UNIVERSITI PUTRA MALAYSIA

A STUDY ON THE MICROFILARIAL PERIODICITY OF DIROFILARIA IMMITIS IN NATURALLY INFECTED DOGS

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A STUDY ON THE MICROFILARIAL PERIODICITY OF *Dirofilaria immitis* IN NATURALLY INFECTED DOGS

by

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A paper submitted
in partial fulfilment of the requirements
for the degree of Doctor of Veterinary Medicine (DVM)
at the Faculty of Veterinary Medicine and Animal Science,
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January 1986

To my parents

for their love, patience and understanding

ACKNOWLEDGEMENT

I would like to express my sincere thanks to my supervisor, Dr. Rashid bin Ibrahim for his guidance, assistance and encouragement throughout the course of this project.

A special note of thanks is also due to Dr. George Abraham of the S.P.C.A., Kuala Lumpur for his kind cooperation without which this study would not have been possible. Thanks is also due to Dr. Rehana binti Abdullah Sani and Mr. Lee Chu Chong for sharing their time and knowledge related to this project.

Finally, my gratitude also goes to all my friends who have helped in any possible ways during the course of this project.

ABSTRACT

Four dogs positive for the microfilariae of Dirofilaria immitis were used for this study. Cutaneous and venous blood was collected at 3 hourly intervals for 24 hours. The periodicity indices of microfilarial periodicity in the cutaneous blood of 46.2 ± 9.0 was relatively higher than that of the venous blood of 31.4 ± 14.4. Thus, it can be classified as subperiodic. The peak hours of microfilariaemia centered around 16:24 ± 00:24 hours and 21:30 ± 01:54 hours. The variability of the peak hours does not justify the classification of the microfilarial periodicity into diurnal or nocturnal.

There was higher microfilarial density in the venous blood than the cutaneous blood. For the enumeration of microfilariae, the micropipette method was found to be more accurate as compared to the Knott dilution method. Significant correlation between the observed and expected microfilarial periodicity was observed in fifteen out of seventeen cases with correlation of determination values, r^2 ranging from 51.4% to 94.8%.

CONTENTS

1. INTRODUCTION

Canine dirofilariasis is one of the most common parasitic diseases that affect the dog population. Surveys conducted in various location in Malaysia showed prevalence rates ranging from 7% to 70% (4,8,11,16,17). The microfilariae of Dirofilaria immitis and other filariod worms exhibit periodicity. This microfilarial periodicity can be defined as a wave of microfilarial density in the circulating blood in relation to the hour of the day. By postulating that this wave is a simple harmonic type with a wave period of twenty four hours, it can be simulated by the mathematical formula (15), $\acute{Y} = m + a \cos \left[15 (h - \acute{k})\right]^{\circ}$, where $\acute{Y}$ is the expected microfilarial density, m is the mean microfilarial density a is the amplitude of the wave, h is the hour of observation and k is the peak hour.

Since the cheapest and most commonly used methods of diagnosing canine heartworm disease namely the Knott concentration technique, filter yechnique, capillary hematocrit technique and direct smear were based on the detection of microfilariae in the blood (2), knowledge of the wave profile can be used to increase the effectiveness of these tests.

However the limited literature on this subject were from studies carried out in the temperate environment. Thus it is the general purpose of this study to provide more information on the microfilarial periodicity of Dirofilaria immitis in naturally infected dogs under local condition.

This study was conducted with the following objectives:

i) To establish the parameters of microfilarial periodicity of Dirofilaria immitis in the tropical environment.

ii) To determine, if any, significant difference in the microfilarial periodicity in cutaneous and venous blood.

iii) To determine, if any, significant difference between the micropipette method and the Knott dilution method for determining microfilarial periodicity.

iv) To apply the model of Sasa and Tanaka (15), using the mathematical formula, $\acute{Y} = m + a \cos 15 (h - \acute{k})$ to simulate the microfilarial periodicity of Dirofilaria immitis, where $\acute{Y}$ is the expected microfilarial density, m is the mean microfilarial density, a is the amplitude of the wave, h is the hour of observation and k is the peak hour.

2. LITERATURE REVIEW

Various methods have been use to observe the periodicity of microfilariae. Early workers on human filariasis used the simple comparison of the presence or absence of microfilariae in unmeasured smears from blood samples taken by day and by night. Examining multiple blood samples taken at regular intervals throughout the day were found to be not only a more quantitative method for periodicity studies but also enables it to be expressed in a curve form. In previous studies on the microfilarial periodcity of Dirofilaria immitis, enumeration of microfilariae were made

from blood samples taken at different time intervals of two to four hours (3,5,6). Angus (1) made two-hourly observations followed by hourly observations when the microfilarial counts approaches its peak density. A shorter interval of observation will obviously give a more accurate estimation of the peak hour. Observations for the duration of twenty-four hours were sufficient since the wave period is twenty-four hours (15). Nevertheless, some studies have observations for forty-eight hours (3,5).

Enumeration of microfilariae can be made from stained dried blood films (7), McMaster egg-counting chamber with a diluted blood sample (1,10) or using the modified Knott dilution method technique (3). Church et al. (3), used the micropipette method which was basically direct counting of microfilariae in a known volume of blood (5 to 20ul) diluted with distilled water. This method was useful for counting microfilariae in small volumes of cutaneous blood and can also be used for venous blood. The same authors found no significant difference between the micropipette method and the modified Knott dilution method for microfilariae enumeration.

The conventional classification of microfilarial periodicity employed qualitative terms, that is, periodic, subperiodic and non-periodic. A periodic form of filarial species or strain is one where the microfilarial counts were high at a certain time of the day but were rare or absent at another time of the same day. Various reports classified the periodicity of Dirofilaria immitis as subperiodic (3,5,18), where the microfilarial density showed a peak but never disappears altogether from the blood at other times of the day. In the non-periodic filarial

form there is little variation in the level of microfilariaemia throughout the day. Microfilarial periodicity can be further classified as a nocturnal form if the peak microfilarial density occur at night and drop during the daytime or as a diurnal form if the relationship is reversed.

The conventional classification were ambigious and not quantitative. Thus by postulating that the profile of microfilarial periodicity follows a harmonic wave pattern, Sasa and Tanaka (15) introduced the quantitative concept of "periodicity index" which is the standard deviation of the relative microfilarial density to replace the terms periodic, subperiodic and non-periodic. Essentially the periodicity index is a measure of the relative size of the amplitude of the wave. The hour of maximum microfilarial density is more precise than the terms nocturnal or diurnal in describing microfilarial periodicity. The same authors went furthur to apply the mathematical formula $\acute{Y} = m + a \cos[15\ (h - \acute{k})]^{\circ}$, where $\acute{Y}$ is the expected microfilarial density, m is the mean microfilarial density, a is the amplitude of the wave, h is the hour of observation and k is the peak hour to simulate the microfilarial periodicity in their investigation of human filariasis.

Several periodicity studies have shown that the microfilariae of Dirofilaria immitis exhibit nocturnal periodicity (6,9,18), with the peak hour ranging from 19:45 hours to 00:40 hours (18) and 18:00 hours to 21:00 hours (6). Diurnal peaks occuring between 12:00 to 16:00 hours have been reported (5). There were reports of two peaks within a twenty-four hour period. In a study by Wright and Newton (12), two peaks occuring at 01:00

hours and 16:00 hours were observed with the former having a higher microfilarial density. Contrary to this, Angus (1) reported a higher microfilarial density in the diurnal peak at 16:00 hours than the nocturnal peak at 00:00 hour to 01:00 hour. Church et al. (3) concluded from their studies that variation in the peak hours observed, ranging from 18:00 hours to 03:00 hours do not permit it to be classified as diurnal or nocturnal. By analysing the observed data according to the mathematical model proposed by Sasa and Tanaka (15), Church et al. (3) obtained periodicity indices ranging from 24.9 to 53.8 and thus fell into the subperiodic category. There were significant correlation between the observed microfilarial density with the values generated from the mathematical model in seven of the ten studies with correlation of determination values of 78.5% to 95.3%.

Besides the daily fluctuation in microfilarial density there was also seasonal variation (6,13). Higher numbers of circulating microfilariae were found in the warmer months when the mosquito vectors were plentiful(13). Emotional stress during the period under study may produce transient changes in the microfilarial density which can obscure the periodicity (3).

3. MATERIALS AND METHODS

A total of four microfilaria positive dogs were used for this study, identified as dog A, dog B, dog C and dog D. In all periodicity studies, venous and cutaneous blood were collected at three-hourly intervals for 24 hours duration for study I and repeated one week later as study II. In dog D, only study I was done. Microfilarial counts were made from cutaneous and venous

blood in all dogs except in dog C due to its extreme uncooperativeness, the cutaneous blood sample was not obtainable. Dog B was pregnant during study I and whelped four days later. Cutaneous blood was collected after nicking the skin of the ear with the point of a hypodermic needle and drawing the blood into a 20 ul micropipette and treated using the micropipette method given in Appendix I.

