ISOLATION OF AMOEBAE OF THE GENERA *Naegleria* AND *Acanthamoeba* FROM PUBLIC FOUNTAINS IN GALICIA (N.W. SPAIN)

ESPERANZA PANIAGUA CRESPO, MARGARITA MARTI MALLEN, Mª P. MONICA PENAS ARES, Mª CRISTINA ARIAS FERNANDEZ* and Mª PILAR COMBARRO COMBARRO

Departamento de Microbiologia y Parasitología, Facultad de Farmacia, Universidad de Santiago de Compostela, Santiago de Compostela, España

(Received April 9, 1990; revised August 27, 1990)

Abstract. Tests for free-living amoebae in water from 11 public fountains in Galicia were conducted at two seasons of the year, winter and summer. Only one fountain gave a negative result for the presence of amoebae in both samples. Twenty five strains were isolated, 14 belonging to the genus *Acanthamoeba* and 10 to the genus *Naegleria*. The *Acanthamoeba* strains were made up of the species *A. polyphaga, A. quina, A. castellanii* and *A. paradivionensis*. No relation was found between temperature, presence of free chlorine in the water and whether the water was drinkable and the presence or absence of amoebae, nor with the number of strains present in the water samples. The pathogenic capacity of the strains isolated from the genera *Naegleria* and *Acanthamoeba* was tested in vivo. It was found that of the 22 strains that would grow at 37 °C, 4 (3 strains of *Acanthamoeba* and 1 of *Naegleria*) caused the death of a statistically significant number of mice that had been inoculated intracerebrally, and the presence of amoebae was confirmed in the brains of all the animals inoculated. Intranasal inoculation caused less mice deaths than intracerebral inoculation, and less organs were found containing amoebae.

1. Introduction

The presence of amoebae in waters is one of the principal epidemiological factors in the spread of encephalitis produced by amoebae. Many people in Galicia frequently use public fountains for domestic use. This has prompted us to look for and study amoebae that belong to the genera pathogenic in man, *Naegleria* and *Acanthamoeba*, in public fountains.

2. Materials and Methods

Sample collection and processing

Twenty two were taken, 11 in winter and 11 in summer, from fountains located in heavily populated areas with a high number of users. One-liter samples were placed in sterile glass containers and vacuum filtered through 1.2 μm diameter meshes once in the laboratory. The filters were placed upside down into Petri dishes

* Colegio Universitario de Vigo, Apartado 874, 36200 Vigo, España.

Water, Air, and Soil Pollution **53**: 103–111, 1990.

containing 2% non-nutritive agar in distilled water which were incubated at 27 °C for 24 hr, after which the filters were removed and a few drops of a suspension of heat-killed *Escherichia coli* (*E. coli*) were added to each dish (to act as a nutrient for the amoebae that might be left). The Petri dishes were incubated at 27 °C and inspected daily under the microscope, and those with no amoebae after 30 days were deemed negative. The distinct amoebae in the positive Petri dishes were isolated with the aid of an inverted microscope (Molet and Kremer, 1976). Strains were identified using the criteria of Pussard and Pons (1977), Molet and Kremer (1976) and Rafalko (1951).

All the samples were analyzed for bacteria and the free-chlorine level in the water was determined by the orthotoluidine method (Casares, 1977). The parameters employed to determine the degree of bacteriological contamination of the water were: total coliforms (TC), fecal coliforms (FC), *E. coli*, fecal streptococi (FS), sulphite-reducing clostridia (SRC) and heterotrophic bacteria (HB). The count of coliforms and fecal streptococi was done using the most-probable-number method (MPN) (Standard Methods, 1985). The count of *E. coli* was done using the procedure developed by Grunnet and Gundstrup, 1977. Numerical estimation of sulphite-reducing clostridia were carried out using the procedure developed by Wilson and Blair (Bonde, 1977) and the heterotrophic bacteria was made using the spread plate method using bacto nutrient agar medium (Standard Methods, 1985).

