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MÉMOIRES ORIGINAUX

ENTAMOEBA MOSHKOVSKII (TSHALAIA, 1941) : Morpho-biological characterization of new strains isolated from the environment, and a review of the literature

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SUMMARY. After an investigation carried out with samples from several sewage sludges from the town of Pavia (Italy) the AA. report the isolation of two new strains of *Entamoeba moshkovskii*. The usefulness and reliability of two methods, i.e. growth in hypotonic media and the thermoresistance tests *in vitro* for the biological typization of this species are analyzed and discussed. Both methods had already proven useful to characterize the Laredo-type strains of *E. histolytica* too, as reported by other AA.

Entamoeba moshkovskii (Tshalaia, 1941) : caractérisation morpho-biologique de nouvelles souches isolées de l'environnement et revue de la littérature.

RÉSUMÉ. A la suite d'une recherche parasitologique conduite sur différents échantillons d'eau et de boue d'égouts de la ville de Pavie (Italie) a été possible l'isolement de deux nouvelles souches d'*Entamoeba moshkovskii*. La caractérisation morpho-biologique des souches a été effectuée à l'aide de deux méthodes, tels que la culture en milieux hypotoniques et le test de thermorésistance *in vitro*, qui d'après les travaux d'autres AA. s'étaient montrés déjà de bons marqueurs, même pour la différenciation entre *E. histolytica* et les souches Laredo « thermoadaptables ».

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The phylogenesis and, consequently, the exact taxonomic position of *Entamoeba moshkovskii* (Tshalaia, 1941) is still controversial and poorly defined, in spite of the intriguing hypotheses suggested by Meerovitch (19), de Carneri (5), Goldman (14) and reconsidered in particular by Rondanelli *et al.* (26) *(Table I)*.

TABLE I. — Phylogenetic hypothesis of tetranucleate cyst of *Entamoeba* species (Rondanelli *et al.*, 1973)

E. moshkovskii
(free-living species, 24° C-37° C)

↓

E. histolytica Laredo-type
(non-pathogenic species for man ; « thermoadaptable » ; 24° C-37° C)

↓ ↓

E. invadens (species pathogenic for reptiles ; 24° C) — *E. histolytica* (species pathogenic for man and primates ; 37° C)

TABLE II. — Countries, locations and areas of isolation of *E. moshkovskii* (Literature data up to 1981 included)

Country	Location	Area and/or isolation material	References
Australia	Sidney, Glenelg, Adelaïde, Quaker's Hill	Urban and suburban systems	28
Brazil	São Paulo, Parà Goiàs, Minas Gerais Federal District	Depuration station of urban sewage systems. Rivers, lakes, mud	1, 8, 9, 10 12, 18
Canada	St. Ann (Quebec)	Depuration stations of urban sewage	17
Costa Rica	Cartago	id.	27
Equador	Guaiaquil	Mud	2
Italy	Milan, Messina	Irrigation canals and waterways. Urban sewage	3, 4, 23
Malaysia	Singapore	Depuration stations of urban sewage systems	33
Pakistan	Karachi	id.	34
Poland	Gdynia	id.	15
United Kingdom	London	id.	20, 21
USSR	Moskow, Minsk, Leningrad, Tashquent	Mud and irrigation canal. Mud. Catchment basins and lakes	13, 25 30, 31
Uruguay	La Boyada	Rivers and urban sewage systems	12
USA	California, Louisiana, Georgia	Depuration station of urban sewage systems	6, 32

Whether *E. moshkovskii* should be classified as a "naturally" free-living species, or as a parasite of yet undetermined homeothermic or poikilothermic animals, or whether this species should be regarded as ancestrally parasite of animals (fishes ? arthropods ? amphibians ?) and yet capable, for still unknown reasons, to adjust with time to autonomous life in the external environment is still debated.

Isolations from the environment have been so far ubiquitously reported *(Table II)* and in Italy they are mainly due to de Carneri (3,4) and Pennisi *et al.* (23).

In this paper we report the isolation of two new strains from sewage sludges of the town of Pavia (Italy) and the usefulness of the tests of cultivation in hypotonic media and thermoresistance, for the morphobiological typization of this species.

Material and methods

The material (water mixed with mud) collected from the chosen areas was distributed into sterile screw-cap bottles (about 500 ml per bottle).