Venous blood (2-3 ml) was collected from the cephalic vein into 3 ml vacuum tubes containing EDTA as anticoagulant. Microfilariae counts on the venous were made using the micropipette method (Appendix II) and the Knott dilution method (Appendix III).

The microfilarial counts for each sample were converted to percentages of the mean. The standard deviation (SD) of the percentages of the mean is the periodicity index. The equation for calculating the expected microfilarial density for 3 hourly sampling would be $\acute{Y} = 100 + 1.323 \times SD \cos\left[15(h-\acute{k})\right]^{\circ}$. Having estimated the peak hour $\acute{k}$ and SD by computer analysis, it is substituted in the equation to obtain the expected microfilarial density. The fit of the observed data to the estimated data was evaluated using Pearson's product-moment correlation (r). The difference between the mean microfilarial density in the cutaneous and venous blood were analysed using two-way analysis of variance. Comparison between the micropipette method and the Knott dilution method for microfilarial enumeration was made using the two-way analysis of variance also.

4. RESULTS

Table I summarises the mean microfilarial density obtained by using the micropipette method and the Knott dilution method for microfilarial enumeration in the venous blood. The correlation between the periodicity obtained using the two methods is given by the correlation of determination r^2. A plot of the microfilarial density against time for the venous blood from the two methods is shown in Fig. 1 to 4.

Table I: COMPARISON OF MEAN MICROFILARIAL DENSITY BETWEEN THE MICROPIPETTE AND KNOTT DILUTION METHOD AND ITS OBSERVED PERIODICITY CURVE CORRELATION.

STUDY	M	K	M - K (%)	r^2 (%)
A - I	293.0	195.4	50.0**	67.7**
A - II	191.1	134.6	42.0**	0.3
B - I	767.7	694.1	10.6	13.0
B - II	714.9	539.7	32.5**	24.4
C - I	749.7	585.8	28.0**	83.6**
C - II	1004.3	621.1	61.7**	84.5**
D - I	42.6	28.2	50.8**	78.8**

** ($P<.01$); M = Micropipette; K = Knott; r^2 = Correlation of determination.

A comparison of the observed mean microfilarial density between cutaneous and venous blood (micropipette method) is shown in Table II. Correlation between the microfilarial periodicity in the cutaneous and venous blood (micropipette method) is given

by r^2 in the same table. Figure 5 to 7 show the plot of microfilarial density against time for the cutaneous and venous blood for dog A, B and D respectively.

Table II: COMPARISON OF MEAN MICROFILARIAL DENSITY AND CORRELATION BETWEEN CUTANEOUS AND VENOUS BLOOD.

STUDY	M	C	M - K (%)	r^2 (%)
A - I	293.0	302.6	3.2	75.4**
A - II	191.1	115.1	66.0**	10.1
B - I	767.7	467.6	64.2*	4.9
B - II	714.9	579.4	23.4	19.2
D - I	42.6	29.1	46.2**	34.6

* ($P < .01$); ** ($P < .05$); M = Micropipette; C = Cutaneous; r^2 = Correlation of determination.

The observed and expected microfilarial periodicity in the cutaneous and venous blood are presented graphically in Figures 8 to 11. The calculated microfilarial periodicity parameters are given at the top right corner of the corresponding graph. Table III summarises the parameters of microfilarial periodicity in this study.

Table III: SUMMARY OF MICROFILARIAL PERIODICITY PARAMETERS.

Source	Periodicity index + S.D.	k (hours) Nocturnal	Diurnal
Cutaneous	46.2 ± 9.0	20:12	16:24 ± 00:24
Venous	31.4 ±14.4	21:30 ± 01:54	16:48

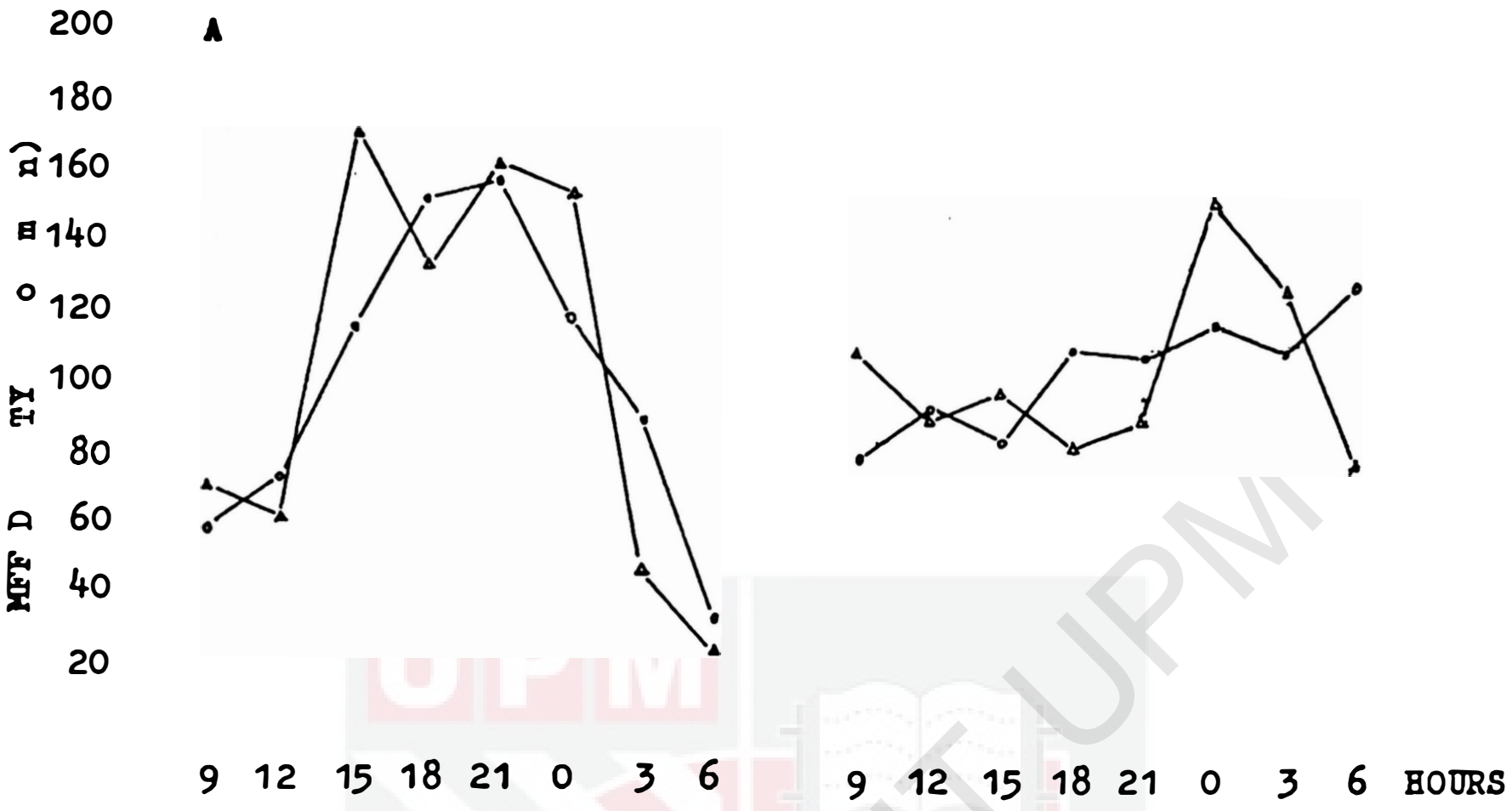


Fig. 1. Observed microfilarial periodicity in venous blood using the micropipette method (o——o) and the Knott dilution method (Δ——Δ). Studies I and II in dog A are represented in graphs A and B.

