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Growth Characteristics, Cytopathic Effect in Cell Culture, and Virulence in Mice of 36 Type Strains Belonging to 19 Different *Acanthamoeba* spp.

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A total of 36 strains belonging to 19 different species of *Acanthamoeba* were compared for temperature tolerance, ability to grow in an axenic medium, cytopathic effect in Vero cell culture, and virulence in mice. Pathogenic strains appeared to belong to different species, whereas pathogenic and nonpathogenic strains occurred in one species. Although growth at high temperatures and readiness to grow axenically indicated a potential for pathogenicity, each such strain had to be tested in cell cultures or laboratory mice to determine whether or not it was virulent. This study was not intended to differentiate *Acanthamoeba* spp., but to provide methods to be used for the specific isolation and identification of pathogenic *Acanthamoeba* strains.

Taxonomic criteria employed for the differentiation of *Acanthamoeba* species are primarily based on morphology of cysts (7, 8, 10). The species designation is based in a few exceptions on more characters, of which serology is of utmost importance (18). Furthermore, serological methods distinguished different species established on a morphological basis, whereas in some instances several species had to be placed together in one group (14, 16). Recently, antigenic differences between *Acanthamoeba* strains referred to the same species have been reported (A. Riany, Ph.D. thesis, University of Lyon, Lyon, France, 1979), so that their identity is put into question. *Acanthamoeba* are important free-living amoebae because they can cause serious diseases in humans.

Acanthamoeba belonging to the species *A. culbertsoni, A. castellanii,* and *A. palestinensis* have been identified postmortem in human brains by immunofluorescence (IF) (17), whereas *A. polyphaga* are isolated from human eye infections (6).

Extensive physiological tests have been applied to different *Acanthamoeba* strains to identify the species (5). The results show that physiological tests are overlapping different species. Also, the tests demand too much effort to be used during ecological studies. Furthermore, we demonstrated in this work that a species identification gives no answer to the pathogenicity problem, and other tests have to be devised.

We have examined 36 strains belonging to 19 different species of *Acanthamoeba* and one strain of *Comandonia operculata* (9). They were compared in virulence and methods that might indicate the pathogenicity of strains. This comparative study was performed to find reliable methods for the isolation and identification of pathogenic *Acanthamoeba* spp.

MATERIALS AND METHODS

The designations of type strains and their origin are summarized in Table 1.

C. operculata were also tested in this study, because they constitute the only other genus that has been included in the family *Acanthamoebidae* (9).

The amoebae were mostly sent to our laboratory growing on an agar layer with bacteria, except for strains OR, 76-2252, 76-2253, 7327, SH 522, 7329, and 7418 which were grown axenically in Casitone-glucose-vitamins medium (CGV) (16).

Upon receipt, amoebic cultures were transferred to 1.5% nonnutrient agar spread with living *Escherichia coli* (NNE) (2) and incubated at 30 and 37°C. When growth occurred at 37°C, strains were tested at 40°C. After growth on NNE, a piece of agar with the migrating ring of dividing amoebae was transferred to serum-casein-glucose-yeast extract medium (SCGYEM) as described for *Naegleria* previously (2) and incubated at 30 and 37°C.

Amoebae received axenically were transferred directly to SCGYEM. Since laboratories wanting to isolate pathogenic *Acanthamoeba* spp. will also try to isolate *N. fowleri,* we have chosen to use the same axenic medium which allows the identification of pathogenic *N. fowleri* (2). By doing so, technicians untrained in amoebic morphology can transfer all isolates to this particular axenic medium. They do not have to identify the genus of an isolate before knowing which medium is to be used.

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TABLE 1. *List of strains*

Species	Strain designation	Origin	Reference	Received from[a]
A. astronyxis	Ray	Water, USA	16	8
A. castellanii	Neff	Soil, USA	16	8
	76-2252	Water, France	Derr-Harf[b]	3
	76-2253	Water, France	Derr-Harf	3
A. comandoni	7324	Water, France	Derr-Harf	3
A. culbertsoni	A-1	Cell culture, USA	16	1
	HA	Cell culture, USA	10	7
	KA	Cell culture, USA	10	7
A. divionensis	AA-2	Soil, France	10	5
A. echinulata	378	Compost, France	10	5
A. griffini	S-7	Seawater sediment, Connecticut	11	6
A. hatchetti	BH-2	Brackish water sediment, Maryland	12	6
A. lenticulata	PD 2	Medicinal pool, France	7	5
	2802	Water, France	10	3
	7327	Water, France	10	3
A. lugdunensis	L3a	Swimming pool, France	10	5
	SH 565	Human feces, France	10	3
A. mauritaniensis	1652	Soil, Morocco	10	5
	SH 197	Human feces, France	10	3
	SH 522	Human feces, France	10	3
A. palestinensis	Reich	Soil, Israel	16	8
A. paradivionensis	AA-1	Soil, France	10	5
A. polyphaga	P23	Water, USA	10	8
	7329	Water, France	Derr-Harf	3
	7418	Water, France	Derr-Harf	3
	Bitzer	Eye infection, USA	6	2
	Garcia	Eye infection, USA	6	2
A. pustulosa	GE3a	Swimming pool, France	10	5
A. quina	Vil 3	Swimming pool, France	10	5
	L1a	Swimming pool, France	10	4
	L3b	Swimming pool, France	10	4
A. rhysodes	1534 CCAP	Soil, England	16	8
	R4c	Swimming pool, France	10	4
A. royreba	OR	Cell culture, USA	18	7
A. triangularis	SH-621-1	Human feces, France	10	5
	SH-1551	Human feces, France	10	3
C. operculata	L5c	Swimming pool, France	9	4

