indicates ~ 15- 18kDa proteins that are responsive for drought tolerance

On the other hand, under water deficit condition, RWC had significant negative correlation with the total chlorophyll content, proline ($r = -0.569$, $p<0.05$) and protein biosynthesis ($r = --0.830$, $p<0.05$). Meanwhile, the negative interrelationship of RWC with proline is presumed because under a normal condition, RWC and proline are in equilibrium and when the plants are exposed to stress, the amount of RWC tends to decrease while proline accumulates. Hein *et al*. (2003) observed that the accumulation of proline was inversely correlated to the P_5cs mRNA detected, and further suggested that a short-term accumulation might be a good indicator of rice osmotic stress tolerance. On the contrary, a decline in the accumulations of RWC and proline indicated the severity of dehydration, whereas osmotic adjustment might fail to maintain the turgor in the affected tissues. Out of several biochemical indices of water deficit injury, the accumulation of proline and the decline in protein synthesis in plants have been widely reported (Irigoyen *et al.,* 1992). In more specific, drought stress was found to disturb protein metabolism and production pathway, and hence was substituted by the accumulation of proline. Hence, under a water deficit condition, the synthesis of proline was found to be associated with protein hydrolysis. The results of the present study are in accordance with the findings of Irigoyen *et al.* (1992), as well as Ashraf and Iram (2005). Meanwhile, protein profile of SDS-PAGE revealed the presence of low molecular protein (~ 15.5 – 18.0 kDa) and high molecular protein (~ 250 kDa).

A protective role of exogenously supplied proline in relation to cell membrane stability and mechanism of its action were documented in many papers (Blum, 1981; Rudolph *et al*., 1986; Smirnoff and Cumbes, 1989; Bandurska, 1998; and Schurr *et al.,* 2000).

As presented in Table 1, the negative association obtained for RWC with the total chlorophyll content ($r = -0.872$, $p<0.05$), chlorophyll stability index ($r = -0.222$, $p<0.05$), proline ($r = -0.569$, $p<0.05$) and protein ($r = -0.830$, $p<0.05$) indicated that the expression of RWC in the plant tissue was determined by several characteristics such as osmotic adjustment, cell membrane stability, and accumulation of compatible solutes like proline. Meanwhile, the reduction in RWC, due to drought stress, was found to affect photosynthetic machinery, cell membrane stability, and proline synthesis.

TABLE 1
Pooled phenotypic correlation coefficient (r) among physiochemical traits for the controlled and drought stress conditions in rice plants

	TC	CSI (%)	RWC (%)	Proline (mg/g) FW	Protein (mg/g) FW
TC	1.000	0.150 0.603**	0.22 -0.872**	0.202 0.259	-0.275 0.899**
CSI (%)		1.000	-0.276 -0.222	0.018 0.135	0.099 0.086
RWC (%)			1.000	0.735* -0.569**	0.543* -0.830**
Proline				1.000	0.273 0.341
Protein					1.000

- Drought stress values are indicated in bold letter
- Correlation coefficients significance at *p = 0.05 level and ** p = 0.01 level

TABLE 2
Genetic variation, Heritability (%) and Genetic advance of physiochemical parameters under controlled and drought stressed conditions in rice

Parameters	Genetic variance		Heritability (%)		Genetic advance	
	Control	Stress	Control	Stress	Control	Stress
Relative water content (%)	130.32	585.44	28.29	97.66	3.96	49.26
Total chlorophyll (mg/ml)	38.96	44.35	97.13	89.99	12.67	13.01
Chlorophyll stability index	3.28	263.79	57.6	73.12	2.83	28.61
Proline content (mg/g) FW	70.96	89.20	96.91	89.20	17.08	35.38
Protein content (mg/g) FW	6.18	0.50	92.56	98.21	4.93	1.44

Estimates of Genetic Variance, Heritability and Genetic Advance

Estimates of genotypic variance offer scope for crop improvement, while heritability and genetic advance enable us to make prediction about the progress of selection. Determination of genotypic variance, broad sense heritability and genetic advance (Table 2) indicated that under water stress condition, RWC % (585.44) and CSI % (263.79) had a very high genotypic variance, followed by proline (89.20) which was moderate. These variations could effectively be utilized for screening drought tolerance at an early stage in rice. The effectiveness of selection is in turn dependent on the percentage of broad sense heritability. A high heritability was noticed for all the physiochemical traits involved in the study, indicating that the physiochemical traits possess inherent potential for drought improvement. Progress in selection is therefore determined by high estimates of genetic advance. In the current study, a moderate genetic gain was recorded for RWC (%), CSI (%) and proline content, respectively.

From the foregoing results, it is obvious that the physiochemical traits undertaken in this study were affected, either positively or negatively, by water deficit condition. A sharp decline in RWC (%), total chlorophyll content, CSI (%) and protein was noticed, whereas the accumulation of proline was more prominent. The correlation coefficient analysis revealed the presence of interrelationship between RWC (%), Proline and Protein, as well as the total chlorophyll content and CSI (%) under a water deficit condition. All the physiochemical parameters involved in the study had high heritability but moderate genetic advance which indicate the scope for further selection and improvement of rice for drought tolerance. Therefore, the parameters viz., total chlorophyll content, chlorophyll stability Index, relative water content, proline accumulation, and protein synthesis are sufficient enough to provide reliable laboratory screening indictors to screen for drought tolerance in rice breeding programmes. Moreover, these parameters are fair enough to distinguish between tolerance and susceptible, and these parameters could therefore be used as a selection criterion for drought stress improvement.

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Adsorption and Absorption of Cu in *Trichoderma atroviride*

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ABSTRACT

Conventional methods for removing heavy metals from polluted waters, using chemical precipitation, sludge separation, chemical oxidation or reduction, and ion exchange, have been uneconomical and are weak processes. An alternative technique is the use of fungi as bioremediating agents. A strain of *Trichoderma atroviride*, isolated from a river passing through the metal polluted Serdang industrial area, was studied for its uptake and tolerance to Cu. This study found that the uptake capacity of *T. atroviride* for Cu ranged from 0.77 to 11.20 mg/g in Potato Dextrose Broth in liquid media over the Cu concentration range of 25 to 300 mg/L. The isolate showed 50.3 to 85.4% adsorption and 9.6 to 47.1% absorption. These adsorption and absorption values are comparable to any good bioremediators for Cu found in the literature. This study suggests that *T. atroviride* is a potential bioremediator of Cu. However, further studies are still needed to confirm its practical use as a bioremediating agent under field conditions.

Keywords: *Trichoderma atroviride,* fungus bioremediation, adsorption and absorption of Cu

ABBREVIATIONS

C	:	Centigrade
Cu	:	Copper
DDW	:	Double distilled water
EDTA	:	Ethylenediamine tetraacetic acid
g	:	Gram
mg	:	Milligram
µg/g	:	Microgram per gram
mg/l	:	Milligram per litre
PDB	:	Potato Dextrose Broth
RBA	:	Rose Bengal Agar

INTRODUCTION

Rapid industrial development has led to an increased discharge of industrial effluents, which usually contain heavy metals in concentrations well beyond the permissible limits, into the environment (Ahuja *et al*., 2001). Cu is a ubiquitous metal present in the environment and is the most common contaminant of industrial effluents such as those produced by mining and metal processing (Anand,

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2006) or those used in vineyards, ranging from the application of fertilizers, dumping of agricultural, and municipal wastes. Cu is also an element essential for all living organisms as a co-factor for a variety of enzymes; however, an excess of this element can be mutagenic and can cause the appearance of highly reactive oxygen radicals (Zapotoczny *et al.,* 2006). An excess of Cu can disrupt the ecological status of biota, whereas the occurrences of heavy-metal resistant micro-organisms in the soil and water of industrial regions have been reported (Aleem, 2003). In order to survive in such an adverse stressful situation, the micro-organisms develop mechanisms which confer upon them resistance against Cu by accumulating high amounts of heavy metals, either intracellulary or extracellulary or by a combination of both mechanisms (Ahuja *et al*., 2001).

Fungi are a versatile group as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations (Anand, 2006); recently, they are considered as the best bioremediators for polluted waters purification (Savvaidis, 1998) in comparison to conventional methods for removing dissolved heavy metals from aqueous solution, such as by chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, electrochemical treatment, membrane technologies, reverse osmosis, filtration, adsorption on activated carbon, and evaporative recovery (Lopez and Vazquez, 2003).

Trichoderma atroviride (family: Hypocreaceae) is a potential bioremediator in this area of research with regard to its frequent presence in high polluted areas, and for which just a few investigations have been carried out on. On the other hands, there has been no report of bioremediation by this particular isolate in this region. Thus, the objective of this study was to determine the adsorption and absorption of Cu by *T. atroviride* under laboratory conditions.

MATERIALS AND METHODS

Metal Analytical Procedure in the Sediment Samples

The top 3-5 cm of surface sediments were collected from metal polluted site at Kuyoh River Industrial Area and were placed in an acid-wash polyethylene bag and frozen (-10 °C) prior to analysis. They were later dried at 105 °C for at least 16 h until a constant dry weight was obtained (Tanner *et al.,* 2000). The samples were then passed through a stainless steel sieve of 63 µm size and vigorously shaken to produce homogeneity. The dried samples were then weighed and digested in a combination of concentrated nitric acid (Anala R grade, BDH 69 %) and perchloric acid (Anala R grade, BDH 60 %) in the ratio of 4:1; first at a low temperature of 40 °C for 1 hour and then at 140 °C for at least 3 hours (Yap *et al*., 2002). The digested samples were then diluted to 40 ml with double distilled water (DDW) and filtered through Whatman No. 1 filter paper into acid-washed polyethylene bottles, where they were stored until ready for Cu determination using a flame Atomic Absorption Spectrophotometer (AAS) (Perkin-Elmer Model AAnalyst 800).

T. Atroviride *Isolation*

For isolation, the collected sediments were mixed with a little double distilled water (DDW) in sampling polyethylene bottles (50 ml) and kept in a refrigerator at 7 ºC in the Mycology laboratory. The dilution was then carried out based on Lopez and Vazquez (2003). Rose Bengal Agar (RBA) was used for the direct isolation of fungal colonies from the sediment solution, where they were counted as Colony Forms Unite (CFU), while Potato Dextrose Agar (PDA) was used for sub-culturing and storage media.

Estimation of Cu in the Culture Filtrate

Estimation of Cu in the biomass was performed according to the procedure described by Ahuja *et al*. (2001). The concentrations of Cu in the

liquid cultures were measured with a flame AAS before inoculation by the fungus. After the growth, the residual Cu concentration in the liquid cultures filtrate was also estimated. Metal uptake was estimated as the amount of Cu (mg) per unit of mycelium dry weight (g) (Lopez and Vazquez, 2003).

$$Q = \frac{C_i - C_f}{M} \times V$$

Where Q= Cu uptake (mg metal/mg biomass), Ci= initial Cu concentration (mg/l), Cf= final Cu concentration (mg/L), M= quantity of dry biomass (mg), V= suspension volume (ml).

Localization of Cu in T. Atroviride

The amount of Cu associated with the cell biomass was fractionated as the ethylenediamine tetraacetic acid (EDTA) washable fraction was present on the cell surface and the EDTA non-washable fraction was also present intracellularly. The biomasses were washed first with DDW and next with 10 ml of 0.5 mM EDTA for 30 minutes. It was then centrifuged, while the supernatant was collected and the pellet was dried and used for the Cu estimation through wet digestion. Based on this method (wet digestion), the entire dried and weighed biomass from each conical flask was taken in a digestion tube. The biomass was digested in 3 ml of acid mixture of nitric acid (AnalaR grade, BDH 69%) and perchloric acid (AnalaR grade, BDH 60%) in the ratio of 6:1 at 100 °C for 1 hour. These samples were subsequently made up to 5 ml with DDW. Digested samples were diluted with DDW to a certain volume for Cu determination using a flame AAS (Ahuja, 2001).

RESULTS AND DISCUSSION

Cu Concentrations in the Surface Sediment of Sg. Kuyoh River

Based on the findings by Yap *et al*. (2002), there are advantages in the use of sediment samples to assess human impacts on the aquatic environment. Firstly, sediment plays a main role in the transport of metals. Secondly, it is frequently used to identify sources of pollution spatially and temporally. Thirdly, sediment can be used to locate the major sink for heavy metals and these elements are persistent in the marine environment.

The mean of total Cu concentration, based on the aqua-regia method in the sediment of Kuyoh River Industrial Area (the micro-organism's habitat), was 347.64 µg/g.

Growth of T. Atroviride *in Presence of Cu in Liquid Medium*

Results showed that the increase in the concentrations of Cu (i.e. from 0 to 300 mg/L) decreased the levels of fungal biomass (*Fig. 1*). There is an almost constant note of decrease of biomass between 0 and 200 mg/L concentrations of Cu, but there is a remarkable decrease of fungal biomass at 300 mg/L. Lopez and Vazquez (2003) reported a similar finding for *T. atroviride*, except that the dramatic decrease was observed at 350 mg/L and a complete absence of growth at 400 mg/L, as compared to this study which still observed a little biomass at 400 mg/L. This could be due to the different media used. PDB was used as the liquid medium in the present study, while Lopez and Vazquez (2003) used Sabouraud liquid medium (Scharlau) supplemented with 1% peptone and 2% dextrose. They used Peptone, rather than other nitrogen-containing substrates, because of its comparatively low metal binding capacity. Thus, the metal availability of the liquid medium increased and the lethal concentration of their experiment was limited to 350 mg/L compared to the final result of 400 mg/L found in the present study.

Cu Uptake by T. Atroviride *in Liquid Media*

Based on the data presented in *Fig. 2*, it can be seen that by increasing the concentrations of Cu from 0 to 300 mg/L, the Cu uptake also increased, whereas the levels of biomass were found to decrease based on *Fig. 1*. Moreover, the maximum biosorption (11.20 mg/g) from the

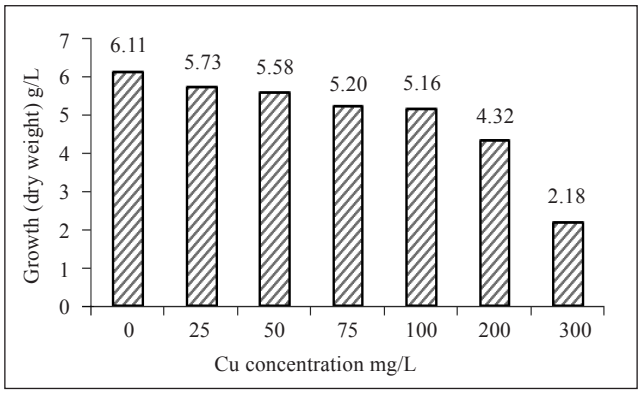

Fig. 1: Biomass weight of T. atroviride *in the presence of different Cu concentrations in PDB*

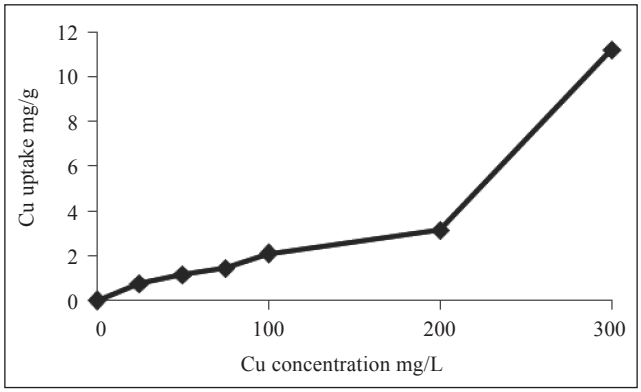

Fig. 2: Cu uptake of T. atroviride *at elevated Cu concentrations (mg/L) in liquid medium*

metal solution occurred at a concentration of 300 mg/L. This concentration is close to the Cu level in sediment of the sampling site at Kuyoh River (346.91 mg/L), while the minimum biosorption occurred at the concentration of 25 mg/L (0.77 mg/g). There are significant differences between the concentrations below and above 100 mg/L. From 0 to 100 mg/L, the uptake increased with a slow constant speed as similarly found by Wang and Chen (2006). They found that the uptake rate of the metal had increased along with increasing the initial concentration, if the amount of biomass was kept unchanged. From 100 to 300 mg/L, on the other hand, *T. atroviride* showed a sudden increase up to the maximum uptake (300 mg/L). The increase in the uptake might often be associated with toxicity and/or increased permeabilization of cell membrane on the account of further binding of the metal to exposed intracellular sites (Gadd, 1990) in relation to the decreased biomass with elevated Cu concentrations.

There are a few studies on biosorption of heavy metals by species of the genus of *Trichoderma*. Anand *et al*. (2006) reported 17 mg/L Cu removal from the liquid medium with 100 mg/L initial Cu concentration by *T. viride* at 30 ºC over a 72 hours incubation period. This finding is different from the result gathered in the present study (10.93 mg/L Cu removal by *T. atroviride* at 100 mg/L of Cu in liquid medium with 7 days incubation). Some researchers have demonstrated factors such as contact time, biomass dosage, temperature, and

pH influence biosorption of metals (Yan and Viraraghavan, 2003). Variations in the tolerance of metals for different or the same species of a genus might be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different fungi (Zafar *et al.,* 2006).

Localization of Cu in T. Atroviride

EDTA could remove 50.3 to 72.9% of Cu from the cell surface of *T. atroviride,* when this metal was present in the range of 25 to 75 mg/L and 81.2 to 85.4% in the range of 100 to 300 mg/L (*Fig. 3*). The mentioned percentage of the Cu removal in *T. atroviride* indicated that most of the Cu was surface bound. However, as clearly depicted in *Fig. 3*, only 50.3% could be removed at 25 mg/L of Cu concentration. This could be due to the fact that a good amount of mycelia was formed at this concentration, and that metal was adsorbed and entrapped in the deeper layers of the hyphal network. Therefore, it was difficult to extract the metal with EDTA (Anand *et al*., 2006). Using a scanning electron microscope, Zapotoczny *et al*. (2006) also observed a gradual increase in the thickness of the cell wall due to toxicity of Cu on *Acremonium pinkertoniae.*

According to Anand *et al*. (2006), 48.15% and 80 to 85% of Cu could be removed using *T. viride* in the media of 50 mg/L and 100 to 200 mg/L, respectively. It seems that both species of the same genus show a similar response to Cu concentrations but the Cu percentage removal by *T. atroviride* demonstrated more adsorption at low concentration and less adsorption at higher concentration of Cu in comparison with *T. viride*.

Biosorption of toxic metals is based on the ionic species associating with the cell surface or extra-cellular polysaccharide, proteins and chitins (Zafar *et al.,* 2006). Different species/ strains and cell types of the same fungus vary in terms of their chemical composition of the cell wall due to variation in their uptake capacities (Mowll and Gadd, 1983).

As shown in *Fig. 3*, around 2.7 to 5% of Cu in the liquid medium was removed by washing. These values were obtained by the assumption of differences between the levels of Cu removal from the liquid medium by fungus and the addition of adsorption and absorption values obtained by the wet digestion method.

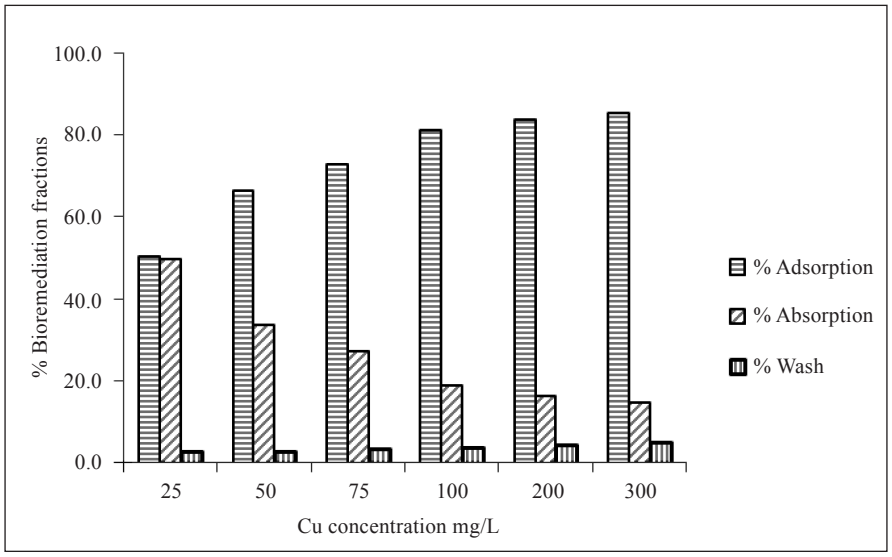

Fig. 3: Cu removal percentage by T. atroviride *from the liquid medium at elevated Cu concentrations*

Anand *et al*. (2006) reported 5% of Cu removal from mycelia form washing hyphal during the experiment, without any report of initial Cu concentration in the liquid medium, whereas the result of washing in this study showed 2.7 to 5.0% of Cu loss in the range of experiment using 25 to 300 mg/L.

This assumption seems to be more accurate than what Price *et al.* (2001) reported in their experiment. They treated the fungal mycelium with 0.1 N HCl to remove any heavy metals adsorbed into the mycelium and then all the remaining heavy metals with the biomass after the treatment was assumed to be absorbed intracellularly. However, both assumptions can not clearly present the real level of washing during the experiment. Therefore, there is a need to use more accurate methods such as cell fraction analysis in order to show the final heavy metal removal by fungi over the period of the experiment.

CONCLUSIONS

The *T. atroviride* showed the ability to bind Cu into its cell wall surface and this appeared to be the main mechanism of metal tolerance in the present study. It plays this role by showing 50.3 to 85.4% adsorption and 9.6 to 47.1% absorption. Binding of Cu onto the cell surface (or adsorption) immobilized the metal making it less available in the medium, thereby reducing its toxicity. This allowed the organism to further resume its normal growth (Anand *et al*., 2006). This study revealed 2.7 to 5.0% Cu loss in the range of 25 to 300 mg/L due to washing, based on the wet digestion method. However, further studies should be designed to elucidate the mechanisms of metal tolerance by *T. atroviride*.

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Correlations between Speciation of Zn in Sediment and Zn Concentrations in Different Soft Tissues of the Gastropod Mollusc *Telescopium telescopium* Collected from Intertidal Areas of Peninsular Malaysia

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ABSTRACT

The aim of this study was to relate the Zn level in the different tissues of *Telescopium telescopium* to the Zn levels of surface sediment of the gastropod habitat. Zn concentrations were determined in the different soft tissues (foot, cephalic tentacle, mantle, muscle, gill, digestive caecum and remaining soft tissues) of *T. telescopium* and in the sediments collected from 17 intertidal areas of Peninsular Malaysia, where the snails were collected. Total Zn concentrations and speciation of Zn of the surface sediment were correlated with the Zn concentrations measured in the different soft tissues of *T. telescopium*. The results showed that significant ($p < 0.01$) correlations were observed between Zn concentrations in mantle, muscle, gill, and remaining soft tissues with non-resistant Zn in sediment; Zn concentration in gill with resistant Zn in sediment; mantle, muscle, gill, and remaining soft tissues with acid reducible Zn in sediment; gill and remaining soft tissues with oxidisable-organic Zn in sediment. The pattern of Zn distribution showed that digestive caecum of *T. telescopium* in all 17 sites always contained the highest concentration of Zn, except for Kuala Sg. Ayam. Therefore, the present results generally supported the use of different soft tissues of *T. telescopium* as a more accurate biomonitoring organ for Zn, besides the total soft tissues.

Keywords: ***Telescopium telescopium*, different soft tissues, intertidal area, Peninsular Malaysia**

INTRODUCTION

During the past few years, studies on the distributions, concentrations, and functions of heavy metals in gastropod tissues have been stimulated by several factors. The accumulation of several heavy metals, especially by aquatic organisms, has drawn attention to their essential role in many life processes, e.g. Zn is particularly involved in enzyme functions and respiratory functions (Spronk *et al*., 1971). Several researchers (Bu Olayan and Thomas, 2001; Conti and Cecchetti, 2003; Dang *et al*., 2005; Ireland and Wootton, 1977; Shiber and Shatila, 1978; Taylor and Maher, 2003; Walsh *et al*., 1995) have reported the use of whole soft tissues of snails for biomonitoring studies. These researchers have investigated heavy metal concentrations in the whole soft tissues of gastropod molluscs but no emphasis has been given to the different soft tissues of gastropods. Some of the gastropods that inhabit rocky shores or sediments fulfil most of the requirements of good biomonitors (Raibows *et al.*, 1990); however, it is important that they accumulate metals in proportion to metal availabilities in the environment (Ying *et al*., 1993). In addition to that, Ying *et al*. (1993)

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suggested that in the case of sediment-dwelling gastropods, only the bioavailability fraction of metals in sediment can have an impact on accumulation. Nevertheless, bioavailability is not the only factor which influences the metal concentrations in marine organisms (Bryan and Hummertsone, 1986; Rainbow *et al*., 1990; Rainbow, 1997), the physiological differences between the different soft tissues and metal-fractionation in sediment are also influential. Other studies (Luoma and Bryan, 1978; Luoma, 1983; Yap *et al*., 2002; Ying *et al*., 1993) have examined various molluscs species and demonstrated significant correlations between metal concentrations in organisms and metal concentrations extracted from the sediment by various extractants. Therefore, this present study focussed on the correlation between the total concentrations of Zn in different soft tissues in snails and fractionated heavy metals in sediment.

In general, Zn concentrations in sediments and tissues of aquatic organisms are usually elevated in the vicinity of smelters and other point sources of Zn, and decrease with increasing distance (Ward *et al*., 1986). Moreover, sediment acts as an important reservoir for heavy metals which leads to the following question: to what extent are sediment-bound metals available for uptake by living organisms? (Mountouris *et al*., 2002). In most circumstances, the major part of the anthropogenic metal load in the sea and sea bed sediments and organisms has a terrestrial source from mining and intensive aquaculture and municipal wastewaters, untreated effluents, harbour activities, urban and agricultural runoff along major rivers, estuaries and bays (Dalman *et al*., 2006).