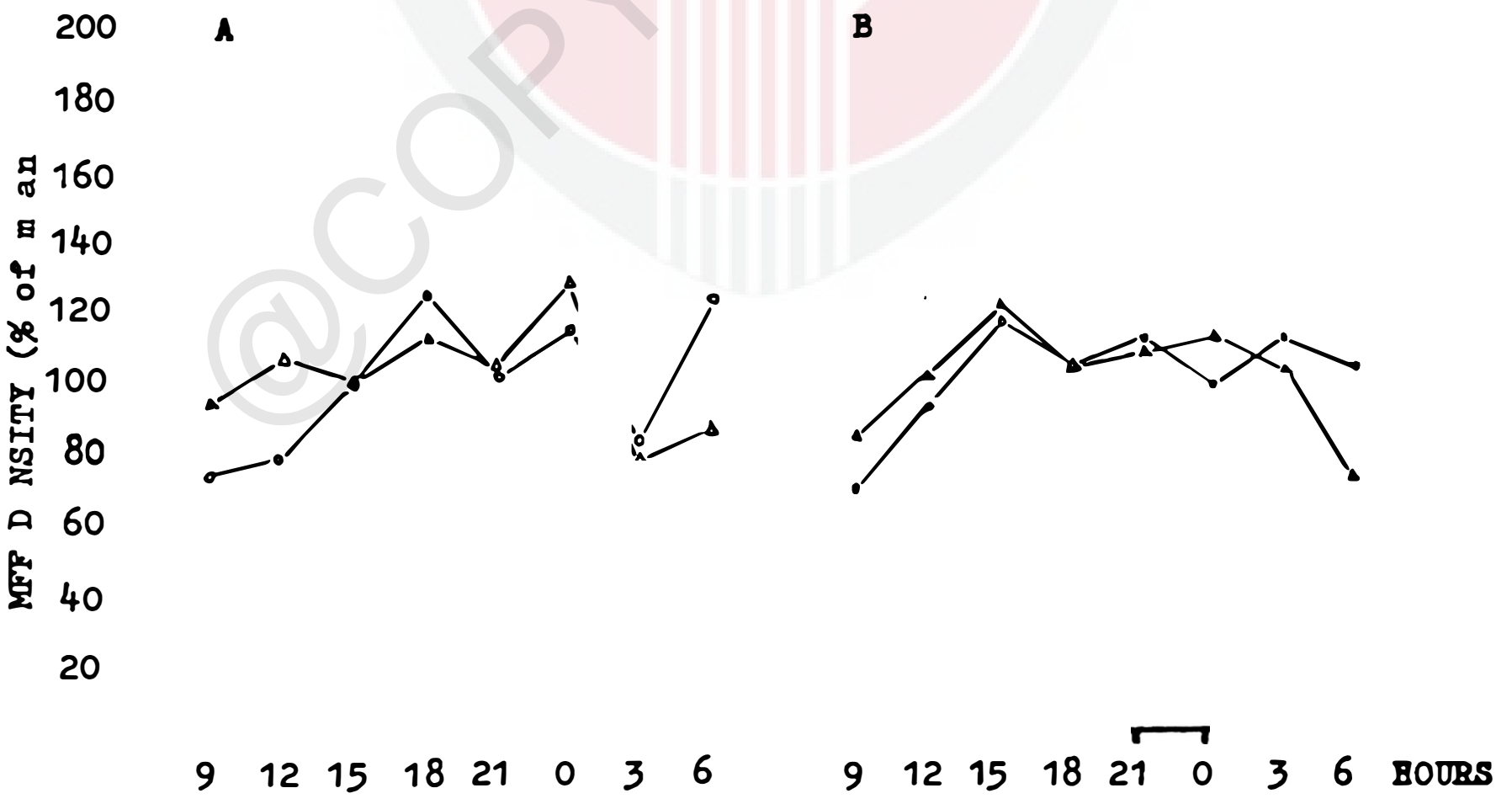


Fig. 2. Observed microfilarial periodicity in venous blood using the micropipette method (o o) and the Knott dilution method (Δ——Δ). Studies I and II in dog B are represented in graphs A and B respectively.

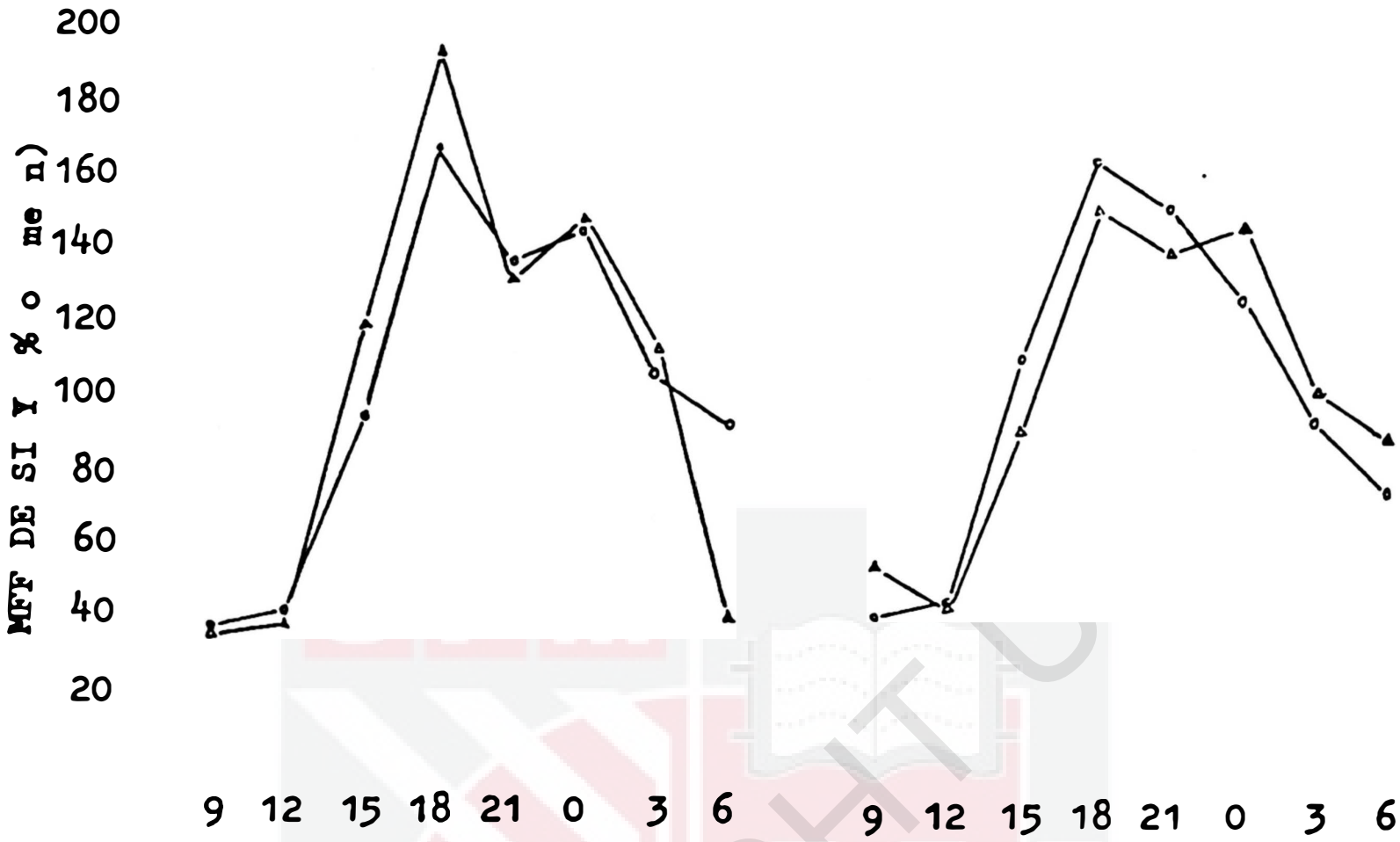


Fig. 3. Observed microfilarial periodicity in venous blood using the micropipette method (o——o) and the Knott dilution method (▲——▲). Studies I and II in dog C are represent in graphs A and B respectively.