According to Félix-Silva and Mayrink (12), following homogenization in cylinders the samples were allowed to sediment spontaneously for several hours in the laboratory ; in this way the formation of three layers was routinely observed : at the bottom the mud with the thickest waste material, then an intermediate layer mostly consisting of water and fine residues, then an upper layer with water and the lightest waste material. After sedimentation, a few drops from each layer of the material were examined under the phase contrast microscope to detect any vegetative and/or cystic forms of *Entamoeba* spp.

Subsequently, seeding in specific media for *Entamoeba* spp. (Jones and Boeck-Drbohlav) was carried out, using a slight modification of the methods proposed by Félix-Silva and Mayrink (12) *(fig. 1)*. All the culture tubes, seeded in duplicate, were incubated at both 24° C and 37° C.

For the thermoresistance and thermoadaptability tests, the methods suggested by Siddiqui (29), Entner and Most (7) and Pennisi *et al.* (22) were followed, incubating the strains at 43° C, 41° C, 24° C and 4° C in Jones medium.

The results obtained were compared with those yielded by standard reference strains of *E. histolytica* (Meah strain), *E. histolytica* Laredo-type (Huff strain), *E. invadens* (Rodhain strain) and *E. moshkovskii* (Bizzozzero strain).

For the test of survival in hypotonic medium the protocol of Pennisi *et al.* (22) and previously proposed by Richards *et al.* (24) for the Laredo-type strains of *E. histolytica* was used.

The tested strains and the reference ones were grown in Jones medium, previously diluted in serial concentrations with sterile bidistilled water.

Again according to Pennisi *et al.* (22) growing in tap water previously filtered through Millipore® 0.45 μm and in sterile bidistilled water attempted, and survival times were recorded.

All the strains, except *E. histolytica*, were incubated at 24° C.

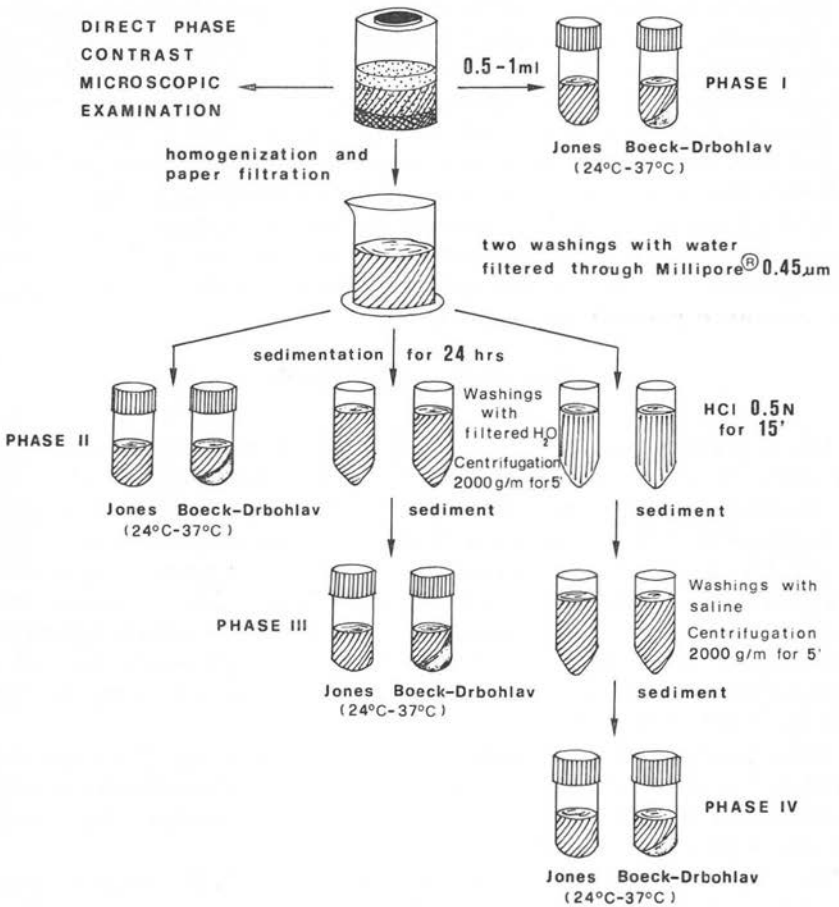

FIG. 1. — Techniques of *in vitro* isolation of *E. moshkovskii* (modified Félix-Silva and Mayrink method, 1974)

Results and discussion

Table III summarizes the results obtained with regard to isolation attempts. Out of 20 samples taken from 20 sewage sludges of Pavia, 8 (40 %) proved completely negative in both media (Jones, Boeck-Drbohlav) and at both incubation temperatures (24° C-37° C).

