J Vet Clin 22(2) : 79-83(2005)

Quantitative Analysis of the Periodicity of *Dirofilaria immitis* in Dogs with Various Microfilarial Densities

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Abstract: Six dogs including 4 dogs living in different geographic locations of Kangwon province and 2 client-owned dogs were used to determine the periodicity of microfilariae of *D. immitis* using a trigonometric model. The calculated periodicity index was ranged from 25.6 to 95.5% with mean of 57.6%, and the estimated hour of peak was approximately 21:00 hrs (range, 20:04 - 21:29 hrs) and minimum counts at 09:00 hrs (range, 08:04 - 09:29 hrs). Correlation coefficient between the observed and the expected count from the model varies depending on dogs, ranging from 6.4 to 49.2%. Based on this study, the periodicity of microfilariae of *D. immitis* was considered as nocturnally sub-periodic for all dogs employed. This result is in consistent with previous report in peak hour but different in minimal hour, indicating that further studies on the periodicity need to be performed to better understanding the dynamics of the periodicity and to help practitioners in the choice of the time for examination of the dogs.

Key words : *Dirofilaria immitis*, Model, Periodicity, Dogs.

Introduction

Canine heartworm disease, due to infection with *Dirofilaria immitis*, is a potential zoonotic parasite transmitted by vector mosquitoes[10] and occurs worldwide in tropical, sub-tropical and temperate zones[8,9]. In Korea, human filariasis has almost been eradicated except in a very restricted coastal area. In contrast, canine dirofilariasis is widely prevalent with varying reported prevalence of greater than 40% in certain localities[19].

Periodicity, the circadian rhythm commonly called the microfilarial periodicity, is defined as a cyclical rise and fall in microfilaria numbers in the circulating blood over time and further classified as nocturnal or diurnal depending on what time of day maximum numbers of microfilaria are present in peripheral blood[7]. A filarial species has been called a nocturnal form if the microfilarial counts increase at night and drop during the daytime, or diurnal form if the relationship is reversed. Also, the term non-periodic, sub-periodic or periodic refers to the grade of differences between the maximum and the minimum counts. Periodicity index, on the other hand, is the coefficient of variation over 24 hrs counts and is a quantitative measure of the character formerly called by terms such as periodic or subperiodic, and the best estimate of the peak hour, which correspond to the rather ambiguous terms such as diurnal or nocturnal[17].

Many adult heartworms were identified ultrasonographically within pulmonary artery, but traditionally, the diagnosis is made by inspecting blood samples for microfilariae using light microscopy or detecting specific antigens released from the adult worm into the peripheral blood[6,16,21]. A serious problem in making diagnosis is the dirofilariasis without microfilaremia (occult dirofilariasis), due to single-sex infection, low numbers of microfilariae, presence of immature adults, or immune-mediated clearance of microfilariae[14]. Thus, study on the periodicity is important in better understanding the nature of dirofilariasis and predicting times when maximum microfilaria counts are obtainable for possible use in clinical diagnosis.

Many studies have been performed to determine the types of microfilarial periodicity using simple impressions from the crude data[3], observing the curves of a number of cases infected with the same form of filaria[23], plotting periodicity curves from data collected from multiple samples[5], empirical or theoretically derived mathematical models[15,17]. Because the individual microfilarial counts are subject to great variations, especially when the values are small, the use of single counts such as those of the peak hour is not recommended from the statistical point of view. Thus more reliable measures of periodicity have been developed for this purpose. Microfilarial periodicity is observed in many filarial infections[7]. The periodicity shown by *D. immitis* is more controversial than that of other filarioids, because microfilariae appear to be a periodicity superimposed on this which varies with geographic location[2].

Since the first report on the periodicity of *D. immitis* identified in a southern area of Korea[15] as being low grade nocturnal with the maximal time of 21:00 hrs and the minimal time of 11:00 hrs no further investigations have been reported in localities where the prevalence of *D. immitis* infection is different. For better understanding of geographic variations of the microfilariae in Korea we analyzed the periodicity in the peripheral blood of infected dogs living in different environmental conditions, using a mathematical model.

