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Colonies of trypanosomatids on agar plates: the tool for differentiation of the species and isolates

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Summary

Trypanosomatids unlike many other protozoans may grow on axenic liquid and solid media providing cultures of high density. It allows to investigate the colonies size and shape polymorphism. In the present study colonies structure was investigated under scanning electron microscopy. The comparison of colony diameter distribution in 5 species of trypanosomatids was also studied. The density of cell packing varies in the colonies of different species. In cultures where the single size class of colonies predominates some selective factors eliminate the cells which could form colonies of certain diameter. Such factor may be connected with a procedure of isolation or with cultivating conditions. All data obtained allow to discriminate various isolates of trypanosomatids by phenotypic characters of the colonies and to use this features in taxonomy of trypanosomatids.

Key words: Trypanosomatidae, insect and plant trypanosomatids, systematics, colonies on solid media

Introduction

Trypanosomatids unlike many other protozoans may grow on axenic liquid and solid media. The high density of the cells in cultures (10^8–10^9 cells/ml) allows to use a wide spectrum of biochemical and molecular methods as well as techniques which are rather rarely used in protistology. One of such approaches is the investigation of colonies size and shape polymorphism.

First attempts to use solid media for trypanosomatids cultivation were made in the twenties (Nöller, 1917; Nieschulz, 1922, 1924) and then iterated sporadically for limited number of species (Senekjie, 1944; Newton, 1956; Keppel and Janovy, 1977, 1980; Goldberg and Chiari, 1980; Lucic and Nadazdin, 1982; Wittner et al., 1982). The clonal nature of colonies was demonstrated for insect trypanosomatids *Crithidia harmosa, Herpetomonas megaseliae* and *Crithidia oncopelti* (Keppel and Janovy, 1977; Haetsky, 1982).

C. harmosa H. megaseliae were found to differ from each other by colonies shape (hemispherical or flattened) and it was proposed that colonies shape may be of a taxonomic value (Keppel, Janovy, 1977). Later the colonies polymorphism has been studied on 8 isolates of trypanosomatids from insects collected in the North-West Russia (Podlipaev, 1985; Podlipaev et al., 1990, 1991). Several distinctive phenotypes were determined as heritable ones: for example hemispherical or amoeba-like colonies were found to be such characters for *Leptomonas peterhoffi* (Podlipaev, 1985); they remained stable during at least 5 years of cultivation. Some isolates were marked by peculiar set of phenotypes displayed on solid media and characters of their colonies were used for taxonomical purposes (Krylov et al., 1985; Podlipaev, 1985; Podlipaev et al., 1990, 1991).

Giant multinuclear cells, described in liquid cultures of insect trypanosomatids (Podlipaev, 1985) have been discovered in colonies of *Crithidia oncopelti* and *Leptomonas peterhoffi* by transmission electron microscopy (Skarlato and Malysheva, 1987; Malysheva and Skarlato, 1989).

Colonies size has never been used for discrimination of trypanosomatids isolates except the case with colonies of *Blastocrithidia miridarum* that are much bigger than those of *Leptomonas rigidus* having just different size distribution (Podlipaev et al., 1991).

For *Leishmania donovani* it was shown that colonies having been initiated from promastigotes are indistinguishable from colonies originated from another life cycle stage – amastigotes (Keppel and Janovy, 1980). On the contrary, *Wallaceina inconstans* demonstrates three definite heritable types of colonies, which differ from one another by cell composition (Podlipaev et al., 1990). Cells in different *Wallaceina* colonies coincide to different stages of *Wallaceina* life-cycle in the host as well as to different cell morphotypes in culture.

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Colonies seem to serve as a promising tool for delineation of trypanosomatids species and isolates as it was used earlier for numerous microorganisms.

In the present study we investigated colonies characters of new species and isolates of monogenetic trypanosomatids.

