Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 43 (1986)
Heft: 2

Artikel: Detection of "Dirofilaria immitis" microfilariae in peripheral blood : a quantitative comparison of the efficiency and sensitivity of four techniques

Autor: Feldmeier, H. / Bienzle, U. / Schuh, D.

DOI: https://doi.org/10.5169/seals-313620

Acta Tropica 43, 131–138 (1986)

[1] Landesinstitut für Tropenmedizin, Berlin
[2] Bernhard-Nocht-Institut für Schiffs- und Tropenkrankheiten, Hamburg
[3] Institut für Medizinische Statistik und Dokumentation, Universität Erlangen

Detection of *Dirofilaria immitis* microfilariae in peripheral blood

A quantitative comparison of the efficiency and sensitivity of four techniques

H. Feldmeier[1], U. Bienzle[1], D. Schuh[1], R. Geister[2], I. Guggenmoos-Holzmann[3]

Summary

Four techniques for the detection and quantification of *Dirofilaria immitis* microfilariae in peripheral blood were compared, namely the conventional thick smear, thick films prepared by cytocentrifugation, filtration of blood through polycarbonate membranes, and densitiy gradient centrifugation followed by membrane filtration. The efficiency of the methods and their sensitivity was assessed by determining quantitatively the recovery of known numbers of microfilariae from defined volumes of blood. Polycarbonate membrane filtration either alone or in combination with density gradient centrifugation showed an efficiency of 0.9 and 0.8, respectively, and reached 100% sensitivity with microfilariae densities of $\geqslant 10$ microfilariae per ml of blood. Conventional thick smears and cytocentrifugation were of considerably lower efficiency (0.02 and 0.03, respectively) and reached 100% sensitivity with more than 200 microfilariae per ml.

Key words: microfilariae; membrane filtration; efficiency; sensitivity.

Introduction

The diagnosis of filariasis and the identification of microfilariae is based on the demonstration of filariae in blood, lymph or connective tissue. Numerous techniques have been devised for the detection of microfilariae in peripheral blood.

Correspondence: Dr. H. Feldmeier, Landesinstitut für Tropenmedizin, Königin-Elisabeth-Strasse 32–42, D-1000 Berlin 19

The concentration of parasites by filtration through cellulose acetate membranes was introduced by Bell (1967) and modified by Chularek and Desowitz (1979). More recently, polycarbonate membranes have been used (Dennis and Kean, 1970). Comparative studies proved that the filtration techniques are superior to all other techniques (Desowitz et al., 1973; Southgate, 1974; Abaru and Denham, 1976). However, since all investigations were performed with unknown densities of microfilariae, comparison of the efficiency was only possible on a qualitative but not on a quantitative level.

We therefore designed a series of experiments which permitted the quantitative evaluation of the efficiency of four techniques to detect known numbers of microfilariae in a defined blood volume. A mathematical formula was used to compare the efficiency of the methods, taking into account inherent characteristics such as the blood volume examined and the loss of microfilariae due to the techniques.

Materials and Methods

Preparation of microfilariae densities

Venous blood from beagles infected with *Dirofilaria immitis* was drawn into EDTA-coated Venoject tubes (Terumo, Tokyo, Japan). Red blood cells were haemolysed with distilled water and then washed 3 times (2000 rpm, 5 min) with Hank's balanced salt solution (HBSS). The supernatant was removed and one drop of the microfilariae rich sediment was transferred to a microscope slide. Under an inverted microscope a defined number was aspirated into a microcapillary of 6.6 µl volume. The capillary was then emptied into a polystyrene test tube and examined again to detect remaining microfilariae. The tube was filled with different volumes of blood from a microfilariae free human donor and then used for investigation.

A preliminary series of experiments was carried out with 1, 3, 5, 10, 20 and 40 microfilariae in 1 ml, 3 ml and 5 ml of blood, respectively. For quantitative comparison of the four techniques 20, 40 and 60 microfilariae were then dispersed in 1 ml of blood.

Thick blood film

Twenty µl of a blood sample with a known number of microfilariae were spread on a microscope slide over a surface of approximately 6 cm^2, dried for 24 h at room temperature and then haemolysed with distilled water for 3 min. The slides were stained with Giemsa stain for 30 min.

Cytocentrifugation

One ml of blood with a known number of microfilariae was haemolysed with distilled water. After washing 3 times with HBSS the supernatant was removed, leaving 100 µl of sediment. Two 50 µl aliquots of the sediment were centrifuged at 400 rpm for 10 min in a cytocentrifuge (Shandon, Great Britain). Before staining with Giemsa stain the slides were allowed to dry over night.