Materials and Methods

Animals and collection of blood sample

Six dogs, including 4 German shepherds(age range, 2-3 year-

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old; dog No. 1-4) reared in different geographic locations (Hwacheon, Daegu, Chuncheon, and Hwengseong) and 2 client-owned Yorkshire Terriers (2.5 year-old each; dog No. 5-6) from Chuncheon, were used in the periodicity study, the former being outdoors and the latter being indoors. All dogs were confirmed as being infected with *D. immitis* by PCR technique using specific primer[18]. According to their owners, all dogs have no history of traveling out of their born area or receiving heartworm prophylaxis. All dogs were maintained in a normal room environment under natural daylight conditions and fed with commercial ration and water *ad libitum*.

The dogs were screened by an ELISA kit (Snap™, IDEXX, USA) and microscopic test using plain microhematocrit capillary tubes (Chase Scientific, USA). Briefly, for antigen-positive dogs, the tube was centrifuged for 3 min at 1000 rpm and then observed over the upper side of the buffy coat to detect microfilariae. By using an automatic pipette, 100 ml of blood was taken from the ear vein at 2 hrs intervals over the period of 24 hrs beginning at 07:00 hrs. The vaselin was applied on the surface of the venipuncture site for easier collection of blood. After collection of the blood, thick smear was prepared on three clean glass slides and dried completely. The dried thick blood films were dehemoglobinized in water, fixed in methanol for 5 min and dried completely. The slides were stained with 0.02% Brilliant Cresyl Blue (Sigma, Germany) for an hour and washed in water and dried again. The numbers of microfilariae were counted by examining the three stained films for each animal.

Mathematical model and statistical analysis. The periodicity of microfilarial density in the peripheral blood was mathematically analyzed by the trigonometric method of Sasa and Tanaka[17] as expressed by the following formula: $Y=100+1.347xDxcos15(H-K)^0$, where Y is the expected microfilarial count at the hour H, D is the difference of density between the peak hour and the mean (called periodicity index by Sasa and Tanaka[17]), and K is the estimated hour of maximum microfilarial density over 24 h. Pearson's correlation coefficient was used to test for the degree of relationship between the observed microfilarial counts and the expected density of microfilariae estimated from the formula. The Statistical Analysis System Version 8.1 (SAS, Cary, NC) was used for the analysis.

Results

The summary profiles of the 6 dogs examined in this study, according to analytical components are presented in Table 1. The calculated periodicity index was ranged from 25.6 (dog No. 6) to 95.5% (dog No. 3) with mean of 57.6% and the estimated hour of peak was approximately 21:00 hrs (range, 20:04–21:29 hrs) and minimum counts at 09:00 hrs (range, 08:04–09:29 hrs). Correlation coefficient varies depending on dogs, ranging from 6.4 to 49.2%, with no significant difference between observed and expected counts. Periodicity profiles in dogs (Nos. 1-6) used in the present study are shown in Fig 1. Scatter diagram of 6 forms of *D. immitis* according to the periodicity index and the best estimate of peak hour are shown in Fig 2, in together with data from Rhee *et al*[15].

Discussion

The degree of the periodicity of microfilaria in the peripheral blood has been classified conventionally into the five qualitative groups: nocturnally periodic, nocturnally sub-periodic, diurnally periodic, diurnally sub-periodic, and aperiodic[12]. Aperiodic forms show a minimal change in peripheral density throughout the day. Sub-periodic forms, while continuously present in the blood, exhibit periods of high and low density. Periodic forms are alternately rare/absent or at high density in the blood.

Rhee *et al*[15] fitted observed counts to an empirical wave model, in which there were great discrepancies between the actual observed counts and expected counts generated by the model. Of the many approaches for obtaining a periodicity as stated above, the trigonometric method was chosen in the present study since goodness of fit of the model was well evaluated[1,20]. The mean periodicity index of 57.6% and the estimated peak hour at 21:00 hrs with continuous presence in the peripheral blood over the 24 h indicates the nocturnal sub-periodic nature of microfilaremia for all dogs. Exceptionally, mean total count for dog No. 3 was very low, and the estimated peak hour was about 20:04 hrs. Sasa and Tanaka[17] suggested that the periodicity curves drawn from data obtained from carriers with higher microfilarial densities are more reli-

Table 1. Summary profiles of 6 dogs infected with *Dirofilaria immitis*, according to analytical components

Components	Dog number					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Estimated peak hour	21:03	21:01	20:04	21:29	21:01	21:02
Estimated minimum hour	09:03	09:01	08:04	09:29	09:01	09:02
Mean total count	513	39	4	2,332	3,758	30
Periodicity index	76.4	58.9	95.5	60.6	28.3	25.6
Theoretical maximum count	1,040	70	8	4,236	5,188	40
Theoretical minimum count	0	9	0	428	2,327	20
Correlation coefficient*	0.492	0.276	0.242	0.234	0.064	0.483

*Correlation coefficient between observed and expected count.