Pathogenicity studies

All the isolated strains were submitted to survival tests at 37 and 42 °C. These were carried out on Petri dish cultures during 15 days. After this, subcultures of the surviving amoebae were made in order to test their growing capacity at these temperatures. Each of the strains that had been capable of surviving at 37 °C were studied for their pathogenic capacity in vivo in lots of seven CD-1 mice using both intracerebral (i.c.) and intranasal (i.n.) inoculations (Cerva, 1967a, b; Madrigal *et al.*, 1985). The i.c. dose was 3000 to 4000 trophozoites suspended in a volume no greater than 30 μL, and 25 000 to 35 000 trophozoites in a volume no greater than 80 μL were administered for the i.n. inoculation. A control lot of seven animals was inoculated in both cases. The i.c. inoculated animals were examined daily for 15 days, after which the animals that had not died spontaneously were killed and their brains removed. I.n. inoculated mice were monitored for 30 days and the brain, lung and liver were removed.

(a) A macroscopic examination to look for evident lesions. If these were found, a specimen from the lesion was seeded in 2 general bacteriological media, Levine and Blood-Agar, to see if the lesion could have been of bacterial origin.

(b) A microscopic examination to look for amoebae in the organs. This was carried out by dividing the organ in two parts. One was cultured in Petri dishes on solid medium with 2% non-nutritrive agar and the other in a tube with a liquid medium (SCGYEM medium, De Jonckheere, 1977).

3. Results

This study has only revealed one fountain, denominated PF_3, that was negative to the presence of free-living amoebae in both the samples taken, which represents a positivity of 90.9%.

Tables I and II show the overall results for each of the samples, with respect to the number of strains for each sample, the presence or absence of residual chlorine and the results of the bacteriological analysis. Upon analyzing these results it can be seen that the presence or absence of limax amoebae and the number of strains present in a sample have nothing to do with the presence of residual chlorine nor with the bacteriological characteristics of the water.

Table III shows the names of the species isolated, differentiating between the two seasons in which samples were taken, as well as the designation given to each one of the isolated strains. Twenty five strains were isolated overall, one belonging to the genus *Valhkampfia*, 10 to the genus *Naegleria* and the other 14 to the genus *Acanthamoeba*. The most abundant species in the samples belong to the genus *Acanthamoeba*, however it is noteworthy that the genus *Naegleria* occurs in 9 of the 11 fountains analyzed for at least one of the seasons when samples were taken. The commonest *Acanthamoeba* species is *A. quina* which was isolated from 8 of the 11 fountains analyzed.

All the strains were able to flourish at 37 °C, with the exception of three, CF_1V, CF_2N and CF_3N, but none could do so at 42 °C.

Tables IV, V, VI and VII show the results obtained after the i.c. and i.n. inoculations. For the in vivo pathogenicity study of the strains capable of surviving at 37 °C, the

TABLE I

General characteristics of the fountain waters-analysed

Sample	Number of amoeba species	Temperature (°C)		Residual chlorine		Bacteriological diagnosis for consumability	
		W	S	W	S	W	S
CF_1	3	9	14	–	–	Non drinkable	Non drinkable
CF_2	1	11	15	+	+	Drinkable	Drinkable
CF_3	2	13	17	–	–	Drinkable	Drinkable
CF_4	2	12	16	–	–	Non drinkable	Non drinkable
CF_5	2	11	15	–	–	HR	HR
PF_1	3	12	18	+	+	Drinkable	Drinkable
PF_2	3	13	18	–	+	HR	Drinkable
PF_3	0	11	17	+	+	Drinkable	Drinkable
PF_4	3	12	16	+	+	Drinkable	Drinkable
PF_5	2	12	15	–	–	HR	HR
PF_6	4	13	19	–	–	Non drinkable	Non drinkable

W = Winter, S = Summer.
HR = Allowed by National Health Regulations (Boletín Oficial del Estado, 1982).
Non drinkable, Drinkable (Boletín Oficial del Estado, 1982).

TABLE IIa

Bacteriological test results of water samples from fountains in winter

Sample	TC 100 mL^{-1}	FC 100 mL^{-1}	*E. coli* 100 mL^{-1}	FS 100 mL^{-1}	SRC 100 mL^{-1}	HB mL^{-1} 35 °C	HB mL^{-1} 22 °C
CF_1	1.1×10^3	9.0	9.0	7.0	3.0	4.5×10^2	1.4×10^3
CF_2	<2	<2	<2	<2	0.0	<10	<10
CF_3	<2	<2	<2	<2	0.0	<10	<10
CF_4	<2	<2	<2	<2	6.5×10^1	4.0×10^1	5.8×10^1
CF_5	4.0	<2	<2	<2	0.0	<10	<10
PF_1	<2	<2	<2	<2	0.0	<10	<10
PF_2	<2	<2	<2	<2	0.0	2.0×10^3	2.8×10^3
PF_3	<2	<2	<2	<2	0.0	2.6×10^1	3.0×10^2
PF_4	<2	<2	<2	<2	0.0	1.0×10^1	4.0×10^1
PF_5	<2	<2	<2	<2	0.0	8.6×10^2	1.2×10^3
PF_6	4.0	4.0	4.0	<2	5.2×10^1	6.4×10^2	5.6×10^3

