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QUANTITATIVE STUDIES ON THE EMERGENCE OF *ONCHOCERCA VOLVULUS* MICROFILARIAE FROM SKIN SNIPS

ISAO TADA[1], ISAO IWAMOTO[2] AND TEFERRA WONDE[3]

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Abstract: The authors made studies on the emergence of *O. volvulus* microfilariae from skin snips in order to assess accurate MFD in onchocercal infections from quantitative view point. The results obtained are as follows: Skin snips should not be teased into small pieces, but the intact snips should be incubated for a longer period than the teased ones. The distribution of microfilariae in a minute skin area is quantitatively even in most cases. This finding suggests the usefulness of this method to compare MFD of the adjacent skin regions to each other. However, the comparison of MFD with extremely different-sized snips should be avoided. There were no significant changes in MFD by warming skin surface.

For the diagnosis of human onchocerciasis, the skin snip method has been widely used as an essential and standard method. During a period of epidemiological survey of onchocerciasis in Ilubabor Province, Ethiopia, the present authors attained the conclusion that the microfilaria density (MFD) of skin snips obtained was highly affected by the teasing process and incubation time. For example, when the biopsies were teased by the technique recommended by several previous workers, there were some microfilariae which were still migrating out from the newly cut surface of the fragmented tissue 15 to 20 minutes after incubation. Small numbers of microfilariae newly released were observed from time to time by additional incubation. Furthermore, many microfilariae were found torn into small pieces so that they were immobile. These findings suggested the possibility that the teasing process caused mechanical damage to the microfilariae in the biopsies. This process naturally might lead to an inaccurate MFD. It was inconvenient for the authors to assess the densities of microfilariae under several chemical stimulants, unless a standard method was established. For this reason, the authors made quantitative studies on the emergence of *Onchocerca volvulus* microfilariae from skin snips in order to establish a standardized method for the skin snips which could be of epidemiological

1 Dept. of Medical Zoology, Faculty of Medicine, Kagoshima University, Kagoshima, Japan (Present address: Dept. of Medical Zoology, Kanazawa Medical University, Uchinada, Ishikawa, Japan). 2 Dept. of Parasitology, Institute for Tropical Medicine, Nagasaki University, Nagasaki, Japan (Present address: Dept. of Internal Medicine, in same Institute). 3 Dept. of Medical Zoology, Imperial Central Laboratory & Research Institute, Addis Ababa, Ethiopia.

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and experimental importance.

Materials and Methods

In Abdella, Ilubabor Province of Ethiopia, the experiments were performed, the first one in August and the second in November, 1971. Our preliminary survey with skin snippings revealed that the microfilaria rate was 80.7% in male adults from this village. Volunteers were then picked among these adults and brought to the Dabana Missionary Station for the experiments.

All of the experiments were undertaken at room temperature; in August, it ranged from 18.0 to 23.0 C., and in November, from 17.5 to 21.5 C.

Every skin snip was taken from the left buttock of volunteers with a needle and a surgical blade. The detailed technique to obtain the skin snip was described by Duke (1962). When multiple snips were needed from one volunteer, every snip was taken 1 cm apart from the others. Thereafter, skin snips were placed into drops of physiological saline on slides.

The slide was then placed in an optical apparatus which magnified and projected the shape of the skin snip on a piece of paper put on the top of the apparatus. The area of the skin snip was obtained by counting the number of smallest sections (1 mm^2) encircled by the outline of the projected biopsy. One square millimeter of the actual skin snip area was equivalent to 49 mm^2 on the paper. The precise area of the biopsy was obtained by calculation in mm^2. The measured snips in physiologic saline were incubated at room temperature for a determined time and were transferred to the saline on the next slide carefully with a small forceps. This process was repeated continuously. The number of microfilariae which were left behind was immediately counted under 50 $\times$ magnification of a binocular microscope. The microfilaria density (MFD) was calculated by dividing the total number of microfilariae which were released from one snip by the individual snip area in mm^2.

The microfilariae found from these volunteers were identified as those of *Onchocerca volvulus* from the morphological feature of the stained specimens and from the measurement of its anatomical landmarks (Iwamoto et al., 1972).