On the contrary, of the 12 positive samples (60 %) 10 showed the development of *limax* amoebae recognized as belonging basically to the *Hartmannella, Saccamoeba* and *Naegleria* spp. ; in two cases there was also development of strains of *Entamoeba* spp. both at 24° C and at 37° C and in one case the development of 1 strain of *Enta-*

TABLE III. — Summary of the samples collected and their positivity.

A) *Sewage sludges*	B) *Water from rivers and waterways*
1) negative	1) positive 24° C-37° C *Hartmannella* sp.
2) positive 24° C-37° C *Hartmannella* sp.	2) negative
3) positive 24° C-37° C *Hartmannella* sp.	3) negative
4) positive 24° C *Entamoeba* sp. ; positive 24° C-37° C *Hartmannella* sp. and *Saccamoeba* sp.	4) positive 24° C *Hartmannella* sp. and *Saccamoeba* sp.
5) negative	5) negative
6) positive 24° C-37° C *Entamoeba* sp. ; positive 24° C-37° C *Hartmannella* sp.	
7) positive 24° C *Entamoeba* sp. ; positive 24° C-37° C *Naegleria* sp. and *Saccamoeba* sp.	
8) negative	
9) positive 24° C-37° C *Entamoeba* sp. and *Hartmannella* sp.	
10) positive 24° C *Entamoeba* sp. ; positive 24° C-37° C *Hartmannella* sp.	
11) negative	C) *Water from irrigation canals*
12) negative	
13) positive 24° C *Entamoeba* sp.	1) negative
14) positive 24° C-37° C *Valhkampfia* sp.	2) positive 24° C-37° C *Hartmannella* sp.
15) negative	3) negative
16) positive 24° C-37° C *Entamoeba* sp.	4) negative
17) negative	5) positive 24° C-37° C *Hartmannella* sp. and *Naegleria* sp.
18) negative	
19) positive 24° C *Entamoeba* sp. ; positive 24° C-37° C *Saccamoeba* sp.	
20) positive 24° C-37° C *Entamoeba* sp. and *Hartmannella* sp.	

moeba sp. only at 24° C. Of the other two specimens, found positive *in vitro* only for amoeba belonging to the genus *Entamoeba*, only one proved interesting in connection with our investigations, since cultures were positive at both incubation temperatures. On the whole, therefore, our of 20 samples, there were 3 strains of *Entamoeba* capable of growing *in vitro* both at 37° C and at room temperature.

Ten other samples were collected from waterways and irrigation canals flowing through the urban and suburban areas of our town. Of these, 6 were negative, while the 4 samples wich proved positive to *in vitro* culturing exhibited the development of *limax* amoebae only.

Table IV summarizes the results of the thermoresistance tests, with reference to the mean survival time of each strain.

As it can be seen, of the three strains (called PV-I-06, PV-I-09, PV-I-20) of *Entamoeba* spp. isolated by us, two (PV-I-09 and PV-I-20) showed a behaviour similar to those of Laredo-type "thermoadaptable" *E. histolytica* and *E. moshkovskii* both at high (41° C-43° C) and at low (4° C) temperatures.

TABLE IV. — *In vitro* thermoresistance tests (Jones medium) : 41° C - 43° C - 24° C - 4° C.

	E. histolytica	*E. histolytica* Laredo-type	*E. moshkovskii* *Entamoeba* sp PV-I-09 PV-I-20	*E. invadens*	*Entamoeba* sp PV-I-06
Detection of vital vegetative and/or cystic forms (subcultures in fresh medium after max 72 hrs at 43° C-41° C)	~ 72 hrs 41° C	~ 56 hrs 41° C	~ 56 hrs 41° C	~ 16 hrs 41° C	~ 24 hrs 41° C
	~ 48 hrs 43° C	~ 16 hrs 43° C	~ 16 hrs 43° C	~ 8 hrs 43° C	~ 8 hrs 43° C
Detection of vital vegetative and/or cystic forms (subcultures in fresh medium after max 5 days at 24° C and 4° C)	+ 24 hrs 24° C	+ 24° C	+ 24° C	+ 24° C	+ 24° C
	— 4° C	~ 50 days 4° C	~ 50 days 4° C	~ 60 days 4° C	> 60 days 4° C

TABLE V. — Culture tests in hypotonic media (Jones medium ; Millipore® filtered tap water ; bidistilled water)

	E. histolytica 37° C	*E. histolytica* Laredo-type	*E. moshkovskii* *Entamoeba* sp PV-I-09 — PV-I-20	*E. invadens*	*Entamoeba* sp. PV-I-06
Resistance in diluted Jones medium (1/4, 1/8, 1/16, 1/32, 1/64, 1/80, 1/100)	positive at 1/4, 1/8, 1/16 dilutions for max 48 hrs	+	+	+	+
Resistance in filtered tap water	—	~ 48 hrs	~ 48 hrs	> 48 hrs	> 48 hrs
Resistance in bidistilled water	—	~ 48 hrs	~ 48 hrs	> 48 hrs	> 48 hrs

Only one strain, unidentified PV-I-06, reveal different survival times (only at high temperatures) which did not coincide with any of those of the reference species used by us.