Material and methods

List of trypanosomatids is presented in Table 1. Methods of culture isolation and cultivation were described earlier (Haetsky, 1982; Podlipaev, 1985; Podlipaev and Frolov, 1987).

Trypanosomatids from insects were cultured in BHI (Brain Hart Infusion) medium (Difco) with hemin (10 mg/ml) and 2% of Difco agar for solid medium. Isolates from plants were maintained in Grace's medium (Gibco) with 10% heat-inactivated fetal calf serum. 10-days colonies were used for analysis.

For scanning electron microscopy colonies were fixed with 2% OsO_4 and material was subjected to the critical-point drying method using acetone/CO_2 system. Colonies were coated in platinum with Hitachi 21PC32 apparatus and examined with a Hitachi S570 microscope.

Results and Discussion

The clonal essence of colonies may be noted from the first cells divisions on agar plates (Fig. 1).

When the humidity is high enough for colonies development the flagella of cover cells are moving (Figs 2, 3).

Colonies of *Leptomonas seymouri* show the inner rosette-like structures, differing from those, described earlier (Podlipaev, 1985; Podlipaev and Frolov, 1987) (Fig. 4). These features may be the evidence of the inner structure of colony.

The density of cell packing varies in the colonies of different species – it is high in the *Leptomonas* sp. P (Fig. 5), somehow lower with the small gaps between the cells in *L. peterhoffi* (Fig. 6) and the most friable in *L. seymouri* (Fig. 8).

Table 1. List of trypanosomatids

Name	Host	Host order and family	Place of isolation	Comments
Crithidia acantocephali Hanson et McGhee, 1961	*Acanthocephala femorata*	Hetmiptera: Coreidae	USA	Received from E. Camargo
Crithidia guilhermei Soares et al., 1986	*Phaenicia cuprina*	Diptera: Calliphoridae	Brazil	Received from E. Camargo
Herpetomonas roitmani (Fiorini et al., 1989)	*Ornidia obesa*	Diptera: Syrphidae	Brazil	Received from E. Camargo
Herpetomonas muscarum Leidy, 1856	*Musca domestica*	Diptera: Muscidae	?	Received from D. Maslov
Leptomonas peterhoffi Podlipaev, 1985	*Nabicula flavomarginata*	Hemiptera: Nabidae	North-West Russia	
Leptomonas samueli Carvalho, 1973	*Zelus leucogrammus*	Hemiptera: Triatominae	Brazil	Received from E. Camargo
Leptomonas seymouri Wallace, 1977	*Dysdercus suturellus*	Hemiptera: Pyrrhocoridae	USA	Received from D. Maslov
Leptomonas rigidus Podlipaev et al., 1991	*Salda littoralis*	Hemiptera: Saldidae	North-West Russia	
Leptomonas sp. P	*Panorpa communis*	Mecoptera: Panorpidae	North-West Russia	Isolated by S. Podlipaev in 1988
Phytomonas serpens 1G (Gibbs, 1957)	*Lycopersicon esculentum*	Plantae: Solanaceae	Brazil	Received from. M. Dollet
Phytomonas sp. EM1	*Euphorbia pinea*	Plantae: Euphorbiaceae	France	Received from. M. Dollet
Phytomonas sp. Hart1	*Cocos nucifera*	Plantae: Palmaceae	French Guiana	Received from. M. Dollet
Phytomonas sp. Mz1	*Elais guinensis*	Plantae: Palmaceae	Colombia	Received from. M. Dollet