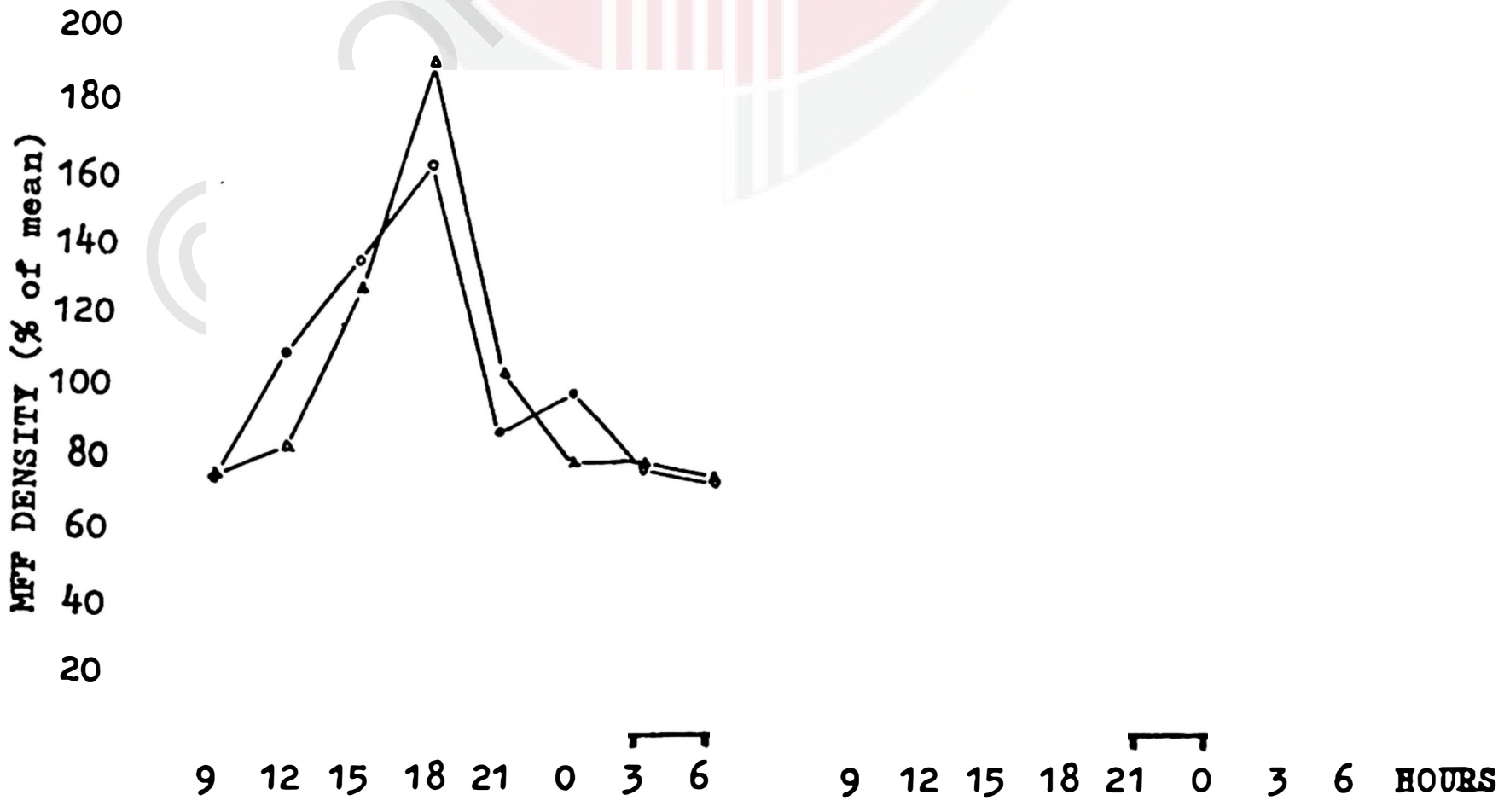


Fig. 4. Observed microfilaeial periodicity in venous blood uisng the micropipette method (o——o) and the Knott dilution

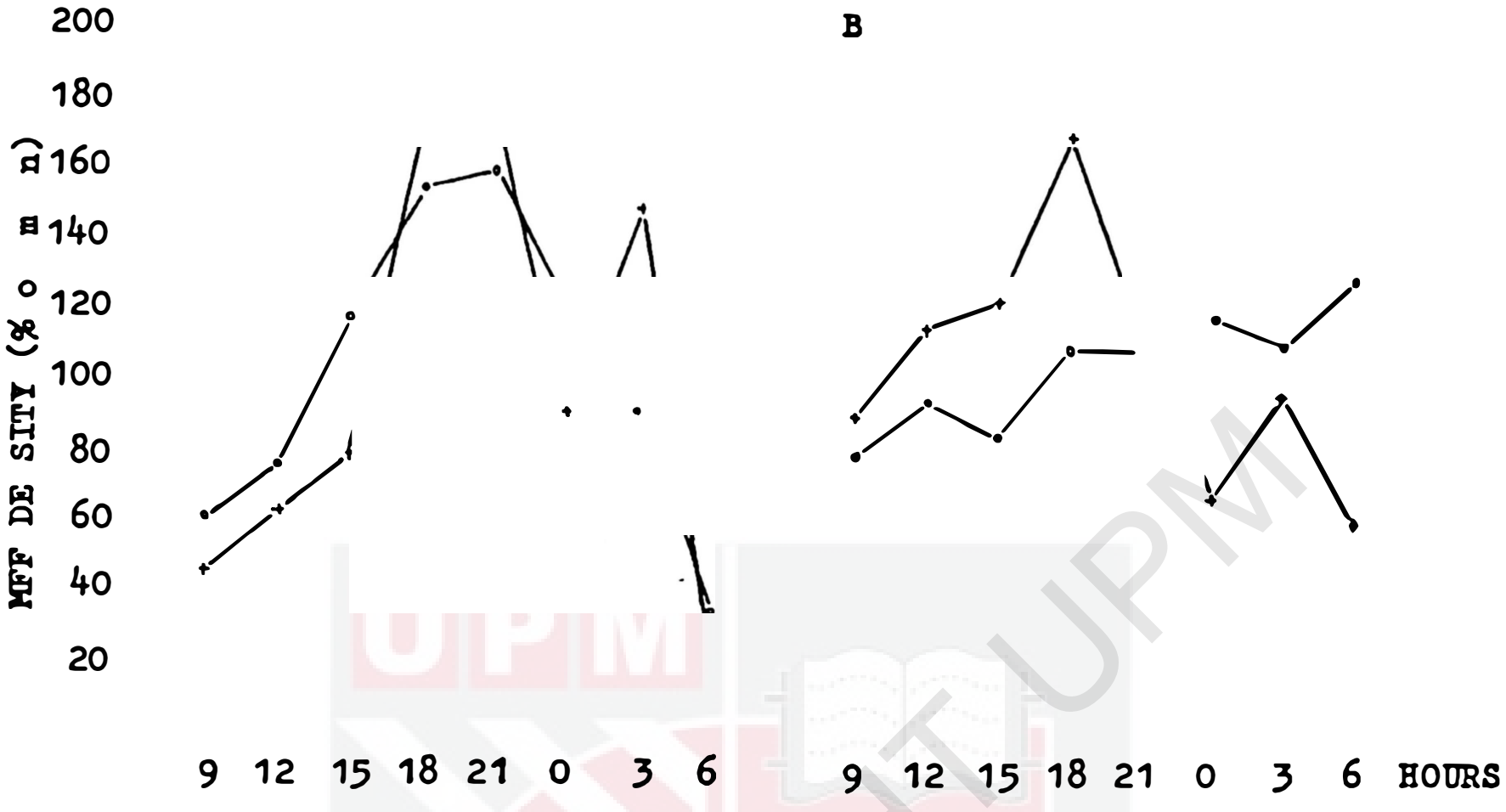


Fig. 5. Observed microfilarial periodicity in venous (o o) and cutaneous blood (+——+). Studies I and II of dog A are represented in graphs A and B respectively.