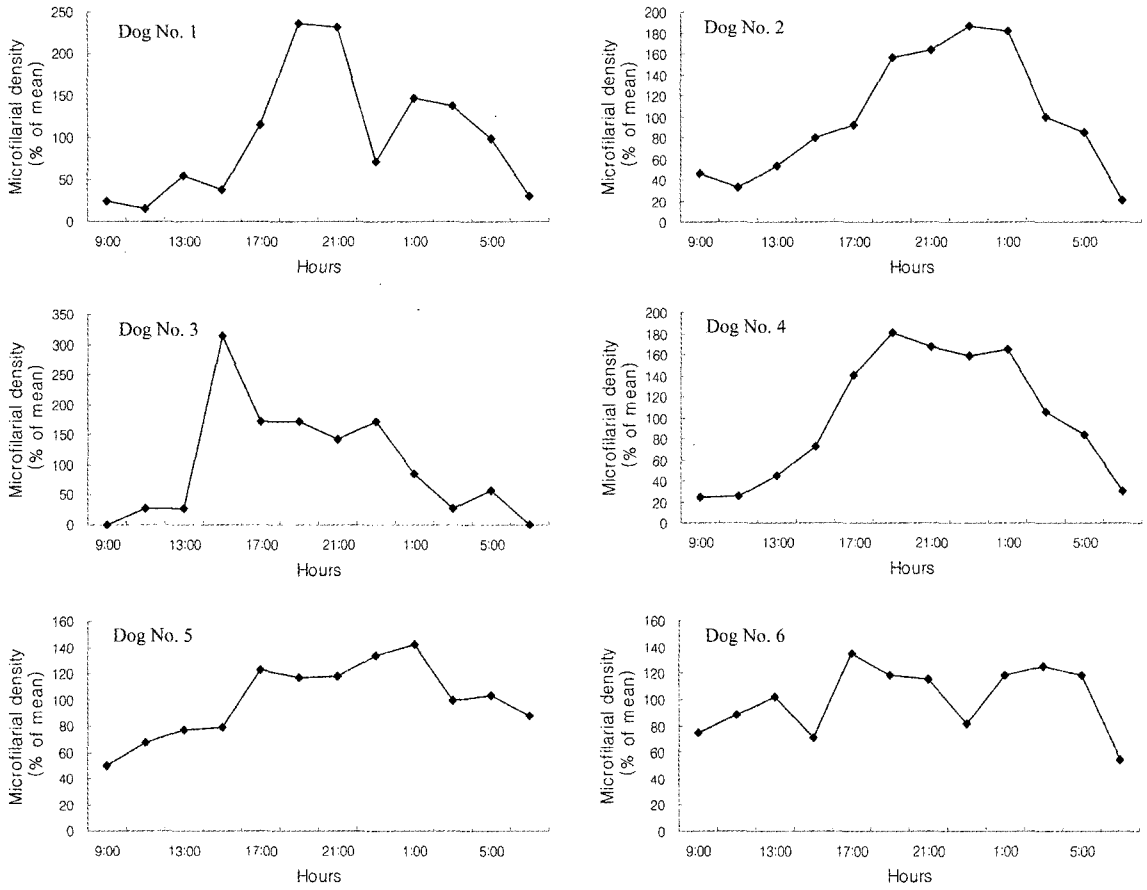

Fig 1. Periodicity profile of *D. immitis* microfilariae in dogs (Nos. 1-6) used in the present study.

