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Periodicity exhibited by *Dirofilaria immitis* microfilariae identified in dogs of Korea

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Abstract: Microfilarial periodicity of *Dirofilaria immitis* (the dog heartworm) was determined at two hr intervals for 72 consecutive hrs in 10 naturally infected war dogs, 3-9 years old, in Korea to facilitate harvest of the microfilariae for possible use in laboratory works and to elucidate further the periodicity of the microfilaria depending on geographic location. Although the periodicity had been observed as being lowgrade nocturnal, maximal microfilarial counts were found at 21:00 hr and minimal at 11:00 hr, giving rise to an evident peak in fluctuation of the larval counts. This is the first record of the periodicity of the microfilariae identified as *D. immitis* in Korea.

Key words: *Dirofilaria immitis*, microfilarial periodicity, dog, Korea

INTRODUCTION

Dirofilaria immitis, the dog heartworm parasitic in the right ventricle and pulmonary artery of the dog, fox, wolf and various other wild carnivores, is common in warm countries particularly in the tropics (Rhee, 1987). In Korea the prevalence rates have been shown to be 23%, 17% or 28% in dogs by means of microfilarial detection, autopsy or antigen test, respectively (Rhee, 1966; Rhee and Rim, 1970; Lee *et al.*, 1996).

Periodicity is a well-known phenomenon which occurs with many filarioid worms, and various hypotheses put forward to explain periodicity have been comprehensively reviewed by Oishi (1959), Hawking (1967), Katamine (1972) and Masuya (1976). Females of *D. immitis* are ovoviviparous and the naked microfilariae may be found in the blood at all times, but there appears to be a periodicity superimposed on this which varies with geographic location (Rhee, 1987). The microfilarial periodicity of *D. immitis* in the peripheral blood stream has already been reported in different countries by various investigators mentioned in discussion. However, the periodicity as well as precise identification of *D. immitis* microfilariae in Korea, so far, has not been recorded. In order to facilitate harvest of the microfilariae for possible use in immunological, laboratory diagnosis and chemotherapeutic studies, the present investigation was designed to examine this aspect. It can be further elucidated the wave pattern of the microfilaria depending on different localities.

MATERIALS AND METHODS

Ten war dogs, 3-9 years old, with varying levels of the microfilariae of *D. immitis* were used in the experiment from June to July 1998. The dogs were screened by a modified Knott's test from 35 individuals in an air base

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of the Republic of Korea Air Force, born and reared only in Korea. In counting the degree of the microfilariae by using an automatic pipette, 0.1 ml of blood was withdrawn from the ear vein at 2 hr intervals over a period of 72 consecutive hrs and thickly smeared on three clean glass slides, respectively. After complete drying the thick blood films, they were dehemoglobinized in water, fixed in methanol, and stained with 0.02% brilliant cresyl blue for one hr. A count was made of arithmetic mean of the numbers of worms in the three thick blood films from each animal and stage. Meanwhile, the differentiation of microfilariae was based on the morphologic characteristics and on the distance of certain fixed points from the anterior extremity as percentages of the total length as a criteria for identification (Rhee, 1987).

All examinations for 72 hrs were repeated, at weekly interval, at least twice with similar results. Data obtained as percentages of mean microfilarial counts at 2 hr intervals were analyzed by Non-Linear Regression procedure in SAS package.

RESULTS

As shown in Table 1, dimension of the naked microfilariae differed between wet and dry preparations. They were 309.2 ± 5.32 × 7.4 ± 0.55 μm in a modified Knott's method fixed in 2% formalin and 255.2 ± 9.11 × 6.2 ± 0.48 μm in dry preparation fixed in methanol. The microfilariae fixed in 2% formalin had a long straight tail and a tapering cranial end. The percentage distance from the anterior end to the fixed points is depicted in Table 2. Thus the mean percentages of the distance of these reference points were: nerve ring, 23.2;

Table 1. Comparison of morphological characteristics of *Dirofilaria immitis* microfilariae by modified Knott's test and dry preparation

Characteristics	Modified Knott's test (wet)[a]	Dry preparation[b]
Length (μm)	309.2 ± 5.32	255.2 ± 9.11
Width (μm)	7.4 ± 0.55	6.2 ± 0.48
Cranial end	tapered	tapered
Tail	straight	some winding

[a]fixed in 2% formalin; [b]fixed in methanol.
Each value represents the mean of 10 determinations per individual with the standard deviations.

