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Daytime variation in the density of *Onchocerca volvulus* microfilariae in human skin*

DAVID B. THOMAS,[1] ROBERT I. ANDERSON,[2] & ANNE A. MACRAE [3]

One aim of this study was to determine whether a corneal-scleral punch biopsy instrument provides skin specimens of sufficiently uniform size so as to eliminate the need to weigh or measure the specimens in order to provide a reproducible index of the density of Onchocerca volvulus *microfilariae in the skin. A second aim was to determine whether the density of microfilariae in the skin varies with the time of day. The reports of others on the latter are contradictory. Duplicate skin specimens were taken from 10 people four times during one day with a corneal-scleral punch biopsy instrument. Each specimen was weighed and measured, and the number of microfilariae were counted. Although the specimens were not of uniform weight or surface area, the number of microfilariae per specimen was as good an index of the density of larvae in the skin as the number of microfilariae per mg, or per mm² of skin. This may not be true when other methods of procuring skin specimens are utilized. A universally acceptable standard method is advocated. The density of microfilariae in the skin approximately doubled from morning to midday, and began to decline by early evening.*

With some predominantly blood-borne species of microfilariae, the number per blood smear is known to vary with the time of day. Thus, the microfilariae of *Brugia malayi* are most readily found in blood obtained during the hours of night; *Wuchereria bancrofti* microfilariae are also found in the blood mainly at night, except in the Polynesian Islands where no temporal periodicity occurs; the microfilariae of *Loa loa* are usually found during the day; and those of *Dipetalonema perstans* and *Mansonella ozzardi* exhibit no periodicity.

The microfilariae of *Dipetalonema streptocerca* and *Onchocerca volvulus* are normally found in the skin. Duke et al. (3), in studies carried out in Cameroon on both the " Sudan-savanna " and " forest " strains of *O. volvulus*, observed that the number of microfilariae per mg of skin increased significantly during the hours after noon. Their findings are in contrast to those made in Upper Volta by Lartigue (unpublished report to WHO, 1966), who detected the lowest number of *O. volvulus* microfilariae in the skin at midday. These contradictory observations suggested the need for further investigation.

In the present study, the density during the daylight hours of microfilariae of *O. volvulus* in human skin was determined, and the different methods used for quantifying microfilariae in the skin were compared and their precision assessed.

MATERIAL AND METHODS

This study was performed as an adjunct to a broadly based epidemiologic investigation [4] conducted in the village of Baoussi on the Adamaoua plateau in north central Cameroon, an area endemic for onchocerciasis.

Seven men and three women between 31 and 64 years of age who had microfilariae of *O. volvulus* in biopsies of their skin were studied. By means of a Holth corneal-scleral biopsy punch with a 1-mm bite, duplicate specimens of superficial skin were taken from the lumbar region of the back at 08 h 00,

* From the Department of Epidemiology, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205, USA. Supported by Contract No. DADA17-72-C-2060, US Army Research and Development Command, Washington, D.C.

[1] Assistant Professor of Epidemiology.

[2] Associate Professor of Epidemiology and International Health.

[3] Research Associate, Department of Epidemiology and International Health.

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11 h 00, 14 h 00, and 17 h 00 on a single day. The duplicate specimens taken at the same time were obtained from areas separated by no more than 2 cm. The 2 specimens taken at each of the 4 times of day were obtained alternately from the left and right sides of the body, and always from areas more than 10 cm apart. Each skin specimen was placed in a drop of 0.85% saline on a welled microscope slide and examined under a compound microscope at low power. The microfilariae in each specimen were identified and counted.

The skin specimens were stored in individual test tubes containing 10% formalin solution. They were subsequently blotted, dried in air, and weighed on an analytical balance in the laboratory. All specimens appeared elliptical. The major and minor axes of each were measured with a Bausch and Lomb precision magnifier with a 15×0.1 mm division transparent scale. The surface area (A) of each was estimated by the formula

$$A = \pi ab$$

where a is one-half the major axis and b is one-half the minor axis. For each specimen, the number of microfilariae per mg and per mm² of skin were calculated.