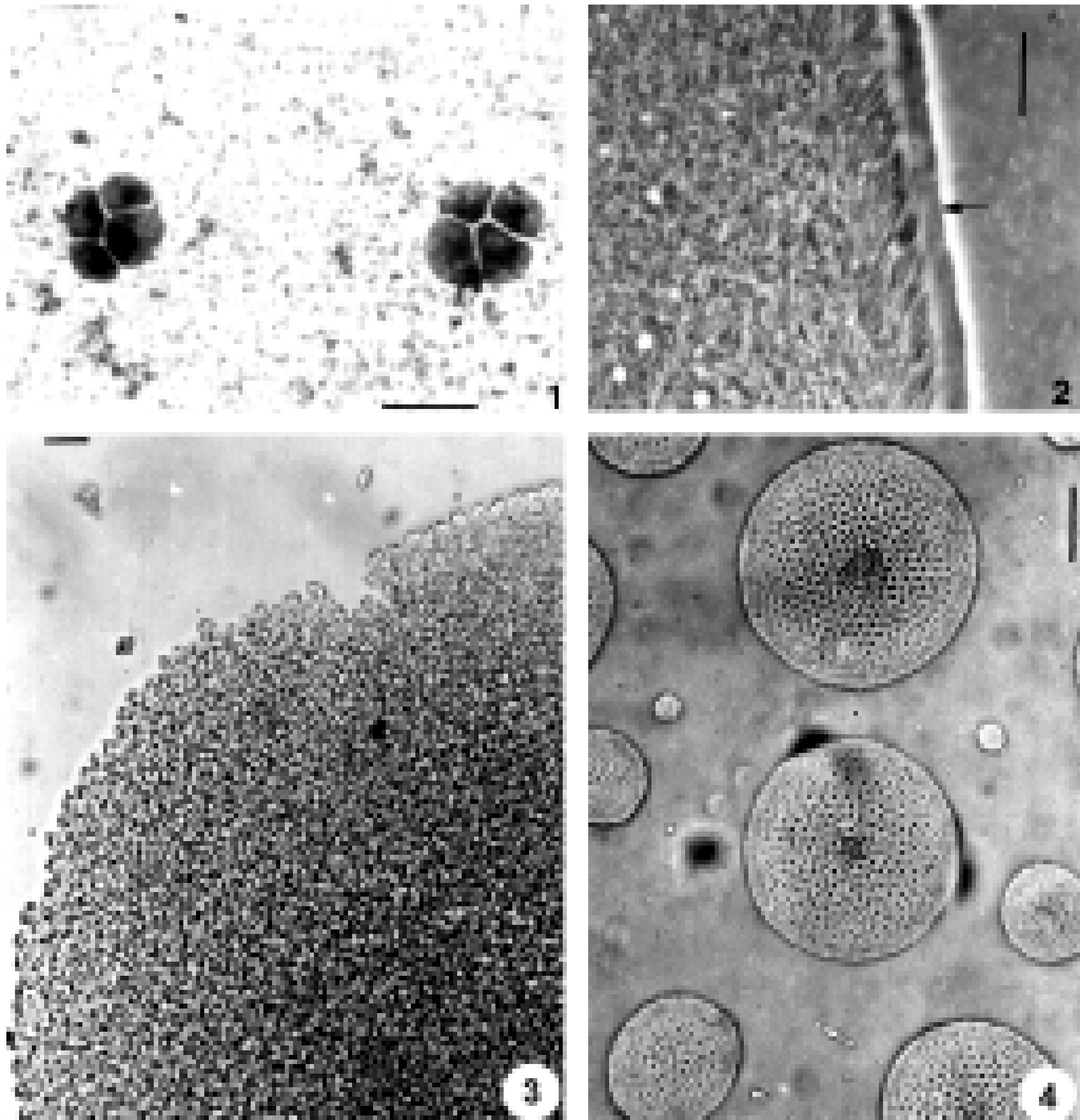

Fig. 1. Cells of *Leptomonas peterhoffi* on agar plate, 24 hours after inoculation. Giemsa staining. Scale bar: 10 µm.
Figs 2, 3. Border of *Blastocrithidia gerricola* alive 10-days colonies. Arrow – moving flagella. Scale bar: 20 µm.
Fig. 4. Alive *Leptomonas seymoury* 10-days colonies with inner rosette-like structures. Scale bar: 0.1 mm.