Many organisms, including plankton, molluscs and fish, can act as biomonitors by accumulating pollutant metals as a function of the metal concentrations in their environment (Foulkes, 1990). *Telescopium telescopium* (Family: Potamididae) is focused in this study because it fulfils most of the recommended criteria for a good biomonitor including sedentary lifeform, accumulative of metal concentrations, easy sampling and wide geographical distribution (Kang *et al*., 1999; Yap *et al*., 2006: 2007). For this study, the samples of sediment and the snails, *T. telescopium* were collected from 17 intertidal areas of Peninsular Malaysia. These were analyzed for Zn concentrations in an attempt to recognize subtle pollution effects and anthropogenic influences. The objective of this study was to correlate the Zn levels in the different tissues of T. telescopium and the Zn levels in the surface sediments.

MATERIALS AND METHODS

Sampling and Sample Preparation

The description of each site is given in Table 1. Snails were collected from 17 geographical sites along the south western intertidal area of Peninsular Malaysia (*Fig. 1*). Stations shown on the map were chosen because the west coast of Peninsular Malaysia receives many industrial and domestic effluents from the surrounding areas. The samples were kept in an ice chest and brought to the laboratory. About 6-21 individual snails from each station were used for the analysis. The mean length and width of the shell of the snails were measured. As in the previous collections, the snails were not kept in the laboratory in any attempt to purge contents, and this was to avoid the possibility of contamination. Therefore, they potentially contained gut contents, but were considered to represent only a small proportion of the total body metal content (Rainbow, 1987: 1998; Rainbow and Blackmore, 2001), given the characteristics of snails as particularly strong trace metal accumulators.

Metal Analyses

About 9-15 individuals of *T. telescopium* from each site were dissected and pooled into seven different soft tissues, namely foot, cephalic tentacle, mantle, muscle, gill, digestive caecum, and remaining soft tissues (‘rest’).

All the snails and sediment samples were dried at 80 °C for 72 h until constant dry weights were achieved. Three replicates of each different part of soft tissues and shells of snails were

TABLE 1
The description of sampling sites

No of sites	N	Date	GPS	Sampling sites	Height	SE	Width	SE
					Min	Max	Min	Max
1.	9	12th January 2007	N 04º 55' 89.6" E 100º 26' 79.1"	Kuala Gula (KG)	8.60 8.03	0.11 9.33	4.28 3.98	0.07 4.91
2.	13	25th February 2006	N 04° 14' 44.3" E 100° 41' 35.6"	Kg Setiawan (KS)	6.58 5.82	0.14 7.08	3.18 2.95	0.04 3.41
3.	6	25th February 2006	N 04° 14' 53.8" E 100° 42' 09.1"	Kg Deralik (KD)	5.65 4.51	0.15 6.67	3.24 2.78	0.07 3.75
4.	8	27h February 2006	N 04° 16' 46.0" E 100° 39' 50.2"	J. Permaisuri Bainun (JPB)	5.35 4.62	0.07 5.84	2.83 2.45	0.04 3.05
5.	8	16th August 2006	N 03º 0' 22.94" E 101º 18' 22.5"	Pulau Indah (PI)	8.98 8.55	0.13 9.48	4.52 4.38	0.05 4.69
6.	10	24th February 2006	N 03° 13' 14.6" E 101° 18' 19.5"	Kg Pantai Jeram (KPJ)	7.81 6.85	0.12 8.35	3.71 3.42	0.04 3.94
7.	21	20th March 2006	N 03° 10' 20.0" E 101° 18' 1.4"	Sg Janggut (SJ)	8.35 8.02	0.08 8.81	4.56 4.01	0.12 5.26
8.	10	7th January 2006	N 02º 36' 19.41" E 101º 42' 11.51"	Sg Sepang Besar (SB)	4.96 4.73	0.06 5.29	2.89 2.75	0.06 3.25
9.	9	15th September 2006	N 02º 35' 57.52" E 101º 42' 31.41"	Bagan Lalang (BL)	7.64 7.31	0.06 7.94	3.72 3.39	0.05 3.95
10.	10	18th August 2006	N 02º 36' 4.11" E 101º 41' 7.79"	Sg Sepang Kecil (SK)	8.41 7.27	0.17 8.96	3.89 3.63	0.07 4.25
11.	12	28th April 2006	N 02° 34' 49.2" E 101° 49' 34.4"	Kuala Lukut Besar (KLB)	8.74 7.55	0.16 10.74	4.73 3.9	0.09 5.28
12.	6	28th April 2006	N 02° 33' 42.2" E 101° 48' 00.2"	Kuala Lukut Kecil (KLK)	9.20 8.83	0.08 9.47	4.68 4.21	0.21 5.93
13.	17	29th April 2006	N 01° 52' 21.0 E 102° 44' 16.5	Sg Balang Laut, Muar (SBL)	7.83 7.53	0.07 7.97	3.90 3.63	0.08 4.15
14.	15	29th April 2006	N 01° 45' 12.5" E 102° 55' 45.4	Kuala Sg Ayam, Batu Pahat (KSA)	7.15 6.37	0.13 7.83	3.51 2.93	0.07 3.82
15.	11	29th April 2006	N 01° 41' 07.2 E 103° 05' 54.6"	Pantai Punggur, Pontian (PP)	8.47 7.95	0.14 8.98	4.16 3.98	0.04 4.35
16.	11	30th April 2006	N 01° 26' 05.8" E 101° 56' 02.4"	Kg Pasir Puteh, Johor Baharu (KPP)	9.03 8.56	0.13 9.66	4.82 4.56	0.04 4.97
17.	8	15th December 2006	N 06º 12' 55.21" E 102º 14' 14.21"	Tumpat, Kelantan (T)	7.30 6.62	0.18 8.15	3.08 2.96	0.04 3.29

then digested in concentrated nitric acid (BDH: 69%). The dried sediment samples were crushed using a mortar and pestle and sieved through a 63 µm aperture stainless steel sieve and were shaken vigorously to produce homogeneity (Yap *et al*., 2002). For the analyses of the total Zn concentrations in the sediment samples, three replicates were analyzed using direct aqua-regia method (Yap *et al*., 2006). About 1 g of each dried sample was digested in a combination of concentrated HNO_3 (AnalaR grade; BDH 69%) and $HClO_4$ (AnalaR grade; BDH 60%), in the

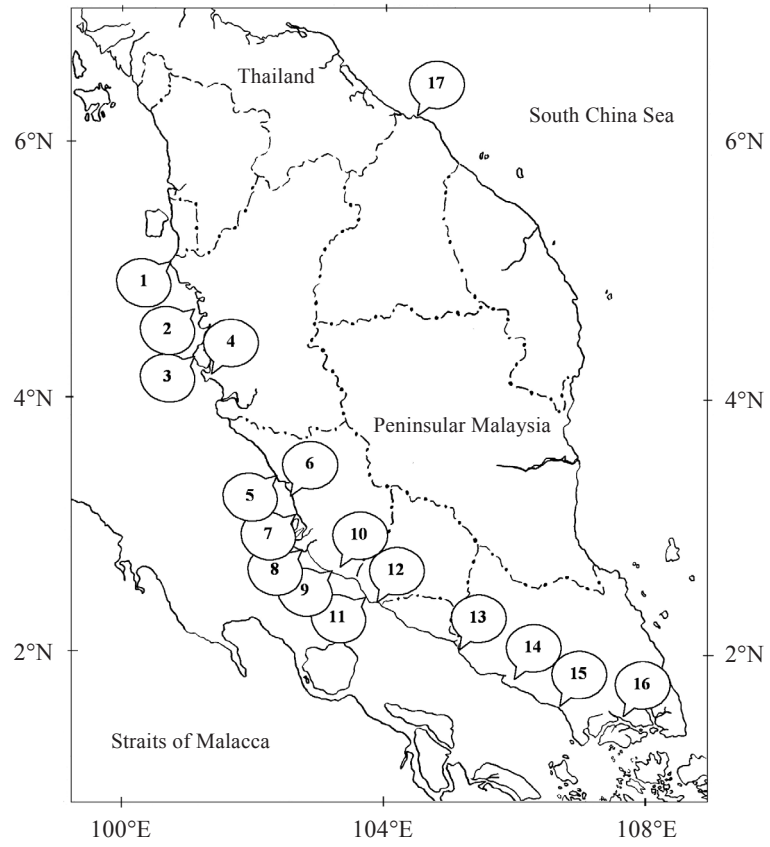

Fig. 1: Map showing 17 sampling locations (in numbers) of the intertidal areas of Peninsular Malaysia (the names of all sampling sites in numbers are given in Table 1)

ratio of 4:1. The snail and sediment samples were put into a hot-block digester first at a low temperature (40 °C) for 1 hr and they were fully digested at 140 °C for at least 3 hrs (Yap *et al*., 2002). Geochemical fractions of Zn in the sediments were obtained using the modified SET (Sequential Extraction Technique) described by Badri and Aston (1983). The four fractions 'easily, freely or leachable or exchangeable (EFLE)', 'acid reducible', 'oxidisable-organic' and resistants were employed (Yap *et al*., 2002). Two replicates for each fraction were analyzed. In the four fractions considered, the extraction solutions and the conditions employed are as follows:

(1) EFLE: About 10g of sample was continuously shaken for 3 hours with 50 ml 1.0 M ammonium acetate (NH_4CH_3COO), pH 7.0, and at room temperature.

(2) 'Acid reducible': the residue from (1) was continuously shaken for 3 hours with 50 ml 0.25M hydroxylammonium chloride ($NH_2OH•HCl$) acidified to pH 2 with HCl, at room temperature.

(3) 'Oxidisable – organic': The residue from (2) was first oxidized with 30 % H_2O_2 in a water bath at 90 °C – 95 °C. After cooling, the metal released from the organic complexes was continuously shaken for 3 hours with 1.0M ammonium acetate (NH_4CH_3COO) acidified to pH 2.0 with HCl at room temperature.

(4) 'Resistant': The residue from (3) was digested in a combination of concentrated nitric acid (AnalaR grade, BDH 69 %) and perchloric acid (AnalaR grade, BDH 60 %) as performed in the direct aqua-regia method.

The prepared samples were then analyzed for Zn using an air-acetylene flame Atomic Absorption Spectrophotometer (AAS) Perkin Elmer Model A- Analyst 800. The sample concentrations are presented as µg/g dry weight (dw).

Quality Assurance

To avoid possible contamination, all the glassware and equipment used were acid-washed. To check for contamination, procedural blanks were analyzed after every five sample (Yap *et al.*, 2006). Meanwhile, the quality control samples, made from standard solutions of Zn, were analyzed in every sample to check for the metal recoveries. The accuracy of the digestion analysis procedure was evaluated by the analysis of NIST 1566a Oyster tissue, NRCC Dolt-1 Dogfish liver and MESS-3 Marine sediment, as depicted in Table 2.

STATISTICAL ANALYSIS

The data were analyzed using SPSS 15.0, i.e. the coefficient of correlation (r) between variables; Pearson's correlation was performed to determine the relationship of different soft tissues of snail and metals concentration in sediment based on untransformed data. Analysis of variance (ANOVA) was used to calculate the interaction of different soft parts and sampling location on the metals concentrations of snail, *T. telescopium*. The ANOVA analysis revealed significant differences for the factor 'sampling locations' on the Zn concentrations of the sediment and snail bodies (different soft parts). The hierarchy of the mean values and relationship to homogeneous subsets (mean values within are not significantly different) were determined with Student-Newman-Keuls test. Homogeneous subsets are marked with letters (a-j) (Table 3). All the statistical tests were performed at the significance level of $P < 0.05$.

RESULTS AND DISCUSSION

The concentrations of Zn in all geochemical fractions in the surface sediments are given in Table 5 and *Fig. 2*. The Zn concentration in the sediment collected from Kuala Sg. Ayam (297.46 ± 0.81µg/g dw) was significantly higher compared to the other sites ($P < 0.05$). However, the resistant fraction for Zn from Kuala Sg. Ayam was the dominant fraction. It is assumed that the Zn concentrations in the sediment derived greatly from the geochemical background rather than anthropogenic inputs. With respect to the Zn concentrations of the surface sediments from Kg. Pasir Puteh, Kuala Lukut Besar, and Sepang Kecil, the Zn non- resistant fraction (EFLE, acid reducible, and oxidisable-organic) was found to contain more than 70%, and these sites are most likely to be influenced by the untreated domestic effluent. Henkin *et al.* (1984) classified Zn as a typical indicator of industrial and domestic wastes.

The highest concentration of EFLE fraction of Zn was found at Kg. Pasir Puteh, which was significantly higher ($P < 0.05$) than the other sites. Kuala Sg. Ayam showed the highest

TABLE 2
Comparison of Zn concentration between measured values by using AAS and CRM values

	Dogfish	Mussel	Sediment
Measured	100.26	129.21	145.03
CRM	86.60	137.00	159.00
Recovery (%)	115.77	94.31	91.21

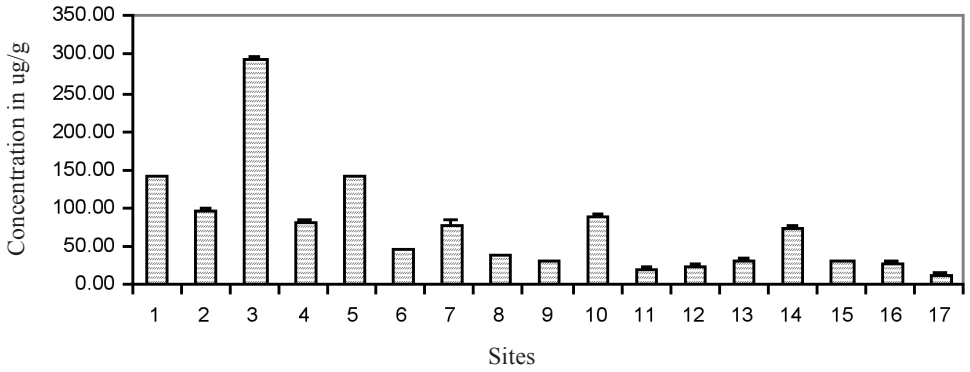

Fig. 2: Mean Zn concentration of sediment collected from 17 intertidal area of Peninsular Malaysia

level of acid reducible fraction, oxidisable-organic fraction, and resistant fractions of Zn in sediment.

The concentrations of Zn in digestive caecum, gill, muscle, and foot were commonly higher than those in the sediment. For example, Zn in the digestive caecum of gastropods from Bagan Lalang averaged three times higher when compared to the total Zn concentration in the sediment. In addition to that, digestive caecum were always found to accumulate high concentrations of Zn in the 17 sampling locations (Table 5), except for the population from Kuala Sg. Ayam in which the gill accumulated higher concentrations of Zn. Other tissues such as cephalic tentacle, remaining soft tissues, and gill showed low concentrations of Zn, but not in any consistent pattern. Based on the findings of the present study, some important points can be noted. According to Deb and Fukushima (1999), metals may be in high concentrations in the gills, intestine and digestive glands of gastropod. These organs have relatively high potential for metal storage and accumulation (Altindag and Yigit, 2005). Unlike this report, Zn concentrations in foot, mantle, and muscle did not show significant difference, but Zn found in the gill and digestive caecum of *T. telescopium* was markedly higher ($P < 0.05$) than other different soft tissues because as an essential trace element, Zn is known to act as an enzyme cofactor in over 200 enzymes with important biological functions regulating many physiological processes including DNA synthesis, behavioural response, and reproduction (Vallee and Auld, 1990). The high level of Zn in the digestive caecum could be related to its functions in biochemical mechanism within a particular tissue, regardless of the environmental exposures of the organisms to the metals (Turoczy *et al*., 2001) and physiological differences between those different soft tissues rather than proximity to human activity or other environmental variables (Park and Presley, 1997). In general, the accumulation and storage of trace metals (e.g. Zn) in common biomonitors, such as bivalve and gastropod molluscs, are strongly associated with the level and metal binding capacity of metallothioneins or other detoxificatory systems in their tissues (Roesijadi, 1992; Dallinger *et al*., 2004a, b; Yap *et al*., 2006).

Table 3 shows that the highest Zn concentrations were found in the foot from Jambatan Permaisuri Bainun, followed by Kuala Sg. Ayam and Sg Janggut. The foot from Kg. Pasir Puteh recorded the lowest Zn concentration but the gastropods from this particular site recorded the highest concentrations of Zn in mantle, muscle and remaining soft tissues. For gill, the highest concentrations were found from Kuala Sg. Ayam, followed by Kg. Setiawan and Kg Pasir Puteh, while Bagan Lalang recorded the highest Zn concentration in digestive caecum, followed by Sg. Janggut and Sg. Balang Laut.

TABLE 3
Mean Zn concentrations (µg/g dry weight) in the different soft tissues of *Telescopium telescopium* collected from 17 intertidal areas of Peninsular Malaysia

No	Site	Foot		CT		Mantle		Muscle		Gill		REST		DC	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1.	KPP	49.22 ± 1.74	a	36.81 ± 0.97	a	105.57 ± 1.17	h	133.39 ± 2.27	f	144.48 ± 2.58	h	246.57 ± 5.49	h	282.59 ± 6.33	c,d
2.	PP	82.48 ± 4.56	c,d,e	63.37 ± 0.57	c	71.69 ± 1.94	c,d,e	78.19 ± 3.62	b,c,d	72.99 ± 4.26	f	30.92 ± 0.10	a,b	194.55 ± 3.51	a,b
3.	KSA	91.21 ± 1.11	e	71.78 ± 0.82	e,f	80.49 ± 6.92	e,f,g	87.61 ± 7.23	d,e	402.78 ± 11.58	j	77.13 ± 1.84	f	180.08 ± 6.74	a,b
4.	SBL	70.68 ± 1.10	b,c	51.27 ± 1.45	b	63.98 ± 2.52	b,c	50.08 ± 1.29	a,b	19.86 ± 0.81	a	62.69 ± 1.49	d,e	284.02 ± 2.76	c,d
5.	KLK	84.90 ± 0.69	d,e	66.43 ± 4.42	c,d,e	82.12 ± 0.87	f,g	88.60 ± 1.32	d,e	26.75 ± 0.43	a,b	71.51 ± 3.39	e,f	211.18 ± 1.26	a,b,c
6.	KLB	82.60 ± 1.50	c,d,e	71.17 ± 2.23	d,e,f	84.72 ± 3.40	f,g	87.98 ± 1.24	d,e	40.30 ± 0.89	b,c	67.94 ± 1.13	d,e,f	201.29 ± 8.01	a,b
7.	SB	69.66 ± 1.72	b,c	60.93 ± 3.71	c	68.58 ± 1.93	c,d	80.58 ± 1.60	b,c,d,e	74.12 ± 0.28	f	99.34 ± 4.63	g	220.56 ± 2.57	a,b,c,d
8.	BL	71.06 ± 1.02	b,c	50.96 ± 2.19	b	58.09 ± 0.40	a,b	69.44 ± 0.64	b	48.22 ± 1.02	c,d	26.33 ± 1.56	a	394.69 ± 66.04	e
9.	SK	64.42 ± 0.31	b	63.39 ± 0.10	c,d,e	75.23 ± 1.74	d,e,f	84.86 ± 0.79	c,d,e	35.57 ± 1.12	a,b,c	59.61 ± 1.68	d	243.86 ± 0.79	b,c,d
10.	KPJ	61.75 ± 1.01	b	50.27 ± 0.20	b	50.66 ± 3.07	a	52.51 ± 4.26	a	55.11 ± 2.04	d,e	23.23 ± 1.60	a	144.78 ± 0.50	a
11.	SJ	91.15 ± 1.28	e	64.68 ± 1.85	c,d,e	78.93 ± 1.41	d,e,f	84.94 ± 1.93	c,d,e	70.46 ± 0.65	e,f	31.27 ± 1.14	a,b	292.54 ± 5.01	d
12.	PI	77.32 ± 0.85	c,d	64.61 ± 2.11	c,d,e	68.76 ± 1.32	c,d	83.87 ± 0.43	c,d,e	70.19 ± 3.55	e,f	38.37 ± 0.99	b	170.21 ± 2.80	a,b
13.	KD	78.82 ± 1.67	c,d,e	62.46 ± 1.38	c,d	61.36 ± 0.75	a,b,c	73.85 ± 1.69	b,c	61.45 ± 2.01	d,e,f	31.49 ± 2.30	a,b	277.97 ± 2.43	c,d
14.	KS	79.10 ± 0.82	c,d,e	71.24 ± 1.65	e,f,g	58.71 ± 2.08	b	91.15 ± 2.15	e	175.80 ± 9.60	i	22.76 ± 1.35	a	215.86 ± 2.73	a,b,c,d
15.	JPB	126.29 ± 9.45	f	75.25 ± 0.33	f	71.10 ± 0.27	c,d,e	87.12 ± 1.41	d,e	93.87 ± 3.31	g	46.27 ± 3.61	c	272.18 ± 4.45	c,d
16.	KG	84.62 ± 0.38	d,e	70.86 ± 0.52	d,e,f	89.13 ± 0.46	g	75.26 ± 2.93	b,c	94.20 ± 0.73	g	50.03 ± 0.74	c	194.97 ± 2.23	a,b
17.	T	71.88 ± 1.27	b,c	53.50 ± 0.28	b	70.19 ± 1.00	c,d	70.75 ± 0.17	b	23.67 ± 1.55	a	70.00 ± 0.03	e,f	278.83 ± 2.59	c,d

Objects indicated with different alphabet: $p < 0.05$, objects indicated with same alphabet: $p > 0.05$ (the same alphabet showed that the means do not differ from one another, $p > 0.05$). Homogeneous subsets resulting from one-way ANOVA and Student Newman Keuls test ($p < 0.05$) are donated with letters $^{a-j}$

These findings are similar with the ones found in the previous study by Yap *et al*. (2007) on *Perna viridis*. According to Wang and Rainbow (2005), the uptake of Zn by marine invertebrates is also dependent on the excretion of accumulated Zn in their bodies. On the other hand, the Zn uptake from the solute phase was proportional to the ambient Zn concentration (Wang *et al*., 1996; Chong and Wang, 2000). Wang and Wong (2003) also found that in *Perna viridis*, regulation was mainly achieved by a change in the Zn assimilation from the dietary phase which might dominate the overall Zn accumulation in mussels. However, the accumulation and tissue distribution of Zn in various organs of the *T. telescopium* should be investigated to elucidate the suitability of this snail as a biomonitor of Zn pollution through experimental field or laboratory work.

The correlations between different geochemical fractions of Zn in sediment and Zn in the different soft tissues of *T. telescopium* are shown in Table 2. Significant correlations were also found between Zn in mantle ($r = 0.595$, $P < 0.01$)), muscle ($r = 0.768$, $P < 0.01$) and remaining soft tissues ($r = 0.910$, $P < 0.01$) and EFLE fraction of Zn (Table 4). Zinc found in mantle, muscle, gill, and remaining soft tissues was found to be significantly correlated with acid reducible fraction ($r = 0.374$, 0.516, 0.695 and 0.627, $P < 0.01$) (Table 4). Significant correlations were also found between Zn in gill, and remaining soft tissues and oxidisable- organic fraction of Zn ($r = 0.390$, 0.462, $P < 0.01$). Zn in gill was also found to be significantly correlated with the resistant fraction of Zn in the sediment. For Zn in digestive caecum of *T. telescopium,* no significant correlation ($P > 0.05$) was found among the EFLE, acid-reducible and oxidisable-organic fractions, except for negative correlation ($r = -0.296$, $P < 0.05$) with the resistant fraction of Zn in sediment. The general trend of Zn concentrations in different soft tissues was digestive caecum > gill > muscle > foot > mantle > remaining soft tissues > cephalic tentacle, which is probably the result of both exposure and ability to regulate Zn. The data presented in Table 3 show that the digestive caecum accumulated the highest concentration of Zn as compared to other tissues ($P < 0.05$). Even though the digestive caecum always accumulated higher concentrations of Zn in 16 sampling locations out of 17, the Zn levels in this digestive caecum did not significantly correlate with any of the sedimentary fractions of Zn. This could be due to the bioavailability of Zn to the digestive caecum not reflecting the Zn contamination of the sampling sites, as represented by the surface sediments (Yap *et al*., 2002). A strong correlation ($P < 0.01$) between Zn in mantle,

TABLE 4
Pearson correlation coefficients between EFLE, acid reducible, oxidisable-organic and resistant of Zn concentration in sediment with different parts of soft tissues correlations

	EFLE	AR	OO	Resistant	Foot	CT	Mantle	Muscle	Gill	REST	DC
AR	0.649**	1									
OO	0.359**	0.751**	1								
Resistant	0.020	0.670**	0.772**	1							
Foot	-0.455**	-0.274	-0.172	0.124	1						
CT	-0.539**	-0.214	-0.190	0.149	0.730**	1					
Mantle	0.595**	0.374**	0.259	0.106	-0.034	-0.024	1				
Muscle	0.768**	0.516**	0.200	0.059	-0.056	-0.035	0.729**	1			
Gill	0.225	0.695**	0.390**	0.741**	0.183	0.252	0.219	0.349*	1		
REST	0.910**	0.627**	0.462**	0.110	-0.420**	-0.514**	0.707**	0.714**	0.192	1	
DC	0.107	-0.144	-0.219	-0.296*	-0.031	-0.369**	-0.043	0.031	-0.244	0.097	1

Note: Data based on 17 sites of intertidal area of Peninsular Malaysia. Level of significance ** $P < 0.01$ level and *$P < 0.05$ level

TABLE 5
Order of Zn concentrations in the different soft tissues of *T. telescopium* from each sampling location

No.	Sites	Patterns
1.	Kg Pasir Puteh	DC > REST > Gill > Muscle > Mantle > Foot > CT
2.	Pantai Punggur	DC > Foot > Muscle > Gill > Mantle > CT > REST
3.	Kuala Sg Ayam	Gill > DC > Foot > Muscle > REST > Mantle > CT
4.	Sg Balang Laut	DC > Foot > Mantle > REST > CT > Muscle > Gill
5.	Kuala Lukut Kecil	DC > Muscle > Foot > Mantle > REST > CT > Gill
6.	Kuala Lukut Besar	DC > Muscle > Mantle > Foot > CT > REST > Gill
7.	Sepang Besar	DC > REST > Muscle > Gill > Mantle > Foot > CT
8.	Bagan Lalang	DC > Foot > Muscle > Mantle > CT > Gill > REST
9.	Sepang Kecil	DC > Muscle > Mantle > Foot > CT > REST > Gill
10.	Kg Pantai Jeram	DC > Foot > Gill > Muscle > Mantle > CT > REST
11.	Sg Janggut	DC > Foot > Muscle > Mantle > Gill > CT > REST
12.	Pulau Indah	DC > Muscle > Foot > Mantle > Gill > CT > REST
13.	Kg Deralik	DC > Foot > Muscle > CT > Gill > Mantle > REST
14.	Kg Setiawan	DC > Gill > Muscle > Foot > CT > Mantle > REST
15.	J P. Bainun	DC > Foot > Gill > Muscle > CT > Mantle > REST
16.	Kuala Gula	DC > Gill > Mantle > Foot > Muscle > CT > REST
17.	Tumpat	DC > REST > Muscle > Mantle > Foot > CT > Gill

muscle, gill, and remaining soft tissues and non-resistant sedimentary fraction (total of EFLE, acid reducible fraction, and oxidisable organic fraction) was found, suggesting that *T. telescopium* is a good biomonitor of Zn contamination besides Zn bioavailability. Other studies (Luoma and Bryan, 1978; Luoma, 1983; Ying *et al*., 1993; Yap *et al*., 2002) have examined various mollusc species and demonstrated significant correlations between metal concentrations in organisms and metal concentrations extracted from the sediment by various extractants.