TC= Total Coliform, FC= Fecal Coliform, FS= Fecal Streptococcus, SRC= Sulphite-reducing Clostridia, HB= Heterotrophic bacteria.

TABLE IIb

Bacteriological test results of water samples from fountains in summer.

Sample	TC 100 mL^{-1}	FC 100 mL^{-1}	*E. coli* 100 mL^{-1}	FS 100 mL^{-1}	SRC 100 mL^{-1}	HB mL^{-1} 35 °C	HB mL^{-1} 22 °C
CF_1	4.0	4.0	4.0	7.0	3.0	4.0×10^2	5.5×10^3
CF_2	<2	<2	<2	<2	0.0	<10	<10
CF_3	<2	<2	<2	<2	0.0	<10	<10
CF_4	<2	<2	<2	<2	5.5×10^1	4.5×10^1	5.0×10^1
CF_5	4.0	<2	<2	<2	0.0	<10	<10
PF_1	<2	<2	<2	<2	0.0	<10	<10
PF_2	<2	<2	<2	<2	0.0	<10	<10
PF_3	<2	<2	<2	<2	0.0	2.0×10^1	3.0×10^2
PF_4	<2	<2	<2	<2	0.0	<10	<10
PF_5	<2	<2	<2	<2	0.0	2.0×10^3	1.0×10^3
PF_6	4.0	4.0	4.0	<2	6.5×10^1	8.0×10^2	1.2×10^3

TC= Total Coliform, FC= Fecal Coliform, FS= Fecal Streptococcus, SRC= Sulphite-reducing Clostridia, HB= Heterotrophic bacteria.

TABLE III

Free-living amoebae isolated from fountain waters in the two samples taken.

Sample	Strain designation	Species name	
		Winter	Summer
CF_1	CF_1V		*Vahlkampfia* sp.
	CF_1A	*A. quina*	
	CF_1B		*A. quina*
CF_2	CF_2N		*Naegleria* sp.
CF_3	CF_3N		*Naegleria* sp.
	CF_3A	*A. quina*	
CF_4	CF_4N	*Naegleria* sp.	
	CF_4A		*A. quina*
CF_5	CF_5N	*Naegleria* sp.	
	CF_5A	*A. quina*	
PF_1	PF_1N_1	*Naegleria* sp.	
	PF_1N_2		*Naegleria* sp.
	PF_1A		*A. quina*
PF_2	PF_2N		*Naegleria* sp.
	PF_2A		*A. quina*
	PF_2B	*A. castellanii*	
PF_4	PF_4N	*Naegleria* sp.	
	PF_4A	*A. polyphaga*	
	PF_4B		*A. quina*
PF_5	PF_5N		*Naegleria* sp.
	PF_5A	*A. quina*	
PF_6	PF_6N		*Naegleria* sp.
	PF_6A	*A. paradivionensis*	
	PF_6B	*A. polyphaga*	
	PF_6C		*A. paradivionensis*

TABLE IV

Pathogenic strains of *Acanthamoeba* spp. administered by i.c. inoculation.

Strain	Mortality in mice (days after inoculation)	Macroscopic lesions	Dish culture	Culture in SCGYEM medium
		B	B	B
CF_1A	s,s,s,s,s,s,s	0	4	3
CF_1B	s,s,s,s,s,s,s	0	4	2
CF_3A	s,s,s,s,s,s,s	0	0	0
CF_4A	3,4,s,s,s,s,s	0	4	2
CF_5A	2,4,s,s,s,s,s	0	3	2
PF_1A	s,s,s,s,s,s,s	0	7	5
PF_2A	s,s,s,s,s,s,s	0	4	3
PF_2B	3,3,4,4,s,s,s	3	4	4
PF_4A	2,2,3,3,4,s,s	4	7	7
PF_4B	s,s,s,s,s,s,s	0	2	0
PF_5A	3,4,s,s,s,s,s	0	6	5
PF_6A	s,s,s,s,s,s,s	0	2	0
PF_6B	2,3,3,4,4,s,s	3	6	5
PF_6C	s,s,s,s,s,s,s	0	1	0

B = Brain, S = surviving.