At the surface of the colonies of *Leptomonas* sp. P the rare free flagella take place, their number is considerably higher in *L. peterhoffi* and many of them extend above the colony surface (Fig. 7). In *L. seymouri* the majority of the cells retains typical promastigote form and the upper covering layer is not expressed in the colony (Fig. 8).

The upper cells in the colonies of *L. peterhoffi* form the dense covering layer, the inner cells are packed crumbly, many of them bearing the free flagellum (Figs 9, 10).

Till now the colonies polymorphism of about 15 species and isolates of insect trypanosomatids has been investigated (Keppel and Janovy, 1977; Krylov et al., 1985; Podlipaev, 1985; Podlipaev et al., 1990, 1991). Newly investigated cultures (*Leptomonas samueli, L. seymouri, Herpetomonas muscarum, H. roitmani, Crithidia acanthocephali and C. guilhermei*) show the same spectrum of colony phenotypes as it was mentioned before: 1) hemispherical large; 2) hemispherical small; 3) amoeba-like and 4) branched (Podlipaev, 1985; Podlipaev et al., 1991). Plant trypanosomatids (*Phytomonas serpens* 1G; *Phytomonas* sp. EM1; *Phytomonas* sp. Hart1 and *Phytomonas* sp. Mz1) display monomorphic hemispherical colonies only.

Besides the discrete character of the form, trypanosomatid colonies may differ by their size. The comparison of colony diameter distribution in 5 species of