RESULTS

Table 1 shows the mean, standard deviation, median, and range of values for 4 measures of specimen size (weight, length, width, and area), and for 3 measures of microfilarial density (microfilariae/specimen, microfilariae/mg skin, and microfilariae/mm² skin), based on all 80 skin specimens. The specimens are unimodally distributed with respect to all 7 variables, and skewed toward the higher values (the means are greater than the medians). There is a broad range of values for all the variables except for the widths of the specimens.

Table 1. Size of 80 skin specimens, and numbers of microfilariae per specimen, per mg of skin, and per mm² of skin.

	Mean	Standard deviation	Median	Range
specimen size				
weight (mg)	0.47	0.20	0.40	0.10–1.15
length (mm)	1.57	0.34	1.46	1.0 –2.5
width (mm)	1.08	0.10	1.03	0.7 –1.3
area (mm²)	1.34	0.38	1.27	0.60–2.55
microfilariae per:				
specimen	38.9	22.7	34	6–96
mg skin	90.0	53.5	70	20–240
mm² skin	29.4	16.3	26	7–74

Precision of measurements

The skin specimens were taken in duplicate to provide data needed to determine the precision (reproducibility) of a single measurement. The index of precision used is the standard error of a single measurement (technical error), which is calculated according to the formula

$$S_r = \sqrt{\Sigma d^2/2k}$$

where d is the difference between the first and second measurements for each pair of measurements, and k is the number of pairs of measurements, i.e., 40.

The technical errors associated with the above 7 variables are shown in column 1 of Table 2. If a single skin specimen taken with a 1-mm corneal-scleral punch weighed x mg, then one could say with 95% confidence that a second specimen similarly obtained would not differ in weight from x mg by more than 1.96 times the technical error (i.e., by more than $1.96 \times 0.11 = 0.22$ mg). The technical errors for the length, width, and area of specimens, and for the number of microfilariae per specimen may be used for similar estimates of reproducibility.

If a single specimen contained y microfilariae per mg of skin, then one could say with 95%

Table 2. Technical error and coefficient of variation of the weight, length, width, and area of skin specimens, and of the number of microfilariae per specimen, per mg, and per mm² of skin.

	Technical error	Coefficient of variation
specimen size		
weight	0.11 mg	0.23
length	0.25 mm	0.16
width	0.08 mm	0.07
area	0.28 mm²	0.21
microfilariae per:		
specimen	8.37 mf[a]/specimen	0.22
mg skin	30.95 mf/mg	0.34
mm² skin	7.17 mf/mm²	0.24

[a] mf = microfilariae

confidence that the true number of microfilariae per mg of skin in the area from which the specimen was taken lay between the observed value, y, plus and minus 1.96 times the technical error ($1.96 \times 30.95 = 60.66$ microfilariae per mg). The technical error associated with the number of microfilariae per mm² of skin may be used in a similar manner.

The coefficient of variation (CV) was calculated for the 7 variables using the formula

$$CV = S_r/\bar{X}$$

where "$\bar{X}$" is the mean value for the variable (Table 1). These are shown in column 2 of Table 2. Because this is a dimensionless statistic the values shown in the table may be compared.

The specimens varied much more in length than in width, and these differences in length led to considerable variability in the area of the specimen as well. These differences presumably resulted from variation in the size of the bites taken. Values for the width of the specimen are primarily a function of the size of the instrument, which was constant. Variability in the specimen's weight is undoubtedly also related to differences in length; the thickness of the specimen may also vary and influence weight, but this was not investigated. The coefficients of variation for the weight and area of specimens are nearly equal. The numbers of microfilariae per specimen and per mm² of skin show the same degree of variability; but the number per mg appears to be a less precise measure of microfilarial density.