Results

1. The effect of teasing the skin snip on the emergence of microfilariae

Three snips were taken from each of the 6 volunteers (from T–1 to 6). The first snip was torn into small pieces with needles for about 30 seconds (snip P), the second one, coarsely torn into two pieces (snip M) and the third one (snip G) was not teased. Skin snips were then incubated for approximately 22 hours and the MFD obtained is shown in Table 1. The result of this experiment has been already reported in a brief article by the present authors (Tada et al., 1973). The highest MFD is seen almost in snip G. On the other hand, the snips P, which were torn into small pieces due to the previously recommended technique released less microfilariae than the others. When the highest MFD from 3 snips is described as 1.00, the relative MFD of the other two snips are calculated by proportions.

The relative MFD in average is as follows: 0.92 in snip G, 0.79 in M and 0.50 in P, respectively. In contrast to the method recommended by various previous researchers, the present data clearly show that skin snips should not be torn into pieces to assess the accurate MFD.

TABLE 1 The effect of teasing the skin snips on the MFD

Case No.	MFD		
	Snip type* G	M	P
T-1	16.6 (0.61)***	27.3** (1.00)	17.4 (0.64)
T-2	11.8 (1.00)	10.7 (0.91)	6.4 (0.54)
T-3	21.2 (1.00)	11.7 (0.55)	9.1 (0.43)
T-4	4.0 (0.89)	4.5 (1.00)	1.6 (0.36)
T-5	111.9 (1.00)	84.7 (0.76)	89.6 (0.80)
T-6	46.1 (1.00)	23.7 (0.51)	10.8 (0.23)
Average of the relative MFD (6 cases)	0.92	0.79	0.50

* Snip type: G, non-teased; M, coarsely teased; and P, teased into small pieces

** The under-lined count shows the highest MFD among the 3 snips from the same individual

*** Relative MFD: When the highest MFD of a snip among the 3 snips is determined as 1.00, the relative MFD of the others is obtained by proportional calculations

In this experiment, as shown in Table 2, the number of microfilariae released were counted right from the beginning every 20 minutes to 120 minutes, and every 60 minutes from 120 to the end of the incubation. The end of incubation ranged from 8 to 22 hours depending on the emergence of microfilariae from individual snips. The table shows the cumulative percentage of microfilariae from 3 types of snips in association with the incubation time while cumulative percentage of microfilariae was apparently the lowest in snip G at any incubation time. For example, snip P released almost 90% of microfilariae in average at 80 minute incubation, while only 67.6% of microfilariae emerged from snip G in average. This fact does not contradict the above mentioned conclusion that skin snips should not be teased, but it may indicate that the living microfilariae would emerge easily and quickly within a short time from the teased snips. But it should be also beared in mind that teased skin snips could lead to a wrong conclusion. The teasing would hinder the accurate MFD of each skin snip by causing mechanical damage to the microfilariae in the skin.

TABLE 2 The effect of teasing on the recovery rate* of microfilariae in individual snip types arranged by incubation time

Incubation time (min.)	Cumulative percentage of microfilariae released from skin snips (average of 6 cases)		
	Snip type** G	M	P
20	36.2	50.4	61.4
40	50.0	65.5	76.1
60	60.1	77.0	84.0
80	67.6	81.8	89.7
100	72.0	85.6	92.3
120	75.6	88.9	94.2
180	84.6	93.8	96.0
240	86.9	96.5	99.1
300	91.7	97.1	99.4
360	93.4	99.1	99.9

* Recovery rate (in percent): The ratio of microfilaria count at individual incubation time to the total count of microfilatiae obtained from the identical snip

** Snip type: as shown in Table 1

From this experiment, it is concluded that skin snips should not be teased, in particular for the quantitative assessment of MFD in human skin.