CONCLUSIONS

Significant ($p < 0.05$) correlations between Zn concentrations in different soft tissues of *T. telescopium* (mantle, muscle, gill, and remaining soft tissues) and some geochemical fractions of Zn in the sediment were found. These results suggest that selected soft tissues of *T. telescopium* could be used as biomonitoring organs for Zn pollution in the intertidal area of Peninsular Malaysia. However, further validation is still required based on the field and laboratory studies. Furthermore, biochemical and molecular studies should be conducted in future in order to establish *T. telescopium* as a good biomonitor of Zn for intertidal area in Malaysia.

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A Survey on Orchids in Selected Trails of Gunung Nuang Forest Reserve

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ABSTRACT

Gunung Nuang, which is the highest mountain peak in Selangor at 1493 m, marks the meeting point of three Malaysian states, namely Negeri Sembilan, Pahang, and Selangor. Gunung Nuang Forest Reserve comprises of lowland dipterocarp, hill dipterocarp, upper hill dipterocarp, and montane dipterocarp forest, covering 3.9 % of Selangor land area. Starting from 2005, several collection trips were made to observe and document the flora from the family of Orchidaceae. As a result, 27 species of orchids were found on the selected trails, while 11 taxa remain unidentified. From the collected specimens, 34.9% of Selangor's orchids were represented by the results, indicating the rather high species diversity in the area. From the collections, five species are new records to Selangor. All the specimens were identified based on their morphological vegetative features and floral structures.

Keywords: Orchidaceae, orchid diversity, Gunung Nuang, Selangor

INTRODUCTION

Gunung Nuang (1493 m) is the highest mountain peak in the district of Hulu Langat, Selangor. It is situated at the northern border of Lentang Forest Reserve, Pahang. Hulu Langat Forest Reserve is a watershed forest. The vegetation of Gunung Nuang showed changes with altitudinal differences (Sahibin *et al*., 2005). Gunung Nuang exhibits a rich diversity of plants including many traditional medicinal herbs. The forest belongs to the tropical rainforest with high annual rainfalls and dense flora and fauna.

There was no attempt to list all the vascular plant flora of the Peninsular Malaysia in a single published list until Turner in 1995. He prepared a catalogue enlisting all the vascular plants in Peninsular Malaysia and Singapore by recording details of localities. This compilation was done by referring to several renowned publications of plants in Malay Peninsular. In this checklist by Turner (1995), 169 species of orchids were classified as common and widespread species in Peninsular Malaysia, but no exact locality was stated. A total of 105 orchid species were found and recorded in Selangor.

As for Gunung Nuang, there has been no attempt, at least in the recent times, to list the possible occurrence of plants from the family of Orchidaceae. This forest is rich in the diversity of flora and fauna but certain parts have been developed into industrial and residential areas, especially the lowland areas of the mountain. This is so because Hulu Langat Forest Reserve has not attained the status of permanent forest reserve.

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SITE DESCRIPTION AND METHODS

Gunung Nuang

Gunung Nuang is situated at the latitude of 101° 50'E and the longitude of 3° 14'N. It is bordering with Pahang in the north and Negeri Sembilan in the west. In general, Gunung Nuang is a part of the formation of the Main Range, which is running from southern Thailand to the south of Negeri Sembilan. The average temperature for the forest is 20.3 °C while humidity ranges from 90-100%, with the average as high as 99.77% recorded at the altitude of 1350 m (Nizam *et al*., 2005).

Hulu Langat Forest Reserve covers approximately 31,378 ha and this makes up 3.9 % of the land area in Selangor. Gunung Nuang Forest Reserve is a part of the Hulu Langat Forest Reserve with the highest peak of the area. A portion of the lowland areas to the land altitude of 720 m was once logged in the early 1960's (Latiff, 2005).

Gunung Nuang Forest Reserve exhibits an important role as watershed forest for the state of Selangor, where water enters into the main rivers such as Sungai Langat and several smaller rivers which are channelled into two reservoirs, namely Semenyih Reservoir and Pangsun Reservoir (Nizam *et al*., 2005).

The study sites covered Compartment 63 – 67, in which each compartment has at least a river flowing in the site. The samplings of the present study were done along the riverbanks and also some inland trails which were about 300 m away from the river. The land elevation of the selected trials ranges from approximately 100 m to 700 m above the sea level (a.s.l.).

Methods

Field explorations and specimen collections of orchids were carried out in the selected trails in the forest reserve between January 2005 and November 2006. Most of the specimens were photographed in the natural habitat and their characteristics were documented. Fresh specimens collected for identification were preserved into herbarium specimen via standard herbarium procedure. All herbarium specimens were deposited in the Herbarium of Biology Department, Faculty of Science, Universiti Putra Malaysia. Some of the plants collected were brought back to UPM and cultivated as *ex situ* conservation in the green house.

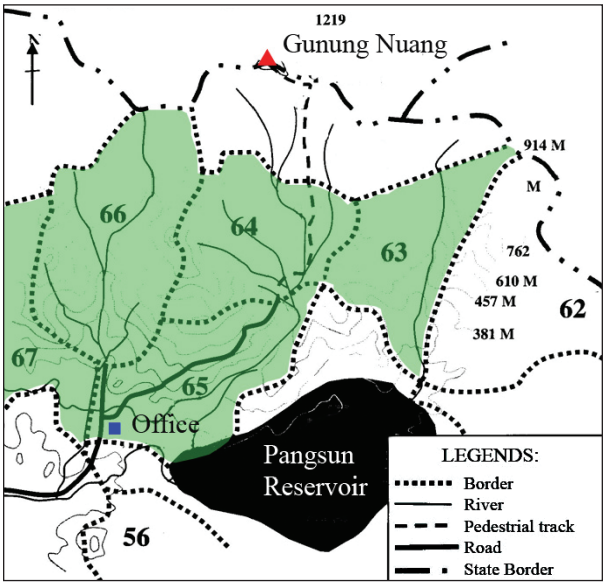

Fig. 1: Sampling collection areas in Gunung Nuang Forest Reserve, Selangor

RESULTS AND DISCUSSION

A total of 38 orchid species, belonging to 23 genera, were identified during this study (Table 1). Of the collected specimens, 27 were identified into their respective species level, and the remaining 11 were only identified to the genus level as the specimens were incomplete, due to lack of flowers. Five species collected, namely *Dendrobium anosmum*, *Liparis lacerata*, *Pennilabium struthio*, *Plocoglottis plicata*, and *Pomatocalpa spicata*, are new records for Selangor (Table 1). Therefore, a total percentage of 34.9% from Selangor's orchids were found in the study site. These results clearly show the great diversity of orchids for a small area of less than 31,378 hectares.

Based on the observations of the collected specimens, there were not many differences between the collected species and the cited species in the reference book. Nevertheless, some minor morphological characters were found to slightly differ in terms of the colour and sizes of the flowers, length and width of the leaves, and the plant habitat. Despite that, the key characters which identify them into their respective genus still remain similar between the collected species and the cited ones.

The orchid species found in this study comprises of terrestrials, epiphytes, and lithophytes. Most of the orchids were collected in the shady areas of the forest with the altitude of 250 m to 700 m a.s.l. Most of the epiphytic orchids were growing abundantly on tree trunks

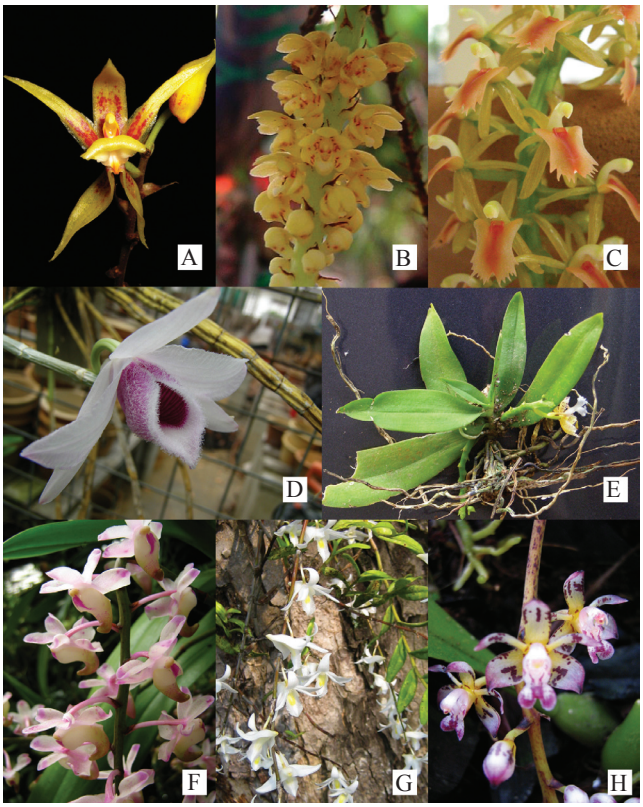

Plate 1: New Record and Orchids of Gunung Nuang. A.* Plocoglottis plicata*, *B.* Pomatocalpa spicata*, *C.* Liparis lacerata*, *D.* Dendrobium anosmum*, *E.* Pennilabium struthio*, *F.* Aerides odorata, *G.* Dendrobium crumenatum, *H.* Thecostele alata

TABLE 1
Orchids collected from Gunung Nuang, Selangor

No.	Species	Descriptions
1	*Acriopsis liliiflora* (Koenig) Ormerod	Epiphytic to 30 cm tall; on riverside trees
2	*Aerides odorata* Lour	Epiphytic to 40 cm tall; on semi-shaded tree trunks; flowers sweetly scented
4	*Agrostophyllum glumaceum* Hook.*f.*	Epiphytic clump to 30 cm tall; on riverside trees
3	*Agrostophyllum majus* Hook.*f.*	Epiphytic clump to 70 cm tall; on exposed trees
5	*Bulbophyllum patens* King *ex*Hook.*f.*	Creeping epiphyte; on riverside trees
6	*Bulbophyllum* sp. 1	Creeping epiphyte; on semi-shaded trees
7	*Bulbophyllum* sp. 2	Creeping epiphyte; on Semi-shaded trees
8	*Bulbophyllum* sp. 3	Creeping epiphyte; on riverside trees
9	*Calanthe ceciliae* Rchb.*f.*	Terrestrial to 50 cm tall; on shaded slopes and riverbanks
10	*Ceratostylis subulata* Blume	Epiphytic clump; on shaded branches near riverside
11	*Coelogyne* sp. 1	Epiphytic to 20 cm tall; on shaded trees
12	*Coelogyne* sp. 2	Terrestrial to 50 cm tall; on rocks by the rivers
13	*Coelogyne* sp. 3	Epiphytic to 30 cm tall; on tree trunks
14	*Corymborkis veratrifolia* (Reinw.) Blume	Terrestrial to 180 cm tall; on the shaded slopes; flowers slightly scented
15	*Cymbidium* sp.	Epiphytic to 50 cm tall; on fallen logs
16	*Dendrobium acerosum* Lindl.	Epiphytic to 15 cm tall; on semi-shaded trees and fallen trees
17	*Dendrobium anosmum* Lindl.*	Epiphytic to 50 cm tall; on tree trunks
18	*Dendrobium concinnum* Miq.	Epiphytic to 15 cm tall; on semi-shaded trees and fallen trees
19	*Dendrobium crumenatum* Sw.	Epiphytic to 1 m long; on exposed trees; flowers with fresh scent
20	*Dendrobium truncatum* Lindl.	Epiphytic to 15 cm tall; on semi-shaded trees and fallen trees
21	*Flickingeria convexa* (Blume) A.D. Hawkes	Creeping epiphytic; on semi-shaded trees
22	*Liparis lacerata* Ridl.*	Epiphytic clumps to 15 cm tall; on mossy cushion on rock and trees
23	*Oberonia* sp.	Epiphytic to 15 cm tall; on semi-shaded trees and fallen trees
24	*Pennilabium struthio* Carr.*	Epiphytic to 10 cm; on riverside tree branches
25	*Pholidota articulata* Lindl.	Pendulous epiphyte to 50 cm long; on riverside trees
26	*Pholidota imbricata* Hook.	Epiphytic clump to 20 cm tall; on trees and rocks
27	*Plocoglottis plicata* (Roxb.) Ormerod.*	Terrestrial to 50 cm tall; on shaded slopes and riverbanks
28	*Pomatocalpa latifolia* (Lindl.) J.J.Sm.	Epiphytic; short-stemmed; on semi-shaded tree trunks
30	*Pomatocalpa spicata* Breda*	Epiphytic; short-stemmed; on semi-shaded tree trunks
29	*Pomatocalpa* sp.	Epiphytic; on semi-shaded tree trunks
31	*Robiquetia spatulata* (Bl.) J.J.Sm.	Hanging epiphytic; on twigs of canopy
32	*Tainia paucifolia* (Breda) J.J.Sm.	Terrestrial to 60 cm tall; on semi-shaded riverbanks
33	*Thecostele alata* (Roxb.) C.S.P. Parish & Rchb.*f.*	Epiphytic clumps about 20 cm; on semi-shaded trees
34	*Thelasis pygmaea* (Griff.) Blume	Epiphytic clumps; on shaded trees with mosses
35	*Thrixspermum centipeda* Lour.	Hanging epiphytic to 10 cm long; on exposed trees
36	*Thrixspermum* sp.	Hanging epiphytic to 30 cm long; on exposed trees
37	*Vanilla griffithii* Reichb.*f.*	Climber; on trees in fairly open places
38	*Vanilla* sp.	Climber; on trees in fairly open places

* New records to Selangor

near the streams and high humidity areas. Nevertheless, the higher region towards the peak of the mountain was not covered and might possibly be explored in future research.

As for *Plocoglottis plicata*, there are no records of the orchid collections by Seidenfaden and Wood (1992) and Turner (1995). However, it was stated to be distributed in Peninsular Malaysia by Comber (2001). This particular genus might be grouped together as *Plocoglottis javanica* because of the similarity in the colour of the flower. Nevertheless, some distinguished characters, such as the distribution of red spots and the curvature of petals and sepals, differentiate *P. plicata* from *P. javanica*. Further observation and collections can be made in order to confirm the existence of the former species in Peninsular Malaysia. This species, *P. plicata,* is widespread in Gunung Nuang, particularly in the semi-shaded areas near the streams.

CONCLUSIONS

Gunung Nuang Forest Reserve shows a great diversity of orchids. A total of 38 species from 27 genera were collected in the selected trails. Meanwhile, 5 orchid species were documented as the new records for Selangor. This finding indicates the richness in the diversity of orchids in Selangor, particularly in Gunung Nuang Forest Reserve. These results have thus increased the number of orchid species documented in the state of Selangor.

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Diagnostic Cytology of Neoplastic Lesions in Dogs

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ABSTRACT

Cytology is a reliable and minimally invasive method of obtaining tissues for diagnosis. It is a rapid method to be used for differentiating inflammatory from neoplastic disease. Inflammation is easily differentiated from neoplasia based on the presence of inflammatory cells. Ulcerative neoplasm is always secondarily infected by bacteria, and thus inflammatory cells could also be observed. Cytologically, neoplastic cells are classified into three major categories based on certain common cytological features (Meinkoth *et al*., 2008), namely round, epithelial, and mesenchymal cells (Meinkoth *et al*., 2008). Cytological images and specific features of neoplastic lesions in dogs from cytology smear, which were obtained from the Veterinary Haematology and Clinical Biochemistry Laboratory, Universiti Putra Malaysia (UPM), were illustrated and briefly described. Round cell tumour was identified as round and discrete neoplastic cells with specific cytoplasmic features, whereas epithelial tumour was identified as clusters of neoplastic cells with closed cytoplasmic attachment, and mesenchymal tumour was identified as spindle neoplastic cells with indistinct cytoplasmic border.

Keywords: Cytology, dog, neoplasia

INTRODUCTION

Cytology is a quick diagnostic tool to be used to differentiate between inflammatory and neoplastic conditions. Inflammatory conditions are easily diagnosed by examining the presence of inflammatory cells. Chronicity of the inflammatory conditions is then evaluated through evaluating the percentage of polymorphonuclear (PMNs) and mononuclear cells (MNC). In acute inflammation, the percentage of PMNs is approximately 70%. For sub-acute or chronic active inflammation, the percentage of PMNs is more than 50%, and for chronic inflammation, more than 50% of the inflammatory cells are MNC. Neoplastic conditions are diagnosed by examining the presence of neoplastic cells. Malignant neoplastic cells are easily differentiated from benign or hyperplastic cells through identification of the criteria of malignancy. It is important to note that the differentiation of benign and hyperplastic cells can only be performed via histopathological examination. Cytologically, neoplastic cells are divided into three groups, namely round or discrete cell, epithelial, and mesenchymal tumour. The specific criteria of each group of tumours will be briefly described and illustrated.

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MATERIALS AND METHODS

Cytology samples, submitted to Veterinary Haematology and Clinical Biochemistry Laboratory, Universiti Putra Malaysia (UPM), were examined. The samples were stained with Wright's stain and examined using a light microscope. Images were captured using a digital camera and ACT2U Imaging Software, and they were compared to the images illustrated and described by Cowell and co-workers (2008).

RESULTS AND DISCUSSION

Round cell tumour, such as lymphosarcoma, mast cell tumour, melanoma and transmissible venereal tumour (TVT), is identified as a group of discrete cells with specific cytoplasmic features. The cytoplasmic features are used to differentiate the different types of round cell tumours. For instance, lymphosarcoma (*Fig. 1*) has a scanty basophilic cytoplasm as compared to histiocytoma and malignant histiocytosis (*Fig. 2*) with abundant pale to basophilic and vacuolated cytoplasm. Differential diagnosis of lymphosarcoma is always included in dogs with clinical signs of generalised lymphadenopathy. Mast cell tumour is usually and easily differentiated from other types of round cell tumours based on the presence of purplish granules in the cytoplasm (*Figs. 3, 4, and 5*). Meanwhile, well to poorly differentiated mast cell tumour (*Fig. 4*) is usually diagnosed from a cutaneous ulcerated mass. The one with intermediate to poorly differentiated (*Fig. 5*) is usually involved or metastasised to the lymph nodes and/or internal organs, such as spleen. Diagnosis of intermediate to poorly differentiated mast cell tumour is challenging when a rapid stain, such as Diff quick, is used. Mast cell granules are poorly stained using these types of stain. Melanoma is usually diagnosed from ulcerated lesions in the oral cavity and limbs (*Fig. 6*). It is a greater imitator which yields cell populations that may appear discrete, epithelial, or mesenchymal. The cytoplasmic features of this tumour, however, made it easily distinguished from other types of round cells tumours. The dusky blue-black pigments or granules are presence in the cytoplasm and/or in the background of the smear. Another type of round cell tumour which is usually diagnosed in dogs is TVT (image not shown). It is usually present in external genital organs.

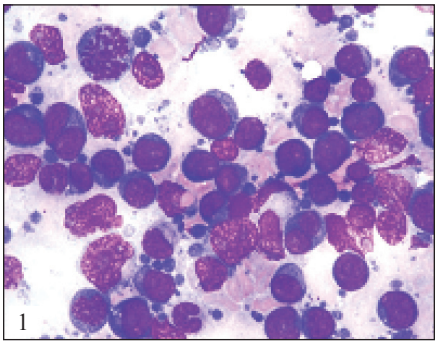

Fig. 1: Fine-needle aspiration (FNA) of a lymph node from a dog with lymphosarcoma containing numerous lymphoblasts and lymphocytes with prominent criteria of malignancy. The neoplastic cells have a scanty basophilic cytoplasm. One abnormal mitotic figure is present. Numerous cytoplasmic fragments (lymphoglandular bodies) are present in the background of the smear

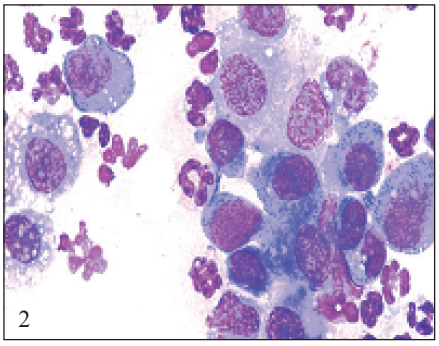

Fig. 2: Pleural fluid from a dog with malignant histiocytosis. The pleomorphic neoplastic cells characterised by marked anisocytosis, cytoplasmic vacuolation, anisokaryosis, multiple nucleoli, and irregular chromatin clumping. Numerous neutrophils are present

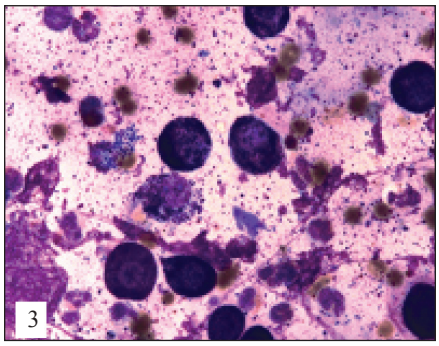

Fig. 3: Impression smear of an ulcerated wound from a dog with well-differentiated granulated mast cell tumour. The neoplatic cells are uniform and the nuclei are almost obscured from view because of the many coarse purple granules

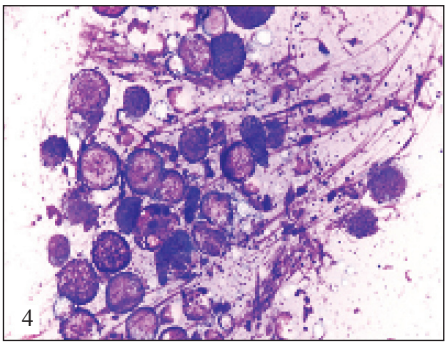

Fig. 4: Fine-needle aspirations (FNA) of a cutanoeus mass from a dog with mast cell tumours. The smears are highly cellular, containing a mixture of well to intermediately differentiated granulated mast cells, and inflammatory cells

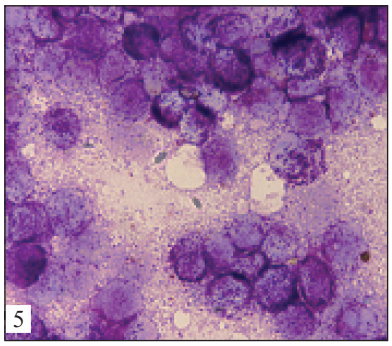

Fig. 5: Fine-needle aspirations (FNA) of an enlarged spleen from a dog with mast cell tumours. The smears are highly cellular, and contain a mixture of intermediate to poorly differentiated granulated mast cells. The neoplastic cell nuclei are stained very pale blue because of the heavy degree of granulation and lack of stain penetration to the nucleus

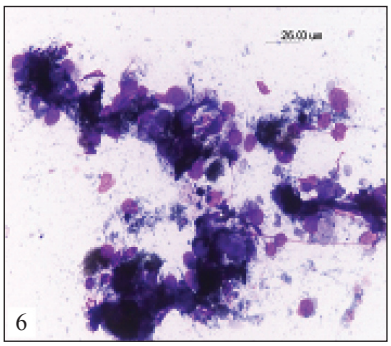

Fig. 6: Fine-needle aspiration (FNA) from an oral cavity of a dog with melanoma. The cellular detail in most of the intact cells is obscured by the pigmentation, preventing evaluation of these cells. Nuclei can be seen in some cells which appear partially ruptured. Numerous free melanin granules are present in the background of the smear

The cytoplasm of the neoplastic cells contains numerous clear vacuoles and smoky grey in colour. The vacuoles may also be freely presence in the background.