Relationship between specimen size and number of microfilariae

The contingency coefficient is a measure of the correlation between two variables that is not influenced by differences in time or between subjects. It is based on the relative differences between the values for duplicate specimens taken from the same individual at the same time. Table 3 shows the data used to calculate the contingency coefficients that depict the relationship between the weight of the specimen and the number of microfilariae per specimen, and the relationship between the area of the specimen and the number of microfilariae per specimen. In 21 of the 40 pairs of specimens (52.5%) the relative differences in the weight and in the number of microfilariae corresponded; the relative differences in the area and in the number of microfilariae corresponded in 19 of the pairs (47.5%).

The chi square (χ^2) with four degrees of freedom

Table 3. Distribution of 40 pairs of skin specimens by the relative differences [a] in their weight and area, and in the number of microfilariae

		Differences in the no. of microfilariae			
		−	0	+	total
differences in the weights of specimens	−	7	1	7	15
	0	2	4	3	9
	+	2	4	10	16
total		11	9	20	40
differences in the areas of specimens	−	6	2	6	14
	0	3	3	4	10
	+	2	4	10	16
total		11	9	20	40

[a] − = value for the second specimen of a pair is less than that of the first by > 10 %.
0 = value for the second specimen of a pair differs from that of the first by ⩽ 10 %.
+ = value for the second specimen of a pair exceeds that of the first by > 10 %.

based on the data for the weights and the number of microfilariae is 8.22 ($0.05 < p < 0.10$). The corresponding contingency coefficient (C) is 0.41, as calculated by the formula:

$$C = \sqrt{\chi^2/(k + \chi^2)}$$

where k is the number of pairs, 40. The chi square based on the data for the areas of the specimens and the number of microfilariae is 4.52 ($0.30 < p < 0.50$), and the corresponding contingency coefficient is 0.32.

Values for the contingency coefficient that approach unity indicate a strong correlation. The numbers of microfilariae are therefore not highly correlated with the weights or areas of the specimens. The difference between the two contingency coefficients is not appreciable.

Relationship between microfilariae per specimen and microfilariae per mg and per mm²

The number of microfilariae per skin specimen is well correlated with the number of microfilariae per mg of skin (correlation coefficient = 0.73), and with the number of microfilariae per mm² of skin (correlation coefficient = 0.87). These correlation coefficients were calculated from values for all 80 specimens.

Table 4. Mean values (for each of 10 individuals from whom duplicate biopsies were taken four times on one day) of the weights and surface areas of skin specimens, and the numbers of microfilariae per specimen, per mg skin, and per mm² skin

Individual	Mean skin specimen size		Mean no. of microfilariae per:		
	weight (mg)	area (mm²)	specimen	mg skin	mm² skin
1	0.83	1.31	43.5	53.4	34.4
2	0.63	1.61	43.6	80.3	31.7
3	0.49	1.33	36.9	75.1	27.2
4	0.33	1.18	22.8	73.8	19.4
5	0.43	1.29	65.5	157.4	51.5
6	0.36	1.24	22.6	68.6	19.1
7	0.58	1.82	37.4	69.3	21.3
8	0.31	1.05	15.3	48.4	13.8
9	0.44	1.48	67.1	150.0	44.1
10	0.31	1.14	34.6	123.4	31.1
average	0.47	1.34	38.9	90.0	29.4

Variability among persons and with time.

Columns 1 and 2 of Table 4 show respectively the mean weight and area of the 8 specimens taken from each subject, and columns 1 and 2 of Table 5 show respectively the mean weight and area of the 20 specimens taken from the 10 individuals at each of the 4 times of day. Two-way analyses of variance revealed that both the weight and area of the specimens varied significantly among the 10 participants ($p < 0.01$), but that the slight variation in values for these 2 variables with time was not significant. These observations are consistent with the clinical impression that specimens were more easily obtained from people with thin, dry skin than from those whose skin was thick and oily.

Table 5. Mean values at four different hours of day (based on duplicate specimens from 10 individuals at each time) of the weights and surface areas of skin specimens, and the numbers of microfilariae per specimen, per mg skin, and per mm² skin.