Polycarbonate membrane filtration

Polycarbonate membranes of 25 mm diameter with 3 or 5 µm pores as well as swin-lock filter holders were obtained from Nuclepore Corp. (USA). 15 ml of distilled water and a defined volume of blood with a known number of microfilariae were aspirated into a 20 ml plastic syringe. The syringe was vigorously shaken to haemolyse the red blood cells and adjusted vertically on top of the filter holder. Filtration was facilitated by means of a waterjet pump. Using the same syringe the membrane was rinsed with distilled water (three times 20 ml), followed by blowing air (two times 20 ml) through the filter. The polycarbonate membrane was transferred to a slide, dried and stained with Giemsa stain.

Density gradient centrifugation

Density gradient centrifugation followed by membrane filtration has been described elsewhere (Feldmeier et al., 1981). In brief, a density gradient was achieved by high-speed centrifugation (26,000×*g*, 45 min) using an iso-osmolar Percoll solution (Pharmacia, Freiburg, Germany) of a density of 1.090 g/ml. Blood with a known number of microfilariae was carefully layered on top of the Percoll gradient, followed by centrifugation with 2000×*g* for 20 min. The microfilariae containing layer was then filtered through a polycarbonate membrane (pore size 5 µm) as described above.

Statistical analysis

Microfilariae counts are assumed to follow a Poisson distribution (Sasa, 1976). The probability of a positive parasite count in a sample of v ml blood can therefore be estimated by $p = 1 - e^{-v \cdot d}$ where d is the microfilariae density per ml. When a given technique is applied beside the sample volume v an intrinsic loss of parasites has to be taken into account. The rate of microfilariae actually detectable by the technique is called recovery rate. The efficiency of a method can be described by a product $r \cdot v$ of recovery rate and volume examined. The rate of positive microfilariae counts, i.e. the sensitivity of the technique is then a function of the microfilariae density d and the efficiency $r \cdot v$. Hence, sensitivity can be defined as

$$\% \text{ Sensitivity } S = 100 \cdot (1 - e^{-(r \cdot v) \cdot d})$$

The homogeneity of observed counts was assessed by the Snedecor-Irwin test.

Results

Polycarbonate membrane filtration

A series of experiments was performed with a wide range of microfilariae densities in order to analyse the relationship between microfilarial density, pore size and number of microfilariae recovered. The results are shown in Table 1. Homogeneity of the data distribution was scrutinized for each pore size (3 µm and 5 µm, respectively) and for each test volume (1 ml, 3 ml and 5 ml) by the Snedecor-Irwin test. Homogeneity was confirmed not only within all groups, but also when the results of the tests with different pore size and test volume were combined ($\chi^2 = 27.954$, $v = 29$, $p = 0.520$). From a total of 1738 microfilariae seeded into 132 blood samples 1504 microfilariae were encountered on the membranes. The average recovery was 0.865 per seeded microfilaria.

Quantitative comparison of the techniques

For the quantitative evaluation of the techniques standard concentrations of 20, 40 and 60 microfilariae in one ml were prepared. The results are shown in Table 2.

In a thick blood film only 0.02 ml from a sample of one ml is used for screening for microfilariae. Thus the low efficiency of the technique is mainly due to the small test volume. The efficiency of the cytocentrifugation is slightly better than that of the thick film. However, the diagnostic advantage of this method is the perfectly preserved morphology of the parasites. For the polycarbonate membrane filtration the whole blood sample volume was used in the experiment. Therefore the efficiency of 0.889 is due to a reduced recovery rate, which is largely independent of the microfilariae density (see also Table 1). If a

Table 1. Recovery of microfilariae by polycarbonate membrane filtration using membranes with 3 µm and 5 µm pore size

Pore size	Sample volume (ml)/ Number of experiments	Microfilariae seeded per experiment	Microfilariae recovered per series of experiments		Test on homogeneity
			observed/ expected	mean	
3 µm	1/5	1	4/ 5		
	2/5		4/ 5		
	1/5	3	11/ 15		
	2/5		12/ 15		
	1/5	5	23/ 25		
	2/5		17/ 25	684/790 = 0.866	p = 0.143
	1/5	10	46/ 50		
	2/5		42/ 50		
	1/5	20	91/100		
	2/5		86/100		
	1/5	40	178/200		
	2/5		170/200		
5 µm	1/4	1	4/ 4		
	3/4		3/ 4		
	5/4		3/ 4		
	1/4	3	11/ 12		
	3/4		10/ 12		
	5/4		9/ 12		
	1/4	5	17/ 20		
	3/4		17/ 20		
	5/4		16/ 20	820/948 = 0.865	p = 0.800
	1/4	10	35/ 40		
	3/4		35/ 40		
	5/4		36/ 40		
	1/4	20	69/ 80		
	3/4		62/ 80		
	5/4		68/ 80		
	1/4	40	142/160		
	3/4		142/160		
	5/4		141/160		

density gradient centrifugation is performed before polycarbonate membrane filtration the recovery of microfilariae is significantly lower ($p = 0.03$), but much higher than that of the thick film and the cytocentrifugation method ($p < 0.001$).