2. The distribution of microfilariae in the skin

To assess the changes in MFD for some quantitative studies, the distribution of microfilariae should be even in some small skin regions. Based on this viewpoint, the authors examined if the microfilariae were evenly distributed in minute skin regions or not by comparing the MFD of 3 skin snips from each of 8 volunteers (from N–1 to 13). The skin snips were taken in a triangular shape, 1 cm apart from each other from the left buttock of volunteers. Those snips were then incubated for 24 hours and the individual MFD obtained and the average MFD and percentage deviation of the individual MFD of three snips from the average one are shown in Table 3. The MFD of 3 different snips highly coincided with each other in most of the cases examined. Fig. 1 clearly shows that in case of the subjects whose MFD are below 10, the maximal measurement error of MFD is 20% or more. On the other hand, the error is markedly reduced in proportion to the increase of the average MFD. For this reason, it is quite appropriate to use volunteers whose MFD is 20 or more for the purpose of quantitative studies in order to minimize the measurement error within 10%. In this experiment, however, the MFD of case N–8 unexpectedly fluctuated notwithstanding its proper MFD in its average. This finding may suggest the rare presence of uneven distribution of microfilariae even in closely adjacent skin regions. However, generally speaking, in onchocercal infections, it may be concluded that the MFD is uniform in small skin regions.

TABLE 3 Comparisons of the MFD in minute skin regions

Case No.	Snip No.	Snip area in mm²	Mf* count per snip	MFD (x)	Average MFD (M)	Difference of individual MFD from the average one	
						Difference in MFD ($x-M$)	Difference in percentage**
N-1	1	7.37	256	34.74	36.34	−1.60	−4.40
	2	6.49	238	36.67		+0.33	+0.91
	3	6.94	261	37.61		+1.27	+3.49
N-4	1	8.43	189	22.42	22.08	+0.34	+1.54
	2	7.47	164	21.95		−0.13	−0.59
	3	6.08	133	21.88		−0.20	−0.91
N-5	1	5.59	127	22.72	21.25	+1.47	+6.92
	2	6.45	123	19.07		−2.18	−10.26
	3	6.24	137	21.96		+0.71	+3.34
N-6	1	5.49	230	41.89	42.83	−0.94	−2.19
	2	3.94	172	43.65		+0.82	+1.91
	3	5.33	229	42.96		+0.13	+0.30
N-8	1	5.02	201	40.04	54.69	−14.65	−26.79
	2	3.86	192	49.74		−4.95	−9.05
	3	5.02	373	74.30		+19.61	+35.86
N-9	1	5.16	278	53.88	55.20	−1.32	−2.39
	2	4.18	237	56.70		+1.50	+2.72
	3	6.16	339	55.03		−0.17	−0.31
N-10	1	6.18	53	8.58	9.01	−0.43	−4.77
	2	3.61	27	7.48		−1.53	−16.98
	3	3.65	40	10.96		+1.95	+21.64
N-13	1	7.29	174	23.87	27.28	−3.41	−12.50
	2	7.55	221	29.27		+1.99	+7.29
	3	6.69	192	28.70		+1.42	+5.21

* Mf: microfilaria

** Difference in percentage: $\frac{x-M}{M} \times 100(\%)$. The highest absolute value of the percentage among the 3 snips was regarded as the measurement error and graphically shown in Fig. 4

3. Relation between incubation time and the emergence of microfilariae

The authors tried to examine the relation between incubation time and the emergence of microfilariae in non-teased skin snips, which were taken from 4 persons whose MFD was as follows: 272.0 in Tm-1; 63.2 in Tm-2; 160.6 in Tm-3; and 22.0 in Tm-4 cases, respectively. Each fresh skin snip was quickly transferred successively to the next slide at intervals of 1 minute during the incubation period ranging from 1 to 20 minutes, and then at 60 minute intervals from 1 to 6 hours. The size of the skin snip was measured after 20 minute incubation in this experiment. The skin snips were incubated for 19 to 30 hours until no more microfilariae were observed. In the experiment which was performed in August, only a single skin snip was examined which was taken from 4 volunteers. Fig. 2 shows the emergence of microfilariae from a single skin snip during an incubation time ranging from one to 360 minutes. In November, however, a similar experiment was repeated