Time of day	Mean skin specimen size		Mean no. of microfilariae per:		
	weight (mg)	area (mm²)	specimen	mg skin	mm² skin
08 h 00	0.47	1.42	22.7	53.6	16.3
11 h 00	0.55	1.43	51.6	105.5	36.7
14 h 00	0.43	1.19	42.0	105.9	34.4
17 h 00	0.44	1.34	40.0	94.9	30.1
average	0.47	1.34	38.9	90.0	29.4

Columns 3, 4, and 5 of Table 4 show respectively the mean numbers of microfilariae per specimen, per mg, and per mm² of skin for each subject. The same columns in Table 5 show the mean values of these 3 variables for the 4 times of day. Two-way analyses of variance showed that all 3 indices of microfilarial density varied significantly ($p < 0.01$) both among individuals, and with time. The numbers of microfilariae in the skin increased from 08 h 00 to 11 h 00, changed little by midafternoon, and declined somewhat by 17 h 00.

For each variable, the analysis of variance only tests whether the mean value at any one time is significantly different from the values for the other three times. Table 6 shows, in addition, that the temporal variation in the density of microfilariae in the skin was fairly consistently observed in all 10

Table 6. Number of individuals (out of 10) with an increase in 3 measurements of microfilarial skin density from morning [a] to midday [b], and the number with a decrease from midday till evening [c]

Measurement	No. with an increase from morning to midday	No. with a decrease from midday to evening
microfilariae/skin specimen	10 ($P < 0.001$)	8 ($P > 0.05$)
microfilariae/mg skin	9 ($P < 0.05$)	6 ($P > 0.05$)
microfilariae/mm² skin	10 ($P < 0.001$)	7 ($P > 0.05$)

[a] Based on 2 specimens taken at 08 h 00.
[b] Based on 2 specimens taken at 11 h 00 and 2 taken at 14 h 00.
[c] Based on 2 specimens taken at 17 h 00.

subjects, although the probability level of significance of 5% was not reached by the number with a decrease from midday to evening. This is true for all three measures of microfilarial density.

DISCUSSION

Punch biopsy instruments have been advocated for use in epidemiologic studies of onchocerciasis in the belief that they provide skin specimens of relatively uniform size (1, 5). The advantage of such uniformity is that the specimens would not have to be weighed or measured, and the counts of microfilariae per specimen made in the field would immediately provide a reproducible index of their density in the skin.

Although all the specimens in this study were collected by one investigator (DBT) using a single instrument, they were not of uniform size. They varied in both weight and area from one individual to the other, which is probably in part attributable to differences in skin thickness and texture. In addition, the specimens taken from the same individual at the same time also exhibited considerable variation in size. The areas of the specimens, and undoubtedly also their weights, varied because of differences in their length, a function of the size of the bites taken. Further investigations are required to determine if the size of the bite can be rendered more uniform.

In spite of the lack of uniformity in specimen size, the number of microfilariae per specimen was still at least as good an index of the number of larvae in the skin as the number per mg, or the number per mm^2 of skin. This is supported by four pieces of evidence: 1) the coefficient of variation (reproducibility) of the microfilariae per specimen was smaller than that of the microfilariae per mg, or per mm^2; 2) there was only a low degree of correlation between the number of microfilariae per specimen and the specimen's weight or area; 3) the number of microfilariae per specimen was well correlated with the number per mg or per mm^2 of skin; 4) the variability between individuals and with time in the density of microfilariae in the skin was demonstrated as well by the number of microfilariae per specimen as by the number per mg or mm^2 of skin. It is concluded that it is unnecessary to weigh or measure skin specimens taken with the Holth corneal-scleral biopsy punch with a 1-mm bite. Further studies should be carried out to determine if this is also true for specimens taken with larger instruments of the same type.