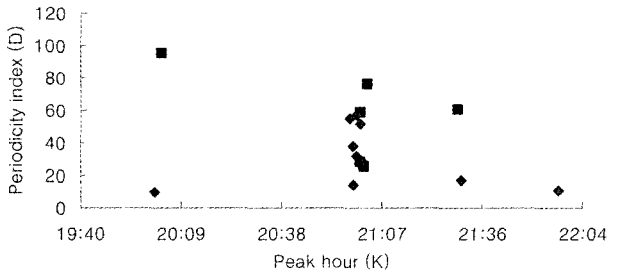

Fig 2. Scatter diagram of various *Dirofilaria immitis* according to the periodicity index (D) and the best estimate of peak hour (K). Squares represent the data from this study and diamonds from Rhee *et al*[20].

able than those with lower densities. As shown in Fig 2, the periodicity indices in the present study were higher than that of previous study[15], in which authors did not calculate index so that based on the raw data we calculated mean periodicity index of 31.3% (range, 9.7-57%). Explanations for this different periodicity pattern between the two studies are: use of different parasites of Dirofilaria immitis, inter-dog variation with different environmental conditions and even circadian variation within dog. Similar nature of the periodicity has been published in dogs[13,24] and cats[12]. Church *et al*[4] reported that *D. immitis* microfilaremia is subperiodic, but cannot be categorized as diurnal or nocturnal because of the variation in the time of peak microfilarial density. On the other hand, both diurnal and nocturnal peaks have been observed in the US[2] and Australia[11]. The influence of different animal hosts on microfilarial periodicity has also been reported in experimentally infected mice, with nocturnal subperiodic microfilareia[7].

The highest density in the blood may occur either diurnally or nocturnally and represents an evolutionary adaptation by filarial worms to maximize microfilarial uptake at mosquito vector activity times[12]. The periodicity if microfilaria has been closely correlated with feeding activity of the vector which acts as the intermediate host[22,26]. The relationship between the density of vector mosquitoes and seropositivity in dogs has never been studied in the country. Previous studies have revealed that different forms exist even within the same filarial species[25]. Furthermore, the correlation coefficient (range, 6.4-49.2%) in this study was not significantly different between observed and expected counts, indicating that variation in the observed counts may not be explained by the expected microfilarial counts. Similar findings were reported by Church *et al*[4], who reported correlation coefficients of 4.5-95.3%. Thus attempts to explore better mathematical model and other factors influencing the variation are needed in the future study.

In conclusion, although the periodicity of *D. immitis* used in this study has shown great variations depending on the dogs, nocturnal sub-periodic nature of microfilaremia was seen for all dogs. Further studies on the periodicity, which seems to vary in different geographic locations, need to be performed to better understanding the dynamics of the periodicity and to help practitioners in the choice of the time for examination of the dogs.

Acknowledgements

This Study was financed by the Institute of Veterinary Science, Kangwon National University.

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개심장사상충의 정기 출현성에 관한 정량적 분석

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요 약: 본 연구에서는 주거환경이 다른 6두의 개를 대상으로 일정한 시간간격으로 채혈하여 만든 염색표본에서 필라리아자충의 수를 측정하여 정기 출현성을 파악하고자 trigonometric 모형을 사용하였다. 모형에 근거하여 계산된 주기성 지수 (periodicity index)는 평균 57.6% (범위: 25.6-95.5%)로 나타났다. 혈액 내 필라리아자충의 수가 최고에 이르는 시점은 21:00 (범위: 20:04-21:29), 최저에 이르는 시점은 09:00 (범위: 08:04-09:29)로 나타났다. 실체로 관찰된 자충의 수와 모형에 의하여 계산된 기대치와의 상관계수는 6.4-49.2%로 자충의 수에 따라 차이를 보였다. 본 연구에 사용된 6두의 실험견 모두 nocturnally sub-periodic 양상을 보였다. 이러한 결과는 기존의 연구에서 보고된 혈중 자충의 수가 최대에 도달하는 시간은 동일하였지만 최저에 이르는 시간은 차이가 있는 것으로 나타났다. 국내 개에서 조사 대상지역에 따른 정기 출현성에 차이가 있는지를 명확히 증명하고 심장사상충의 역동성을 보다 정확하게 이해하기 위해서는 광범위한 조사가 필요할 것으로 사료된다.

주요어: 심장사상충, trigonometric 모형, 정기 출현성, 개