Table 2. Percentage distance from the anterior end to the fixed points of *Dirofilaria immitis* microfilariae in dry preparations

Case No.	NR[a]	EP[b]	EC[c]	G1[d]	AP[e]	LTC[f]
1	23.1 ± 0.73	33.7 ± 0.92	38.4 ± 1.27	71.5 ± 1.41	80.9 ± 1.59	87.3 ± 1.10
2	23.2 ± 0.81	34.1 ± 0.86	39.0 ± 0.80	69.5 ± 1.25	79.4 ± 1.60	89.6 ± 1.40
3	24.0 ± 0.95	34.1 ± 0.98	39.8 ± 0.62	70.7 ± 1.40	79.9 ± 1.08	90.7 ± 1.28
4	22.9 ± 1.07	33.1 ± 0.82	38.1 ± 0.94	68.0 ± 1.23	78.0 ± 0.89	89.6 ± 0.97
5	23.1 ± 0.85	33.4 ± 0.55	38.7 ± 0.67	68.6 ± 1.07	78.5 ± 1.24	89.6 ± 1.20
6	22.9 ± 0.25	33.2 ± 0.89	38.5 ± 0.55	68.3 ± 0.98	77.6 ± 0.82	90.0 ± 0.66
7	23.3 ± 0.48	34.1 ± 0.66	38.9 ± 0.80	69.2 ± 1.61	78.6 ± 2.00	89.9 ± 1.24
8	23.3 ± 0.45	33.6 ± 0.51	38.9 ± 0.41	69.2 ± 1.09	79.0 ± 1.05	89.8 ± 1.14
9	22.9 ± 0.83	33.0 ± 0.80	38.3 ± 0.77	68.7 ± 0.98	79.1 ± 0.93	89.7 ± 0.77
10	23.2 ± 0.45	33.4 ± 0.52	38.5 ± 0.47	68.8 ± 0.98	79.2 ± 1.09	89.9 ± 0.92
Mean	23.2 ± 0.31	33.6 ± 0.40	38.7 ± 0.45	69.3 ± 1.03	79.0 ± 0.89	89.6 ± 0.83

[a]nerve ring; [b]excretory pore; [c]excretory cell; [d]first genital cell; [e]anal pore; [f]last tail cell.
Each value represents the mean of 10 determinations with the standard deviations.

Table 3. Mean microfilarial counts of *Dirofilaria immitis* at 2 hr intervals in dogs from Korea