The second category of neoplastic lesion which can be cytologically dignosed is epithelial neoplasm. It is identified as clusters of epithelial cells characterised by large pleomorphic neoplastic cells with a closed cytoplasmic attachment (*Figs. 7, 8, 9, 10, 11, and 12*). Examples of these epithelial cells tumours are squamous cell carcinoma (SCC) (*Figs. 7 and 8*), nasal carcinoma (*Fig. 9*), metastatic carcinoma (*Fig. 10*), mammary gland adenoma (*Fig. 11*), and Sertoli cell tumour (*Fig. 12*). Squamous cell carcinoma is usually seen in the oropharyngeal

organs. Two types of SCC, well- and poorly differentiated, are generally diagnosed in dogs. Well-differentiated SCC (*Fig. 7*) is characterised by fully cornified superficial cells with prominent perinuclear vacuolation. In the presence of inflammatory cells, biopsy is necessary to rule out epithelial hyperplasia or dysplasia. Meanwhile, poorly differentiated SCC is characterised by large round neoplastic epithelial cells with abundant, pale to basophilic cytoplasm (*Fig. 8*). Perinuclear vacuolation and cytoplasm showing angular borders could also be observed. Cytology of malignant neoplastic epithelial cells, which were obtained from the nasal cavity (nasal carcinoma), is easily distinguished from benign neoplastic epithelial cells (nasal adenoma). *Fig. 9* clearly demonstrates the malignancy criteria of nasal epithelial cells including coarse granular chromatin, macronuclei, marked multinucleolar nuclei, high nuclear to cytoplasmic ratio, anisokaryosis, and poikilocytosis. Similar malignancy criteria are used to distinguish metastatic carcinoma (*Fig. 10*) from other types of metastatic neoplastic cells, such as lymphosarcoma and mast cell tumour, obtained from the body cavity fluids. The example of benign epithelial neoplastic cells or hyperplasia is shown in *Fig. 11*. The cells obtained from an enlarged mammary gland are lacking cytologic criteria for malignancy. Histopathology of the enlarged mammary gland must be performed for a definitive diagnosis. Sertoli cell tumour (*Fig. 12*) is the tumour of the epithelial cells of testes. It can be mistaken from the round cell tumour, particularly TVT. Based on the location of the affected organs, and the presence of highly pleomorphic neoplastic cells with abundant small and very distinct vacuolated cytoplasm, finely reticulated nuclear chromatin and small multinucleolar nuclei, Sertoli cell tumour, is easily identified.

The third category of neoplastic lesion which could be cytologically diagnosed is mesenchymal neoplasm, which includes fibrosarcoma (*Fig. 13*), osteosarcoma (*Figs. 14 and 15*) and chondrosarcoma (*Fig. 16*). It is characterised by oval to round neoplastic cells with indistinct cytoplasmic borders. Mesenchymal tumour is hardly diagnosed cytologically. In cases of advanced stage of neoplastic conditions, in which the neoplastic cells are able to aspirate from the affected organs or areas, rapid diagnosis could be made. Meanwhile, fibrosarcoma is identified as a spindle cell with oval to round nucleus and tapering long cytoplasmic tails. *Fig. 13* shows fibrosarcoma cells with prominent

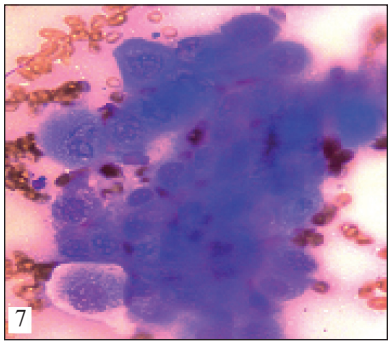

Fig. 7: Fine-needle aspiration (FNA) from an oral cavity of a dog with well-differentiated squamous cell carcinoma. Note the cluster of pleomorphic epithelial cells show perinuclear vacuolation and large nonpyknotic nuclei, despite a mature cornified appearance the cytoplasm

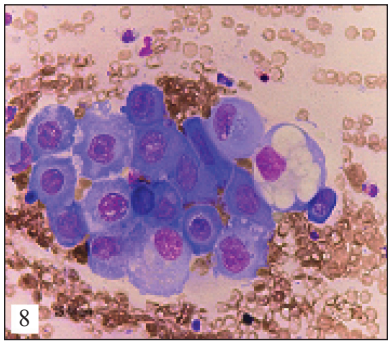

Fig. 8: Fine-needle aspiration (FNA) from an oral cavity of a dog with poorly differentiated squamous cell carcinoma. Note that the cluster of large pleomorphic epithelial cells showing perinuclear vacuolation and large nonpyknotic nuclei with a noncornified appearance of the cytoplasm

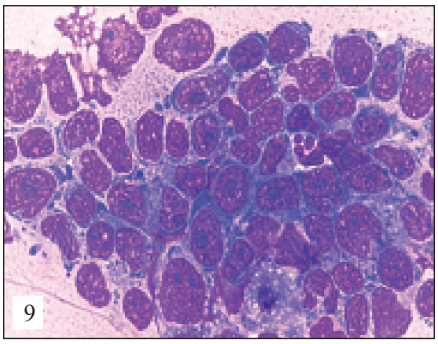

Fig. 9: Nasal wash of a dog with nasal carcinoma. There are a cluster of neoplastic epithelial cells with prominent criteria of malignancy with a few inflammatory cells

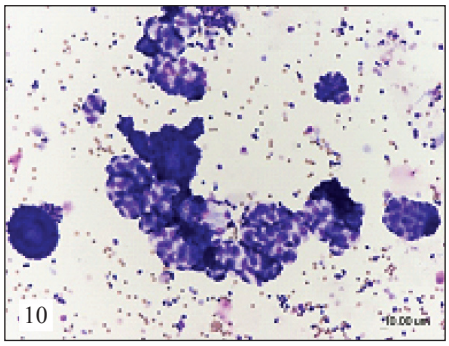

Fig. 10: Abdominal fluid of a dog with metastatic carcinoma. There are numerous aggregates of a homogenous population of large neoplastic epithelial cells with criteria of malignancy which show acinar-like structure

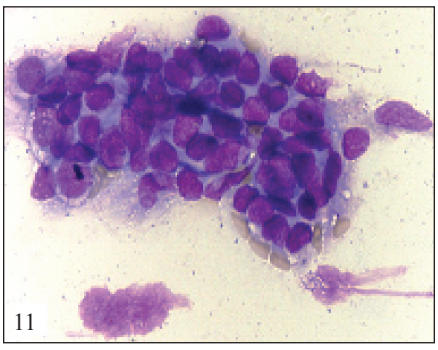

Fig. 11: Fine-needle aspiration (FNA) from enlarged mammary gland of a dog with hyperplasia or adenoma. These epithelial cells have little or no cytoplasmic vacuoles and minimal cellular atypia, emphasising the need for histopathologic confirmation when neoplasia is suspected

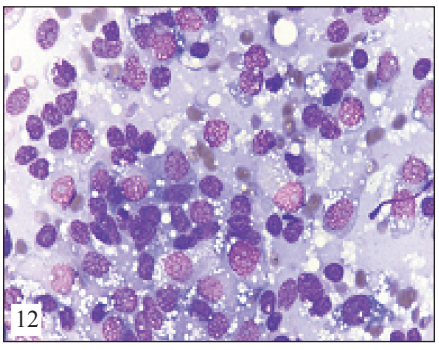

Fig. 12: Impression smear from the ulcerated testes of a dog with Sertoli cell tumour. There are clusters of neoplastic epithelial cells with abundant vacuolated cytoplasm showing prominent criteria of malignancy

criteria of malignancy which is as similarly described by Tyler *et al*. (2008), including multinucleolar nuclei, cytoplasmic basophilia, increased nuclear to cytoplasmic ratio, enlarged nucleoli, and mild to marked variation in cellular, nuclear, and nucleolar size and shape. In contrast to fibrosarcoma, spindle shape of osteosarcoma (*Figs. 14 and 15*) and chondrosarcoma (*Fig. 16*) cells is less prominent, mainly due to the lack of tapering long cytoplasmic tails. The major characteristics of these malignant bone tumours are osteoid and chondroid, which are characterised by eosinophilic matrix in the background of the smears. Osteoid is identified as fibrillar eosinophilic matrix, in which the neoplastic mesenchymal cells are interspersed on the matrix (*Fig. 14*). Chondroid is identified as homogenous eosinophilic matrix, in which the neoplastic mesenchymal cells are embedded in the matrix (*Fig. 16*). Another example of

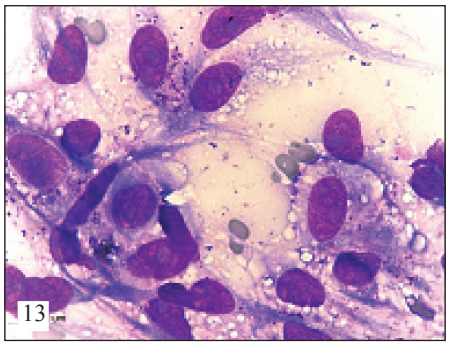

Fig. 13: Fine-needle aspiration (FNA) from an oral cavity of a dog with fibrosarcoma. There are numerous neoplastic spindle cells with prominent criteria of malignancy. Note that most cells are extremely elongated with thin, tapered cytoplasm

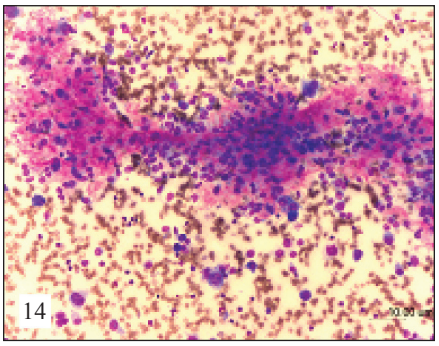

Fig. 14: Fine-needle aspiration (FNA) from osteolytic bone of a dog with osteosarcoma. Low-magnification image shows numerous neoplastic mesenchymal cells which appear to be embedded in a brightly fibrillar eosinophilic matrix

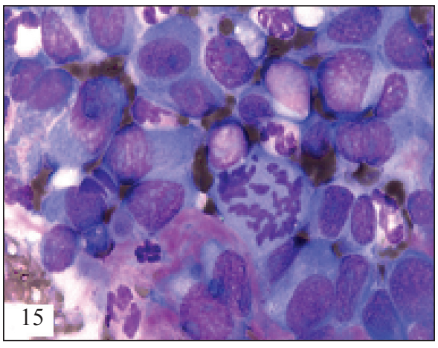

Fig. 15: Fine-needle aspiration (FNA) from osteolytic bone of a dog with osteosarcoma. High-magnification image (magnification from Fig. 14) shows numerous neoplastic cells showing prominent criteria of malignancy. One abnormal mitotic figure is present

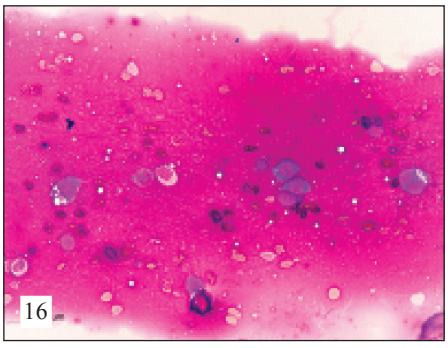

Fig. 16: Fine-needle aspiration (FNA) from osteolytic bone of a dog with chondrosarcoma. Low-magnification image shows a few individual neoplastic mesenchymal cells with prominent criteria of malignancy which appear to be embedded in a brightly homogenous eosinophilic matrix

mesenchymal tumour which can cytologically be diagnosed is haemangiosarcoma (image not shown). The main constraint for diagnosis of haemangiosarcoma is the presence of iatrogenic blood contamination on the smear. However, one should be remembered, fine needle aspiration (FNA) of an enlarged spleen suspected with haemangiosarcoma is contraindicated.

CONCLUSIONS

Cytology is a rapid tool for diagnosis of neoplastic lesions in dog. Neoplasia is easily distinguished from inflammation based on the types of cell. Further differentiation of malignant from benign neoplastic cells was performed via examination of the criteria of malignancy. The neoplastic cells are classified into three different groups

based on the origin of the cells including round, epithelial and mesenchymal cells. Meanwhile, the specific criteria of the tumour cells, which are either round cell, epithelial or mesenchymal tumours, are described.

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Algor Mortis Pattern in Dogs, a Guide to Estimation of Time of Death

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ABSTRACT

Although differing methods of estimation of time of death in human forensics have been well documented, there exists paucity of information in the veterinary field. With little accuracy, veterinary pathologists rely on gross post-mortem changes which include autolysis, rigor mortis, livor mortis, and putrefaction in estimating time of death in animals. This study assessed the pattern of temperature drop in six mongrel dogs using commonly available thermometer. Rectal and hepatic temperatures were taken for eight to eleven hours after death at an average ambient temperature of 29 °C (24 °C to 34 °C). Both organs revealed strong regression models which were harnessed to provide a mathematical guide to estimating time of death in the early hours (six to seven hours). Linear model of temperature drop pattern change was considered less cumbersome for field use. The rates of drop were extremely irregular during the study period. This work substantiates the use of algor mortis as an adjunct in estimating time of death in dogs.

Keywords: Algor mortis, dog, hepatic, pattern, rate, rectal, time of death

INTRODUCTION

Estimation of time of death has been a widely investigated area in the human forensic field for a very long time with varying approaches and results. Thus, Mathematical equations and models have been studied by researchers to explain the dynamics of heat exchange between the body and surrounding after death. Earlier studies considered such interactions to follow the Newton's law of thermodynamics which was truer for spherical bodies than the human or animal's. Subsequent work, however, showed unique nature of human and animal bodies thermodynamic properties in life and after death which revolve around a wide range of variables such as surface conductance, wind speed and direction, as well as body weight, ambient temperature, and temperature at death. Marshall's two-exponential model was the first concrete and concise work on such relationship (Baccino *et al*., 1996) which presumes that post-mortem rectal temperature fall follows a thumb rule of 1.5 °F per hour. This formed the basis for improvement by Henssge (1988) that culminated in the development of the nomogram based on a single rectal measurement.

However, Henssge considered other variables, including body weight and varying degrees of ambience, as well as effect of wind, clothing, surface conductance, and irradiation. Though his work is currently the most widely accepted and practical of all on time of death estimation, the nomogram is still opened to questions of precision and repeated accuracy on single rectal measurements (al-Alousi *et al*., 2002) at different ambience. Inevitable flaws in

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any method proffered have pushed researchers to think of more accurate, practical, and relatively inexpensive methods of estimating time of death. The other methods employed include post-mortem biochemical assays (Myo-Thaik-Oo *et al*., 2002) and changes in proteomics such as calmodulin concentration. Leucogram histological changes (Dokgoz *et al*., 2001), nucleic acid (Haas *et al*., 2009), eye orbit and epaxial muscle temperature (Kaliszan *et al*., 2005) changes have been deeply studied over time.

Nevertheless, time of death estimation has received little attention in the veterinary field until quite recently with the emergence of veterinary forensics as a new field (Cooper and Cooper, 2008; Cooper, 2008). Information on this particular subject in animals is thereby rare, with those available being either impractical or requiring sophisticated temperature measuring instruments such as the thermocouple (Erlandsson and Munro, 2007; Kaliszan *et al*., 2005) microwave (Mall *et al*., 2005) and infrared thermometers. There is also insufficient information on the rate of temperature drop of these organs in animals. However, the overwhelming reliance on gross post-mortem changes, including autolysis, rigor mortis, livor mortis and putrefaction, for the estimation of time of death in animals is marred with gross inaccuracy. This study is designed to aid field veterinary forensic pathologists in estimating time of death in animals within early hours with a reasonable degree of accuracy using commonly available and inexpensive thermometers. The authors also intended to bring forth the pattern of temperature changes after death and this was done by assuming that there is no impact of external factors.

MATERIALS AND METHODS

Six mongrel dogs were utilized for the study under an average ambient temperature of 29 °C, after barbiturate euthanasia. Stirring thermometer (AST 5C, Malaysia), with a temperature range of -20 °C to 50 °C (error ± 0.2 °C), was used for rectal and hepatic temperature measurements every thirty minute for seven to eleven hours post-mortem. Animals were placed on the left lateral recumbency, while hepatic temperature readings were obtained following a stab wound on the mid-ventral half of the right thoraco-abdominal wall along the costo-chondral length of the last rib. Thermometers were inserted to a depth of 4 cm (rectal) and 5 cm (into the costo-chondral incision). Individual organ temperature drop pattern was plotted against time as well their mean values and rates of drop. Relevant regression analysis and equations (exponential and linear) which best fit the pattern were generated for each data acquired using the Microsoft Excel 2007 software. Verification of the formulae was done using the random necropsy cases with known time of death. Necessary correction factor, based on the findings from seven random tests, were introduced to the formulae using the mean differences in the actual and calculated times. All the protocols used in this study were approved by the institution's animal care and use committee (ACUC 08R30/July 08–Jun 09).

RESULTS AND DISCUSSION

Both the rectal and hepatic temperature drop patterns revealed strong exponential and linear regression relations (*Figs. 1-4*). In this study, peaks and short plateaus existed in all organs studied and lasted for an average of seventy minutes at periods which were closely matching the ambient temperature. It took a total of 26 ± 8 hours for the body temperature to reach ambience. The general irregularities observed in the rates of drops of all the organs studied which consisted of spikes and very short plateaus might be due to the inconsistent fluctuations in the environmental temperature (24 °C to 34 °C).

An exponential equation which best fitted the changes in the temperature of the rectum post-mortem was with a strong coefficient of correlation (R^2) value of 0.991 (*Fig. 1*); however, the difference in the strength compared to the linear pattern was very small and it hence enabled us to choose a linear model equation for estimating the time of death in dogs for its

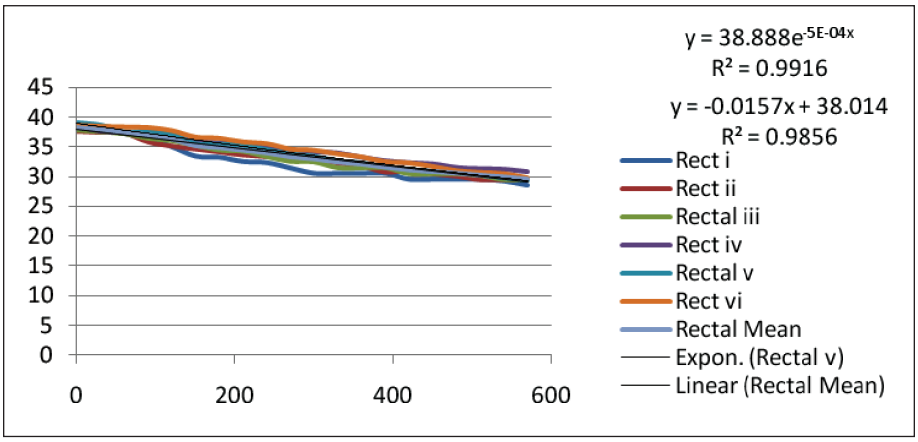

Fig. 1: Post-mortem rectal temperature pattern for six dogs at 29 ^{0}C ambient temperature over time

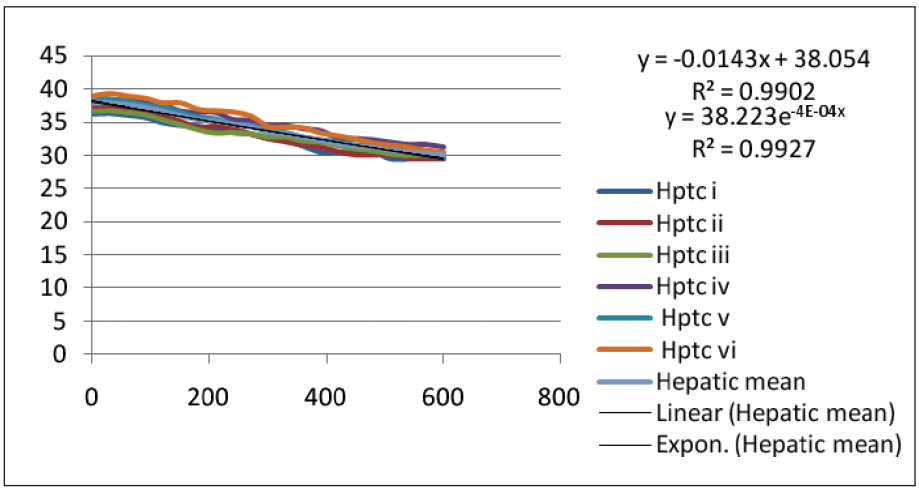

Fig. 2: Post-mortem hepatic temperature pattern for six dogs at 29 ^{o}C ambient temperature over time

mathematical and practical ease. The equations are represented as follows;

$Y = 38.18e^{-5E-0x}$ $R^2 = 0.991$
(Exponential equation)

$Y = -0.015X + 38.01$ $R^2 = 0.985$
(Linear equation)

[If Y is the measured rectal temperature R and X is time of death T], then T can be rewritten as:

$$T = 2534 - 0.015R$$

The exponential pattern of drop for the rectal temperature is similar to the findings of Erlandsson and Munro (2007) and Kaliszan (2005) in beagle dogs and pigs, respectively. However the rates of drop were inconsistent with their findings and did not follow the 1.5 °F

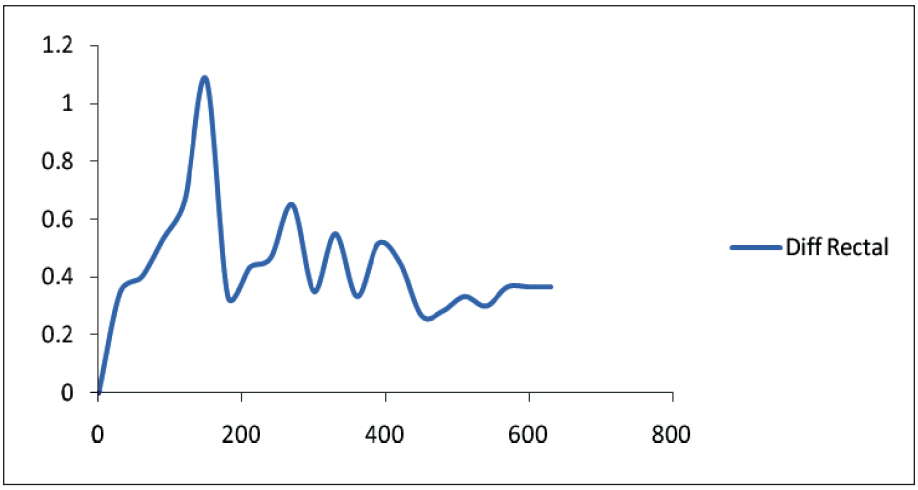

Fig. 3: Mean rate of drop of the post-mortem rectal temperature over time showing an irregular pattern and a polynomial model

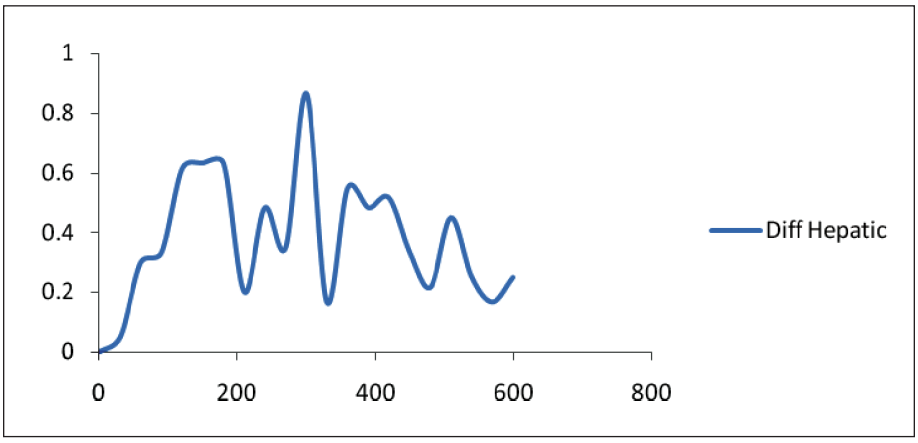

Fig. 4: Mean rate of drop of postmortem hepatic temperature over time showing an irregular pattern and a polynomial model

per hour drop rate rule of thumb. This may have resulted from the different measuring instrument types used, thus thermocouple versus stirring thermometer (our study) as well as the region where the studies were done, temperate for the former and topics for ours. It is well documented that the rate of drop in the temperate regions may be faster and relatively more consistent than those of the tropics, where a possible rise in post-mortem body temperature may be noticed, coupled with very short plateaus or log periods (al-Alousi, 2002). However the resulting equations generated from this study may prove to be more useful in estimation of time of death at the early hours (≈ 7 hours).

Hepatic temperatures appeared to be more insidious in the pattern of drop and might be helpful in cases where both rectal and external auditory canal temperatures are already at par with the ambient temperature. It expressed an

TABLE 1
Calculated time of death using linear regression equation and corrective factor of mean difference for the rectal temperature

Rectal temperature °C	Calculated time (min)	Actual time (min)	Difference (min)	Corrected time (min)
35	201	120	81	117
32.7	354	270	84	270
32.1	394	300	94	310
31.4	441	270	171	186
29.5	567	450	117	483
34.2	254	300	46	170
31.3	447	540	93	363

Mean difference = 84.1 minutes

TABLE 2
Calculated time of death using linear regression equation and corrective factor of mean difference for the hepatic temperature

Hepatic temperature °C	Calculated time (min)	Actual time (min)	Difference (min)	Corrected time (min)
34.9	225	120	105	142
33.2	347	270	77	264
32.8	375	300	105	292
33.2	347	270	77	270
30.5	539	450	89	456
34.5	253	300	47	170
31.6	461	540	79	382

Mean difference = 82.7 minutes

exponential fall over time ($R^2 = 0.992$), and a strong linear correlation coefficient which was summarized by the following equations:

$Y = 38.22e^{-4E-0x}$ $R^2 = 0.992$
(Exponential equation)

$Y = -0.014X + 38.05$ $R^2 = 0.990$
(Linear equation)

[If Y is measured hepatic temperature H and X is time of death T] then T can be rewritten as:

$$T = 2718 – 0.014H$$

The liver being a large well vascularized and highly metabolic abdominal organ may account for higher core temperature values over time and resultant insidious drop rate in comparison to the rectal and external auditory canal. Its inconsistent rate of drop, as well as technical problems of accessibility and possible crucial forensic evidence distortion rendered it less practical for field purposes, though it showed a stronger correlation with time and ability to get a reading even at points where rectal and external auditory canal temperatures are at par with the ambient temperature.