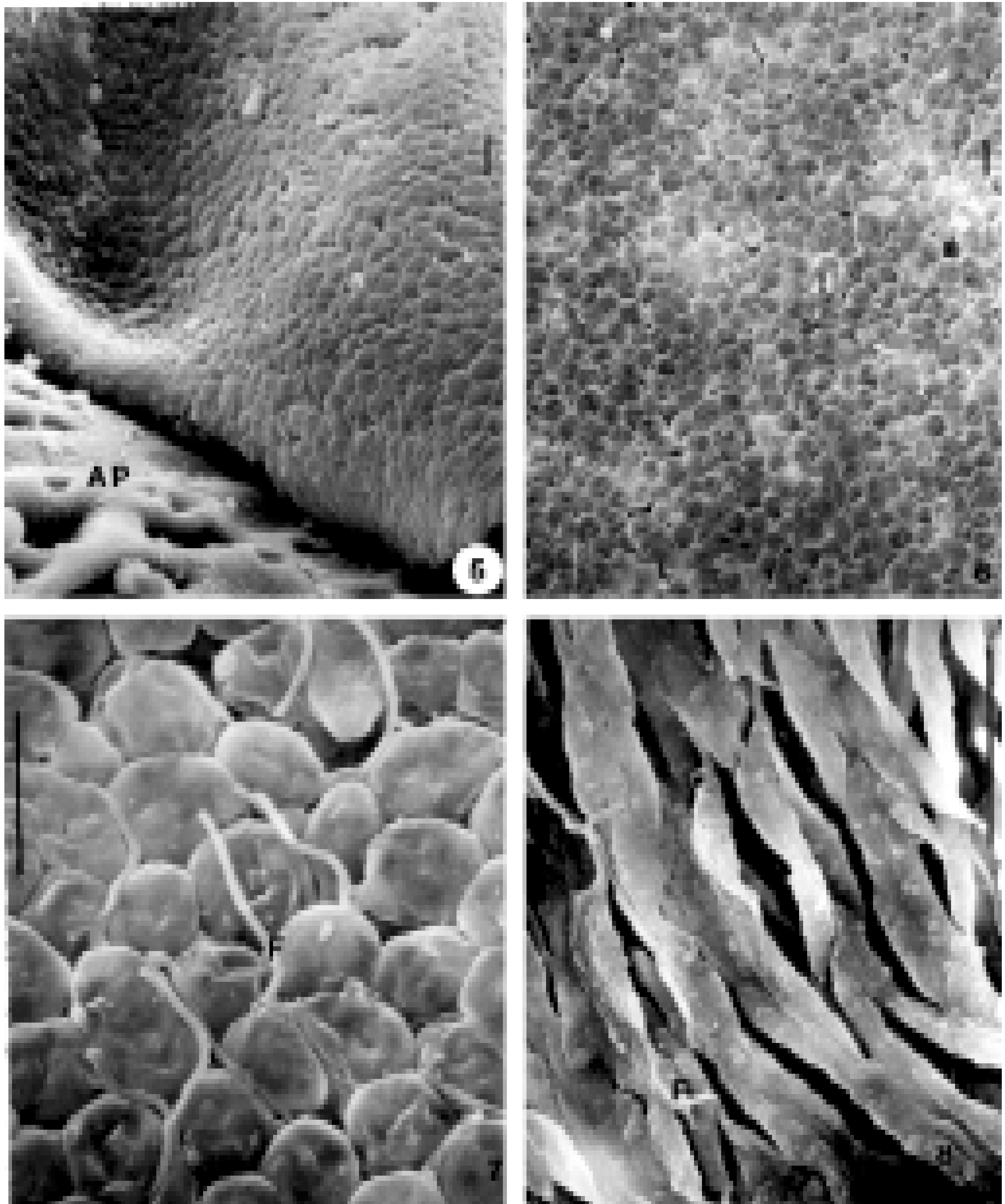

Fig. 5. 10-days colony of *Leptomonas* sp. P on agar plate (AP), scanning electron microscopy (SEM). Scale bar: 10 µm.
Figs 6, 7. Upper surface of *Leptomonas peterhoffi* 10-days colonies (SEM). F – free flagella. Scale bar: 10 µm.
Fig. 8. Upper surface of *Leptomonas seymoury* 10-days colonies (SEM). F – free flagella. Scale bar: 10 µm.

trypanosomatids shows that in *Leptomonas rigidus* and *Crithidia guilhermei* the mode at the area of 0.1–0.3 mm can be noticed, whereas in *Crithidia acantocephali* and *Leptomonas seymouri* it may be found at 0.4–0.6 mm area. *Herpetomonas muscarum* has bimodal distribution of this size parameter, modes lying at the same areas (Fig. 11)**.** The bimodal distribution of colony size in *H. muscarum* as well as the difference of average diameter of other spe-