[a] 1, Culture Collection of Algae and Protozoa, Cambridge, England; 2, D. Jones, Baylor College of Medicine, Houston, Tex.; 3, B. Molet, Institut de Parasitologie, Strasbourg, France; 4, P. Pernin, Departement de Parasitologie et de Pathologie Exotique, Lyon, France; 5, M. Pussard, Institut National de la Recherche Agronomique, Dijon, France; 6, T. Sawyer, Northeast Fisheries Center, Oxford, Md.; 7, A. Stevens, Veterans Administration Medical Center, Gainesville, Fla.; 8, E. Willaert, Veterans Administration Medical Center, Gainesville, Fla.

[b] Ph.D. thesis.

When growth occurred only at 30°C in SCGYEM after transfer from monoxenic culture, strains were transferred from axenic culture at 30°C to a new tube at 37°C.

Axenically growing amoebae were tested for cytopathic effect (CPE) in Vero cell cultures at 30 and 37°C. The concentration of amoebae was determined with a hemacytometer (Bürker); 1.5×10^5 amoebae were transferred to Vero cell cultures in tissue culture flasks (Falcon) with 75 cm^2 of surface area. The Vero cells had been cultured 3 to 4 days in 30 ml of Medium 199 with Earle's salts (Flow Laboratories). Strains that were not growing axenically were tested for CPE after growth on bacteria, and the same amoebic concentration was used. The cell cultures were screened daily for the appearance of CPE and amoebic growth for 3 weeks. The destruction of the whole monolayer was taken as endpoint, and the number of days since inoculation was recorded.

Strains showing CPE after transfer from NNE were inoculated from Vero cell culture to SCGYEM. All axenic growing strains were tested for pathogenicity by means of intracerebral (i.c.) inoculation in five mice, and the CPE was compared with virulence. In cases where at least three mice died after i.c. inoculation, amoebae were instilled intranasally (i.n.) in another set of five mice.

Amoebae were counted in a hemacytometer, and

2.10^4 amoebae were inoculated into mice. The presence of amoebae in brain and lungs was confirmed by culturing these organs on NNE, immediately after death or 3 weeks postinoculation for surviving mice.

RESULTS

The results of laboratory tests are summarized in Table 2. Considering each species separately, it was only in *A. culbertsoni* and *A. lugdunensis* that all strains investigated were found to be highly virulent (three and two strains, respectively, were available). In *A. lenticulata, A. polyphaga,* and *A. quina*, on the contrary, pathogenic as well as nonpathogenic strains were identified. In *A. castellanii, A. mauritaniensis,* and *A. rhysodes* (three, three, and two strains, respectively, were available), no strains were found with severe pathogenicity in test animals. The two strains of *A. triangularis* are probably nonpathogenic, although no i.c. inoculation was performed because they did not grow axenically. Of the other species, only one strain was available,