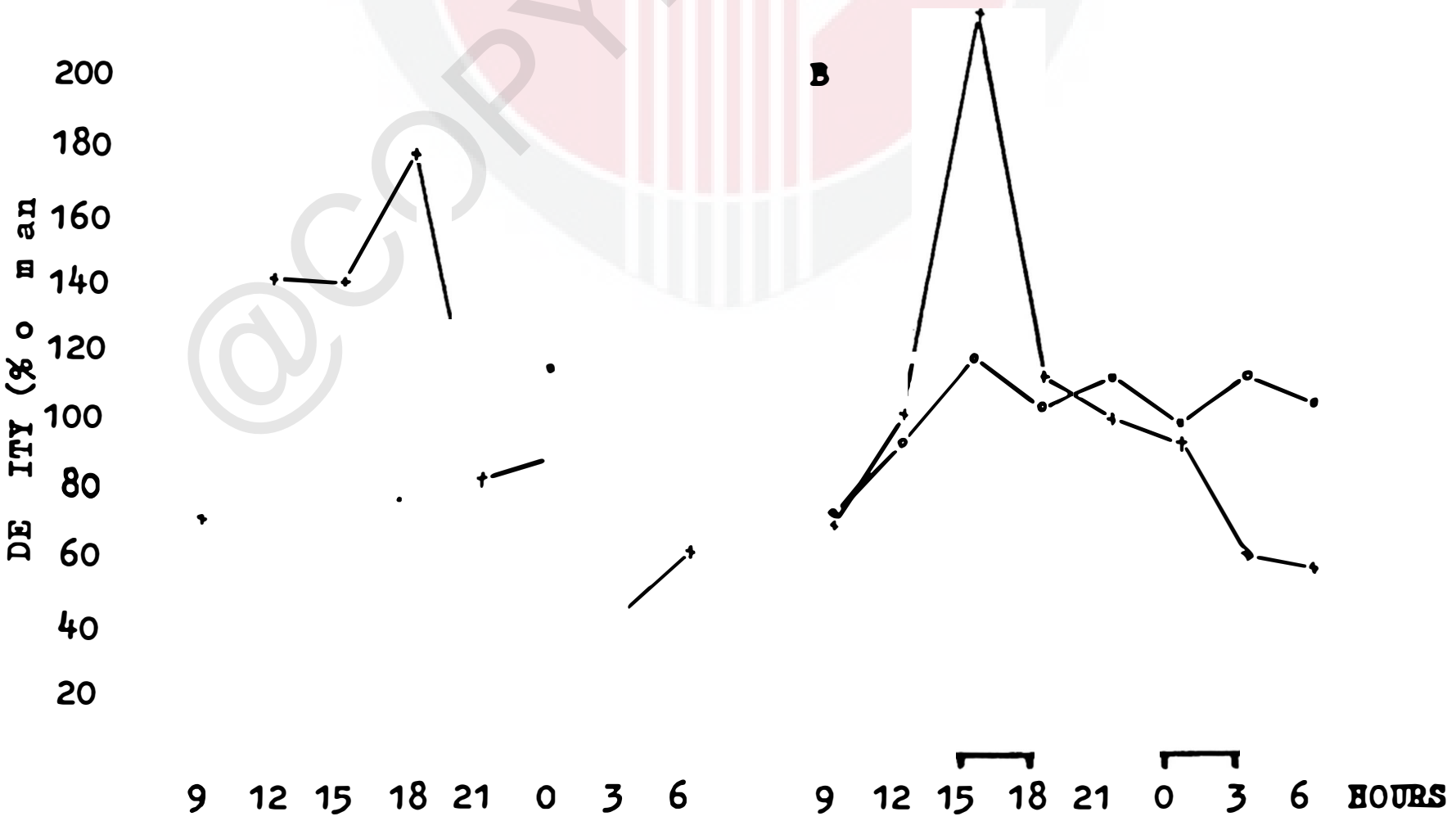


Fig. 6. Observed microfilarial periodicity in venous (o——o) and cutaneous blood (+——+). Studies I and II of dog B are represented in graphs A and B respectively.

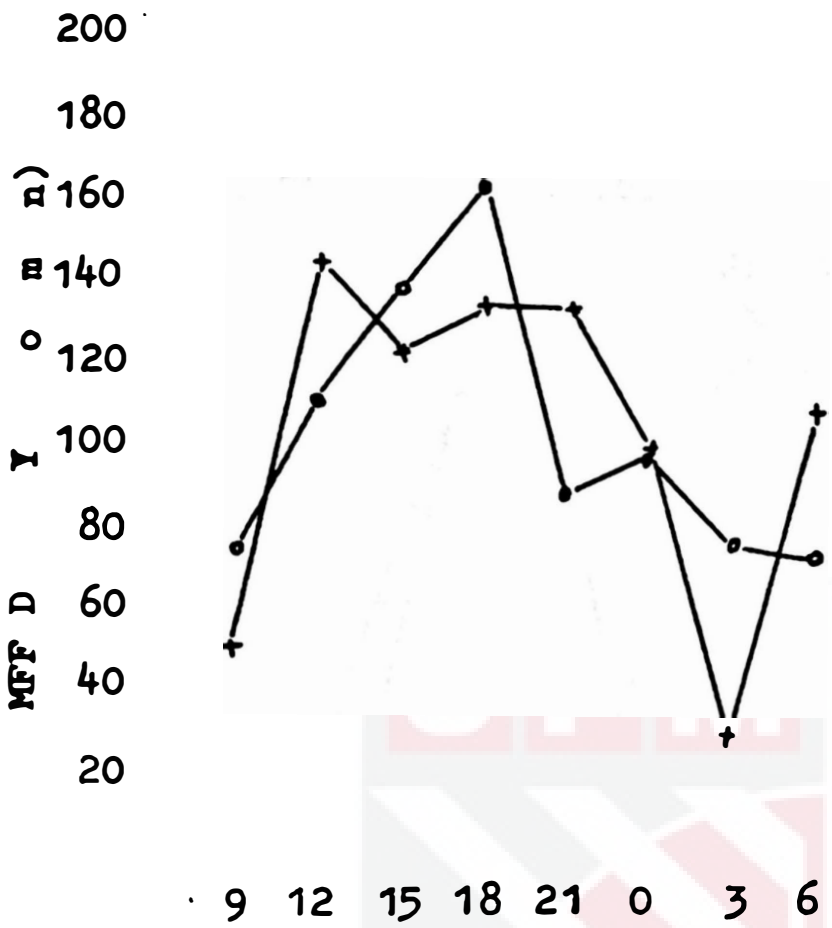
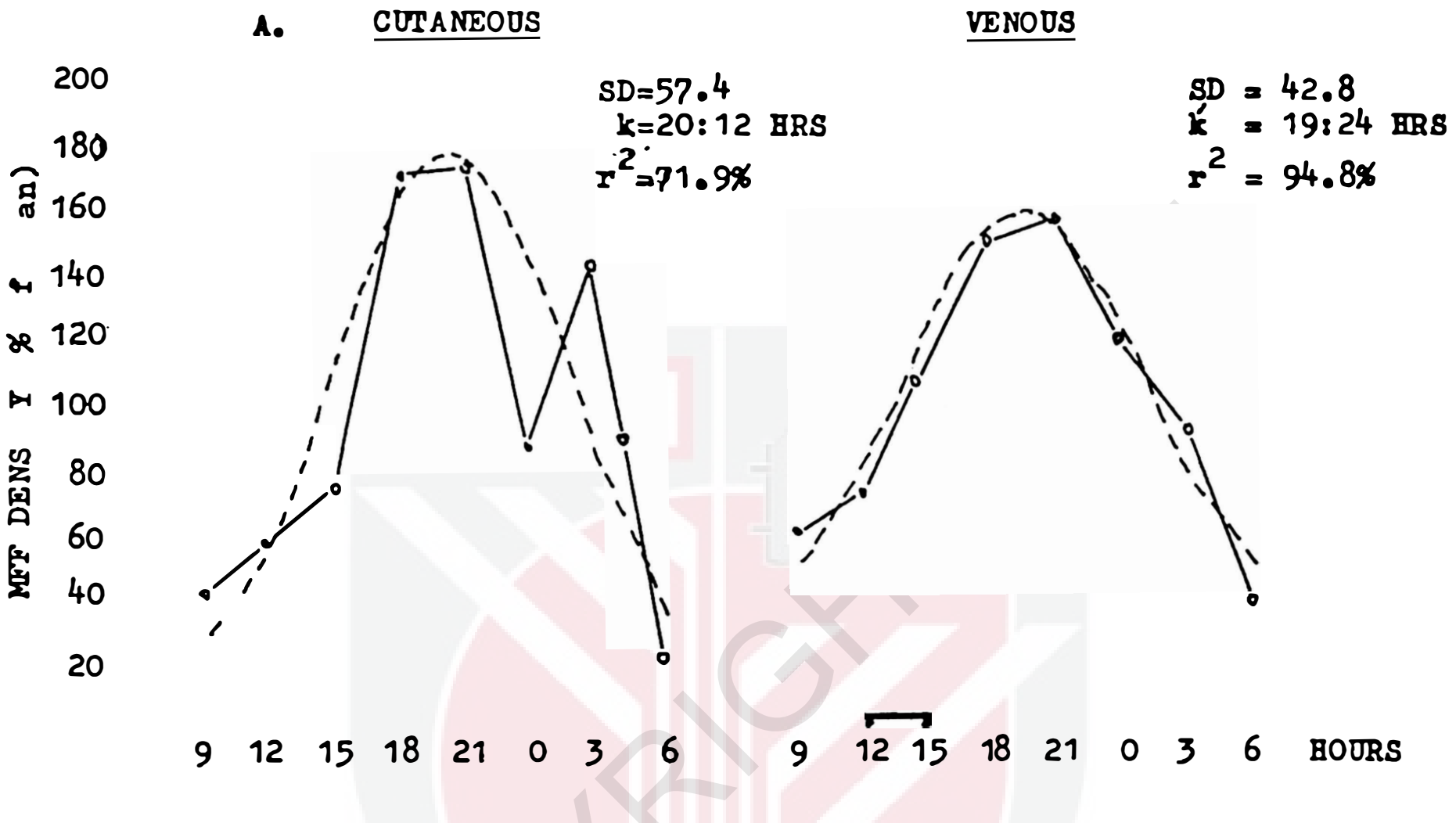


Fig. 7. Observed microfilarial periodicity in venous (o——o) and cutaneous blood (+——+) in Study I of dog D.