Therefore, factors such as post-mortem anaerobic metabolism, bacterial gas and heat production, hypothermia, hyperthermia, as well as pre-mortem dietary, and biochemical values might play a vital role in such observed irregularities. This is also the view of Henssge (1988) and Erlandsson and Munro (2007).

Therefore, the authors theorized that the short plateaus observed might have resulted from a balanced interaction of the heat production due to the post-mortem anaerobic metabolism and the rate of heat loss to the surrounding. In addition, this might coincide with the period where the body temperature closely matched the actual ambient temperature in the present study. The subsequent seemingly linear drop pattern which might be observed could be as a result of the fall in the ambient temperature forcing the body to cool in proportion to that fall till it attained ambience.

Verification of the formulae showed a good estimate of the time of date in the early hours, with the widest error gap observed in the rectal finding. The mean difference from all the calculated values and the actual time of death was calculated and introduced to the formulae as a negative corrective factor. This enabled a near accurate approximation of time since death calculation at 29 °C, which could be further substantiated by gross post-mortem changes, was observable at the early periods. The formulae were then re-written as follows;

$$T = 2450 – 0.015R$$

$$T = 2635 – 0.014H$$

Tables 1 and 2 show the estimated times of death using the corrected and un-corrected linear models. Its margin of accuracy is limited to early post-mortem periods (five to seven hours) for the rectal and hepatic methods. Errors from these estimations were largely due to the pulling of values towards the extremes of the mean used as the corrective factor. It is therefore advised that both the corrected and un-corrected models be utilized at a given point in time and further validated by gross findings.

CONCLUSIONS

Algor mortis rate could be used as a good index for the estimation of time of death in dogs. Both exponential and linear regression equation models showed a strong correlation between the organ temperature cooling patterns and time at an average ambient temperature of 29 °C. A linear model appeared mathematically easier for quick use in the field to calculate an estimated time since death in dogs using commonly available thermometers for the rectal and post-mortem temperatures.

These models are based on a constant ambient temperature of 29 °C and a weight range of 12 to 16 kg for logistic reasons, especially that of controlling and maintaining ambient temperatures at conceivable practical ranges. Such errors of temperature rounding and those attributable to the measuring thermometer would require further studies to rationalize.

Meanwhile, the rate of drop of body temperature post-mortem is irregular at an average ambience of 29 °C, thereby rendering its use ineffective and largely inaccurate in the estimation of time of death in dogs. The temperature drop plateau existed at intervals for a very short period lasting barely two hours in dogs. More intense studies are required to establish the use of algor mortis rate in the estimation of time of death in dogs and other species in order to standardize the use of the drop patterns and rates.

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Pathological Changes in the Lungs of Calves Following Intratracheal Exposure to *Pasteurella multocida* B:2

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ABSTRACT

Pasteurella multocida B:2 is an etiological agent of hemorrhagic septicemia in cattle and buffaloes. It is commonly fatal and considered as one of the most economically important cattle diseases in Southeast Asia. This study describes the pathological changes in the lungs of calves following intra-tracheal challenge with wild-type *P. multocida* B:2. For this purpose, six calves of 8-month old were selected and divided into two groups of 3 calves. Calves of Group 1 were challenged with intra-tracheal 5ml inoculum containing 10^9 cfu/ml of wild-type *P. multocida* B:2, while the calves of Group 2 were similarly administered with PBS. All the challenged calves of Group 1 showed slight dullness and were found to be inactive within 72 hours after inoculation, but none died. Meanwhile, their lungs showed petechiations and patches of acute pneumonia affecting few lobules. Histological examinations revealed the presence of haemorrhages into the alveoli, whereas some sections showed thickened inter-alveolar septa due to congestion and the presence of neutrophils. However, pulmonary oedema was absent. *P. multocida* B:2 was successfully isolated from all the calves of Group 1.

Keywords: Pathology, lungs, calves, *Pasteurella multocida* B:2, intra-trachea

INTRODUCTION

Pasteurella multocida B:2 is the etiological agent of hemorrhagic septicemia in cattle and buffaloes (Bain *et al.,* 1982; Verma and Jaiswal, 1998). This peracute disease is manifested by a short clinical course involving severe depression, pyrexia, submandibular edema and dyspnea, followed by recumbent and death (Graydon *et al.*, 1993). It is commonly fatal and is considered as one of the most economically important cattle diseases in Southeast Asia (Benkirane and De Alwis, 2002). Moreover, it is also endemic in most parts of the tropical Asia, particularly India and Southeast Asia leading to high mortality (Bain *et al.,* 1982; Verma and Jaiswal, 1998). One of the routes of the infection is through the respiratory tract, where *P. multocida* B:2 causes mild lesions in the lungs before they enter the blood circulation via the pulmonary capillaries to cause severe septicaemia (Zamri-Saad and Shafarin, 2007). This report describes the pathological changes in the lungs of calves, following the intra-tracheal administration of wild-type *P. multocida* B:2.

MATERIALS AND METHODS

Animals

In this experiment, six clinically healthy local calves of approximately 8 months of age were

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used. Upon arrival to the experimental house, anthelmintic (Ivomectin) was administered subcutaneously at the rate of 0.2 mg/kg body-weight for three consecutive days to control internal parasitism, which had been shown to influence disease development (Zamri-Saad *et al.,* 1994). Concurrently, nasal swabs were collected from all the calves at the time of arrival to ensure that the calves were free of *P. multocida* prior to the start of the experiment. All the calves were observed on a daily basis for the development of clinical signs of any disease and the experiment were started only when all calves were found negative from carrying *P. multocida* and clinically appeared healthy for a period of 2 weeks. The calves were fed daily with cut grasses and supplemented with pellets at the rate of 1 kg/animal/day. Drinking water was available ad libitum.

Bacterial Strain

Wild-type *P. multocida* B:2, which was isolated earlier from cattle that died during an outbreak of haemorrhagic septicaemia, was used. The organism was grown on blood agar overnight at 37 ^{o}C and kept stored at the room temperature.

Preparation of the Inoculums

To prepare the inoculums, the stock culture of wild-type *P. multocida* B:2 was selected and inoculated into 100 ml brain-heart infusion broth. These inoculums were then incubated at 37 °C for 18 h with gentle shaking before viable count of the bacterial concentration was determined using the plate count technique method proposed by Alcamo *et al.* (1997). The concentration was readjusted to give the required final concentration of x10^{9} colony-forming unit (cfu)/ml using sterile phosphate-buffered saline (PBS). These inoculums were freshly prepared for use on the experimental calves.

Experimental Procedure

At the start of the experiment, the calves were divided into two groups comprising of 3 calves per group, and each group was kept separated. The 3 calves of Group 1 were exposed intra-tracheal to 5 ml of the inoculum containing live wild-type *P. multocida* B:2, whereas the calves of Group 2 were similarly exposed to PBS. Following the exposures, all the calves were observed for clinical signs before surviving calves were killed on day 3 of post-exposure. Post-mortem examinations were carried out before the right apical lobe of the lungs were fixed in 10 % buffered formalin for histological examinations, while the lung tissue, heart swab, small intestine, and lung lavage fluid were subjected to bacterial isolation.

Bacterial Isolation

Post-mortem examinations were carried out immediately with special attention given to the changes in the respiratory tract. Following the post-mortem examination, the lungs were lavaged by introducing 1 litre of cold, sterile phosphate buffered saline into the lungs through the trachea. This was followed by gentle massaging of the lung before the fluid was re-collected into a beaker container. The lung lavage fluid was then centrifuged at 1,000 xg for 15 min to remove the debris.

Specimens from the lungs, heart blood, lung lavage fluid, and small intestine were collected for bacterial isolation. These samples were taken aseptically, cultured onto blood agar and incubated at 37 °C for 24 h. Suspected colonies of *P. multocida* B:2 were confirmed by multiplex PCR assay.

The Multiplex PCR assay was conducted using two primer sets which were designed from the sequence of the clones of Zamri-Saad *et al.* (2006); KMTI (KMT1T7-5′- ATCCGCT ATTTACCCAGTGG-3′ and KMT1SP6-5′-GCTGTAAACGAACTCGCCAC-3′) and 6b (KTT2-5′-AGGCTCGTTTGGATTATGAAG-3′ and KTSP61-5′-ATCCGCTAACACAC TCTC-3′). Briefly, 25 µl reaction mixture containing 1 x PCR buffer, 2.0 mmol/L MgCl2, 200 umol/L of each dNTP, 20 pmol of each primer, and 1 U *Taq* DNA polymerase was prepared and one colony was picked from the blood agar plate as a template and re-

suspended in the PCR mixture. The reaction mixture was subjected to amplification in a thermal cycler (Eppendorf) according to the following program: initial denaturation at 95 °C for 4 min, denaturation at 95 °C for 45s, annealing at 55 °C for 45s, extension at 72 °C for 45s, which was repeated for 30 cycles, and a final extension of 72 °C for 6 min. The amplified products were separated by agarose gel electrophoresis (1.0 %) agarose in 1 x TBE) at 70 V for 1 h 30 min and stained with ethidium bromide. The DNA band was observed under UV transillumination and photographed (Alpha Imager).

RESULTS

Clinical Observations

On the 3-day of the experimental period, the challenged calves of Group 1 appeared slightly dull and inactive. However, no other clinical signs associated with haemorrhagic septicaemia were observed. All the control calves appeared healthy and alert prior to and after infection.

Pathology Changes

While none of the control calves had gross lung lesions (*Fig. 1A*), those of Group 1 showed petechiations, particularly in the apical lobes with occasional small dark red discoloration affecting few lobules (*Fig. 1B*).

Histological examinations on the lungs of calves of Groups 1 showed mild to moderate congestion of the capillaries with evidence of hemorrhages into the alveolus (*Fig. 2A*). Neutrophils were observed within the alveoli of many lung sections, while some areas showed thickened inter-alveolar septa (*Fig. 2B*). Similar exudate was observed in some bronchi (*Fig. 2C*). On the contrary, the lungs of the control calves showed no significant changes.

Bacterial Isolation

Pasteurella multocida B:2 was successfully isolated from the lung, heart swabs, small intestine, and lung lavage fluid of the infected calves of Group 1 (*Fig. 3*). However, none of the control uninfected calves had *P. multocida* B:2.

DISCUSSION

Intra-tracheal exposure of calves to live wild-type *P. multocida* B:2 resulted in mild to moderate lesions in the lungs. They were, nevertheless, peracute lesions in the form of slight congestion with petechiations and mild pneumonia observed, following both gross and histological examinations. Similar lesions have been observed in goats following subcutaneous and intra-tracheal exposures (Zamri-Saad and Shafarin, 2007), and in calves following subcutaneous exposure to *P. multocida* B:2 (Graydon *et al.,* 1993).

Nevertheless, the severe pulmonary and subcutaneous odema observed both in calves and goats were not observed in this study. This was due to the inability of wild-type *P.*

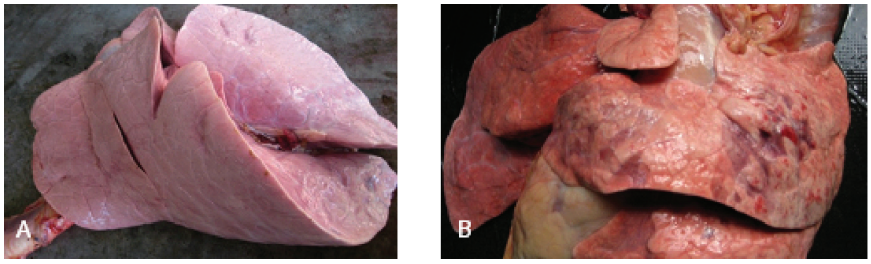

Fig. 1: Lung of a control calf (A) showing relatively normal pink colour without lesion while that of the Group 1 (B) appeared slightly congested with petechiations of the apical lobes and dark-red discolourations affecting several lobules

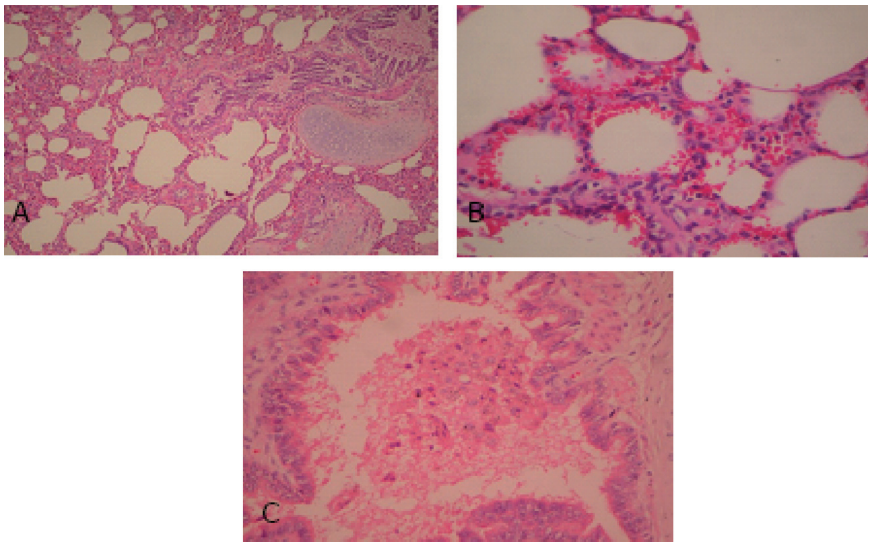

Fig. 2: Photomicrograph of lung of Group 1 calf exhibited congestion, haemorrhages (A; HE x100) and thickened interalveolar septa due to congestion and infiltration by neutrophils (B; HE x40). Photomicrograph of a bronchiole of Group 1 calf showing the presence of exudates consisting of desquamated epithelium, fibrin and few neutrophils.(C; HE x100)

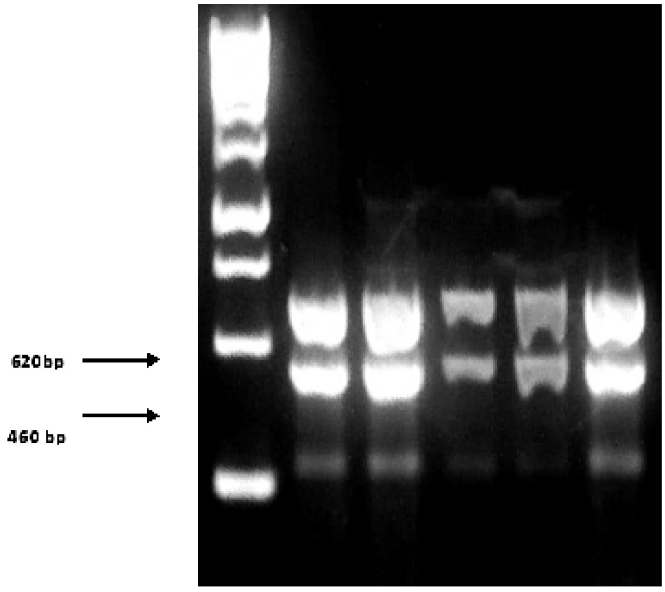

Fig. 3: Photograph of a multiplex PCR products showing bands characteristic of P. multocida *B:2. The presence of double bands in between ~460bp and ~620 bp are indicative of a positive result. Lane 1 = 1kb DNA ladder marker; Lane 2 = positive control; Lane 3 = lung sample; Lane 4 = small intestinal sample; Lane 5 = heart swab sample; Lane 6 = lung lavage fluid sample*

multocida B:2 to cause severe disease in this study. Dexamethasone administration prior to infection has been shown to cause much severe lesions (Shafarin *et al.,* 2009). Stressful condition causing immuno-suppression has been strongly associated with bacterial infection and recognized as the most important factor which can lead to outbreaks of haemorrhagic septicaemia in cattle and buffaloes (Saharee *et al*., 1993). Immunosuppression, through dexamethasone treatment, enhances the ability of *P. multocida* B:2 to prolong colonization and significantly cause more severe lung lesions. Therefore, the failure to cause stress in the calves, through the usage of dexamethasone in this study led to development of only mild and moderate lesions.

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First Case of Pulmonary Acariasis in a Pig-Tailed Macaque in Malaysia

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ABSTRACT

A 15 year-old male, southern pig-tailed macaque (*Macaca nemestrina*), was found to have a history of bloody urine, inappetance, weight loss, and was weak before death. Multiple yellow to gray nodular-like lesions were observed grossly all over the lung. Some of the nodules were flattened and some were raised and there were also cavitations and tubercles-like lesions on the cut surface. Histopathologically, focal granulomatous lesions were seen throughout the lungs, especially those with the presence of mites. There were moderate alveolar macrophages observed with mild eosinophilic, plasma cells, and slight lymphocytes and neutrophilic cells infiltration. There was also the presence of the brownish mite pigments in the lung tissue as well as in macrophages. Diagnosis was made from the morphological observation of intact mites isolated from lung tissues. This is the first report on pulmonary acariasis in Malaysia.

Keywords: Pulmonary acariasis, pig-tailed macaque, diagnosis

INTRODUCTION

Pulmonary acariasis is the most common endoparasite of the respiratory system affecting non-human or old world primates (Andrade *et al*., 2007). There were reports which indicate that pulmonary acariasis is most often found in macaques or *Macaca* genus (Hiraoka *et al*., 2000; Innes *et al.,* 1953). Although very limited number of reports have been published sporadically regarding the incidence of pulmonary acariasis, there has yet to be any case reported from Malaysia.

Pulmonary acariasis is defined as infestation of lung with mites. There are several species of mites which have been known to parasitize the respiratory system of primates. These include *Pneumonyssus simicola, Pneumonyssus duttoni, Pneumonyssus congoensis, Pneumonyssus stammeri, and Pneumonyssus dinoltiga.* Among these species, much attention was given to *Pneumonyssus simicola* as the main cause of pulmonary acariasis (Hiraoka *et al.,* 2000).

MATERIALS AND METHODS

A male, southern pig-tailed macaque, aged 15 years-old, was sent to the Faculty of Veterinary Medicine, Universiti Putra Malaysia for post mortem. Prior to death, the monkey was inappetant, losing weight, and it had haematuria and was very weak.

An examination of the carcass revealed that the monkey was in poor body condition. Grossly, there were multiple pale yellow to gray nodular-like lesions of various sizes found scattered throughout the lungs. Meanwhile, the cut surface of the nodules was found to be raised while some others were flattened indicating cavitations and suggesting tuberculous appearance.

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Portions of the affected lung samples were obtained and immediately fixed in 10% buffered formalin. Paraffin-embedded sections were then routinely stained with haematoxylin-eosin (H&E).

RESULTS AND DISCUSSION

Histopathological findings revealed the presence of mites in multifocal granulomatous lesions which consisted of numerous alveolar macrophages, mild infiltration of eosinophils, plasma cells, and lymphocytes with slight neutrophilic infiltration. The lungs showed fibrinous pleuritis, localized bronchiolitis, peribronchiolitis, focal lobular pneumonitis, as well as some degree of bronchiolectasis and slight edema. This is in agreement with the findings of some other researchers (e.g. Kim *et al.,* 1972; Hirakoa *et al.,* 2000; Andrade *et al*., 2007).

One of the unique and peculiar features of pulmonary acariasis is the consistent findings of pigments as described by Kim *et al*. (2003) and Davis (1945). In this case, there were also depositions of yellow-brown, refractile pigments, whereas many macrophages laden with these pigments were observed. It is believed that these brown pigments are the excretory products of mites which have ingested the host's blood. These findings are similar to those reported by other researchers (Davis, 1945; Innes *et al.,* 1954; Stone and Hughes, 1969; Hiraoka *et al.,* 2000; Andrade *et al*., 2007).

To further confirm the diagnosis in the present study, the fixed lung tissues were obtained and softened before the nodules were teased apart to retrieve the mites which were then fixed in 70% alcohol and mounted in Hoyer's medium. They were later identified as *Pneumonyssus spp.,* based on their morphology. As most reports have assumed that pulmonary acariasis is caused by *Pneumonyssus simicola* without properly identifying the mite prompted, there is a need to ascertain the true identity of the mite behind pulmonary acariasis. Mites are easily identified when both male and female are found and by looking at the extended chelicerae of the male which can be seen even clearer. However, Loos-Frank (1986) suggest that the identification is even difficult since females predominate in ratio of 7 to 8 to 1 male.

Infected monkeys are often asymptomatic and rarely show any clinical signs. Although some monkeys were severely infested by these mites, Innes *et al*. (1954) stated that the most common signs reported were sneezing and coughing. In addition, there was also evidence suggesting that infected monkeys in the wild showed more tolerance to the presence of these mites. However, when subjected to new environment or stressful factors, the monkeys would soon succumb to pulmonary acariasis.

Pulmonary acariasis rarely causes death but more often found as an incidental finding (Stone and Hughes, 1969) and thus makes it more challenging to carry out diagnostic tests on live monkeys. There are cases where the pulmonary acariasis is very severe and it usually occurs concurrently with other diseases or stressful factors like poor nutrition, overcrowding, effect of translocation, and old age or concurrent diseases which can lead to death. In this case, the cause of death is due to pulmonary failure, as a result of pulmonary acariasis, which is characterized by the massive inflammatory cells infiltration and reactions.

Recommended treatment and control measure for pulmonary acariasis is single ivermectin injection (200 microgram/kg). This is according to a research conducted by Joseph *et al*. (1984), whereby monkeys treated with ivermectin showed less inflammation, whereas presence of only dead and fragmented mites was observed as compared to untreated monkeys which were found to have more severe inflammation with live mites seen in the lungs.

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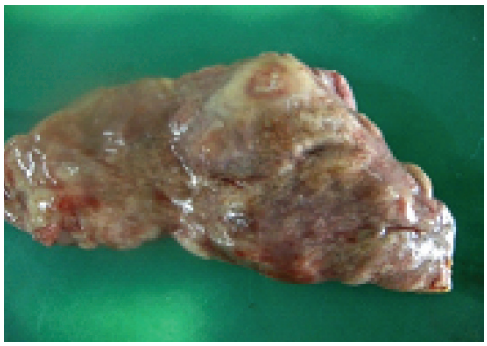

Fig. 1A: Macroscopic appearance of the lung with multiple pale yellow to gray nodular like lesions of various sizes throughout the lung

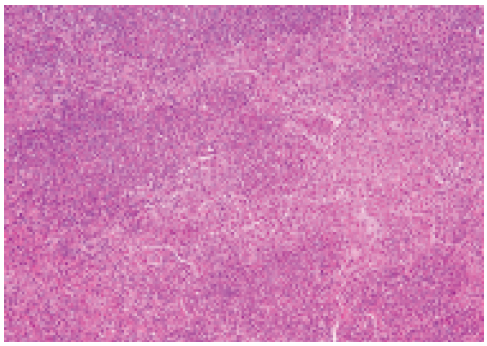

Fig. 1B: Photomicrograph of the lung indicating hypercellularity and minimal air spaces (H&E x100)

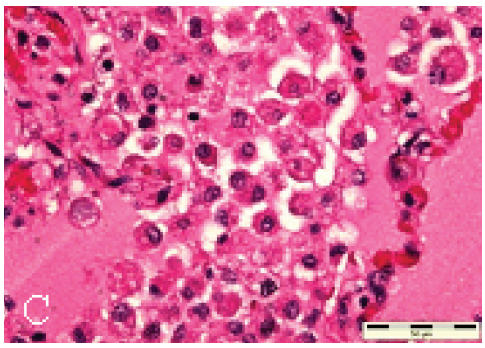

Fig. 1C: Photomicrograph of the lung with evidence of numerous macrophages, eosinophils and plasma cells in oedema fluid (H&E x400)

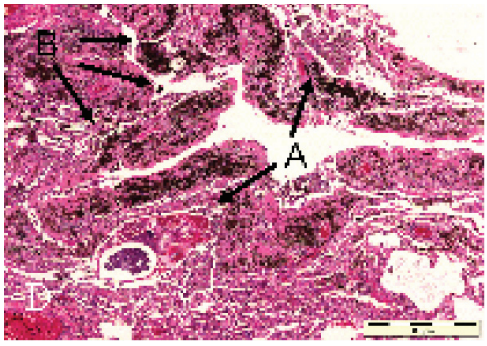

Fig. 1D: Photomicrograph of the lung showing Pneumonyssus spp. *(A) and masses of brown pigments (B) [H&E x40]*

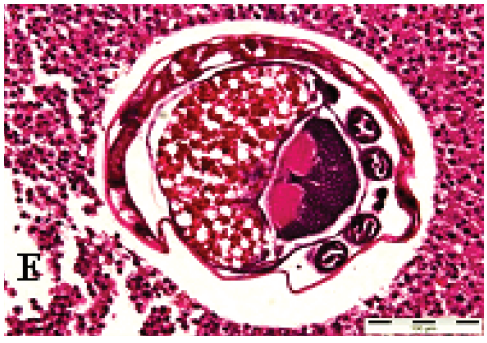

Fig. 1E: Photomicrograph of the lung showing a single mite characterized by exoskeleton, striated muscle and gut segments surrounded by inflammatory cells (H&E x200)

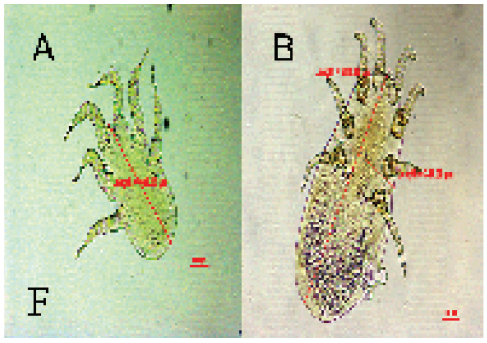

Fig. 1F: Photomicrograph of Pneumonyssus spp. *isolated from the lung and mounted in Hoyer's medium. Six-legged larva (A) and eight-legged adult (B) x 200*

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Emerging Diseases of Goats in Malaysia

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ABSTRACT

Malaysia is aggressively reviving its sluggish small ruminant industry via imports of high and good quality productive goats. However, certain diseases especially the ones which take an insidious course may be missed (rather long incubation period) during quarantine. This paper describes the first definite outbreak of caprine arthritis encephalitis (CAE) and coenuriasis in goats. The disease was confirmed via clinical signs, pathology, and virus isolation (CAE). Further corrective and preventive measures are being discussed.