TABLE 2. *Results obtained with Acanthamoeba type strains*

Species	Strain	Growth on NNE at:			Growth in SCGYEM at:		CPE in Vero cell culture (days) at:[a]		Time of death (days)	
		30°C	37°C	40°C	30°C	37°C	30°C	37°C	i.c.	i.n.
A. astronyxis	Ray	+	−	−	+	−	12[b]	−	S, S, S, S, S[c]	ND[d]
A. castellanii	Neff	+	+	−	+	−	10	−	3, S, S, S, S	ND
	76-2252	+	+	−	+	+	5	−	12, S, S, S, S	ND
	76-2253	+	+	−	+	+	7	−	S, S, S, S, S	ND
A. comandoni	7324	+	−	−	−	−	ND	−[e]	ND	ND
A. culbertsoni	A-1	+	+	+	+	+	7	2	ND	5, 6, 6, 7, 10
	HA	+	+	+	+	+	8	3	2, 3, 4, 4, 5	7, 7, 9, 11, S
	KA	+	+	+	+	+	6	3	3, 3, 3, 3, 4, 5	S, S, S, S, S
A. divionensis	AA-2	+	+	−	+	±[f]	6	8[b]	17, S, S, S, S	ND
A. echinulata	378	+	−	−	−	−	−[e]	ND	ND	ND
A. griffini	S-7	+	−	−	+	−	14[b]	−	S, S, S, S, S	ND
A. hatchetti	BH-2	+	+	+	+	+	8	9	4, 5, 5, 5, 6	9, 10, S, S, S
A. lenticulata	PD 2	+	+	+	+	+	14	10	3, 3, 5, 5, 5	13, S, S, S, S
	2802	+	−	−	+	−	4	−	S, S, S, S, S	ND
	7327	+	+	+	+	+	4	6	3, 3, 3, 4, 5	S, S, S, S, S
A. lugdunensis	L3a	+	+	−	+	+[f]	5	10	7, 7, 8, 10, 14	S, S, S, S, S
	SH565	+	+	−	+	+[f]	7	7	4, 5, 6, 9, S	S, S, S, S, S
A. mauritaniensis	1652	+	+	−	+[g]	±[f]	10[e]	−	S, S, S, S, S	ND
	SH197	+	+	−	+	−	12[e]	−	S, S, S, S, S	ND
	SH522	+	+	−	+	−	9	−	S, S, S, S, S	ND
A. palestinensis	Reich	+	−	−	+	−	14[b]	−	3, S, S, S, S	ND
A. paradivionensis	AA-1	+	+	−	+	±[b]	8[e]	11[a]	3, 17, S, S, S	ND
A. polyphaga	P23	+	+	−	+	−	11	−	S, S, S, S, S	ND
	7329	+	+	−	+	+	11	13	6, 8, 9, 18, S	S, S, S, S, S
	7418	+	+	−	+	+	8	9	S, S, S, S, S	ND
	Bitzer	+	+	+	+	+	7	12[b]	S, S, S, S, S	ND
	Garcia	+	+	±	+	+	7	11	4, 10, S, S, S	ND
A. pustulosa	GE3a	+	+	−	+	+	−	−	S, S, S, S, S	ND
A. quina	Vi13	+	+	−	+	+[f]	8	−	S, S, S, S, S	ND
	L1a	+	+	−	+	−	−	−	S, S, S, S, S	ND
	L3b	+	+	−	+	+[f]	7	9	5, S, S, S, S	ND
A. rhysodes	1534CCAP	+	+	−	+	−	10	−	S, S, S, S, S	ND
	R4c	+	+	−	+	−	7	−	S, S, S, S, S	ND
A. royreba	OR	+	+	+	+	+	11	3	2, 3, 3, 4, 4	S, S, S, S, S
A. triangularis	SH-621-1	+	+	−	−	−	−[e]	−[e]	ND	S, S, S, S, S
	SH-1551	+	+	+	−[g]	−	12[e]	−[e]	ND	S, S, S, S, S
C. operculata	L5c	+	+	−	−	−	ND	ND	ND	ND

[a] At which time the whole monolayer is destroyed.
[b] Very few amoebae were observed.
[c] S, Survivor.
[d] ND, Not done.
[e] After transfer from NNE.
[f] After transfer from SCGYEM at 30°C.
[g] After transfer from Vero cell culture.

so that no conclusion can be reached. More strains of these species should be available for testing. However, only one type strain has been identified until now in some species (10, 18).

With three strains, i.e., KA, 7327, and OR, assigned to three different species, high CPE and mortality of i.c.-inoculated animals were obtained without killing any mouse after i.n. instillation. Especially with strain OR this result is unexpected, as it was found earlier that no pathogenicity in mice occurred with this strain, not even after i.c. inoculation (18). Another five mice were therefore inoculated i.c. with strain OR, and again all died.

A relationship between growth at 40°C and virulence existed for *A. culbertsoni, A. hatchetti, A. royreba,* and *A. lenticulata,* whereas the two strains of *A. lugdunensis* and one *A. polyphaga* showed CPE and killed mice when given i.c., although they did not grow at 40°C. However, one *A. triangularis* and one *A. polyphaga* strain did grow at 40°C but showed no pathogenicity.

No relationship between growth in SCGYEM at 37 or 30°C and CPE in cell culture or pathogenicity in mice was found. However, the most virulent strains adapted most easily to axenic growth at 37°C. Concentrations of 0.3×10^6 to 1.4×10^6 amoebae per ml were obtained in 4 to 7 days and *Acanthamoeba* strains formed cysts in SCGYEM. In comparison, *Naegleria* spp. attain concentrations of 1.0×10^6 to 2.5×10^6 amoebae per ml, and *N. fowleri* form no cysts in SCGYEM (2). Only pathogenic *N. fowleri* give a luxuriant growth at 37°C within 2 to 3 days, whereas nonpathogenic *Naegleria* strains can only be adapted to this medium after many subcultures.