Case	Mean microfilarial counts per 0.1 ml of blood on 24 hr clock for 3 days											
No.	09	11	13	15	17	19	21	23	01	03	05	07
1	62	48	63	110	164	247	374	319	304	289	219	157
	(32)	(24)	(32)	(56)	(84)	(126)	(191)	(163)	(155)	(147)	(112)	(80)
2	3,660	2,941	3,173	3,209	3,294	3,482	4,803	4,339	3,957	3,763	3,700	3,476
	(100)	(81)	(87)	(88)	(90)	(95)	(132)	(119)	(108)	(103)	(101)	(95)
3	792	579	836	839	846	783	898	838	820	805	805	779
	(99)	(72)	(104)	(105)	(106)	(98)	(112)	(105)	(102)	(100)	(100)	(97)
4	5,882	5,079	5,531	5,900	6,436	9,238	7,659	7,380	7,088	6,910	6,092	6,045
	(89)	(77)	(84)	(89)	(97)	(140)	(116)	(112)	(107)	(105)	(92)	(92)
5	3,851	2,376	3,709	4,734	5,984	6,484	7,668	7,390	6,311	5,079	4,322	3,897
	(75)	(46)	(72)	(92)	(116)	(126)	(149)	(143)	(123)	(99)	(84)	(76)
6	8,148	5,037	6,755	6,901	8,861	10,766	13,620	9,873	8,123	7,826	7,020	6,342
	(98)	(61)	(82)	(83)	(107)	(130)	(165)	(119)	(98)	(95)	(85)	(77)
7	526	256	329	357	420	563	1,008	820	655	639	598	551
	(94)	(46)	(59)	(64)	(75)	(100)	(180)	(146)	(117)	(114)	(107)	(98)
8	1,442	1,201	1,257	1,444	1,323	1,516	1,341	1,357	1,492	1,255	1,127	1,060
	(109)	(91)	(95)	(109)	(100)	(115)	(102)	(103)	(113)	(95)	(85)	(80)
9	197	221	293	354	435	625	899	790	239	521	325	233
	(46)	(52)	(69)	(83)	(102)	(146)	(210)	(185)	(56)	(122)	(76)	(54)
10	76	49	61	82	107	187	255	208	187	149	100	85
	(59)	(38)	(47)	(64)	(83)	(145)	(197)	(161)	(145)	(116)	(78)	(66)
Total	24,636	17,787	22,007	23,930	27,870	33,891	38,525	33,314	29,176	27,236	24,308	22,625
	(90.8)	(65.6)	(81.2)	(88.3)	(102.8)	(125.0)	(142.1)	(122.9)	(107.6)	(100.5)	(89.7)	(83.5)

Percentages of mean microfilarial counts per 0.1 ml of blood in each stage on 24 hr clock for 3 days were shown in the parentheses (Asymptotic property, 95%).

excretory pore, 33.6; excretory cell, 38.7; first genital cell, 69.3; anal pore, 79.0 and last tail cell, 89.6.

The chronological changes in the number and percentage of the mean microfilarial counts at 2 hr intervals in peripheral blood of each animal are depicted in Table 3. A significant difference of microfilarial counts was not found among the thick blood films prepared from each animal and stage. Of ten infected dogs, high microfilaraemia — 10^3 unit microfilaria/0.1 ml blood — was observed from five dogs, while low microfilaraemia — 10^2 unit microfilaria/0.1 ml blood — from another five dogs. Maximal microfilarial counts were found at 21:00 hr from eight dogs and at 19:00 hr from another two dogs, while minimal at 11:00 hr except two dogs at 07:00 and 09:00 hr, respectively. The percentage of peak count at 21:00 hr was 142.1 and minimal count at 11:00 hr 65.6. The ratio of the minimum to the maximum of the percentage of microfilarial counts (Max./Min.) was 2.17. The periodicity was observed as lowgrade nocturnal, and in agreement with the equation, $Y = 98.99 + 33.97 \times \mathrm{Sin}\ (15.46 \times X)$, showing that the hour (X) was a parameter (Asymptotic property, 95%; SE, B0-1.342, B1-1.917, B2-0.001).

DISCUSSION

There is no definite theory to explain the mechanism of microfilarial periodicity of many filarioid worms as yet. Much work had been carried out on this phenomenon, and two dominating hypotheses were proposed to explain the mechanism before. One is periodic parturition of larva and the other is of migration of larva itself in the body (Katamine.

1972). Thereafter, a diffuse autofluorescence and numerous fluorescent granules comprising Vitamin A were detected in the highly nocturnal microfilariae of *Wuchereria bancrofti* because the larvae were probably injured by sun light. However, in lowgrade nocturnal larvae of *D. immitis*, were observed less granules (Masuya, 1976). Meanwhile, the physiologic basis for periodicity is unknown; however, several investigators suggest that one or a variety of host factors influence the periodic trend of some microfilaraemias (Hawking, 1967).