DOG - A

STUDY I



STUDY II

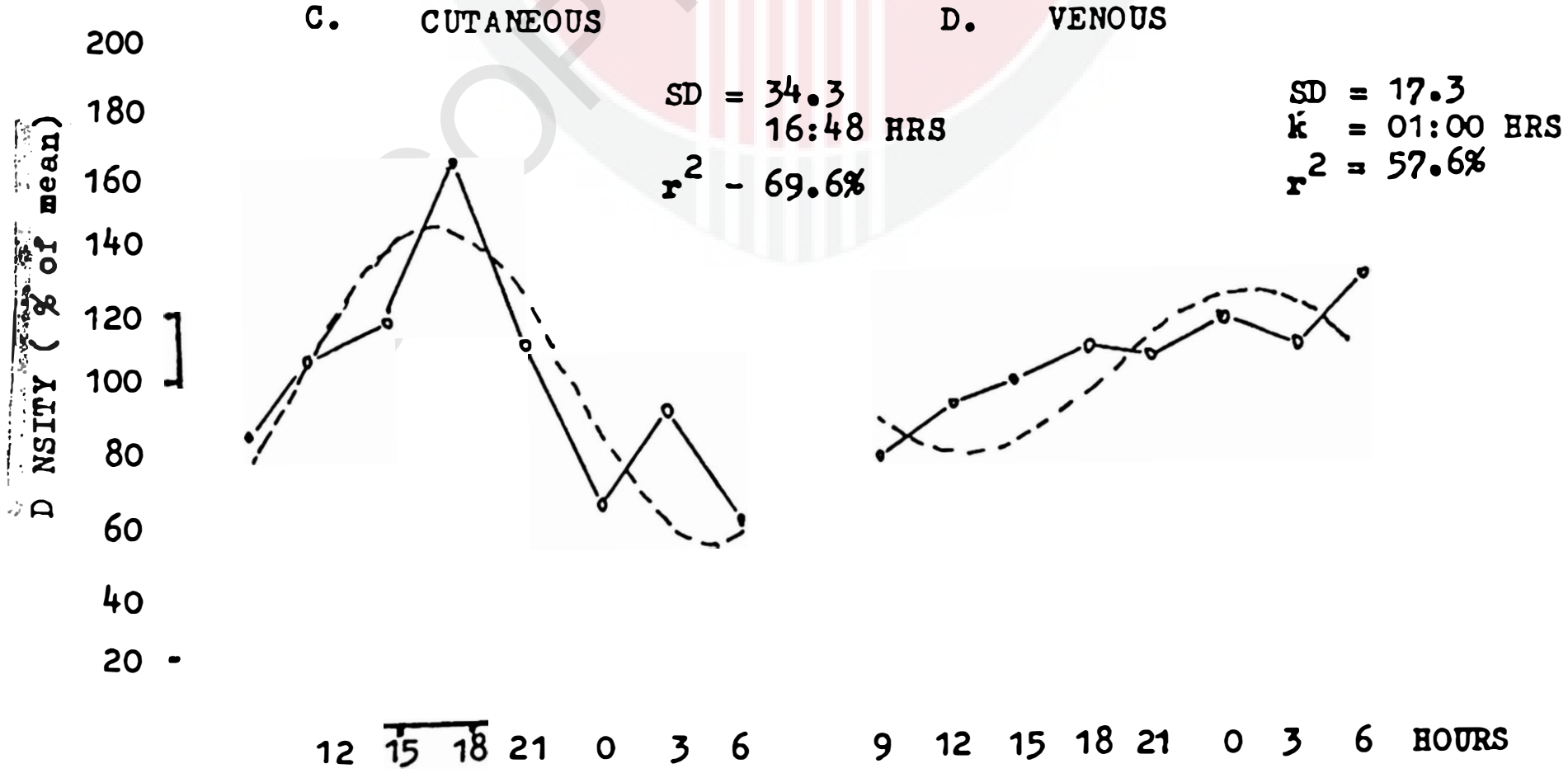


Fig. 8. Observed (o——o) and expected (-----) microfilarial periodicity in dog A.

DOG - B

STUDY I

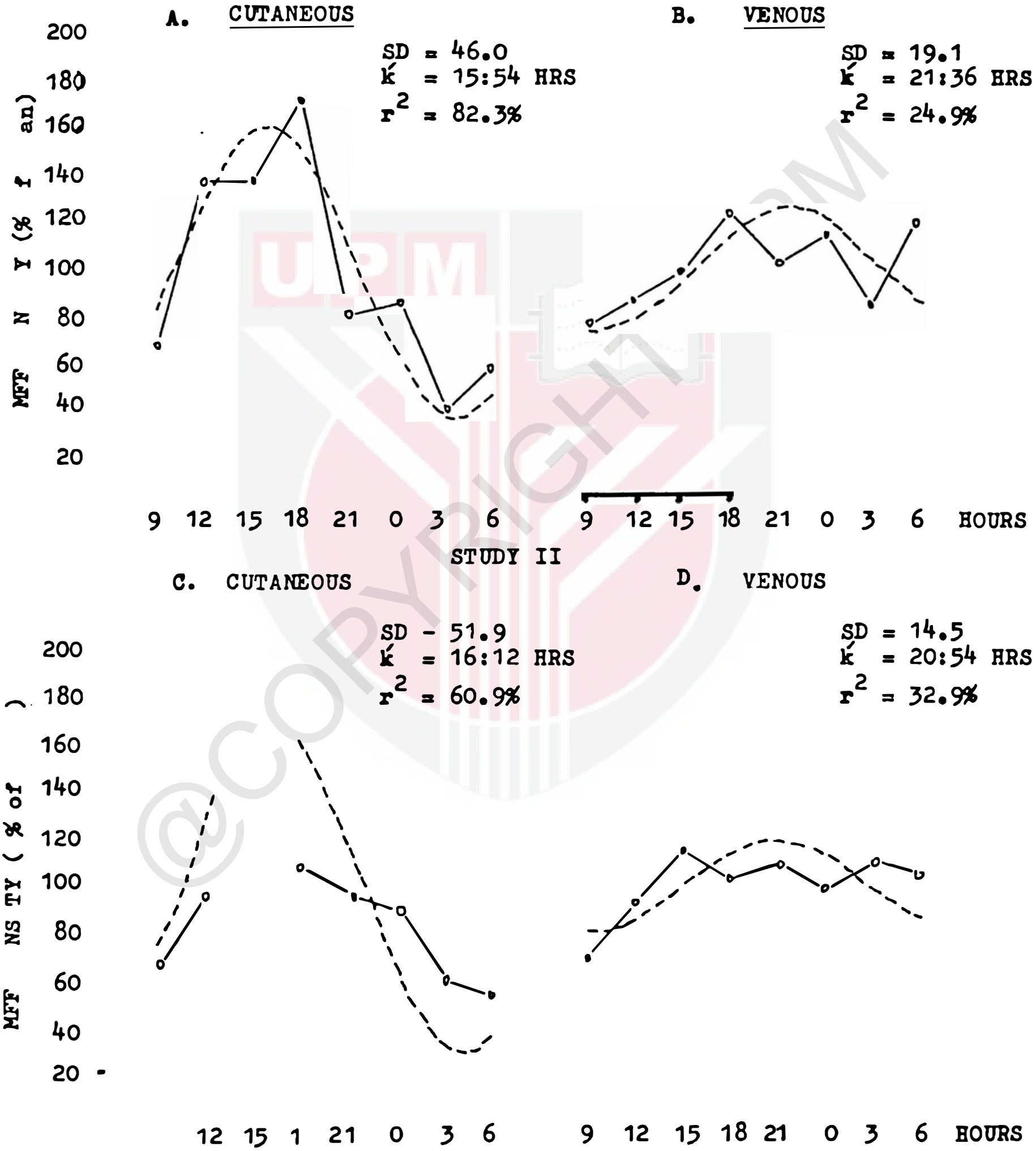
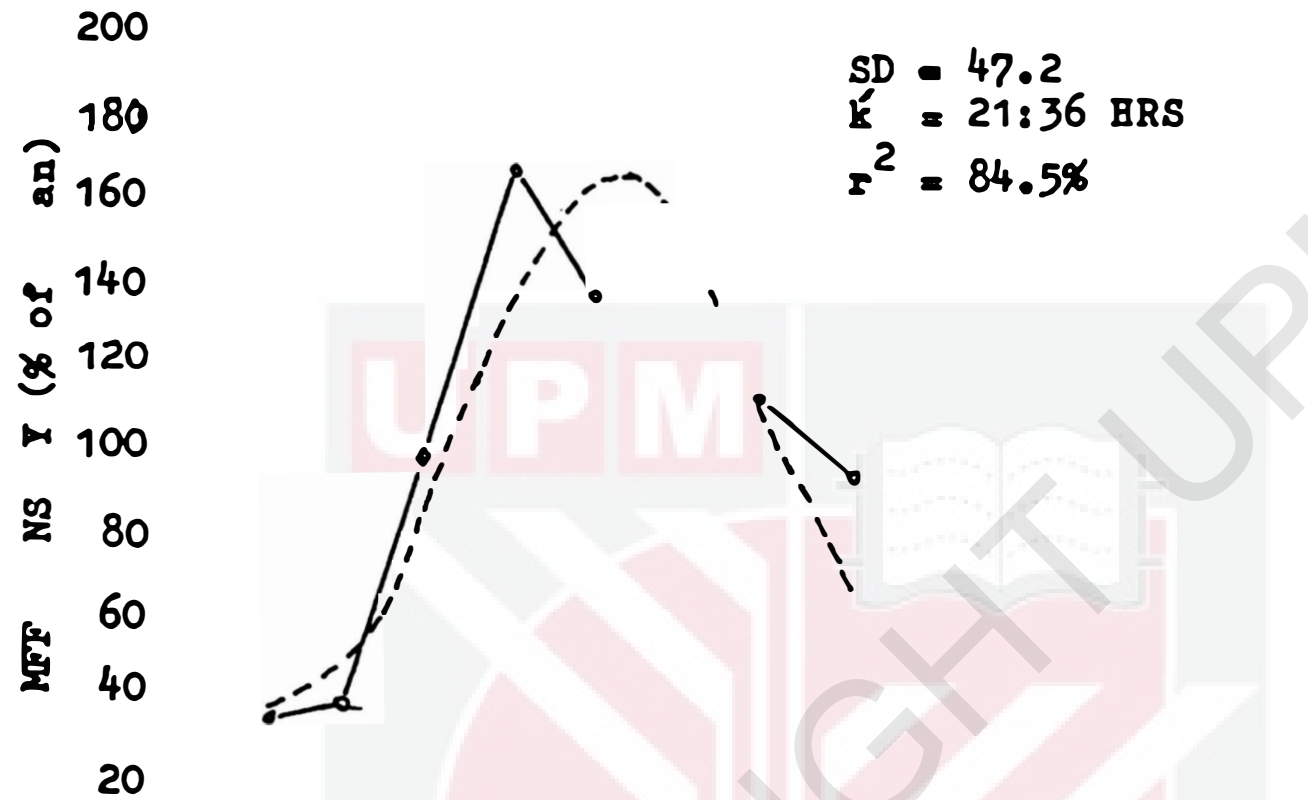