The results of the tests of growth in hypotonic media *(Table V)* confirmed, in agreement with Pennisi *et al.* (22) that the resistance of *E. histolytica* is practically null in filtered, bidistilled water, while survival in Jones medium does not exceed 48 hrs at low dilutions (1/4, 1/8, 1/16).

A clearly different behaviour was on the contrary shown by the other reference species *(E. histolytica* Laredo-type, *E. invadens, E. moshkovskii)* and, what was most relevant with regard to our investigation, by the newly isolated strains.

Indeed, it was demonstrated that in addition to the standard strains, also the *Entamoeba* spp. strains 24° C-37° C isolated by us could survive high dilutions of the culture medium.

A behaviour quite similar to those *E. histolytica* Laredo-type and *E. moshkovskii* was shown by our strain, in particular by PV-I-09 and PV-I-20 also in water filtered through Millipore® and in bidistilled water.

Conclusions

The results obtained by combining the classic microscopic investigations with specific tests such as thermoadaptability and growth in hypotonic media, lead to several interesting conclusions :

1 — The morphology of the vegetative forms and cysts of the two strains PV-I-09 and PV-I-20, studied with a phase contrast microscope and after staining with Heidenhain's iron haematoxylin *(Pl. I, fig. 1-5)* is certainly comparable with the morphological observations made by Neal (21) and Félix-Silva (11).

2 — Our study confirmed that the classic *E. histolytica*, which is pathogenic for man and the Primates, and is able to grow *in vitro* only at 37° C, can be clearly differentiated from the strains of *E. histolytica* labeled as Laredo-type which, though isolated in humans, fail to prove pathogenic at *in vivo* tests and are capable of *in vitro* growth at 37° C, but also at 24° C.

3 — On the other hand, the Laredo-type "thermoadaptable" strains of *E. histolytica* showed a behaviour if not identical at least quite similar to that of *E. moshkovskii*, a species so far isolated only from the environment and also unable to cause experimental infections in the animals, as shown by several authors (2, 8, 9, 10, 12, 15, 16, 18, 21, 25, 31).

4 — Two of the three thermoadaptable strains of *Entamoeba* spp. isolated by us from the environment (PV-I-09 and PV-I-20) showed a behaviour practically identical with that of the *E. histolytica* Laredo-type and *E. moshkovskii* species.

5 — We believe that the strains isolated by us can be reasonably regarded as belonging to the *E. moshkovskii* species, both for their morphological characteristics (mean diameter and morphology of vegetative and cystic forms) and for their biolo-