TABLE V

Pathogenic strains of *Acanthamoeba* spp. administered by i.n. inoculation.

Strain	Mortality in mice (days after inoculation)	Macroscopic lesions			Dish culture			Culture in SCGYEM medium		
		L	B	Li	L	B	Li	L	B	Li
CF_1A	s,s,s,s,s,s,s	3	0	0	3	2	0	2	1	0
CF_1B	s,s,s,s,s,s,s	3	0	0	3	1	0	1	1	0
CF_3A	s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0
CF_4A	s,s,s,s,s,s,s	0	0	0	2	0	0	1	0	0
CF_5A	s,s,s,s,s,s,s	0	0	0	2	0	0	0	0	0
PF_1A	s,s,s,s,s,s,s	0	0	0	2	1	1	1	1	0
PF_2A	s,s,s,s,s,s,s	0	0	0	2	0	2	1	0	0
PF_2B	s,s,s,s,s,s,s	0	0	0	1	0	0	1	0	0
PF_4A	8,12,s,s,s,s,s	3	2	0	5	3	2	5	3	2
PF_4B	s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0
PF_5A	s,s,s,s,s,s,s	0	0	0	3	1	0	2	1	0
PF_6A	s,s,s,s,s,s,s	0	0	0	1	0	0	0	0	0
PF_6B	9,15,s,s,s,s,s	0	0	0	4	1	0	4	1	0
PF_6C	s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0

L = Lung, B = Brain, Li = Liver, S = surviving.

TABLE VI

Pathogenic strains of *Naegleria* spp. administered by i.c. inoculation

Strain	Mortality in mice (days after inoculation)	Macroscopic lesions	Dish culture	Culture in SCGYEM medium
		B	B	B
CF_4N	s,s,s,s,s,s,s	0	2	2
CF_3N	s,s,s,s,s,s,s	0	0	0
PF_1N_1	s,s,s,s,s,s,s	0	0	0
PF_1N_2	s,s,s,s,s,s,s	0	0	0
PF_2N	s,s,s,s,s,s,s	0	2	1
PF_4N	s,s,s,s,s,s,s	0	3	1
PF_5N	4,4,5,6,s,s,s	4	4	3
PF_6N	s,s,s,s,s,s,s	0	3	1

B = Brain, S = surviving.

number of animals in each lot was always of 7 and we have calculated as number statistically significant the 4, adopting a 0.95 confidence level. The cultures in Levine and Blood-Agar media of the organs which presented macroscopic lesions were negative in every case. In the case of i.n. inoculations of *Naegleria* strains it is observed that they caused the no deaths amongst the inoculated mice and that the organs removed from these mice were free from any macroscopic lesions.

TABLE VII

Pathogenic strains of *Naegleria* spp. administered by i.n. inoculation

Strain	Mortality in mice (days after inoculation)	Macroscopic lesions			Dish culture			Culture in SCGYEM medium		
		L	B	Li	L	B	Li	L	B	Li
CF_4N	s,s,s,s,s,s,s,s	0	0	0	2	0	0	1	0	0
CF_5N	s,s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0
PF_1N_1	s,s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0
PF_1N_2	s,s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0
PF_2N	s,s,s,s,s,s,s,s	0	0	0	1	1	0	1	0	0
PF_4N	s,s,s,s,s,s,s,s	0	0	0	2	2	0	2	2	0
PF_5N	s,s,s,s,s,s,s,s	0	0	0	2	2	0	1	1	0
PF_6N	s,s,s,s,s,s,s,s	0	0	0	0	0	0	0	0	0

L = Lung, B = Brain, Li = Liver, S = surviving.