CPE in Vero cells at 37°C was not always followed by virulence in mice with the *Acanthamoeba* type strains. Experimental animals, however, may differ in susceptibility. Although this may have an effect when inoculating i.c., it will have most influence on i.n. results. Therefore, we attributed most importance to the former inoculation route. A 100% mortality i.c. was always related to a total destruction of the Vero cell monolayer within 10 days at 37°C.

DISCUSSION

In this study I have not tried to determine whether each species name is a valid one. On the contrary, my goal was to reassemble different species in two groups, pathogenic and nonpathogenic. I was especially interested in investigating and comparing several new species that have been described during recent years (7, 10, 11, 18) but about whose pathogenic aspect very little is known.

In a separate study (submitted for publication), I have used the same criteria tested and the experience gained in this study to investigate the ecology of pathogenic and nonpathogenic *Acanthamoeba* spp. in thermally polluted discharges and control surface waters.

Although it was found previously and confirmed in this study that all *A. culbertsoni* strains are highly virulent, virulence could not be attributed as a species property to any other *Acanthamoeba* sp., except for *A. lugdunensis.* A recent study by Riany (Ph.D. thesis) suggests, however, that three strains of *A. quina,* which were the same as used in this study, were all virulent for mice i.n. In this study, however, the i.c. results obtained with *A. quina* strains are in good agreement with CPE results at 37°C, indicating that only one strain of *A. quina* is virulent. The study by Riany (Ph.D. thesis) shows differences in enzymatic activities, isoelectric focusing patterns of proteins and immunoelectrophoresis between the three *A. quina* strains. Thus, the species identity of these three strains is put into question.

A. hatchetti that are isolated from seawater also constitute a species in which the only known strain is pathogenic. More isolates of this species, as with other species, need to be available for investigation. When the i.c. virulence results obtained by C. Derr-Harf (Ph.D. thesis, University of Strasbourg, Strasbourg, France, 1977) are compared with mine, there is good agreement in the virulence of *A. lenticulata* 7327 and *A. polyphaga* 7329 and in the nonvirulence of *A. polyphaga* 7418, although for the latter strain I found CPE in cell culture.

The high virulence of *A. comandoni* 7324 and the low virulence of *A. castellanii* 76-2252 and 76-2253 found by Derr-Harf were not confirmed in my experiments.

When comparing the i.n. virulence results of Pernin (Ph.D. thesis, University of Lyon, Lyon, France, 1976) with my findings, the nonvirulence of *A. quina* L1a and *A. pustulosa* GE3a is confirmed. When the results of Riany (Ph.D. thesis) are compared with the present results, the difference in virulence of *A. quina,* as already mentioned, is striking, although Pernin also found one strain tested to be nonvirulent. The nonvirulence of *A. pustulosa* GE3a is in agreement with my results. He also found *A. lugdunensis* L3a nonvirulent i.n.; I found this strain highly pathogenic i.c. with CPE in Vero cell cultures. All the investigators mentioned have found a high virulence for *A. culbertsoni.* Because species identification gave no foolproof information on the pathogenicity of an isolate, I have attempted to relate virulence to easy-to-perform tests. When a strain grows at 40°C, there is a high probability that it is pathogenic; especially

when strains were virulent i.n., they grew at 40°C. Although all strains of *A. quina* failed to grow at 40°C, which is in agreement with its very low virulence, Riany found all strains virulent for mice i.n. Griffin (4) also finds that moderately virulent *Acanthamoeba* spp. do not grow at 40°C. When a strain does not grow axenically in SCGYEM at 37°C, there is very little chance that it will be virulent.

Except for the two strains of *A. lugdunensis*, virulent strains adapted most easily to the axenic medium. Pathogenicity testing should be performed as soon as possible; as has been shown, strains lose virulence (13) with frequent transfers in axenic culture. CPE in cell cultures was found to be the most reliable criterion for pathogenic strains during our study. Cursons and Brown (1) thought, however, that CPE in Vero cell cultures is an indication of virulence in the genera *Acanthamoeba* and *Naegleria*. A report by De Jonckheere and van de Voorde (3) showed, however, that *Naegleria* strains, closely related to *N. fowleri*, showed no virulence against mice, although a total destruction in Vero cell cultures was observed.

Since the investigation reported here was meant as a tool for ecological studies of pathogenic *Acanthamoeba*, we came to the following conclusion: although ability to grow at 40°C on bacteria and the readiness to grow at 37°C in SCGYEM points to virulence in an *Acanthamoeba* isolate, its virulence can only be measured in cell cultures or laboratory animals. The latter are, however, very different in susceptibility, and the inoculation technique is very difficult to standardize.

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