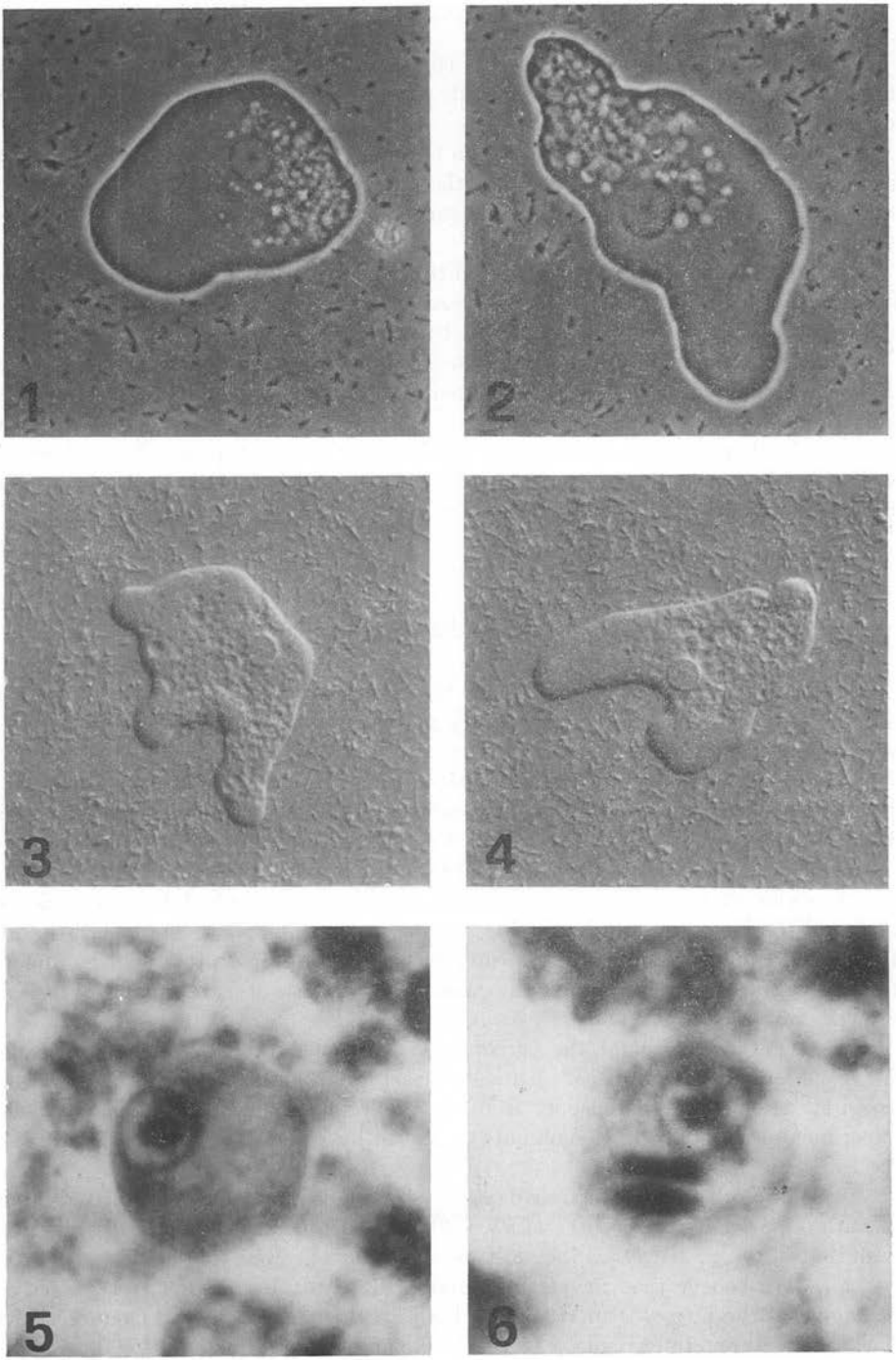

PLANCHE I

gical features (the thermoresistance and hypotonicity tests gave the same results as with the reference strain of *E. moshkovskii*), but also because the Laredo-type strains have so far been isolated from human healthy carriers.

6 — Attempted axenization in Diamond's medium (6) in order to promote a thorough immunological report with preparations of purified antigens are in progress. The demonstration of the immunoelectrophoretic patterns of these new strains with respect to the *E. histolytica* and *E. histolytica* Laredo-type species remains certainly the choice test to positively classify as belonging to the *E. moshkovskii* species not only our strains but also the many other strains isolated from sewage sludges and from the mud of waterways, and so far regarded, until proof to the contrary, as belonging to the *E. moshkovskii* species.

7 — Since all the attempts so far made to experimentally infect several animal species have failed, the hypotheses that *E. moshkovskii* may be regarded as a naturally free-living species, or as an ancestrally parasite species proving able to adjust to autonomous life, presently appear the most plausible ones.

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Plate I

Fig. 1, 2. — *E. moshkovskii*, PV-I-09 and PV-I-20 strains. *In vitro* culture trophozoites (Jones medium). The nucleus with central "like a spot" karyosome and crown-shaped chromatin granules are distinctly seen ; several phagocitic vacuoles can be seen. Phase-contrast, × 1000.

Fig. 3, 4. — *E. moshkovskii*, PV-I-20 strain. *In vitro* culture trophozoites (Jones medium). The nucleus with karyosome and ectoplasmatic pseudopodia can be seen. Interferential contrast, × 630.

Fig. 5. — *E. moshkovskii*, PV-I-09 strain. "Pre-cystic" form with "hipertrophic" nucleus and karyosome (likely pre-mitotic phase). Heidenhain's iron haematoxylin, × 1000.

Fig. 6. — *E. moshkovskii*, PV-I-20 strain. "Immature" mononucleated cyst with two chromatoid bodies and "hipertrophic" karyosome. Heidenhain's iron haematoxylin, × 1000.

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