4. Discussion

There is no doubt that water is a highly favorable medium for amphizoic amoebae, which have been found in many different aquatic environments. It is of concern that these protozoa occur in waters destined for human consumption, particularly in this case when they come from public fountains which are widely distributed in this country. The results obtained lead to the deduction that, under our sampling conditions, limax amoebae are a regular component of the fountains analysed given that some species were found in each, at least for one of the sampling seasons, except in fountain PF_3. The percentage of positive samples (90.9%) is slightly higher than those given in the references consulted, although it can be compared with the values of Madrigal-Sesma *et al.* (1982), Molet *et al.*, (1976), Simitzis-Le Flohic (1976), Lastovica (1980) and Janitsche *et al.* (1982). The majority of the isolated strains belong to the genus *Acanthamoeba*, however it seems of interest that, contrary to the results of these authors, a significant number of *Naegleria* amoebae were found. Nevertheless, no relations could be established between the appearance of these genera and the temperature, the presence of residual chlorine or the bacteriological analysis. This could signify that the amoebae are resistant to the action of chlorine, at least in the concentrations necessary to make water drinkable. These results are in agreement with the observations of Dive *et al.* (1979) for the water supply in France, which led them to conclude that there is no direct relation between amoebae and bacteria.

It is possible that chlorine concentrations in drinking water are insufficient to destroy encysted amoebae, as it is known that the chlorine levels in swimming pools do not kill *Acanthamoeba* cysts (Derreumaux *et al.*, 1974). Those of *Naegleria*, however, are more sensitive to chlorine (De Jonckheere and Van de Voorde, 1977; Derreumaux *et al.*, 1974). This provides an explanation for the greater numbers of this species of *Naegleria* that have been found in swimming pools (Molet *et*

al., 1976; Pernin and Riany, 1980; Madrigal *et al.*, 1984).

With regards to the temperature, one season never differed from the other by more than 6 °C, which might explain the lack of interseasonal variation between the number and type of strains found in the samples.

The commonest species isolated was *A. quina*, found in 8 of the fountains analyzed, allowing us to suppose that it is widely distributed. This has also been put forward by Pussard and Pons (1977) who describe *A. quina* as a very common species.

Concerning the pathogenicity studies, 3 (21.43%) of the *Acanthamoeba* strains studied showed some virulence in mice. This is a rather lower percentage than that observed by De Jonckheere (1979) who found 70% of *Acanthamoeba* strains to be pathogenic, although it should be born in mind that he considered strains capable of producing the death of experimental animals after just i.c. inoculation to be pathogenic. Others have obtained results which conflict with those of this author; Dive *et al.* (1978), tested 101 strains of *Acanthamoeba* and did not find any to be pathogenic.

Two of the strains that we isolated, PF_2B and PF_6B, belonging to *A. castellanii* and *A. polyphaga*, respectively, produced the death of a significant number of i.c. and i.n. inoculated animals. These results agree with the views of other authors on the pathogenicity of certain strains of these species (Cerva, 1967a, b, 1971; Derr-Haff *et al.*, 1978; Proca *et al.*, 1973; Visvesvara *et al.*, 1975). The results with *A. quina* were different, some strains gave negative results in all the tests, whereas others were found in the organs taken from the animals, and a few i.c. inoculated strains were even fatal in some of the mice. These differences lead us to suspect that, amongst species of *Acanthamoeba*, there is no clear cut correlation between the pathogenicity of a strain and the species it comprises.

The macroscopic analysis of organs taken from the test animals after i.n. inoculation has revealed that the organ most affected in every case was the lung. However, the brain was also affected in many instances. Therefore, it can be supposed that amoebae belonging to the genus *Acanthamoeba* tend to lodge in the lung of a host when given by i.n. inoculation. This is not to say that they are purely pneumotropic, even when Kasprzak *et al.* (1974) and Madrigal *et al.* (1985) observed that certain strains of this genus had this tendency after i.n. inoculation.

The only virulent isolate of genus *Naegleria* (PF_5N) obtained was found to be much less pathogenic than strains of *Naegleria fowleri* isolated by other authors since inoculations with similar doses of these strains caused very high mortalities, up to 100% in some cases (Martínez *et al.*, 1971; Newsome and Arnold, 1985), while in our isolate the mortality was only partial in i.c. inoculations and null in i.n. inoculations. Furthermore, pathogenic *N. fowleri* strains usually show a predilection for the brain which has not been seen in this experiment.

Although we have not discovered high percentages of pathogenic amoebae nor even highly pathogenic species we do not wish to present these results as being very optimistic, since, as stated by De Jonckheere (1981), all levels of pathogenicity should be taken into account.