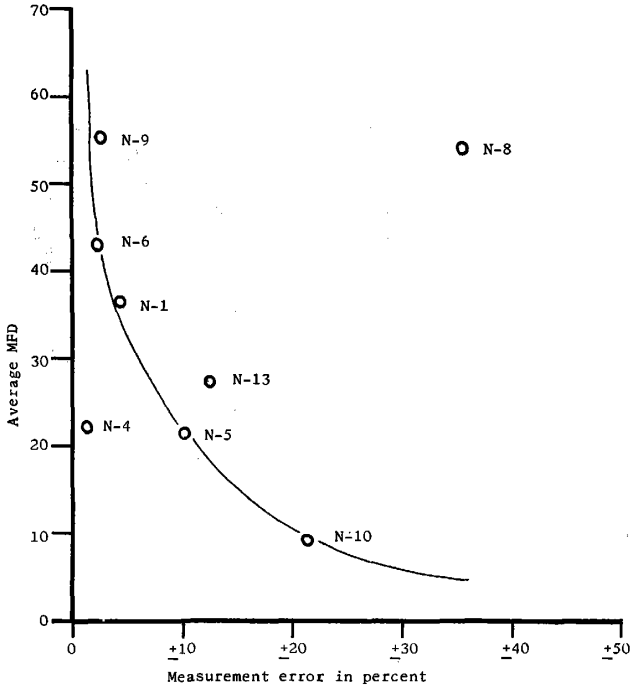

Fig. 1 Relation between the measurement error and the average MFD of 3 biopsies from the same subject.

Measurement error: $\frac{a-M}{M} \times 100\ (\%)$

(M; arithmetic mean of MFD from 3 snips, a; the farthest MFD from M)

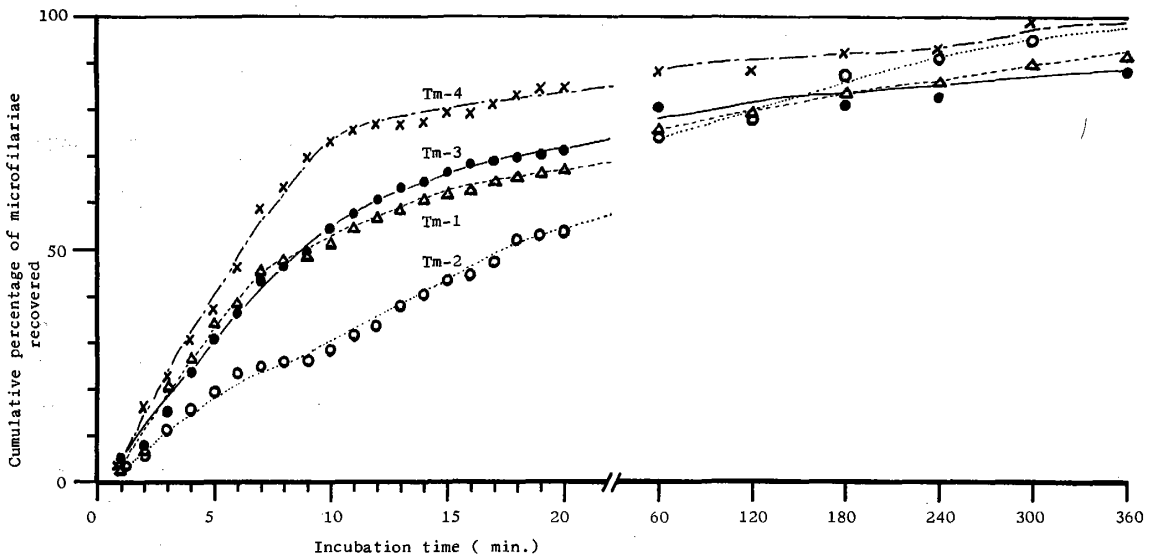

Fig. 2 The emergence of microfilariae from the skin snips of 4 volunteers (fromTm-1 to 4).

by using 3 snips from one volunteer to check the previous result. The result of the latter experiment is shown in Table 4 (group N), in which the transfer of skin snips was made 7 times in the whole incubation only to see the general tendency of the