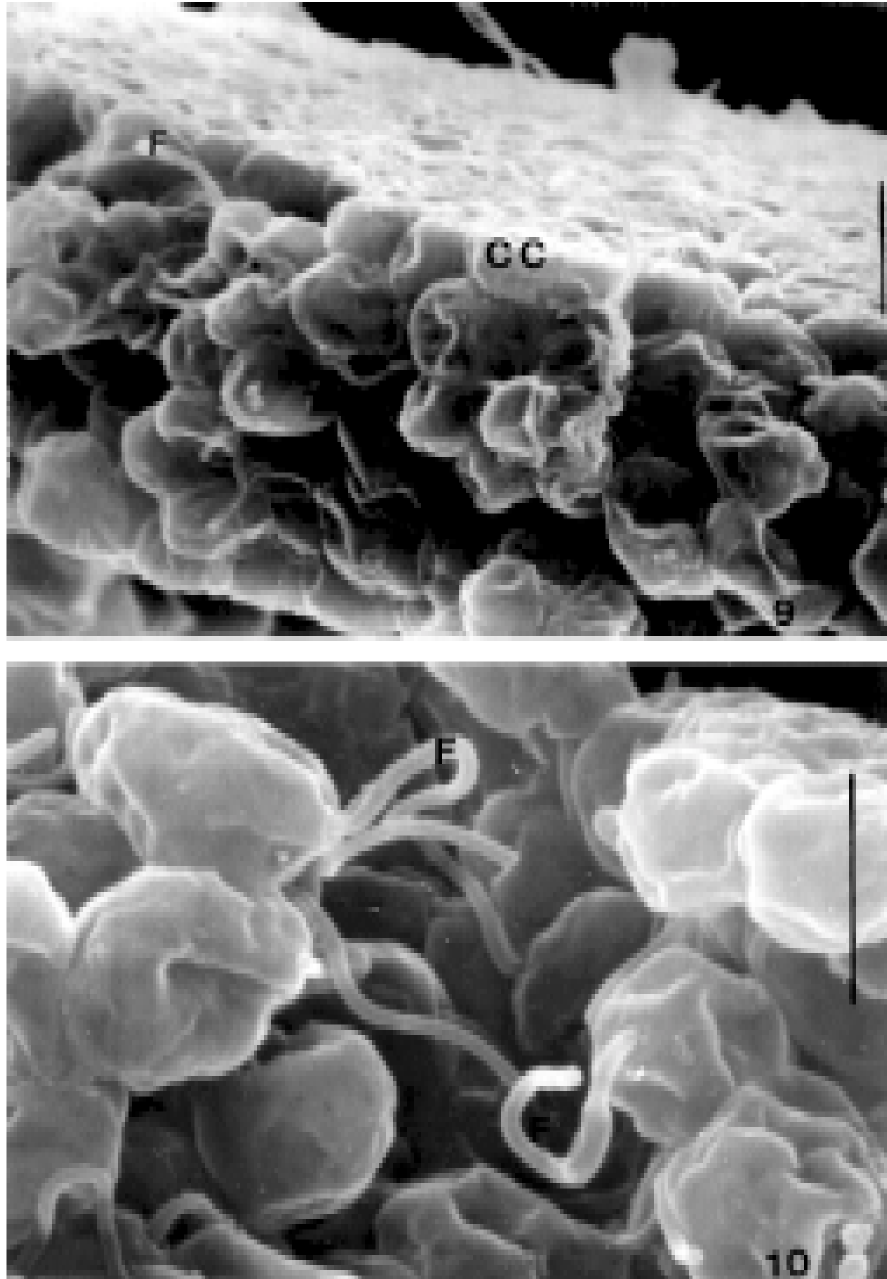

Fig. 9. Crack of the *Leptomonas peterhoffi* 10-days colony border (SEM). CC – cover cells. F – free flagella. Scale bar: 10 μm.
Fig. 10. Inner cells in *Leptomonas peterhoffi* 10-days colony (SEM). F – free flagella. Scale bar: 10 μm.

cies mentioned above are of good statistical significance at the confidence level $P<0.01$.

The described distribution of colony size remains stable during at least two years of cultivation. Thus we can confirm that in the species studied there are two heritable phenotypes differing by sizes of colonies. In *H. muscarum* colonies of both types are phenotypically expressed, while in other species under discussion – only one of them. It may be considered, therefore, that in cultures, where the single size class predominates some selective factors eliminate cells which could form colonies of certain diameter. Such factor may be connected with a procedure of isolation or with cultivating conditions. Colony size distribution in *C. guilhermei* and *L. rigidus* possesses a positive asymmetry, therefore these cultures have enough cells, which can form rather big colonies. It may be suggested that in the case of selection the number of such cells may increase and the second mode of size distribution could be obtained. In this connection it is worth saying that cultures with small colonies predominance were isolated in 1986 (*Crithidia quilhermei)* in Brazil (Soares et al., 1986) and in 1987 (*Leptomonas rigidus)* in North Russia (Podlipaev et al., 1991) – in about 20 years after cultures of *Crithidia acantocephali* (Hanson and McGhee, 1961) and *L. seymouri* (Guttman, 1966; Wallace, 1977) were obtained.