References

Boletín Oficial del Estado (España): 1982, Real Decreto 1423/1982, del 18 de Junio. Boletín número 154 del 29 Junio 1982.

Bonde, G. F.: 1977, *Advances in aquatic Microbiology* **1**, Droop and H. W. Janhasch (eds.), Acad. Press, London, pp. 237–364.

Casares, R.: 1967, *Tratado de Análisis Químico*, Ed. Casares, Octava Edición, Tomo III, Madrid, p. 426.

Cerva, L.: 1967a, *Folia Parasitol.* **14**, 171.

Cerva, L.: 1967b, *Folia Parasitol.* **14**, 207.

Cerva, L.: 1971, *Hidrobiología* **38**, 141.

De Jonckheere, J.: 1977, *Appl. Environ. Microbiol.* **33**, 751.

De Jonckheere, J., Van de Voorde, H.: 1977, *Am. J. Trop. Med. Hyg.* **26**, 10.

De Jonckheere, J.: 1979, *Ann. Microbiol.* **130**, 205.

De Jonckheere, J.: 1981, *J. Protozool.* **28**, 56.

Derr-Haff, C., Molet, B., Schreiber, J., and Kremer, M.: 1978, *Ann. Parasitol. Hum. Comp.* **53**, 467.

Derreumaux, A. L., Jadin, J. B., Willaert, E., and Moret, R.: 1974, *Ann. Soc. Belge. Med. Trop.* **54**, 415.

Dive, D., Leclerc, H., Picard, J. P., Telliez, E., and Van Grevelinghe, R.: 1978, *Ann. Microbiol.* **129**, 225.

Dive, D., Picard, J. P., and Leclerc, H.: 1979, *Ann. Microbiol.* **130**, 487.

Grunnet, K. and Gundstrup, A. S. P.: 1977, *Rev. Int. Oceanogr. Med.* **47**, 147.

Janitschke, K., Lichy, S., and Westphal, C.: 1982, *Zbl. Bakt. I. Abt. Orig.* **176**, 160.

Kasprzak, W., Mazur, T., and Rucha, A.: 1974, *Ann. Soc. Belge Med. Trop.* **54**, 351.

Lastovica, A. J.: 1980, *Trans. Roy. Soc. S. Afr.* **44**, 269.

Madrigal-Sesma, M. J., Santillana, I., and Zapatero-Ramos, L. M.: 1982, *Rev. Iber. Parasitol, Volumen especial*, 125.

Madrigal-Sesma, M. J., Santillana, I., and Martínez-Fernández, A. R.: 1984, *Rev. Iber. Parasitol.* **44**, 379.

Madrigal-Sesma, M. J., Santillana, I., and Martínez-Fernández, A. R.: 1985, *Rev. Iber. Parasitol.* **45**, 149.

Martínez, A. J. Clifford, E., Jones, M. M., Duma, R. J., and Rosenblum, M. D.: 1971, *Laboratory Investigation* **25**, 465.

Molet, B. and Kremer, M.: 1976, *Bull. Soc. Sci. Vét. et Méd. Comp.* **78**, 215.

Molet, B., Derr-Haff, C., Schreiber, J. E., and Kremer, M.: 1976, *Ann. Parasitol. Human. Comp.* **51**, 401.

Newsome, A. L. and Arnold, R. R.: 1985, *J. Parasitol.* **71**, 678.

Pernin, P. and Riany, A.: 1980, *Ann. Parasitol. Hum. Comp.* **55**, 491.

Proca, M. I., Lupascu, G. H., and Sterv, D.: 1973, *Archives Roumaines of Pathology and Experimental Microbiology* **32**, 205.

Pussard, M. and Pons, R.: 1977, *Protistologica* **13**, 557.

Rafalko, J. S.: 1951, *J. Morphol.* **89**, 71.

Simitzis-Le Flohic, A. M.: 1976, *Bull. Soc. Pathol. Exot.* **60**, 302.

Standard Methods for the Examination of waster and Waswater: 1985, A.P.H.A., American Public Health Association, A.W.A.W., W.P.F.C., 16th Edit. Join Edit. Board, Washington, D.C.

Visvesvara, G. S., Jones, D. B. and Robinson, H. N.: 1975, *Amer. J. Trop. Med. Hyg.* **24**, 784.