Keywords: Diseases, goats, caprine arthritis encephalitis (CAE)

INTRODUCTION

The Malaysian government through the Department of Veterinary Services has tirelessly made efforts to revive the sluggish small ruminant industry. However, such impressive tasks are further challenged by diseases with long latency, showing no overt signs or characteristically generating very slow sero-converters (Gendelman *et al*., 1985). Thus, the authors wish to report two such diseases diagnosed over the last two years at the Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia. Although caprine arthritis encephalitis and cerebral coenuriosis are presented here, the researchers believe that many more emerging or re-emerging diseases may flow into the country and that everyone is geared to combat them.

Since the first reported case (Cork *et al*., 1974), the original lesions of CAE have extended beyond its name encompassing abnormalities in the udder, lungs, and kidneys. Fortunately, knowledge on its transmission has led to an effective control and possibly prevention of this particular disease (Narayan and Clemens, 1989). Lamentably, factors which contribute to the onset of clinical signs in infected animals remain unknown. Furthermore, infections with CAE virus are likely to persist in the animal despite a high antibody titter (Gendelman *et al*., 1985). All breeds, sexes and age can be affected, and owing to its non-lethality, the course can last for several weeks to months.

On the other hand, coenuriosis (gid, staggers, sturdy), a disease which is caused by metacestode (*Coenurus cerebralis*), i.e. the encysted larval stage of the cestode *Taenia multiceps*, may lead to a fatal course (Verster and Tustin, 1982). As for CAE, any breed, sex or age of goats can be affected. However, the clinical signs are dependent on the localisation of the cysts within the brain and spinal cord leading to no characteristic presenting signs (Dyson and

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Linklater, 1979). More commonly, the disease occurs sporadically in one-to two-year old animals, and more occasionally in older animals (Doherty *et al*., 1989).

MATERIALS AND METHODS

Caprine Arthritis Encephalitis: The farmer of a farm with approximately 2000 heads comprising of Kacang, Jamnapari, and Boer breeds noticed that about 20 of his animals had shown signs of recumbency and/or head tilt (*Fig. 1A*). Four of the goats were brought to UPM for a thorough examination. Out of the four, one was recumbent while the other two showed circling-like gait. In the latter, evidence of head and neck deviation was also noticed. The following day, the recumbent goat was able to be on its feet, while one of the three ataxic goats died. A post-mortem on the dead goat revealed a voluminous lung with beaded consistency without any other striking lesions elsewhere. Histologically, spinal encephalitis (*Fig. 1B*), interstitial pneumonia (*Fig. 1C*), and cord demyelination *(Fig. 1D*) were seen. Based on the microscopic changes, CAE was suspected and synovial fluid was taken from the remaining goats for virus isolation. The VERO culture yielded cytopathic effects similar to those performed by retrovirus. A year later, one of the three remaining goats showed much more severe signs, including somersault movement, and was euthanized. In addition, lesions of encephalitis, interstitial pneumonia and arthritis were also seen.

Coenuriosis (Gid, staggers, sturdy): This was observed in the four goats which were recently imported from South Africa. The only complaint was that three the goats had abnormal gait, became recumbent and later died. The remaining goat was later sacrificed prior to post-mortem which revealed an abnormally soft brain (*Fig. 2A*) and the presence of two cyst-like structures embedded within the mesenteric fat. Histologically, evidence of encephalitis and the presence of calcerous corpuscle (*Fig. 2B*) were also seen within the spinal cord.

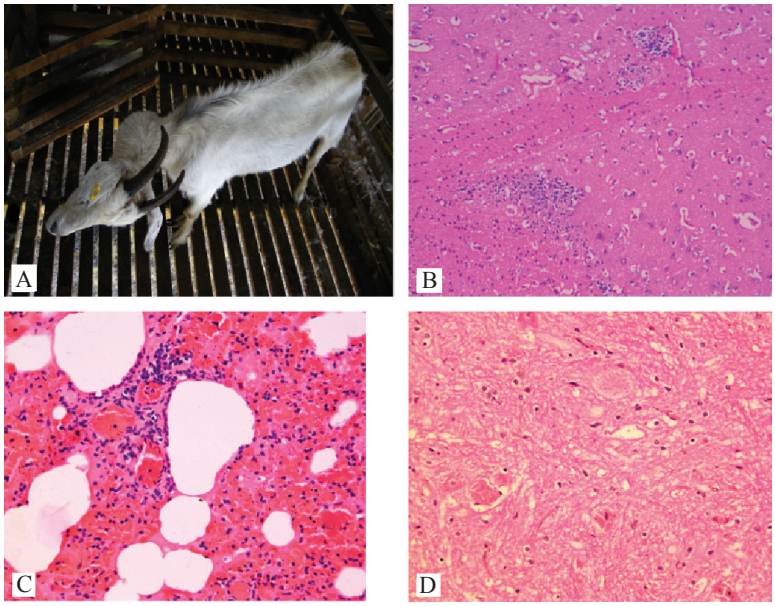

Fig. 1: (A) Severe head tilt in a severely affected buck; (B) Photomicrograph of the brain depicting hypercellularity and foci of lymphocytic aggregation (H&E X 200); (C) Photomicrograph of the lung showing interstitial pneumonia (H&E, X400); (D) Photomicrograph of the spinal cord indicating demyelination and neuronal necrosis (H&E, X400)

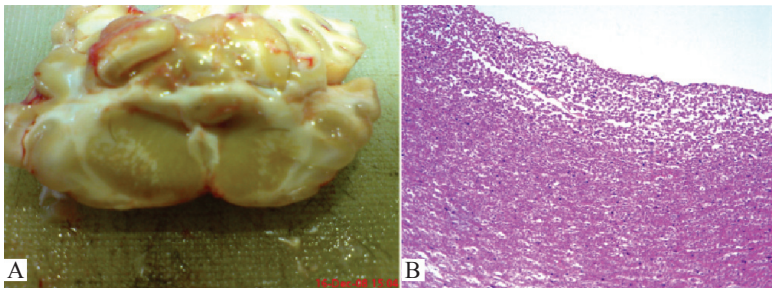

Fig. 2: (A) Photograph of the brain from an affected goat that appeared to be severely softened; (B) Photomicrograph of the spinal cord displaying the presence of calcerous corpsucles (H&E, X400)

RESULTS AND DISCUSSION

Synonymously, one would expect to see either one or both syndromes of arthritis and/or encephalitis in the CAE infection. In the cases reported here, none showed signs of unmitigated arthritis despite harbouring the virus in their joints. Nevertheless, the nature by which infection with the CAE virus escaped one or the other or both syndromes remains a mystery (Rowe and East, 1997). It should be noted that a disease-like CAE endures a tarnishing image to an exporting nation. Although Malaysia (currently) may not supply live goats, the export of *halal* mutton-originated products may suffer a trade embargo. In any way, the confirmed diagnosis of CAE, as reported here, warrants a vigil mechanism in controlling this disease. This includes infringing newly introduced and abating accumulation of new cases. The effectiveness of such programmes requires the cooperation and commitment of all the parties involved. Apart from a judicious clinical examination, various tests may also be used at the point of entry, such as AGID (Anon, 2008). Ironically, the presence of slow sero-converters may allow potential carriers to pass through the check-point "clean" and initiate a new outbreak. At the farm level, any animals showing clinical signs of CAE should be immediately culled. If culling is not economical, all sero-positive animals should be identified and fostering of newly born kids would offer an effective option. All kids should later be tested (i.e. after six months) and sero-positive individuals should be culled. Likewise, sero-positive animals should be barred from becoming a breeder.

The clinical signs of circling, ataxia, hypermetria, blindness, head tilt, head pressing, paralysis, depression or hyperaesthesia, and stargazing should lead to suspicion of coenurosis. These usually occur sporadically in young and occasionally in older animals (Doherty *et al*., 1989). In the cases presented in this study, all the four goats were older than 3 years and thus would qualify the cases as being occasional. Information on the location of the cyst, based on direction of circling (Saklia *et al*., 1987), is meaningless and controversial (Gogoi *et al*., 1994). Of great epidemiologic or clinical interest is the origin of the infection. If it was from Malaysia, identifying the incriminated dog is therefore an arduous task. Records retrieved from the farm revealed that these goats were in Malaysia for less than 2 months, kept indoors and there were no dogs within the farm. Since the symptoms manifestation of coenurosis is between 2-8 months post-infection, it is likely that the goats were harbouring the infection while they were in South Africa. Although the intradermal gid test maybe used, it should be stressed that it ceded inconsistent and doubtful results (Dyson and Linklater, 1979; Skeritt and Stallbumer, 1984). In this study, the researchers were confronted with the issues of proper disposal of infected carcass to prevent re-establishment of Taenia multiceps life cycle which might pose a zoonotic threat.

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Tissues Thiocyanate (SCN) Concentration and Liver Pathology of Sheep and Goats Fed on Cassava Forages

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ABSTRACT

Cassava leaves are good sources of protein which have a potential to substitute grain concentrate in livestock feed. However, a major constraint in using cassava fodder as animal feed is the presence of hydrogen cyanide (HCN). A study was conducted to compare the cumulative effects of thiocyanate (a product from the detoxification of hydrogen cyanide) at 4 mg and 7 mg HCN/ kg body weight on sheep and goats. Thiocyanate was sourced from the detoxification of hydrogen cyanide in cassava. The tissue thiocyanate concentrations were found to be significantly ($p<0.05$) higher in liver (2.29 µg/mL/g tissue) of goats as compared to that of sheep. Meanwhile, histological examination of the liver revealed the presence of periportal necrosis. In spite of detoxification process of hydrogen cyanide to thiocyanate, it could be concluded that at 7 mg HCN/kg body weight, considerable amount of thiocyanate was retained in the body and accumulated in the liver.

Keywords: Cassava, goats, hydrogen cyanide, liver, periportal necrosis, sheep, thiocyanate

INTRODUCTION

Cassava (*Manihot esculenta Crantz*) is one of the major tuber crops grown in more than 80 countries in the humid tropics. It is also a staple food for at least 500 million people in the tropics (Cock, 1985). Besides its important role in human diets, cassava has also been used as a feedstuff for livestock (Maner and Gomez, 1973). Research on the use of cassava, as an animal feed, has been carried out for almost a century. These studies clearly show the importance of cassava in animal nutrition. Cassava fodder has been proven to be a potential fodder source for ruminant feeding as it contains high contents of crude protein (CP), minerals, and vitamins. Moreover, the leaves of cassava have a CP content ranging from 16.7 to 39.9% dry matter (DM), with almost 85% of the CP fraction present as a true protein (Ravindran, 1991). Supplementing low quality feeds like rice straw or grass with cassava foliage has resulted in increased CP intake, digestion of fibre in the rumen (Khang and Wiktorsson, 2004), increased feed intakes, digestibility of diets and improved live weight gains (Dung *et al*., 2005). Wanapat *et al*. (1997) showed that cassava hay, fed either as

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a whole ration or as a supplement in crop-residue based diets, is a good feed for ruminant. Che (2001) reported that the ruminal degradability of cassava fodder was as high as that of grain concentrates such as maize and soy bean meal at the same outflow rates. Therefore, cassava fodder can be used as a protein supplement to substitute grain concentrates up to 50% in the ruminant diet. However, its widespread use as animal feed is limited by the presence of cyanide, which may affect both health and performance of animals.

It is well established that the toxicity of cassava is due to the release of cyanide from cyanogenic glucosides, linamarin, and lotaustralin. Fresh cassava foliage contains high levels of cyanogenic glucosides, which produce cyanide (HCN) toxin. The cyanogenic glucosides are a group of nitrile-containing plant secondary compounds which yield cyanide following their enzymatic breakdown. All cassava tissues, with the exception of its seeds, contain cyanogenic glucosides linamarin (> 90 % total cyanogen) and lotaustralin (< 10% total cyanogen) (McMahon *et al*., 1995). Meanwhile, the leaves were found to contain the highest cyanogenic glucoside level (5.0 g linamarin/kg fresh weight), whereas roots have approximately 20-fold lower linamarin levels. Cyanogenesis is initiated in cassava when the plant tissue is damaged. Ingestion of cassava can trigger several toxic manifestations due to the release of HCN from cassava cyanogenic glucosides. The incidence of acute poisoning from consumption of cassava is relatively rare since the amount ingested is often low. However, intake of cassava in the long run can lead to toxic conditions as the animal is exposed to the sub-lethal doses of cyanide for a prolonged period. The toxicity of cassava is due to the release of HCN in vivo, which is a potent cytotoxin, exerting a wide range of biological effects.

Chronic cyanide intoxication, due to consumption of sub-lethal doses of HCN, has been reported mainly in pigs and rats (Tewe and Iyayi, 1992) and dogs (Kamalu and Agharanya, 1991). Meanwhile, increased level of thiocyanate in blood was observed in gilt fed on fresh cassava diets (Tewe and Maner, 1981). Hill (1977) reported that with doses of 50 mg/kg body weight linamarin, mortality was produced in experimental rats. Moreover, pathophysiological changes have also been observed in dogs fed with "garri", i.e. a fermented cassava product. Feeding of garri caused a significant elevation of serum alanine aminotransferase (ALT) (Kamalu, 1993) and a reduction in thyroid hormone (Kamalu and Agharanya, 1991). It is important to note that a level of HCN in plant material in excess of 200 ppm is potentially dangerous to livestock. Coop and Blakely (1950) stated that the minimum lethal dose (MLD) for sheep is 2.4 mg/kg HCN body weight and the lethal dose has been estimated to be 4 to 5 mg/kg HCN body weight. In the latter case, the MLD for sheep is in an order of 7 mg/kg HCN body weight for normal eating rates. Ruminants are more susceptible to poisoning by cyanogenic plants than the non-ruminants, while sheep are said to be less susceptible than cattle if actual doses are considered. Nevertheless, reliable information on the toxicity to sheep and goats, caused by the cumulative effects of ingested cyanide, is still very limited (Nambisan, 1994). In addition, information on the comparative tolerance to the cyanogenic compound in ruminant livestock, such as sheep and goats, has not been reported. Therefore, the objective of the present study was to assess the short-term effects of feeding different levels (4 and 7 mg HCN/kg body weight) of HCN in cassava forages on sheep and goats, with the focus on thiocyanate accumulation in selected tissues and liver morphology.

MATERIALS AND METHODS

Animals Management

Nine male sheep (Dorsett Malin crossbred) and nine male of Kambing Katjang goats, aged between 10-12 months old and with an average body weight of 21.7 ± 1.07 kg, were selected in this study. Prior to the experiment, the animals were treated for ecto- and endoparasites and they were also trained to adapt to cassava leaves as a part of their diets. After two weeks

of adaptation period, the sheep and goats were allocated into their respective treatment groups. All the animals were then kept in metabolic crate with free access to drinking water during the experiment.

Preparation of the Feed Samples

Cassava fodder of MM 92 variety was freshly harvested at about 8 weeks of age from the experimental plots at Universiti Putra Malaysia, Serdang, Malaysia. They were chopped, oven dried at 70 °C for 8 hours (to retain as much cyanide as possible in the dry fodder), and pelleted for animal feeding experiment. Sub-samples of the pelleted cassava leaves were grounded up through a 2 mm screen sieve and tested for cyanide content. Hay pellets were obtained from Strategic Livestock Research Centre, MARDI, Serdang.

Feeds and Feeding

During the adaptation period, the animals were fed with hay and concentrate. Meanwhile, the animals in the control group were fed with only hay during the experiment. Those in the other two treatment groups were fed, in addition to hay, with pelleted cassava leaves at 3% DM of their body weight to the required HCN levels. The diets for the three groups were 100% hay (control), 45% cassava + 55% hay (4 mg/kg HCN), and 75% cassava + 25% hay (7 mg/kg HCN). The daily feed was given once at 9.00 a.m.

Experimental Design

Three animals of each species were randomly assigned to each treatment group, namely control (no HCN), low level HCN (4 mg HCN/kg body weight), and high level HCN (7 mg HCN/kg body weight) in a Complete Randomised Design (CRD). The experiment took 3 weeks to be completed. The animals were fed with cassava leaves containing 311.7 ppm HCN (DM basis), with each animal received zero (control), 87 mg/day (low level HCN), and 154 mg/day (high level HCN) HCN during the experiment.

Tissue Sampling

At the end of the experiment, all the animals were slaughtered and the samples of liver, thyroid, kidney, and thigh muscles (semimembranosus) were collected and stored at –20 °C for subsequent thiocyanate determination. Tissues section of liver were sliced to 3 mm and rapidly fixed in 10% buffered formalin for histological examination.

Chemical Analysis

Thiocyanate concentration in tissue samples were determined based on the colourimetric principle using the method proposed by Himwish and Saunders (1948). When ferric ions in acid solutions were added to thiocyanate, a brownish-red complex of ferric cyanate, with an absorption peak at 455 nm, was formed. This complex is a useful indicator of the amount of thiocyanate actually presents in the sample. Meanwhile, tissue samples to be assayed were homogenised and centrifuged at 5000 rpm. The clear supernatants were mixed with ferric nitrate reagent and further centrifuged before read at 455 nm using a spectrophotometer.

STATISTICAL ANALYSIS

Data of tissue thiocyanate were subjected to an analysis of variance (ANOVA), with the aid of SPSS 10 software (SPSS Inc., 1999). The full factorial analysis of variance was used to examine the effects of the treatment groups, as well as the species and their interactions. The difference between the treatment groups and species were tested according to the Duncan's Multiple Range procedure.

RESULTS

Thiocyanate Concentration

The concentrations of thiocyanate (SCN) in various organs of sheep and goats are shown in Table 1. Generally, the SCN concentrations in all the organs (except for the livers of both the treatment groups) were not significantly ($p>0.05$) different as compared to the control group. The

SCN concentration in the liver of goats HCN $_{7mg}$ group was found to be significantly ($p<0.05$) higher than the HCN $_{4mg}$ and the control groups. The same treatment (HCN $_{7mg}$) was given to the sheep, and the SCN concentration was only observed to be significantly different ($p<0.05$) from that of the control, but not with the HCN $_{4mg}$ group. Nonetheless, the SCN concentrations in the liver were not significantly ($p>0.05$) different between the species. The liver concentrations in both sheep and goats of the HCN $_{7mg}$ groups increased by about 20% and 30%, respectively, as compared to the control group.

Histopathology

The HCN $_{4mg}$ groups showed no pathological changes in relation to the control. The histological appearances of the liver in sheep and goats of the HCN $_{7mg}$ groups are presented in *Fig. 1*. Only the histological appearances of the goats are shown because of the similar degree of severity. There was demarcation of normal and necrotic cells. In some areas, necroses are centrilobular and the cells were shrunken.

DISCUSSION

Thiocyanate (SCN) is a less toxic product from the detoxification of hydrogen cyanide in the body and it can be used to determine cyanide loading related to the intake of cassava (Haque and Bradbury, 1999). The SCN concentration was significantly high in the liver of both sheep and goats which were fed with pelleted cassava leaves containing 154 mg HCN. The

TABLE 1
The mean thiocyanate (SCN) concentrations (µg/mL/g tissue) in various organs of sheep and goats in the different HCN treatment groups

Organs	Levels of HCN treatment						S.E.M	Between subjects-effects		
	Control		4 mg/kg HCN		7 mg/kg HCN					
	Sheep	Goats	Sheep	Goats	Sheep	Goats		Tr.	Sp.	Tr.*Sp
Kidney	3.83	3.33	4.51	4.27	5.00	4.34	0.24	NS	NS	NS
Liver	1.82^{c}	1.77^{c}	2.00^{b,c}	1.97^{b,c}	2.19^{a,b}	2.29^{a}	0.05	**	NS	NS
Muscle	1.90	2.18	2.81	2.67	2.69	2.01	0.20	NS	NS	NS
Thyroid	2.49	2.31	2.42	2.24	2.74	2.24	0.09	NS	NS	NS

^{a,b,c}: Means in the same row with different superscript/s differ significantly ($p<.05$)

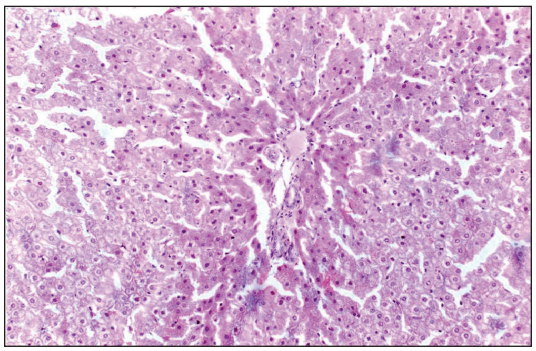

Fig. 1: Photomicrograph of the liver of goats in the HCN $_{7mg}$ group. Swelling and necrotic hepatocytes at the periportal area (arrow) [H&E, x100]

presence of high SCN content in the liver is due to the presence of high rhodanase enzyme (Westley *et al*., 1983) in the liver which is the primary site of detoxification of HCN to SCN (Oke, 1969). Although the levels of SCN in the kidneys of both the treatment groups were numerically higher, they were not significantly different as compared to the control group. Meanwhile, the low SCN content in other tissues could be explained by the fact that the SCN in the body occurred mainly in the tissue fluids such as blood, urine, saliva, gastric juice, and extracellular fluid (Smith, 1968). Thiocyanate penetrates the cellular membranes of different tissues at different rates. According to Moody (1971), the highest penetration has been found in anion concentrating tissues such as thyroid, salivary glands, gastric mucosa, and mammary glands. However, the non-significant finding of the SCN in thyroid gland in the present study was most probably due to the short experimental period.

Several pathological changes have been observed in the liver of sheep and goats, and these support the findings of the previous researchers'. Hepatic necrosis (Kamalu, 1993) was the similar lesion found in both animals of the HCN $_{7mg}$ groups. Periportal necrosis has also been referred to as peripheral lobular necrosis (Popp and Cattley, 1991). It may be evident grossly by its distinctive lobular pattern. However, it is extremely difficult to discern from the gross appearance whether the necrotic area is centrilobular or periportal. Several different reasons have been proposed for the periportal distribution of injury. The periportal area is the first area of the hepatic lobule to be exposed to a toxin being delivered via the blood stream (Huxtable, 1988). This suggests that the periportal hepatocytes may receive the largest dose of the toxin, since cells further down the sinusoid may be partly protected by removal of the toxicant in the periportal area. In some instances, the periportal distribution of hepatotoxicity is apparently due to metabolic zonation, including the greater oxygen tension in the portal than in the central lobular area of the lobule. It is important to note that a metabolic basis accounts for the distribution of centrilobular necrosis compared to periportal hepatocytes. Meanwhile, the centrilobular hepatocytes have much higher concentrations of cytochrome p-450 and associate enzymes (MacLachlan and Cullen, 1991). The distribution of the metabolising system, resulting in a higher concentration of the toxicant in the central lobular region, accounts for the occurrence of centrilobular necrosis. According to Solomonson (1978), liver cytochrome oxidase is not inhibited by cyanide, whereas brain may be the site of lethal action. This is in contrast with the finding of periportal necrosis in the liver. However, Kamalu (1993) demonstrated that the periportal vacoulation found in the liver of dogs fed garri was not due to the cyanide derived from cassava or metabolite thiocyanate. There was another factor detected in cassava, rather than cyanide, i.e. most probably linamarin (major cyanogenic glucosides in cassava) which was responsible for the lesion observed in the liver. However, the results gathered in the present study showed that the periportal necrosis found in the liver is related to the accumulation of thiocyanate in the liver of sheep and goats suggesting that the damage is probably due to presence of thiocyanate.

CONCLUSIONS

This study has shown ingestion of cyanide in cassava forages at high level HCN (7 mg HCN/ kg body weight) particularly in goats resulted in accumulation considerable amount of SCN, in which liver is the target organ for accumulation. In particular, cyanide in cassava fodder might exert a cytotoxic effect on metabolically active tissues such as liver. The effect caused cell death through the selectively poisonous actions of its metabolites cyanide (thiocyanate) which resulted in the necrosis of the liver. Therefore, it could be concluded that animals fed 7 mg HCN/kg body weight were unable to tolerate and affected pathologically.

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Verminous Bronchitis in an Ox

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ABSTRACT

Incidence of verminous bronchitis or pneumonia in Malaysia is an absolute rarity. Recently, lung samples obtained from a field post-mortem case unveiled evidence of this condition in an ox. Gross examination of the fresh unfixed chilled lungs received from Pejabat Veterinar Daerah Kerian, Perak revealed around 20-30 adult whitish worms of 3-5 cm in length and 0.1-0.2 mm in diameter in the bronchi of pneumonic areas. These worms were later identified as *Dictyocaulus viviparous*, a case which has been not diagnosed for the past few years. This finding indicated that the ox might have acquired the infection following exposure to heavy larval challenge. The use of anthelmintic may be helpful if it is given as an early prognosis for clinical condition of cough, and that dyspnoea and pyrexia are guarded. The management of grazing animals and pasture in Malaysia is very critical in preventing recurrence or occurrence of this condition.

Keywords: *Dictyocaulus viviparus*, verminous, bronchitis, ox

INTRODUCTION

Verminous bronchitis is also commonly known as bovine lungworm, parasitic bronchitis, verminous pneumonia, husk or hoose that causes differing degrees of respiratory signs within any affected group such as cough, tachypnoea, and dypsnoea. In severe cases, severe respiratory signs, with pyrexia which is usually fatal within 1 – 4 days later (Taylor *et al*., 2007), are seen. It is caused by several nematodes, but *Dictyocaulus viviparous* is the main agent in cattle and deer. This lungworm belongs to the superfamily *Trichostrongyloidea* and has a direct life cycle (Aeillo and Mays, 1998).