The dog heartworm produces microfilariae that circulate in the peripheral blood stream as well as the blood of all other parts of canine body. There is a tendency towards microfilarial periodicity in a day (circadian rhythm) as well as seasonal periodicity showing a summit in summer throughout a year (Oishi, 1959). This appears to vary in different countries. Thus Tarplee and Bradley (1982) found maximal numbers at midnight in the USA; Euzeby and Laine (1951) found the lowest number at 08:00 hr and the greatest at 20:00 hr in France; Webber and Hawking (1955) found minimum parasitemia at 06:00 hr and maximum at 18:00 hr in a Chinese strain of *D. immitis* in England. Moreover, several investigators in Japan have referred to the periodicity of *D. immitis* as being lowgrade nocturnal (Masuya, 1976), and a distinct nocturnal: maximal numbers were found at 24:00 hr and minimal numbers at 10:00 hr and the number of maximum was 6.5 times of minimal count from 28 dogs naturally infected with *D. immtis* (Oishi, 1959).

On the contrary, Angus (1981) reported that there were both a distinct diurnal (16:00 hr) and lowgrade nocturnal (from 24:00 to 01:00 hr) peaks in the periodicity of *D. immitis* microfilariae in cephalic venous blood of dogs in South East Queensland. There was diurnal periodicity — maximal microfilarial counts of *D. immitis* were found at 11:00 hr and minimal at 22:00 hr in a dog from Tanzania (Matola, 1991). Moreover, the microfilaraemia in a dog experimentally infected with *D. immitis* was diurnally subperiodic with maximum microfilaria numbers between 12:00 and 16:00 hr (Grieve and Lauria, 1983) and Schnelle and Young (1944) observed minimum microfilaraemia at 11:00 hr and maximum at 16:30 hr in the USA.

In Korea, the present observation made on 10 cases has shown nocturnal subperiodicity, giving rise to one evident peak in fluctuation of the larval counts. This observation, along with those of other investigators who described nocturnal periodicity, differed markedly with that of Matola (1991), who found maximal numbers at approximately midday in Tanzania and those of Grieve and Lauria (1983) and Schnelle and Young (1944), who reported diurnal subperiodicity in the USA. However, the present observation is nearly in line with the observations of Tarplee and Bradley (1982) in the USA, Euzeby and Laine (1951) in France, Webber and Hawking (1955) in England, Oishi (1959) and Masuya (1976) in Japan, who found nocturnal periodicity in different countries. Overall, it was concluded that the periodicity of *D. immitis* microfilaria in dogs of Korea exhibited lowgrade nocturnal (Asymptotic property, 95%) and closely resembles that in the strain of Japan. It is likely that this geographical variation appears to be influenced by the environmental conditions such as sun light, atmospheric temperature and humidity upon migration of the larva itself rather than host factors, as indicated by Oishi (1959).

Our present findings and those recorded previously by others indicate that the periodicities of *D. immitis* microfilariae are diurnal, nocturnal or both which may be influenced by the geographical location, although the factors for such variation in the periodicity are still poorly understood.

Interestingly, it has been reported that the prevalence of *Dipetalonema recon-ditum* was associated with that of *D. immitis* in the peripheral blood of dogs in some countries. However, we could found only *D. immitis*, referring to the morphological characteristics in Table 1 and the corresponding description in Table 2 of that given by Newton and Wright (1956), in this study.

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=초록=

한국의 개에서 동정된 개심장사상충 미세사상충의 정기출현성

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1998년 6월과 7월 두 달간에 걸쳐 한국의 한 공군기지의 3-9세령의 군용견 35마리에서 스크리닝한 10마리의 개심장사상충 자연감염 개의 이정맥으로부터 2시간마다 72시간 동안 채혈하여 0.1 ml 혈액의 후층도말 brilliant cresyl blue 표본을 만들어 미세사상충을 동정한 다음 그 수를 계산하였다. 이 실험은 일주일 간격으로 두번 실시하였다. 말초혈액에서 검출된 총 미세사상충의 2시간 간격 평균치에 대한 2시간 간격의 자충수의 백분율을 산출한 바 21시에 최고, 11시에 최저이었으며, 비록 최고치는 최저치의 2.17배로서 낮지만 일봉성 야간정기출현성이 인정되었다 (점근성, 95%).

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