Using a punch biopsy instrument of larger size than that employed in the present investigation, Picq et al. (5) took duplicate skin specimens from 48 persons, placed them in distilled water, and compared the number of microfilariae that emerged after 30 minutes from one specimen of each pair with the number that emerged after 120 minutes from the other. These two time-dependent measures of microfilarial density were well correlated (correlation coefficient = 0.89). This suggests that a larger punch biopsy instrument may provide more precise indices of the density of microfilariae in the skin than the 1 mm instrument used in the present study, although the data of Picq et al. cannot be directly compared with the results of this investigation. Kershaw et al. (4) took multiple skin specimens from a single individual using a needle and razor. The coefficient of variation of the number of microfilariae per mg of skin calculated from their data (0.05 to 0.17) is less than that reported in this study (0.34), suggesting that their method is also more precise than the corneal-scleral punch biopsy method we used. Further studies based on multiple specimens taken from the same individuals by different means should be carried out to compare the precision and accuracy of the different methods for quantifying microfilariae in the skin. Such studies would provide a rational basis for the development of standard methods for measuring the density of microfilariae in the skin. A universally acceptable standard method would greatly facilitate epidemiologic studies of onchocerciasis.

Duke et al. (3) observed that the number of microfilariae in the skin was highest during the afternoon hours, and related this to changes in the temperature/saturation deficiency and the biting density of *Simulium damnosum*. Bull et al. (2) demonstrated *in vitro* that microfilariae migrate toward heat and away from serum and sweat, and hypothesized that the fluctuations noted by Duke et al. resulted from changes in these factors in the subepidermal layers the of skin in response to variations in external temperature and humidity. The results of the present study are consistent with these observations. All three measures of the density of microfilariae in the skin used in this investigation indicate that the density increased from morning to midday, and decreased by early evening. The morning increase was observed in all 10 subjects. The evening decrease was less pronounced and not seen in all the individuals. This is not surprising if the migration of microfilariae in the skin is in response to environmental factors that vary with the time of day. The last specimens were probably taken too early to reflect maximally the onset of nocturnal conditions.

RÉSUMÉ

VARIATIONS DE LA DENSITÉ DES MICROFILAIRES D'*ONCHOCERCA VOLVULUS* DANS LA PEAU HUMAINE AU COURS DE LA JOURNÉE

La présente étude avait deux objectifs: d'une part, étudier les variations du nombre des microfilaires d'*Onchocerca volvulus* dans la peau humaine pendant la journée et, d'autre part, comparer et évaluer les diverses méthodes de numération des microfilaires dans la peau.

Chez 7 hommes et 3 femmes âgés de 31 à 64 ans, porteurs de microfilaires d'*O. volvulus*, on a prélevé, en double, à l'aide d'une pince à biopsie de Holth, des échantillons de peau au niveau de la région lombaire à 8, 11, 14 et 17 heures, le même jour. Les microfilaires ont été identifiées et comptées sur place; les échantillons de peau ont été placés dans une solution de formol à 10%, puis ultérieurement séchés, pesés et mesurés au laboratoire.

Les échantillons prélevés sur le même individu au même moment variaient considérablement en surface et en poids, en raison de la longueur différente des tissus enlevés. Le poids et la surface des échantillons variaient aussi fortement d'un individu à l'autre, mais non en fonction du moment du prélèvement. Le nombre des microfilaires par échantillon et le nombre par mm² de peau fournissaient des indices de la densité microfilarienne d'une précision quasi égale; le nombre de larves par mg de peau s'est révélé une mesure moins reproductible. On ne notait qu'une faible corrélation entre le nombre de microfilaires par échantillon et le poids ou la surface de ce dernier, mais un rapport satisfaisant entre ce nombre et le nombre par mg ou mm² de peau. On en conclut que le nombre de microfilaires par échantillon est un indice de la densité larvaire aussi acceptable que le nombre par mg ou par mm² de peau et qu'il n'est pas nécessaire de peser ou de mesurer les échantillons, dans les conditions de la présente étude. Cette conclusion peut cependant n'être plus valable si l'on utilise d'autres modes de prélèvement, peut-être plus précis. On souligne la nécessité de disposer d'une méthode standard pour mesurer la densité des microfilaires dans la peau.

La densité des microfilaires, mesurée par le nombre de larves par échantillon, par mg ou par mm² de peau, a varié notablement entre individus et aux différentes heures de la journée. Elle a approximativement doublé entre le matin et midi et a commencé à diminuer au début de la soirée.

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