TABLE 4 The emergence of *O. volvulus* microfilariae from skin snips

Incubation time (min.)	The average cumulative percentage of microfilariae released	
	group T* (6 cases)	group N** (8 cases)
15	—	21.4
20	36.2	—
30	—	35.3
40	50.0	—
60	60.1	46.2
80	67.6	—
100	72.0	—
120	75.6	56.5
180	84.6	66.8
240	86.9	72.6
300	91.7	78.3
360	93.4	—

* group T: volunteers examined in experiment 1
** group N: volunteers examined in experiment 2

microfilarial emergence. This table also shows similar observations made in the experiment 1. All of these experiments revealed similar results. In contrast to the results reported by Duke (1962) and to the recommendation by WHO (1966), the emergence of microfilariae from non-teased skin-snips was more prolonged than expected. In the first experiment, the recovery rate of microfilariae at 20 minute incubation revealed that 66.9% in Tm–1, 53.6% in Tm–2, 71.5% in Tm–3 and 84.9% in Tm–4, respectively. In the experiment carried out in November, however, only 35.3% of the total microfilariae were released by 30 minute incubation. On the contrary, the third series of experiments were quite similar to the results of the first one. From these findings, it can be concluded that skin snips should be incubated for at least 5 to 6 hours at a temperature of about 20 C to assess the accurate MFD. From a practical view point, however, it is inconvenient to incubate skin snips for such a long time for the mass examination of human onchocerciasis.

In August, 1971, the authors examined the inhabitants in Dedessa, Ilubabor Province of Ethiopia. Thirty-three microfilaria positives were found out of 54 cases examined with 15 minutes of incubation using the non-teased snip method. Then the skin snips from 21 negatives left were incubated again for 45 minutes longer. Three new positives with a low MFD were found from these negative cases. This fact may also suggest the importance of a longer incubation even for the epidemiological and routine examinations of onchocerciasis to pick up positives with low MFD. Incubation for 1 hour may satisfy the practical purpose of mass examinations when unteased snips are used.

4. Relation between skin snip size and MFD

From each of 5 volunteers (from S–1 to 5), 3 adjacent skin snips of different size were taken to compare the MFD. The authors called the largest snip L, the smallest one S, and the medium one M from their relative difference in size. In this experiment, L, M and S meant the relative size of biopsies taken from the same person. The actual size in mm^2 is seen in Fig. 3. The skin snips were incubated for 8 to 29 hours depending on the emergence of microfilariae. The MFD of each skin snip among the different sizes obtained is also shown in Fig. 3. This experiment clarified that the highest MFD was usually seen in the snip whose area ranged from 5 to 8 mm^2. This finding suggests that one should not compare the MFD of skin snips of extremely different size with each other.

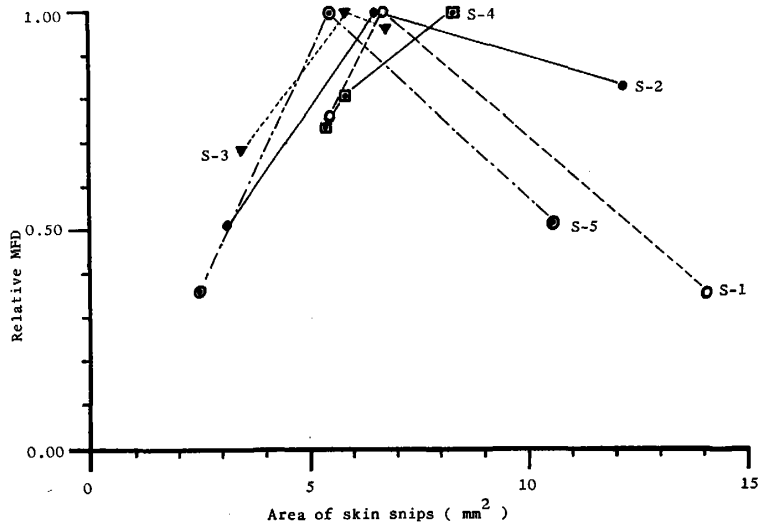

Fig. 3 Relation between skin-snip size and MFD.