Table 2. Comparison of the four methods

Method	Micro-filariae per ml	Number of experiments	Microfilariae expected[1]/ observed[2]	Efficiency[3] r · v	Sensitivity (%)[4]
Thick blood film	20	48	960/ 19	0,020	33,0
	60	18	1080/ 22	0,020	70,4
Cytocentrifugation	20	44	880/ 24	0,027	41,72
	60	4	240/ 8	0,033	86,19
Polycarbonate membrane filtration[5]	20	9	180/160	0,889	100,0
	40	9	360/320	0,889	100,0
Density gradient centrifugation combined with polycarbonate membrane filtration	20	8	160/128	0,800	100,0

[1] Assuming a sample volume of 1 ml per experiment
[2] Observed in the subsamples of technique specific volume
[3] The efficiency of the method is equivalent to the ratio observed : expected
[4] According to the formula $S = 100\ (1 - e^{-(r \cdot v) \cdot d})$; see Materials and Methods
[5] Test results for both pore sizes (3 µm and 5 µm) are combined

Relation between microfilariae density and sensitivity

In order to demonstrate the relationship between microfilariae density and sensitivity, the equation $S = 100 \cdot (1 - e^{-(r \cdot v) \cdot d})$ was applied to a range of hypothetical microfilariae densities (Table 3). The polycarbonate membrane filtration and the density gradient centrifugation approach 100% sensitivity if only 10 microfilariae are present in one ml of blood. In contrast, the sensitivity of the thick smear and the cytocentrifugation increases slowly with increasing microfilariae concentration and approaches 100% for a concentration of 500 microfilariae and 200 microfilariae per ml, respectively.

Discussion

Comparison of techniques used to detect microfilariae has been performed with human or animal blood samples containing unknown numbers of microfilariae. Therefore the information on their efficiency is incomplete. It is, however, desirable to have a quantitative measure of the efficiency of a technique if, for example, in a patient latent microfilaraemia has to be ruled out with a known degree of certainty. In epidemiological surveys this knowledge is equally important to determine the accurate prevalence of microfilariae carriers (Sasa, 1976).

Based on this rationale we designed a series of experiments which enabled us to assess the efficiency and sensitivity of different methods by seeding dis-

Table 3. Sensitivity of thick blood film, cytocentrifugation, polycarbonate membrane filtration and density gradient centrifugation combined with polycarbonate membrane filtration in relation to microfilariae density. The efficiency of the methods is considered to be constant.

Sensitivity (%)[1]				
Microfilariae per ml	Thick blood film[2]	Cytocentrifugation[2]	Polycarbonate membrane filtration[2]	Density gradient centrifugation followed by polycarbonate membrane filtration[2]
	r · v = 0,020	r · v = 0,027	r · v = 0,889	r · v = 0,800
0.02	<0.1	<0.1	1.8	1.6
0.05	0.1	0.1	4.3	3.9
0.1	0.2	0.3	8.5	7.7
0.2	0.4	0.5	16.3	14.8
0.5	1.0	1.3	35.9	33.0
1	2.0	2.7	58.9	55.1
2	3.9	5.3	83.1	79.8
5	9.5	12.6	98.8	98.2
10	18.1	23.7	100.0	100.0
20	33.0	41.7	100.0	100.0
50	63.2	74.1	100.0	100.0
100	86.5	93.3	100.0	100.0
200	98.2	99.6	100.0	100.0
500	100.0	100.0	100.0	100.0

[1] Sensitivity $S = 100\ (1 - e^{-(r \cdot v) \cdot d})$

[2] The efficiency (r · v) is taken from Table 2

tinct numbers of microfilariae into human blood samples. Our results show that a single thick blood film is a very inefficient technique and that a sensitivity of 100%, i.e. at least one microfilaria will be found by microscopical examination, is only achieved if the parasite density is above 500 per ml. This finding is in accordance with the observation of other authors (Partono et al., 1973; Desowitz and Southgate, 1973; Southgate, 1974). Cytocentrifugation for the concentration of microfilariae is somewhat tedious and the efficiency is also low. The excellent preservation of the parasite morphology, however, is an unquestionable advantage. This method may be employed to facilitate distinction between different microfilariae species.

In our experiments almost 90% of the microfilariae seeded into blood were recovered by polycarbonate membrane filtration, regardless whether membranes with 3 μm or 5 μm pores were used. A sensitivity of 100% was found for microfilariae concentrations above 10 per ml of blood, clearly demonstrating the high efficiency of this technique. Our findings confirm the contention of

Dennis et al. (1976), who regarded polycarbonate membrane filtration as the optimal method in the clinical laboratory as well as under field conditions. It is, however, important to note that small microfilariae may be lost when membranes with 5 µm pores are used (Abaru and Denham, 1976). In this case, membranes with 3 µm pores are recommended.