MATERIALS AND METHODS

On 31st March 2009, MVKBT received fresh unfixed organ samples (lungs, liver, heart, spleen and kidney) of an ox from Pejabat Veterinar Daerah Kerian, Perak. The only complaints were innapetant and paleness prior to death. The case only involved 1 animal from a population of 35 heads. No other abnormal signs were noticed and the animal died 3 days later. The cattle were reared on extensive system where animals were allowed to graze.

Gross examination was conducted on a part of the lung sample received and the samples were sent for parasitology. Unfixed lungs, liver, and kidney were sent for bacteriology, while samples fixed in 10% buffered formalin were sent to the histopathology.

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RESULTS

The lung generally showed pneumonic lesions, along with the presence of 20-30 whitish worms (3-5 cm length and 0.1-0.2 mm diameter), within the bronchi. Both the kidney and liver were congested, while the heart and spleen showed no significant gross lesions. The worm was identified as adult *Dictyocaulus viviparous* (*Figs. 1A* and *1B*).

Microscopically, bronchitis, alveolitis, and interstitial emphysema were seen in the lungs. Such cellular infiltration has led to narrowing and collapse of the bronchioles lumina and thickening of the alveolar septa. The larvae could be seen within the bronchioles (*Figs. 2A* and *2B*).

Bacteria of no clinical significance were isolated from the organs. Based on the pathology and parasitology findings, a case of verminous bronchitis due to *Dictyocaulus viviparous* was confirmed.

DISCUSSION

Verminous bronchitis can be caused by several parasitic nematodes, viz. *Dictyocaulus viviparous* in cattle and deer, *D. arnfieldi* in donkeys and horses, *D. filarial, Protostrongylus rufescens* and *Muellerius capillaries* in sheep and goats,

Fig. 1A: Adult Dictyocaulus viviparous *with short trachea (as a difference)*

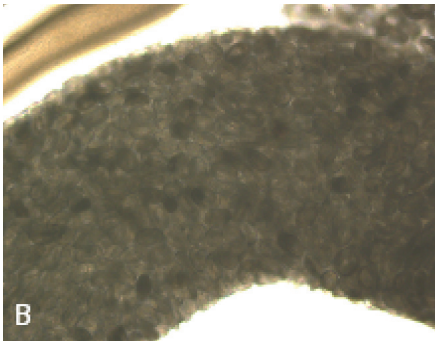

Fig. 1B: Gravid female with millions of ova containing fully developed larvae

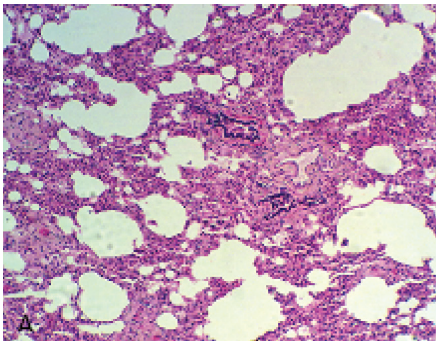

Fig. 2A: Cellular hyperinfiltration of the inflammatory cells causing bronchiolitis and collapsed of bronchioles, as well as alveolitis, thickening of the alveolar septa and interstitial emphysema

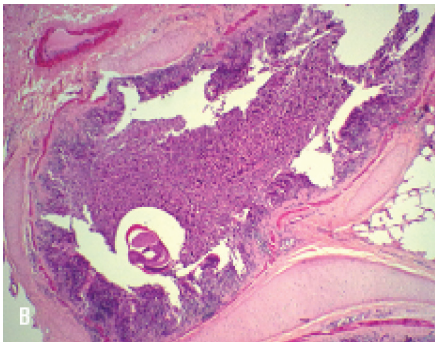

Fig. 2B: Cellular infiltrates of the inflammatory cells temporarily plug the lumina of the bronchus. Please note the cross-section of the lung worms inside the bronchus

Metastrongylus apri in pigs, *Filaroides osleri* in dogs and *Aelurostrongylus abstrusus* and *Capillaria aerophila* in cats (Taylor *et al*., 2007). Diseases caused by the *Dictyocaulus spp*. are of most economic importance since it causes severe outbreaks of parasitic bronchitis in young grazing cattle (Taylor *et al*., 2007).

The female worm is ovoviviparous and has a direct life cycle. The migration of the larvae (L1) from trachea, swallowed into the gastrointestinal tract and passed out into the faeces. It will later moult and become L3 in environment, get ingested again and penetrate the intestinal mucosa, moult in mesenteric lymph nodes and L4 travel up to lungs via lymph and blood circulation and break out the capillaries into the alveoli about 1 week after the infection. The final moult occurs in bronchioles and young adults, before moving up to the bronchi and mature. The pre-patent period is around 3-4 weeks (Aiello and Mays, 1998).

The pathogenesis of Dictyocaulosis can be divided into three phases (Taylor *et al*., 2007), namely the pre-patent phase (around day 8-25), patent phase (around day 26-60) and the post-patent phase (around day 61-90). Pathogenic effects are dependent on their locations within the respiratory tract. The degrees of clinical signs can be classified as mildly affected (intermittent cough particularly during exercise), moderately affected (frequent cough at rest, tachypnoea and hyperpnoea with frequent squeaks, and crackles upon auscultation of lungs) and severely affected (severe tachypnoea and dypsnoea, open mouth breathing with head and neck outstretched, deep harsh cough, salivation, anorexic and sometimes mild pyrexia) which maybe fatal.

Microscopically, during the pre-patent phase, larvae in alveoli will lead to alveolitis, bronchiolitis, and bronchitis. Meanwhile during the patent phase, bronchial epithelium becomes hyperplastic and heavily infiltrated by inflammatory cells, particularly eosinophills. Macrophages and multinucleated giant cells can be seen around the eggs and larvae. There may be varying degrees of interstitial emphysema and oedema. In the post-patent phase, bronchial and peribronchial fibrosis may persist for weeks to months. However, proliferation of the Type 2 pneumocytes, as a result of epithelisation causing interstitial emphysema and pulmonary oedema, can happen and this is frequently fatal and known as the post-patent parasitic bronchitis.

Diagnosis can be made based on the clinical signs, history of grazing as well as the post-mortem findings of the diagnostic presence of adult worms within the bronchi. Faecal sample yielded positive results during the patent phase. Meanwhile, serology (to detect antibodies against *D. viviparous*) can be used in the re-infection case, where seroconversion takes around 4-6 weeks and titre persists for 4-7 months.

An anthelmintic treatment (benzimidazoles, levamisoles or the avermectin/milbemycin) is helpful during early infestation period. However, one should be aware that it may exacerbate the clinical signs with a possible fatal termination in severe diseases. Prognosis is almost guarded for any calves which are dyspnoeic, anorexic, and possibly pyrexic.

The control and prevention of verminous bronchitis is best by immunizing all young calves with lungworm vaccine. However, it is only currently practiced and available in some parts of Europe. In Malaysia, the prevalence of the lungworms in cattle is not critical as compared to the cattle industry in Europe. Thus, the availability and efficacy of the vaccination programs are not yet critical and practical. Furthermore, it does not completely prevent the establishment of small number of lungworms. Therefore, controlling of pasture is very important as the transmissions of the larvae are indicated.

CONCLUSIONS

This is a case of pre-patent phase of *D. viviparous* infection as confirmed by the histopathological findings of cellular infiltration causing alveolitis, bronchiolitis and bronchitis with no obvious coughing noticed in this herd by either farmer of the VA. The mortality and morbidity were also found to be very low as the study only involved an ox. However, this might be the

beginning of an outbreak if proper measure was not taken. In addition, considerations should be emphasised on the clinical signs (cough) as well as poor/false/failure of the exact records of mortality and morbidity. This is especially so since *Dictyocaulosis* may be vague in terms of transmission and epidemiology.

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Poor Reproductive Performance Associated with Skin Injuries of the Male Lesser Mouse Deer

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ABSTRACT

Skin injury at the ventral part of the body and joints of hind limbs are common in lesser mouse deer in captivity, especially when kept in unsuitable cages. These animals showed unstable behaviour due to stress that could lead to their inability to mount during mating. The recovery was long due to the constant in-contact of the lesions to the environment. However, recovery can be aided by antibiotic and supportive therapies, which include fluid and nutritional supplementations which can substitute severe fluid and protein losses. Broad spectrum of antibiotic should be used to prevent secondary bacterial infection.

Keywords: Reproductive performance, lesser mouse deer, skin injuries

INTRODUCTION

Extinction of mammalian species is part of the natural process of evolution and is irreversible. However, it is now occurring at a much higher rate because of human activities such as habitat destruction, over-hunting or competition with introduced herbivores (Holt and Pickard, 1999).

Lesser mouse deer (*Tragulus javanicus*), weighing only 1.6 - 2 kg, belong to sub-order Ruminantia. It has been recognized as the smallest ruminant. Native to Southeast Asia, the wild population of lesser mouse-deer has sharply declined due to illegal hunting and habitat changes resulting from developments in logging, mining, shifting agriculture, and other changes in land use. *Tragulus javanicus* is listed in the red list by IUCN.

Dedicated field investigations of status are urgently warranted, and the species Red List status should be reviewed regularly in light of the current uncertainty and concerns. The magnitude of this phenomenon is illustrated by the 2006 Red List launched by the Species Survival Commission (IUCN-World Conservation Union; http://www.iucnredlist.org/). This document catalogues 1,528 animal species reported as critically threatened, including 162 species of mammals. Therefore, conservation and management of lesser mouse deer is particularly important.

The aim of animal conservation is to maintain biodiversity because removal of a single species can affect the function of global ecosystems (Myers *et al*., 2000). Habitat preservation is one of the best ways to conserve biodiversity (Loi *et al*., 2001). Global conservation can be

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achieved through *in situ* and *ex situ* conservation strategies, whereby the *in situ* conservation strategies enable live populations of animals to be maintained in their adaptive environments. However, these efforts are sometimes insufficient for the propagation of small populations or maintaining adequate genetic diversity. Hence, the *ex situ* conservation strategies have been developed, aimed at establishing a viable population through captive breeding and cryo-preservation of animal genetic resources.

One of the major problems with the implementation of *in situ* and *ex situ* conservation programmes is the lack of understanding of reproductive patterns to maximize reproductive efficiency. Lesser mouse deer can give birth once or twice a year. Puberty is reached at the age of 5-6 months while its females have 16 days estrous cycle with 35-48 hours of estrous duration (Kudo *et al.*, 1997).

Previous studies on the male lesser mouse deer revealed that sperm quality, spermatozoa morphology, and histochemical properties affected the reproductive performance (Haron *et al.,* 2000; Agungpriyono *et al.,* 2005). However, investigating the effects of skin injuries on the reproductive performance of the male lesser mouse deer is also necessary. Therefore, the objective of this report is to describe the skin injuries which have been associated with poor reproductive performance of the male lesser mouse deer.

REPRODUCTIVE PERFORMANCE OF MALE LESSER MOUSE DEER

Lesser mouse deer is believed to be one of the most primitive living ruminants (Whittow *et al.,* 1977; Haron, 2000). Male and female lesser mouse deer in captivity usually reach sexual maturity at 5 - 6 month of age (Robin, 1990). However, attempts at natural breeding of lesser mouse deer in captive are still unsuccessful. This may be due to the lack of information about the animal reproductive behaviour (Haron, 2000). The inability to mount during captivity might be one of the factors which resulted in reduced reproductive performance of the male lesser mouse deer because of poor libido, stress or physical trauma. Sexual appetite or libido is controlled by a neuro-hormonal mechanism which can be affected following any changes in the environment (Arthur *et al*., 1989).

Similarly, stress is a force of nature which externally affects the animals (Panzarino, 2008). Earlier, Selye (1956) concluded that stress is a biological term which refers to the consequences of the failure of animal's body to respond appropriately to physical threats, whether actual or imagined. It is the autonomic response to environmental stimulus which includes a state of alarm and adrenaline production, short-term resistance as a coping mechanism and exhaustion.

Lesser mouse deer is easily under stress, shy, and tend to hide. When threatened, lesser mouse deer rapidly beat their hooves on the ground at a speed of up to 7 times per second, creating a drum roll, bump to the wall of cage (Grzimck, 1994).

Observations on the behaviour of the lesser mouse deer during captivity revealed that these animals passed their time by laying down (*Fig. 1*). At day time, they lay down for a total period of more than 6 hours. Therefore, the skin over metatarsal and hock joint of the hind legs and the sternum showed traumatic injury due to pressure (*Figs. 2-3*). This injury usually takes time to recover since the injured areas are in contact with the ground. Furthermore, the position of the injury affects the ability to mount the female.

PATHOGENESIS OF THE INJURY

Trauma is defined as any body wound or shock produced by a sudden physical injury, such as from accident, injury or impact (Kumar *et al.,* 2003). Traumatic injury has been shown to stimulate an immune response manifested both at the sites of the injury and systemically. The production of both pro-inflammatory and immuno-suppressive cytokines leads to the activation of certain peripheral blood leukocytes as compared to the suppression of others, indicating an attempted differential regulation

Fig. 1: A lesser mouse deer in a laying down position during captivity

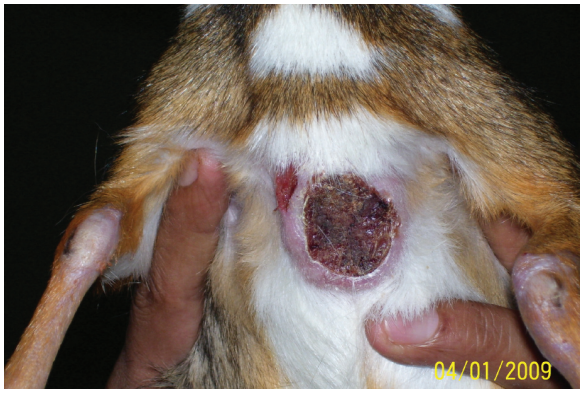

Fig. 2: Traumatic injury in the form of ulceration (arrow) on the skin of sternum

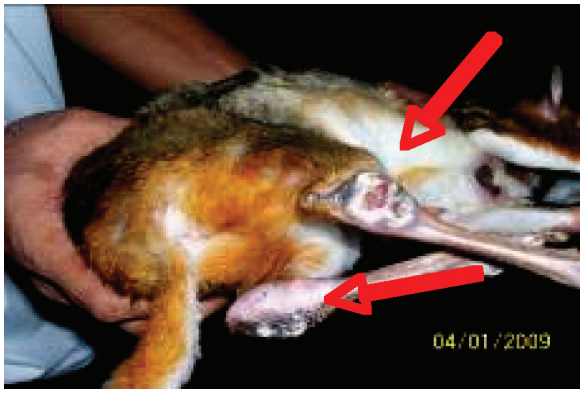

Fig. 3: Skin injury on the hind legs of a lesser mouse deer (arrow)

of immune cell subsets. Hypothetically, the immune reaction at the site of the trauma benefits recovery from injury, whereas a systemic response to self-antigens released after tissue damage could be harmful (Kumar *et al.,* 2003).

When the epidermis is injured, a complex train of events is initiated and this can lead to either regeneration or repair. Skin damage accelerates cell division in the affected part, but the mechanisms of this response are imperfectly understood. It has been suggested that when a wound hormone is released, it stimulates cell division as it diffuses into the area. Another view is that cell multiplication takes place when the normal inhibitors are removed. These inhibiting substances are called chalones. Under normal circumstances, mitosis in the skin is subjected to a circadian rhythm and it is suggested that cell division is normally held in check by the formation of adrenalin-chalone complexes. After injury, it is believed that the inhibitors diffuse away.

The epidermal cells begin to divide after 12-16 h of injury with rapid bursts of mitotic activity. The epithelium in the injured area spreads beneath the surface clot and bridges the gap in the damaged tissues. This new epithelium may considerably thicken and extend well down into the dermis. Meanwhile, an exudative reaction may occur in the area to enable cells and fluid to be brought into the damaged tissue. After several days, mitosis occurs in the connective tissue elements and some of the infiltrating macrophages change their morphology. Collagen is produced from fibroblasts and vascular elements, whereas fibroblasts and collagen proceed to fill in the gaps in the skin. The down-growths of the epithelium are removed by phagocytic activity. The wound is thus filled-in with granulation tissue covered by epithelium. In the late stages of wound healing, contraction of these new tissues occurs and this results in the formation of scar.

The injury recovery in this animal takes a long time because the skin where the injury occurs is always in-contact with the environment. Furthermore, animals with skin injury over joints show unstable and stressful behaviours which lead to the lack in mounting activity during mating.

DISCUSSION

Matsubayashi *et al*. (2003) reported that lesser mouse deer is a solitary individual with a home range that tends to be larger for males than females. The social system of lesser mouse deer is monogamy but the male lesser mouse deer also can be polygamous.

Lesser mouse deer are mainly active during the day and they rest at night. Darlis *et al*. (2001) reported that male and female lesser mouse deer spent 956 and 896 min/day respectively for laying down, 463 and 520 min/day for standing, and 21 and 24 min/day for eating. For lying down, lesser mouse deer select a suitable floor and tend to use the same place if the cage is limited. Therefore, unsuitable floor surface and urine are found to most frequently cause the skin injury on the hind legs and sternum of the lesser mouse deer. Nevertheless, the floor type in the lying area for pig, however, does not affect the proportion of pigs lying down laterally or sternally, or huddling (Salvary *et al*., 2009).

Skin is an outer part which covers the body. It is the largest organ of the integument system made up of multiple layers of epithelial tissues which guard the underlying muscles, bones, ligaments and internal organs. Following skin injury of the limbs, the superficial digital flexor tendon is frequently injured, while 8.6 % injuries are being bilateral (Dahlgren, 2007). Abrasiveness and hardness of the floor are likely to be the major causes of these lesions (Mouttotou *et al*., 1999; Mayer and Hauser, 2001). Jainudeen and Hafez (2000) revealed that the inability to mount is a common disorder encountered in older bulls and boars associated with locomotors dysfunction which arises from dislocations, fractures, sprains, and osteoarthritis lesion of the hind limbs and vertebrae. Similarly, degenerative changes in the articular surface of the stifle and hock joints and exostoses of the thoraco-lumbar vertebrae interfere with mobility and ability to mount, as observed in lesser mouse deer.

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Effects of Omental Pedicle Transposition on Regeneration of Neurotmesis Sciatic Nerve in Rabbit

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ABSTRACT

The present study was aimed at providing an understanding of the role of omental pedicle transposition in peripheral nerve regeneration by utilizing an established rabbit sciatic nerve regeneration model. Twelve adult New Zealand White rabbits (2-2.3kg) were divided into two groups (n=6) and acclimatized for 3 weeks. Complete blood examination, liver and kidney function tests were carried out during this period. In Group A, an end-to-end of sciatic nerve segment anastomosis was done, while that of Group B, the nerve anastomosis wrapped with omental pedicle was performed. The nerve specimens were collected from both groups for histopathological and ultrastructural evaluation after 16 weeks post surgery. Results showed that omental pedicle transpositioned (Group B) had more newly developed nerve fibres and less scar tissue. Ultrastructural examinations showed neuronal sprouting, whereas directions of regenerative nerve fibres were intraneural, but in the end-to-end anastomosis of group B showed that some of nerve fibres had extraneural.

Keywords: Histopathology, nerve regeneration, omental pedicle transposition, ultrastructure

INTRODUCTION

Traumatic peripheral nerve injuries are common in companion animals due to trauma, iatrogenic lesions, and surgical misadventure (Risio, 2005). There are many current conventional techniques of nerve repair such as epineural suturing, perineural suturing, perineurol nerve grafting, and free vascularized nerve grafting (Alluin *et al.*, 2008), often with disappointing results (Saunderland, 1991). Misdirection of regeneration axons is also a factor which may explain poor functional recovery (Brushart, 1991). The omentum has been used in neurosurgery since the early seventies. Successful results have been obtained with the transplant of the omentum to the brain or spinal cord in both animals and humans (Cucca *et al*., 1980; de la Torre and Mussivand, 1993; Pappas *et al.,* 1996).

The omentum has also been used in several situations of ischemia of the extremities, such as Buerger's disease and peripheral vasculopathies, due to presence of lipid fractions in the omentum promoting vascular perfusion and angiogenesis (Goldsmith *et al.,* 1984; Goldsmith *et al.,* 1986). In addition, it has been demonstrated that omental transposition promotes healing, regeneration, and neurons across a transected spinal cord in the experimental studies in cats and human (de la Torre and Goldsmith, Goldsmith and de la Torre, 1992; Goldsmith *et al.,* 2000). The need for a good method of repairing transected nerve was the basis of

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the present study. Thus, the objective of this study was to evaluate morphological changes in omental pedicle transposition technique in regenerating sciatic nerve.

MATERIALS AND METHODS

Laboratory Animals and Surgical Protocol

Twelve male adult New Zealand White rabbits (2-2.3 kg) were divided into two groups (n=6) and they were acclimatized for 3 weeks in individual cages. They were also fed with commercial rabbit pellets and given water *ad libitum*. Complete blood examination, liver and kidney function tests were performed during the period of acclimatization. Meanwhile, broad spectrum antibiotics (Pencillin Streptomycin) and antihelmintic (Ivermectin) were administered prior to the start of the experiment. The experimental procedures were performed as approved by the Faculty's animal care and use committee (08 R13/Dec08-Nov-09). Induction was done by an intramuscular injection of ketamine (Bioketan, Vetquinol), xylazine (Ilium Xylazil. 100) and acepromazine (Calmivet, Vetoquinol) and maintained by on halothane (Isoflurane).

Group A

A 6-8 cm in length caudo-lateral skin incision was made parallel to and 2 cm caudal to the left femur bone. The fascia latae was incised and the biceps femoral muscle was separated from the semitendinosus by blunt dissection. The left sciatic nerve was separated from its surrounding tissue using a pair of ophthalmic scissors and a jeweller's forceps. The nerve was transected using a surgical blade #15.

Nerve Anastomosis

With the aid of a magnifying glass (X3), both ends of the nerve were immediately sutured after transection. An end-to-end anastomosis pattern, using 8-0 nylon suture with simple interrupted suture, was placed in the epineurium and perineurium.

Group B

A similar procedure was performed as in Group A, but the sciatic nerve was wrapped in omental pedicle transposition after an end-to-end anastomosis. The omentum was detached from the transverse colon, and the omental pedicle transposition was done through the abdominal wall muscle by blunt dissection using a pair of artery forceps. The extended omental pedicle was held and pulled through the tunnel between the semi-membranous and adductor muscles. The omental pedicle was then wrapped around the anastomosed sciatic nerve, and fixed to the muscles using two sutures. The two skin incisions were closed in a routine manner with 3-0 Vicryl using the sub-cuticular pattern.

All the animals in both groups were euthanized at 16 weeks post operation by intracardiac injection of pentobarbitone (Dolethal). The anastomosed left sciatic nerve was exposed, examined grossly, and then harvested for histopathological and ultrastructural studies. Three specimens of 1cm in length were collected from the proximal, middle (anastomosis site), and distal segments of the co-opted sciatic nerve. Each specimen was divided into two parts, and these were then fixed and processed in the routine manner for histopathology and electron microscopy, respectively.

RESULTS

Histology

Group A

The longitudinal sections of the proximal nerve stumps demonstrated normal arrangement of the nerve fibres with high concentration of Schwann cells. Occasionally vacuolated, degenerated nerve fibres, and scanty of fibrous tissue were seen perineurally (*Fig. 1a*). The middle portion of the sutured line segment revealed a normal parallel arrangement of the newly regenerated nerve fibres with several vacuolations and degenerated fibrous tissues in the perineural region close to the sutured site (*Fig. 1b*). The distal nerve stump demonstrated parallel distribution of the new nerve fibres. There were

few Schwann cells with several vacuolations, degenerated nerve fibres, and slight fibrosis perineurally (*Fig. 1c*).

Group B

The longitudinal sections of the proximal nerve stumps showed an increase in the number of Schwann cells, and the nerve fibres resembled the normal parallel pattern arrangement of the nerve fibre with normal stain affinity (*Fig. 2a*). The mid portion section revealed an increased number of nerve fibres and Schwann cells, and a slight peripheral fibrosis. (*Fig. 2b*). The section of distal nerve stumps demonstrated occasional vacuolation and fibres degeneration with moderate increased in the number of nerve fibres and Schwann cells (*Fig. 2c*). The cross-section of distal nerve stumps demonstrated a high vascularization close to the omental pedicle (*Fig. 2d*).

Ultrastructure

Degenerated nerve fibres with extra-neural and atrophied band of Bungner bands were observed in the distal segment of nerve in Group A (*Fig. 3a*). Meanwhile in Group B, the Schwann tube structures or basement membrane re-formation and re-distribution (*Fig. 3b*), active Schwann cells and thick myelinated nerve fibres (*Fig. 3c*) were seen.