Fig. 9. Observed (o——o) and expected (- - - - -) microfilarial periodicity in dog B.

DOG - C

STUDY - I

A. VENOUS



STUDY - II

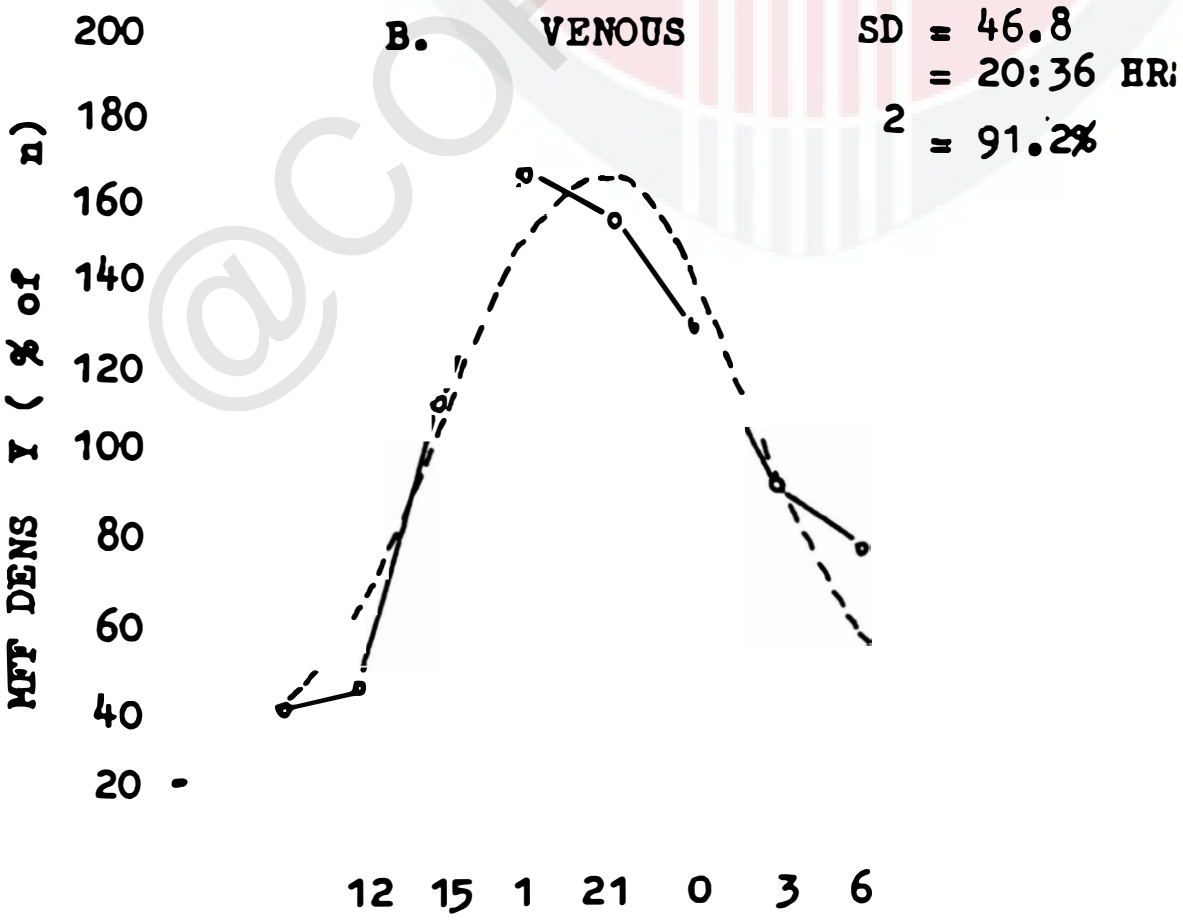


Fig. 10. Observed (o——o) and expected (— — —) microfilarial periodicity in dog C.