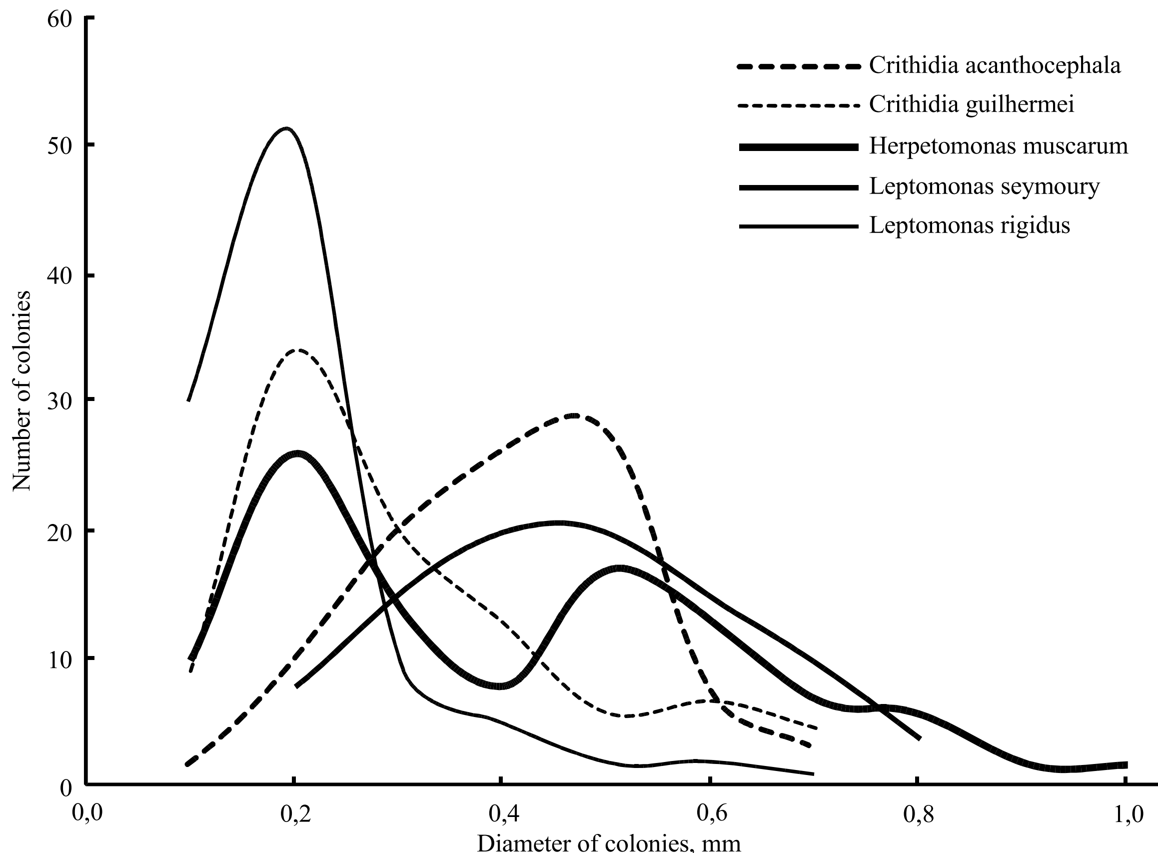

Fig. 11. Colony diameter distribution in 5 species of trypanosomatids from insects.

The procedure of trypanosomatids isolation from a host to culture and the cultivation itself might perform a selective choice of some certain genotypes in parasite population. One of the evidences of such selection is the decreasing number of amoeba-like colonies (Podlipaev, 1985) in culture of *Leptomonas peterhoffi.* They amounted 15 – 20% of the total colonies number just after culture isolation and presented single colonies per Petri dish in 5 years of laboratory cultivation. Therefore laboratory cultures generally do not precisely correspond to natural infection and may reflect the structure of real parasite population rather incorrectly. The latter especially refers to classical cultures (like *Crithidia oncopelti* and *C. fasciculata*) isolated many years ago.

In the case described above we have a simple system of measure characters which seems to be promising for genetic researches of trypanosomatids.

All data obtained allow to discriminate various isolates of trypanosomatids by phenotypic characters of the colonies and to use this features in taxonomy of trypanosomatids.

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