The density gradient centrifugation followed by membrane filtration shows almost the same efficiency as the polycarbonate membrane filtration. As the amount of blood which can be passed through a membrane is not only restricted by the pore size, but also by the occurrence of microclotting due to the use of an inappropriate anticoagulant or an undue delay between venipuncture and processing (Abaru and Denham, 1976), the density gradient centrifugation may be useful if large volumes of blood are to be examined.

Based on the observation of Sasa (1976) that the probability of detecting microfilariae in peripheral blood follows a Poisson distribution we introduced a formula which allows for correction of the intrinsic loss rate and the volume used in a given technique. Thereby it became possible to determine to which extent the first or the second factor was responsible for the efficiency of a technique. Although the loss of microfilariae during staining of a thick film has been reported (Denham et al., 1971; Southgate, 1974), this intrinsic loss is of little influence on the efficiency of the technique. The low product ($r \cdot v = 0.02$) is mainly attributable to the small test volume of 20 µl. Therefore, improvement of the efficiency can only be achieved by increasing the number of thick blood films to be examined. Loss of microfilariae has also been reported in the course of polycarbonate membrane filtration (Abaru and Denham, 1976). However, the loss is negligible for the efficiency of the filtration technique since due to the large test volume a sensitivity of 100% is already achieved at low microfilariae densities (below 10 microfilariae per ml).

In view of these findings we suggest to reevaluate the efficiencies of the various techniques currently used for the diagnosis of microfilariaemia. This will facilitate the choice of an appropriate method for the different types of clinical and epidemiological studies.

Acknowledgments

We thank Dr. Walter, Department of Biochemistry, Bernhard-Nocht-Institute Hamburg, who provided animals infected with *Dirofilaria immitis*. The advice of Prof. Kuhlow and Prof. Büttner is gratefully acknowledged. Data of this communication are part of medical thesis (D.S.).

Abaru D. W., Denham D. A.: A comparison of the efficiency of the nuclepore and millipore filtration system for detecting microfilariae. Southeast Asian J. trop. Med. Pub. Hlth *7*, 367–369 (1976).

Bell D.: Membrane filters and microfilariae: a new diagnostic technique. Ann. trop. Med. *61*, 220–223 (1967).

Chularerk P., Desowitz R. S.: A simplified membrane filtration technique for the diagnosis of microfilaraemia. J. Parasit. *56*, 623–624 (1970).

Denham D. A., Dennis D. T., Ponnudurai T., Nelson G. S., Guy F.: Comparison of counting chamber and thick smear methods of counting microfilariae. Trans. roy. Soc. trop. Med. Hyg. *65*, 521–526 (1971).

Dennis D. T., Kean B. H.: Isolation of microfilariae: report of a new method. J. Parasit. *57*, 1146–1147 (1971).

Dennis D. T., McConnel E., White G. B.: Bancroftian filariasis and membrane filters: are night blood surveys necessary? Amer. J. trop. Med. Hyg. *25*, 257–262 (1976).

Desowitz R. S., Southgate B. A.: Studies on filariasis in the Pacific. 2. The persistence of microfilaraemia in diethylcarbamazine-treated populations of Fiji and Western Samoa: diagnostic application of the membrane filtration technique. Southeast Asian J. trop. Med. Pub. Hlth *4*, 179–183 (1973).

Desowitz R. S., Southgate B. A., Mataika J. U.: Studies on filariasis in the Pacific. 3. Comparative efficacy of the stained blood-film, counting chamber and membrane filtration technique for the diagnosis of *Wuchereria bancrofti* microfilaraemia in untreated patients in areas of low endemicity. Southeast Asian J. trop. Med. Pub. Hlth *4*, 329–335 (1973).

Feldmeier H., Bienzle U., Schuh D.: Combination of techniques for concentration and identification of microfilariae from peripheral blood. Trans. roy. Soc. trop. Med. Hyg. *75*, 251–253 (1981).

Partono F., Cross J. H., Demijati P., Oemijati S.: Evaluation of thick smear, Knott and membrane filtration methods for demonstrating microfilariae in blood. Trop. Geogr. Med. *25*, 286–289 (1973).

Sasa M.: Analysis and evaluation of filariasis survey data. 11 D. The efficiency of detection of microfilariae in human filariasis. University of Tokyo Press, 1976.

Southgate B. A.: A quantitative approach to parasitological techniques in bancroftian filariasis and its effect on epidemiological understanding. Trans. roy. Soc. trop. Med. Hyg. *68*, 177–186 (1974).