5. The effect of warming the skin surface on MFD

Rodger (1957) reported that when the surface temperature fell, microfilariae tended to move more deeply into the dermis. This phenomenon will apparently cause the reduction of MFD in the epidermis. Nelson (1970) stated that in East Africa it had been common practice to apply hot water bottles to the skin to encourage microfilariae to migrate to the superficial layer. These reports seem to indicate that the microfilariae are sensitive to changes of environmental temperature. However, according to the latter author, there are no data to substantiate the validity of this technique. When MFD of the skin is actually increased by warming, the warming technique may have diagnostic validity. In order to assess this point, the present authors examined the MFD of positives before and after warming the skin surface.

The left buttock of volunteers (from N–14 to 20) were covered with a sheet of black-colored polyvinyl immediately after the first snip was removed to exclude the effect of light. The sheet was then heated indirectly by an electric bulb (500 W) about 50 cm apart. The temperature of the sheet was kept at 41 C by adjusting the distance between the bulb and the buttock. The second snip was taken adjacent

to the first one after 15 minutes of warming. The MFD of the two skin snips obtained before and after heating were calculated and compared. The result is shown in Table 5. In cases N–14 and 15, MFD was markedly reduced after warming. On

TABLE 5 The effect of warming the skin surface on the MFD

Case No.		N–14	N–15	N–16	N–17	N–18	N–19	N–20
MFD	First snip* (MFDa)	34.69	21.13	84.28	13.14	31.67	37.61	24.76
	Second snip** (MFDb)	22.99	12.18	88.93	21.69	40.11	38.54	30.41
Difference in MFD		−11.70	−8.95	+4.65	+8.55	+8.44	+0.93	+5.65
Difference in percent***		−33.7	−42.4	+5.5	+65.1	+26.7	+2.5	+22.8

* before warming

** This snip was taken 1 cm. apart from the first one, 15 minutes after the beginning of the incubation.

*** $\frac{\text{MFDb}-\text{MFDa}}{\text{MFDa}} \times 100(\%)$

the other hand, the MFD significantly increased after warming in cases N–17, 18 and 20. No significant changes were observed in cases N–16 and 19. From this brief experiment, it is not easy to reach a final conclusion whether the behavior of the microfilariae was constant or not after warming the skin.

DISCUSSION

In onchocerciasis, teasing of the skin snips has been adopted by most of the previous workers for the quick detection of microfilariae. According to Duke (1962) it was thought essential to tear the snips with needles so that all the contained microfilariae could be freed. He observed about 90% of microfilariae had come out from skin snips after 5 minutes and constant total counts had been obtained at 10–15 minutes. In case of animals infected with *Onchocerca gutturosa,* Nelson et al (1966) showed that many of the microfilariae failed to emerge into the saline unless the skin snips were teased. Lagraulet et al (1967) also lightly teased the biopsies with 2 needles and examined them after 10 minutes of incubation. In contrast to the above quoted results, as the result of the present study shows, the teasing procedure mechanically damaged microfilariae in the skin and caused a reduction in the MFD. This suggests that when the unteased skin snips are incubated for 5 to 6 hours, an accurate MFD will be obtained. Therefore, the present authors conclude that as far as the recovery rate of microfilariae is concerned, torn snips reveal poor and inaccurate MFD.

Some workers did not consider the individual MFD as reliable. Rodger and Brown (1957) used IDF (Individual density figure) and DQ (Density quotient) based on the known anatomical distribution of the microfilaria population and not on microfilaria counts. Because the MFD was shown to be subject to wide variations

in the same site from day to day. Duke (1968) used multiple weighed skin snips to assess the concentration of microfilariae. As microfilariae are not evenly distributed all over the body surface, it seems preferable to use those methods to assess the density of infections. At the same time, it will be necessary to assess the accurate MFD of small skin regions depending on the nature of experiments, such as the effects of treatment, physical and chemical stimulation. Experiment 2 shows that the microfilariae are evenly distributed in small skin areas. This finding may help in the future to perform quantitative studies on the changes in MFD under different conditions.