DOG - D

STUDY - I

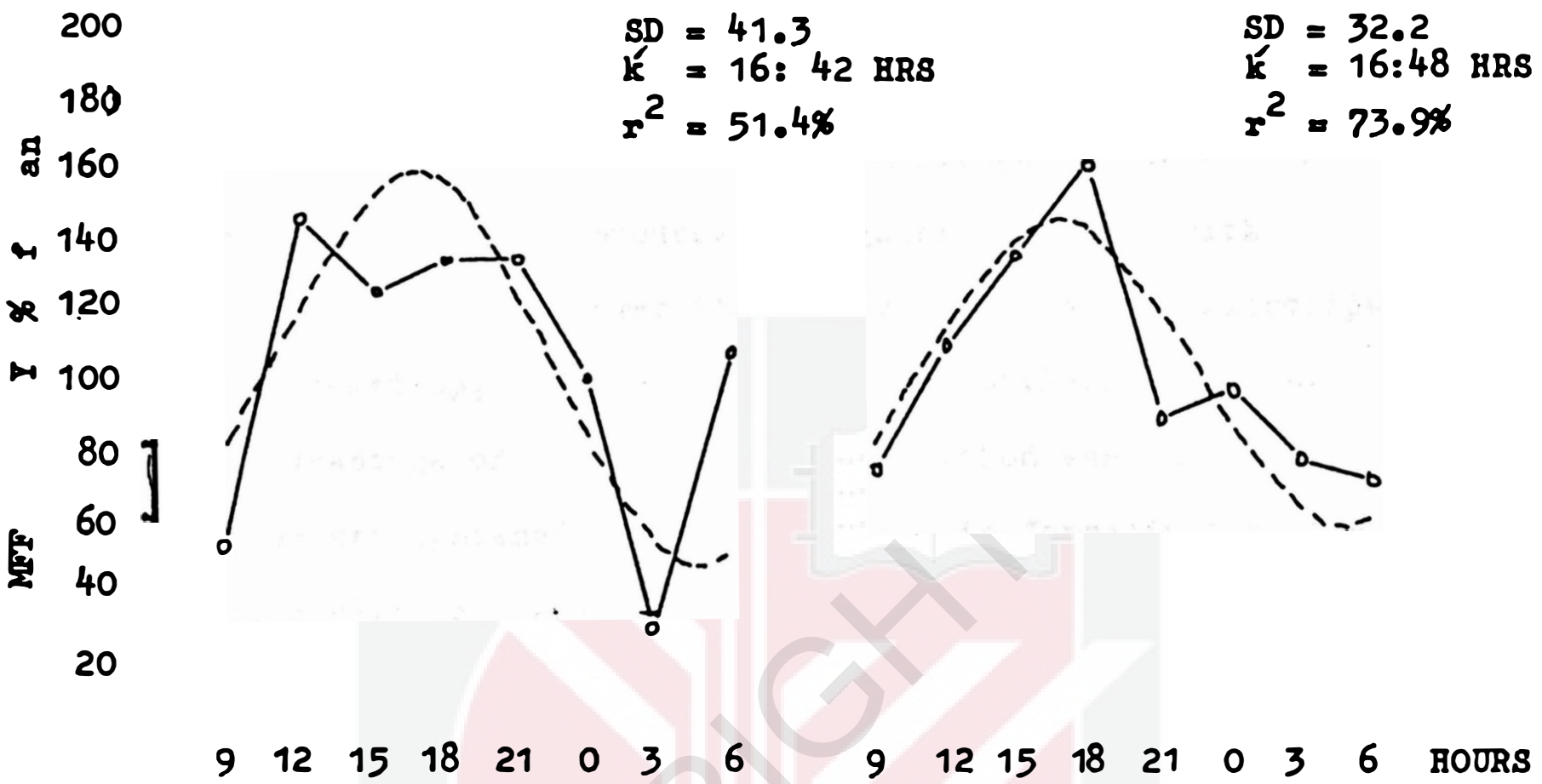


Fig. 11. Observed (o——o) and expected (- - - - -) microfilarial periodicity in dog D.

5. DISCUSSION

Results in Table I showed that the mean microfilarial counts in the venous blood counted by using the micropipette method was significantly higher than that counted using the Knott dilution method by as high as 28.0% to 61.7%. This was consistent with the observation that the use of centrifugation in the Knott dilution method tends to produce aggregates of debris with microfilariae. Clumps larger than the diameter of the micropipette cannot be sucked up, thus causing lower microfilarial counts. The only advantage of the Knott dilution method was that the stained and straightened microfilariae due to formalin fixation were more easily seen and counted. Correlation between the observed microfilarial periodicity obtained using the two techniques was significant in four out of seven cases (Fig. 1A, Fig. 3A and Fig. 4). Thus, the microfilarial counts made by using the micropipette method was used to represent the microfilarial periodicity in the venous blood.

Comparison of the microfilarial density between the cutaneous and venous blood showed a higher microfilarial density in the venous blood. There was no significant correlation between the observed periodicity curves of cutaneous and venous blood except in Study I of dog A (Fig. 5 to 7). Considering the lack of correlation, generalisation should not be made for the microfilarial periodicity parameters of the cutaneous and venous blood although Church et al. (3) reported that there was no difference in the microfilarial periodicity in the cutaneous and venous blood.

The expected peak hour k', which was derived mathematically

(15) would give a better indication of the time of maximum microfilariaemia than the observed peak hour k , which was based on blood sampling at three hourly intervals. The mathematically derived peak hour was a better alternative than the method of increasing the frequency of blood collection as microfilariaemia approaches its maximum density as practised by Angus (1).

As can be seen from Figure 8, in Study I of dog A, the periodicity index of the cutaneous blood of 57.4 was higher than that of the venous blood of 42.8 (Fig. 8A and B). The estimated peak hours in the cutaneous and venous blood were 20:12 hours and 19:40 hours respectively, which were nocturnal peaks. Correlation between the observed microfilarial periodicity of the cutaneous and venous blood was significant at 75.4% (TableIII). However in Study II in the same dog, the periodicity indices of the cutaneous and venous blood at 34.3 and 17.3 respectively were lower than the corresponding values in Study I. There was a diurnal peak hour in the cutaneous blood at 16:48 hours. The periodicity curve of the venous blood in Study II of dog A was markedly different from the periodicity curve of the venous blood in Study I and the cutaneous blood in Study II by having a low periodicity index of 17.3 and a later nocturnal peak hour at 01:00 hours (Fig. 8B, Fig. 8C and D).

The periodicity curves of Study I and II of dog B presented in Figure 9A and B, D and E resembled that of Study II of dog A with the cutaneous blood having high periodicity indices of 46.0 in Study I and 51.9 in Study II with diurnal peak hours at 15:54 hours in Study I and 16:12 hours in Study II. In contrast, the venous blood showed a low periodicity indices of 19.1 in

Study I and 14.5 in Study II with nocturnal peak hours at 21:36 hours and 20:54 hours. The cause of the dissimilarity between the periodicity curves of the cutaneous and venous blood in dog A and B was not known. It showed that the microfilarial periodicity in the cutaneous and venous blood need not be similar. In dog C, the periodicity parameters of the venous blood in Study I and II were quite similar. Periodicity indices were high at 47.2 and 46.8 with nocturnal peak hours of 21:36 hours and 20:36 hours (Fig. 10A and B). In dog D, the periodicity of the cutaneous blood of 41.3 was higher than that of the venous blood but maximum microfilariaemia occured at the same time at 16:42 hours and 16:48 hours which were diurnal peaks.

Thus,from these studies, the periodicity indices of the cutaneous blood ranging from 34.3 to 57.4 were higher than that of the venous blood which ranged from 14.5 to 47.2. Accordingly, the periodicity of Dirofilaria immitis falls into the subperiodic category. Church et al. (3) reported periodicity indices in the venous blood ranging from 24.9 to 53.5. The peak hour can be either nocturnal or diurnal in a particular study of 24 hours duration. In the cutaneous blood, maximum microfilarial density occured diurnally in four out of five studies (Fig. 8C, Fig 9) with a mean of 16:24 ± 00:24 hours. One nocturnal peak was observed in the cutaneous blood at 20:12 hours in Study I of dog A (Fig. 8A). Maximum microfilarial density in the venous blood occured nocturnally at 21:30 ± 01:54 hours, except in dog D at 16:48 hours (Fig. 8B and D, Fig. 9B and D, Fig. 10A and B and Fig. 11B). Thus the peak hours were not confined to the hours of darkness but also occured in the late afternoon. Nocturnal

periodicity with peak hours ranging from 19:45 hours to 00:40 hours (18) and 18:00 hours to 21:00 hours (6) have been reported. Diurnal peak hours of 12:00 hours to 16:00 hours were observed by Grieve and Lauria (5). In this study, there was significant correlation between the observed and expected periodicity curves in fifteen out of seventeen cases with r^2 values ranging from 51.4% to 94.8%. Church et al. (3) obtained significant correlation of 77.8% to 92.3% in seven out of ten cases.

6. CONCLUSION

The periodicity index showed that the periodicity of Dirofilaria immitis falls into the subperiodic category. There is a high degree of variability in the peak hour of microfilariaemia with peak hours occuring at late afternoon and night, thus cannot be categorised as diurnal or nocturnal. Microfilariaemia is more concentrated in the venous blood than the cutaneous blood. The micropipette method was found to be more accurate in enumerating microfilariae as compared to the Knott dilution method. The observed microfilarial periodicity was found to be significantly correlated to the expected microfilarial periodicity.

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APPENDIX III

Knott dilution method for microfilariae enumeration in venous blood.

1. Pipette out 1.0 ml of venous blood with a 1.0 ml volumetric pipette into a 15.0 ml graduated conical glass centrifuge tube.
2. Add 10.0 ml of 2% formalin solution and mix.
3. Stand for 5 minutes for adequate hemolysis and fixation of microfilariae.
4. Centrifuge at 1000 r.p.m. for 5 minutes.
5. Decant the supernatant.
6. Stain the sediment with approximately equal volume of 0.1% methylene blue for 1 hour.
7. Resuspend the mixture in 10.0 ml of 2% formalin and centrifuge at 1000 r.p.m. for 5 minutes.
8. Decant the supernatant to remove excess background stain.
9. Dilute the stained sediment 1 to 5 times with 2% formalin depending on the level of microfilariaemia.
10. Follow steps 3 to 6 in Appendix II.

APPENDIX I

Micropipette method for microfilariae enumeration in cutaneous blood.

1. Pipette out 20 ul of blood with a 20 ul micropipette.
2. Deposit onto a glass slide (48×75 mm). Rinse micropipette three times with distilled water. Add rinsing to the blood on the glass slide.
3. Mix thoroughly with a dissecting needle.
4. Cover with a 40×22 mm coverslip.
5. Count the number of microfilariae under 100× magnification.

APPENDIX II

Micropipette method for microfilariae enumeration in venous blood.

1. Pipette out 0.5 ml of venous blood with a 0.5 ml volumetric pipette into a 3.0 ml test tube.
2. Dilute one to five times with distilled water, depending on the level of microfilariaemia. Mix thoroughly.
3. Pipette out 20 ul of the aliquot with a 20 ul micropipette.
4. Deposit onto a glass slide (45×75 mm).
5. Cover with a 22×22 mm cover slip.
6. Make four replicate counts for each sample under 100× magnification.