DISCUSSION

This study demonstrated that the omental pedicle promoted and improved the rate of functional recovery within a short period of time (16 weeks), as compared to the end-to-end anastomosis. In addition, it was also observed that the omental pedicle enhanced the onset and acceleration of nerve fibres regeneration. The histological examinations showed a plenty of nerve fibres regeneration in the omental pedicle group. Misdirected axonal growth at the repair site might have occurred, leading to poor functional outcome post-operation; this might not necessarily imply total recovery of the nerve function (Sobeski *et al*., 2001; Meck *et al*., 1999). The histological findings, such as nerve fibre density, myelin sheath thickness, number of Schwann cells, and supportive tissues

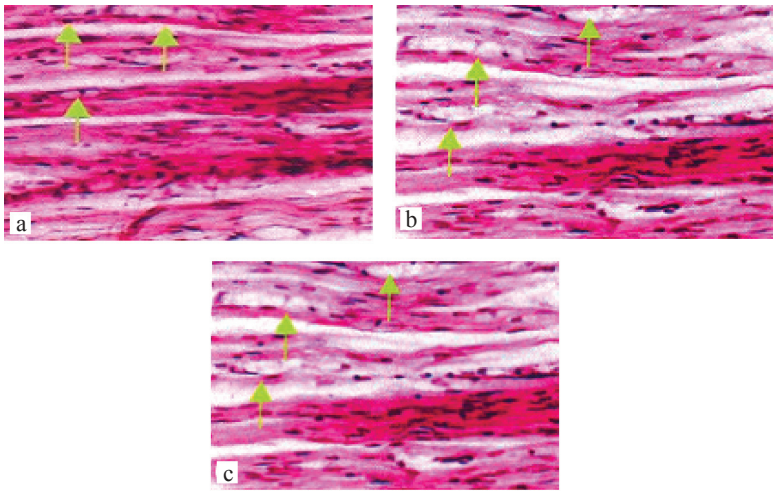

Fig. 1: Photomicrograph of the sciatic nerve in Group A (H&E, X100). (a) Note the proximal part with degenerative nerve fibres (arrows) and poor fibrous tissue perineurally; (b) The middle part contained several degenerated nerve fibres (arrows) and increased number of Schwann cells; (c) The distal part with several degenerated nerve fibres (arrows) and focal concentration of Schwann cells

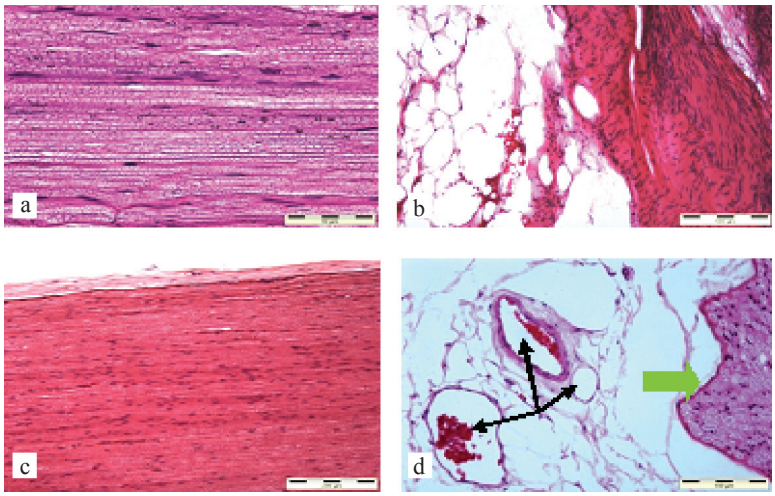

Fig. 2: Photomicrograph of the sciatic nerve in Group B (H&E). (a) No significant pathological changes were seen except for the slight increase in the number of Schwann cells; (b) The middle part showing good axonal alignment, no intra-neural scarring and an increase in the number of nerve fibre and Schwann cells. (c) The distal part has an increase number of Schwann cells. The sciatic nerve showed vascularisation (arrows) of omentum around the anastomosed site (thick arrow) H&E

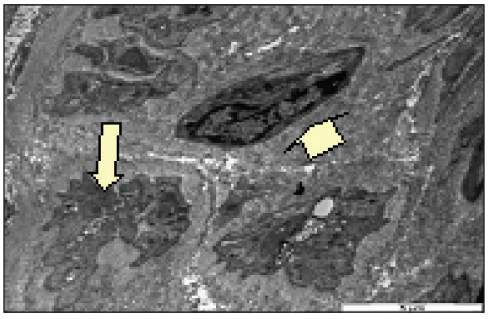

Fig. 3a: Electrograph of ultrastructural of rabbits' sciatic nerve in Group A showing atrophied band of Bungner in distal segment (arrows) and degenerative nerve fibre (arrow heads), Uranyl acetate and lead stain

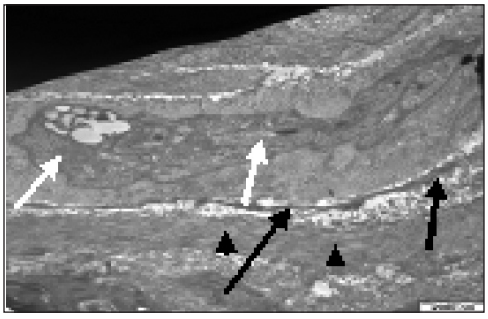

Fig. 3b: Electromicrograph of sciatic nerve treated with omental transposition showing Schwann cells in endoneural tube (white arrows), basement membrane (black arrows) and collagen fibres (arrow heads), Uranyl acetate and lead stain

like blood vessel and fibrous sheath, showed that they played different roles in promoting nerve regeneration. One possible explanation for the improvement in the function of regenerative nerve fibres is that it easily grows throughout the omentum due to lack of intra-neuronal fibrosis (scar formation) and the ability of the omentum to stimulate vascularization (Chamorro *et al*., 1993).

Moreover, omental pedicle also has several advantages for the treatment of transected peripheral nerves as an autogenous transposition,

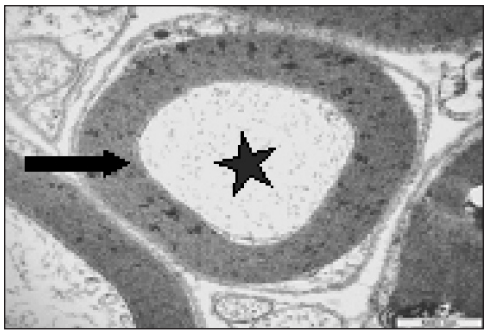

Fig.3c: Photograph of ultrastructural of rabbit sciatic nerve treated with omental transposition showing a good myelin axon (asterisk) with Schwann cells, Axon surrounded by myelin sheath (arrows), Uranyl acetate and lead stain

and does not cause any intra-abdominal defect. It can be extended through minor laparoscopic intervention without complication (Domene *et al*., 1998; Kamei *et al*., 1998) and no injury occurs at the donor site, compared to nerve transplantation.

Ultrastructural examinations showed that the group of omental pedicle demonstrated developed Schwann tube or basement membrane which is very important as a guide for axonal outgrowth due to the presence of extra-cellular matrices such as a collagen, fibronectin, lamanine, and integrine, in addition to active Schwann cells and thick myelinated nerve fibres (Dahluin, 2008). Another study reported that the mechanism of omental pedicle might promote healing when applied to the injured site, because of the rich blood vessel density, blood content, growth and angiogenetic factors, vascular endothelial growth factor (VEGF), chemotactic factors (stromal-cell-derived factor SDF-1α), progenitor cells (WT-1, CXCR4) to clear tissue debris and clotted blood, assuring a fresh blood supply, and thus preventing tissue death by ischemia. Moreover, the re-vascularized injured tissue is thus supplied with a potent mixture of growth factors and progenitor cells which further helps in the recruitment of progenitor cells from the bone marrow and local tissue to accelerate tissue repair (Liberg *et al*., 2007).

In conclusion, omental pedicle promotes healing when it is applied to injured sites because of the rich blood density, blood content, as well as growth and angiogenic factors. The ultrastructural examinations demonstrated developed Schwann tube and thick myelinated nerve fibres which are very important as guides for axonal outgrowth.

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The Role of Oxidative Stress in *Brachiaria decumbens* Toxicity in Sheep

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ABSTRACT

In an attempt to elucidate potential time-dependent oxidative stress mechanisms associated with *Brachiaria decumbens* toxicity in sheep, selected blood malondialdehyde (MD), as peroxidation tissue function biomarker and tissue morphology, were assessed. Six young adult Wiltshire cross bred ram were acclimatized for 3 weeks, where ivermectin injection and liver and kidney function tests were done. Blood samples were collected during the pre-treatment, namely on 3rd, 5th, 7th, 9th, and 11th days of continuous feeding of *Brachiaria.* Meanwhile, clinical signs were monitored and sheep were euthanised on the 7th, 9th, and 11th day, where a post-mortem was performed and relevant tissues were sampled. Results revealed that plasma thiobarbituric acid reactive substances (TBARS), serum (bilirubin total and conjugated, AST, GGT, urea, and creatinine) were significantly ($p<0.05$) increased, whereas total antioxidant potential was decreased ($p<0.05$) in a time dependant manner. Continuous *B. decumbens* feeding induced a time dependent appearance of jaundice, photosensitization, and subcutaneous oedema. Unique intracytoplasmic accumulation of Schmorl's positive greenish lipofuscin granules were observed primarily within the centrilobular hepatocytes and Kupffer cells. This may serve as a histochemical oxidative biomarker in the liver, kidney, brain, and skin. Taken together, this study shows that oxidative stress plays a major role in *B. decumbens* toxicity.

Keywords: *Brachiaria decumbens*, lipid peroxidation, oxidative stress, sheep

INTRODUCTION

Hepatogenous photosensitization (HPS) of sheep, associated with grazing plants containing steroidal saponins, is both economically important and an animal welfare problem (Flaoyen, 1996). *Brachiaria decumbens* (signal grass) intoxication, a disease similar to HPS in small ruminant, has been reported globally (Noordin, 1988; Amaral-de-lemos *et al.,* 1996; Brum *et al.,* 2007). Grasses are high yielding, stoloniferous, and very well adapted to tropical climatic conditions (Loch, 1977) and much preferred by sheep and goats. It has been postulated that *B. decumben*, *per se,* is not toxic but it is converted into hepatotoxic compounds in the rumen of sheep and goats (Noordin, 1988). It was found that diosgenin and its metabolites in *B. decumbens* are hepatotoxic and nephrotoxic (Zhang, 2000).

Oxidative stress is otherwise referred to as "oxidoreductive stress" is an imbalance between the production of oxidizing molecular species (pro-oxidants) and the presence of cellular antioxidants in favour of the pro-oxidants leading to potential damage. Lipid peroxidation is a well-established mechanism of cellular injury and it has been used as an indicator of oxidative stress in cell and tissues. In particular, lipid peroxides

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derived from polyunsaturated fatty acids are unstable and can decompose to form a complex series of compounds. These include reactive carbonyl compounds of which MDA is the most abundant. Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with several models of liver injury (Panozzo *et al.*, 1995; Sergent *et al*., 1995). There are considerable pathologic evidences which indicate the involvement of intrahepatic oxidative stress and subsequently lipid peroxidation in the pathogenesis of this intoxication. There is a dearth of knowledge on the development of pro-oxidant and anti-oxidant status during continuous feeding of *B. decumbens*. Therefore, the objective of this study was to explore the role of oxidative stress in *B. decumbens* toxicity in sheep by measuring or assessing the oxidative parameters and pathological changes in the liver, kidneys, brain, and skin.

MATERIALS AND METHODS

The experimental protocols and ethics were approved by the Animal Care and Use Committee (UPM/FPV/3.2.1.551/AUP-R42). Six clinically healthy young adult (12-14 months) cross-bred Malin rams (20-22kg) were purchased from a local commercial farm in Malaysia. The acquired sheep were kept for an acclimatization period of three weeks, during which they fed on concentrate and non *B. decumbens* fodder with *ad libitum* provision of drinking water. The sheep were dewormed with Ivermectin and both the liver and kidney functions were assessed. Two groups of three animals each were used as a control and *B. decumbens* fed sheep. The treated group was continuously fed with only *B. decumbens* grass (only the green leafy and stem portions), whereas the control was given non *B. decumbens* fodder. Daily monitoring of the development of clinical signs was done while blood samples were collected on day 0, 3, 5, 7, 9, and 11 post feeding. Assays of serum aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT), serum conjugated and total bilirubin, blood urea nitrogen (BUN), and creatinine were done using diagnostic kits (Roche Diagnostic).

The end product of peroxidative decomposition MDA of polyenic fatty acids in the lipid peroxidation process was measured in the serum using the double heating method as described by Placer *et al*. (1966). The SOD activity was measured by pyrogallol oxidation inhibition assay of Marklund and Marklund (1974) using UV/visible spectrophotometer at 330 nm, whereas the activity of GSH-Px in erythrocytes was measured using the DTNB direct method at 422 nm.

Sheep were euthanised on the 7th, 9th, and 11th days (one from each group) and post-mortem was also performed. Samples of the liver, kidney, brain, and skin were collected at necropsy and fixed in 10% neutral buffered formalin, and this was followed by embedding in paraffin wax, sectioning at 5µm and staining with haematoxylin and eosin (H&E). Sections of liver and kidneys were also stained with Schmorl's reaction for lipofuscin, while some sections from photosensitized skin were stained with Masson trichrome for collagen. Data were stated as mean ± standard deviation (SD) and subjected to statistical analysis using the SPSS software package (version 11.0 for windows).

RESULTS AND DISCUSSION

While animals in the control group showed no clinical and pathological changes, variations in the sequences of appearance and severity of clinical signs were observed in the treated group during multiple periods of this study. Signs of photosensitization, which included facial and submandibular oedema and jaundice, were observed as early as the 7th day of post feeding and this conformed to earlier findings (Abas *et al*., 1983). The prominence of jaundice in this study could be due to intra-hepatic cholestasis as indicated by the marked elevation in GGT and unconjugated hyperbilirubinemia. The submandibular, facial, and subcutaneous oedema

was likely to be the result of two factors, namely hypoalbuminaemia (i.e. due to hepatic damage which is strongly related to the severe elevation in AST) and systemic hypertension and congestion (i.e. well-related to the prominence of macula densa of the distal convoluted tubules in the kidney, as shown in *Fig. 1A*). During the late stage of *B. decumbens* sheep toxicity, there were consistent developments of nervous signs such as stamping of forelimbs, head shaking, ataxia, circling movement, and reverse locomotion. These clinical signs were confirmed histopathologically by the presence of status spongiosus in the cerebellum (*Fig. 1B*), a lesion which may have developed as a consequence of hepatic encephalopathy induced by hyperammonianaemia resulting from hepatic failure (Salam *et al.,* 1989).

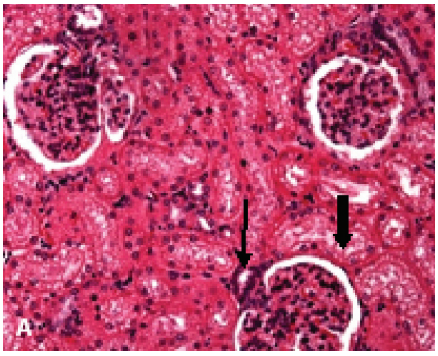

Fig. 1A: Photomicrograph of the kidney of an intoxicated sheep at seven days of post-feeding showing prominence of macula densa of the distal convoluted tubules (thin arrow) with associated glomerular congestion. The renal tubules appear either necrotic or degenerated (thick arrow) [H&E]

Liver, being the target organ of *B. decumbens* toxicity, appeared swollen, bronze-yellow with accentuated lobular pattern, distended gall bladder, greyish pinpoint necrotic areas, and slight thickening of bile duct. Histopathologically, mild bile duct hyperplasia and centrilobular hepatocytes degeneration and necrosis (*Fig. 1C*). The centrilobular region in the liver appeared to be the most vulnerable area for lipid peroxidation as indicated by the gradual aggregations of greenish blue granules of lipofuscin. This may serve as a vital histochemical biomarker of oxidoreductive stress during time dependent *B. decumbens* toxicity. Two criteria render the centrilobular hepatocytes more susceptible to the oxidoreductive stress, namely the minimal oxygen supply and the normally high concentration of detoxifying enzyme content

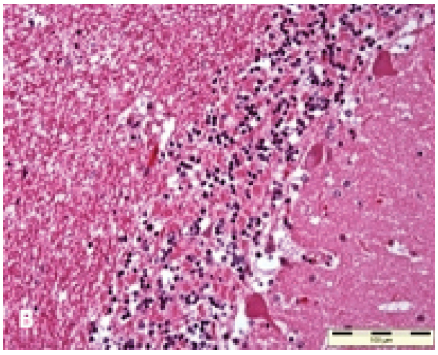

Fig. 1B: Photomicrograph of the cerebellum of an intoxicated sheep at eleven days post-feeding showing vacuolation of the white matter of the cerebellar folium (thick arrow). In the grey matter, the Purkinje cell appears decreased in size and number (thin arrows) [H&E]

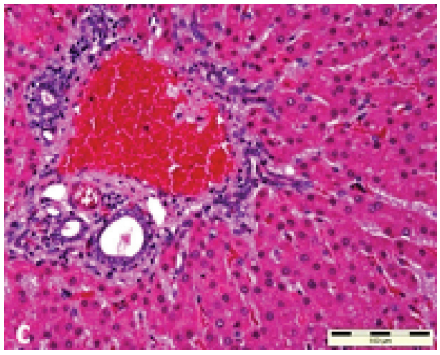

Fig. 1C: Photomicrograph of the liver of an intoxicated sheep, nine days post-feeding showing bile duct hyperplasia (thin arrows), periductal lymphocytic infiltration and severe congestion of the portal vein, hepatic artery and sinusoidal spaces [H&E]

(mixed function oxidase and P-450) (Cheville, 1983). Biochemical and morphological findings were found to be correlated well with evidence of oxidative stress. There is a significant and strong relationship (P>0.001) between the values of AST and that of MDA (*Fig. 2*), and this reflects the role of oxidoreductive stress in the development of hepatotoxic damage in a time dependant manner. These findings quite agree with that of Zhang (2000) who described the AST as a better indicator of the liver function than GGT in cases of *B. decumbens* toxicity in sheep.

As a part of systemic oxidative stress phenomenon, necrotizing dermatitis (*Fig. 1D*) observed in this study may be a consequence of interaction of photoactivated molecules (phylloerythrin) with oxygen, where singlet oxygen or oxygen radicals are generated and leading to disruptions of protein and DNA synthesis, mitochondrial damage, lysosomal damage, and cell death (Allen and Balin, 1989).

The almost parallel increase and decrease of MDA and GSH-Px activities respectively over time indicate a graduation of the severity of tissue damage which is induced by oxidative stress in *B. decumbens* intoxication. Furthermore, this is supported by the variable degree of lipofuscinosis in the liver of the affected sheep (*Fig. 1E*). Likewise, the change in GSH-Px activities (*Fig. 3*) during *B. decumbens* intoxication seems to be important and reflects the glutathione depletion leading to disruption in redox balance. This acts in the normal state to prevent or repair the oxidation of lipids and keep the cellular thiol redox status of liver in the reduced form. Low activity of this enzyme is one of the early consequences of a disturbance of the prooxidant/ antioxidant balance in favour of the former during *B. decumbens* toxicity in this study. During multiple periods of this experiment, insignificant fluctuation in the erythrocyte SOD activites was the overwhelming criterion and it is believed that SOD plays a minimal or negligible role in this intoxication (*Fig. 4*).

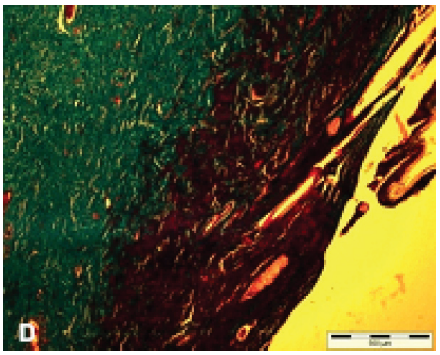

Fig. 1D: Photomicrograph of the skin of an intoxicated sheep at seven days of post-feeding showing red masses of necrotized collagenous tissue which was negatively (purplish) stained while viable parts appeared greenish [Masson trichrome]

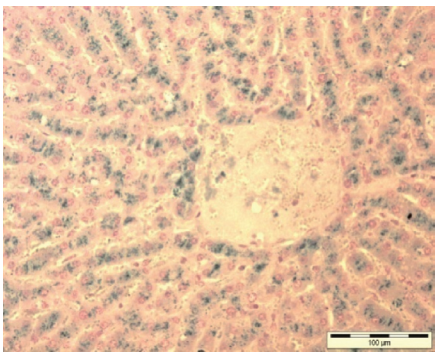

Fig. 1E: Photomicrograph of liver of an intoxicated sheep at eleven days post-feeding showing intra-cytoplasmic dark greenish lipofuscin granules within exhausted hepatocytes, especially in the centrilobular zone [Schmorl]

CONCLUSIONS

These data revealed that the experimental *B. decumbens* sheep toxicity is associated with increased lipid peroxidation in a time dependant manner. We concluded that the oxidative stress in this toxicity is a systemic phenomenon which probably encompasses other tissues and organs.

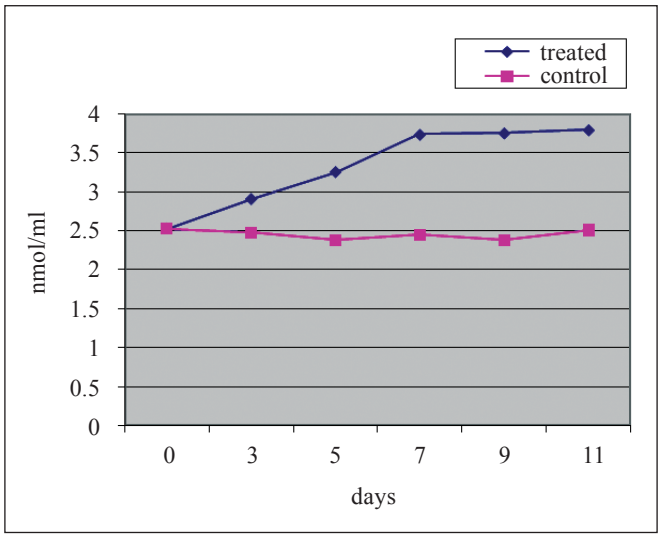

Fig. 2:. Malondialdehyde levels in the serum of sheep during the experimental period

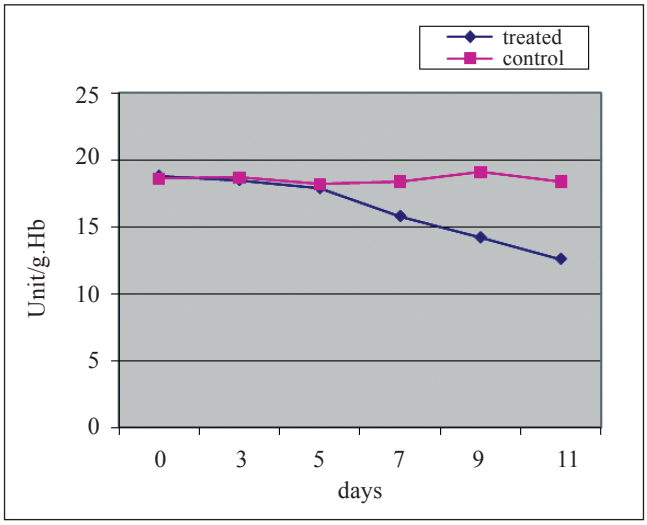

Fig. 3: The activity of erythrocytic GSH-Px in sheep during the experimental period

More importantly, diosgenin the toxic principle of *B. decumbens* can cause not only oxidoreductive stress and subsequently cell injury due to the production of strong intermediate free radicals, but it may also involved in signal transduction and the regulation of gene expression via redox-sensitive mechanisms.

ACKNOWLEDGEMENTS

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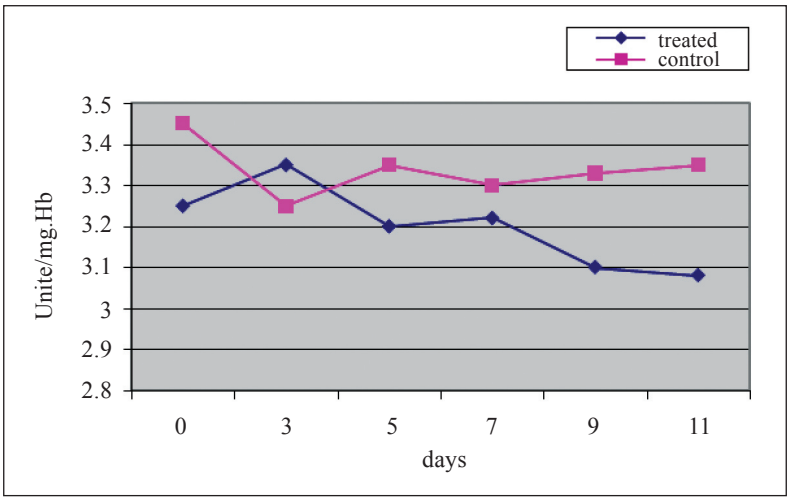

Fig. 4:. Dynamics of erythrocyte superoxide dismutase of sheep during the study period

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     - Tan, S.G., Omar, M.Y., Mahani, K.W., Rahani, M., Selvaraj, O.S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics*, *32,* 415 - 422.
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Selected Articles from the 1st Malaysian Veterinary Pathology Conference 2009

ERRATUM

Erratum for *Pertanika J. Trop. Agric. Sci.* Contents page.

Journal of Tropical Agricultural Science, Vol. 32(2) Aug. 2009: pp. 109-110.

Guest Editor & Guest Editorial Board members

The August 2009 issue of Vol. 32(2), Table of Contents pages **contained errors.** The names of the **Guest Editor** and the **Guest Editorial Board members** were omitted unintentionally.

The names of the Guest Editors and Guest Editorial Board members should be added under the following categories of articles in the corrected version of the Table of Contents.

Selected Articles from the 7th National Genetics Congress 2007
Guest Editorial Board: Wickneswari Ratnam, Tan Soon Guan, Subha Bhassu and Zarina Abd. Latif

Selected Articles from the 3rd Biology Colloquium 2007
Guest Editor: Shamarina Shohaimi

Selected Articles from the UPM Rice Research Colloquium 2008
Guest Editorial Board: Sariah Meon, Mohd. Razi Ismail, Maziah Mahmood, Abdul Shukor Juraimi, Radziah Othman and Halimi Mohd Saud

The Publisher apologizes for the above errors.

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