According to Duke (1962), although the MFD of adjacent snips is usually approximately constant, the assessed densities might vary by as much as 1 : 3 because of the occurrence of pocketing of microfilariae. This is reasonable because the presence of nodules and/or adult worms would disturb the even distribution of microfilariae in the skin. An uneven distribution of microfilariae was also reported in the case N–8 by the present authors. This fact, however, might not indicate the unreliability of MFD because of its rare occurrence. The use of multiple unteased snips will diminish the possible errors of this kind and reveal accurate MFD.

In the present study, the authors did not weigh the skin snips, but measured their surface area. There are apparently some advantages in measuring the area of skin snips: 1) The snip is easily measured in a few drops of saline on a glass slide. This enabled us to avoid time elapsing which frequently causes snips to dry before weighing. 2) The apparatus is easily transferred. On the other hand, in case of weighing, it needs electricity and some adjustment. This is impractical in rural areas. 3) When skin snips are taken almost in a constant thickness, it is considered that the MFD reflects exclusively the 2–dimensioned distribution of microfilariae. This solved one of the inconveniences which are frequently shown in weighing snips that a thin broad biopsy might weigh the same as a narrow deep one which was stated by Rodger and Brown (1957). Lagraulet and Bard (1969) also preferred an estimation of density based upon surface area, because according to them, this method has a theoretical advantage over a method based upon weight of a biopsy which may include some tissue beneath that part of the upper dermis. According to Rodger and Brown, the weighing of biopsies of such small pieces of tissue, the rate of drying in tropical climates and the time elapsing before weighing might all be probable sources of error. The measurement of the surface of skin snips with irregualr outline is somewhat more difficult than weighing them. However, as shown in the experiment 2, this method enabled us to get almost equivalent MFD of some biopsies in minute skin regions. For these reasons, mentioned above, in order to be able to follow the changes of MFD in several experiments, the present method is considered to be of value.

As to the size of skin snips, Duke (1962) used bloodless circular or oval snips about 3–5 mm in diameter and weighing 1–4 mg from volunteers for the skin snip method. Further, Duke et al (1967) concluded that the concentration of mf/mg was independent of the weight within the range from 1 to 3 mg snips based on the examinations of 360 skin snips. In the experiment 4 of the authors, who examined the relation between the size of skin snips and their MFD, the skin snips whose sur-

face area ranged from 5 to 8 mm^2 were considered best. It can be speculated that all the microfilariae will not migrate out in an extremely large snip due to mechanical factors. In extremely small snips, the relative area of the epidermis where microfilariae are usually present might be reduced in comparison with the whole area of the skin snip. Furthermore, the vertical distribution of microfilaria population and the thickness of skin snips seem to have affected MFD of biopsies in different sizes. To overcome this kind of obstacle, the use of a skin punch method (Lagraulet and Bard, 1969) might solve the problem.

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皮膚切片からの *Onchocerca volvulus* マイクロフィラリアの遊出に関する定量的研究

多田　功[1]・岩本　功[2]・テフェラ・ウオンデ[3]

エチオピア南西部のオンコセルカ症患者について皮膚切片からのマイクロフィラリア(mf)遊出を定量的に検討した。これは従来のSkin snip法が定量的でないため，精密なMFD(皮膚内mf密度)を必要とする実験のための検討である。その結果，皮膚片は細切すべきでないこと，更に生理的食塩水中でのインキュベート時間は十分長く行なうべきことが明らかにされた。この方法で近接皮膚領域のMFDを計測した結果，きわめて満足すべき値を得た。唯この際，大きさの極端に異なる皮膚片相互のMFDは多少異なることが判明した。この定量的方法を用いて，皮膚を温めた場合のMFDを測定したが加温による変動はまちまちで一定の成績を示さなかった。

1 鹿児島大学医学部　医動物学教室　2 長崎大学熱帯医学研究所　寄生虫学部門　3 エチオピア帝国中央研究所　医動物学部門