FOLIA PARASITOLOGICA (PRAHA) 28: 97—103, 1981.

DIFFERENT VIRULENCE OF NAEGLERIA FOWLERI STRAINS ISOLATED FROM A SWIMMING POOL

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Abstract. Thirty-three strains of *Naegleria fowleri* were isolated from water in an indoor swimming pool during two years. This population included, in addition to virulent and nonvirulent strains, also forms atypical in their growth properties on artificial media, unstable temperature optimum and low virulence. They were antigenically related to *N. fowleri*. Their pathogenic features disappeared shortly after isolation.

The detection of previous contact of patients suffering from primary amoebic meningoencephalitis (PAME) with water contaminated with virulent amoebae *Naegleria fowleri* (Carter 1972) instigated in many countries protozoological studies of waters from swimming pools (De Jonckheere 1979 a). In Czechoslovakia, this agent was repeatedly isolated due to unusual circumstances in a swimming pool where the highest recorded number of infections occurred (Kadlec et al. 1980).

As it was revealed by the studies of growth and pathogenic properties and antigenic structure, the collection of isolates was heterogeneous. The existence of *N. fowleri* strains with different virulence isolated from natural environment was reported by Willaert et al. (1974), De Jonckheere and van de Voorde (1977), De Jonckheere (1979 b), Lawande et al. (1979) and others.

MATERIAL AND METHODS

The strains were isolated from the environment using the method described by Kadlec et al. (1980) and identified after taxonomic criteria of Page (1967) and Singh and Das (1970).

The strains were cultured on non-nutrient agar with *Aerobacter* suspension (NNA) (Culbertson et al. 1965) and in liquid 2% Bacto-Casitone medium with 10% native rabbit serum (BCS) (Červa 1969). SCGYEM (De Jonckheere 1977) and BCS with 0.5% NaCl (Singh and Das 1970) were used as selective media.

Antigen from axenized strains was prepared from 6-day-old cultures in BCS by repeated washing in phosphate buffer, pH 7.2 and by centrifugation to amoebae concentration of $10^8 \cdot ml^{-1}$. Further dilutions were made if necessary. The cells were disintegrated by freezing and de-freezing. In the strains which could not be axenized the antigen was prepared from a suspension of the culture washed from NNA. This was also washed, concentrated and devitalized as in axenic strains. The antigens were stored at —20 °C.

Males of chinchilla rabbits weighing 1.5—2 kg, without natural antibodies against *Naegleria*, were immunized after Crowle (1973). Antisera obtained by immunization of non-axenized strains were absorbed with *Enterobacter aerogenes* suspension (CCM 2531) to remove homologous antibodies after Wellings et al. (1977). They were also stored at —20 °C.

The indirect fluorescence method (IFAT) was carried out after Červa and Kramář (1973) with 1 : 200 Evans' blue concentration as modified by Willaert (1976). The complement fixation reactions (CFR) were performed after Červa (1967) with the following values of components: antigen (cellular) titre 1 : 32—40, complement 1.25 unit, haemolytic amboceptor 1 : 8000 and sheep erythrocytes 0.2%.

Pathogenetic experiments were performed using the methods described earlier (Kadlec et al. 1980).

RESULTS

Thirty-three strains of amoebae were gradually isolated from various places of a swimming pool during two years. They were identified as *Naegleria fowleri*. Their further diagnosis revealed some differences in the morphology and in growth, pathogenicity and immunologic properties. According to these factors the population of amoebae could be divided into highly virulent, lowly virulent and non-virulent strains.

Morphology. The main diagnostic criterion for the interspecific identification of the genus *Naegleria* is the morphology of cysts (Willaert 1976, De Jonckheere and van de Voorde 1977). The shape and number of cyst pores in all isolates was identical with that of *N. fowleri*, only the sizes were somewhat different in some strains (Table 1).

Table. 1. Measurements of cysts (in μm) in isolated strains compared to HB-1 and 1518/1e strains (after De Jonckheere and van de Voorde 1977)

Strain	Cysts minimum—maximum	Average	Strain	Cysts minimum—maximum	Average
HB-1	8.5—12.5	10.8	559	7.6—10.7	9.8
3224	8.5—12.3	10.2	563	7.6—10.7	9.0
3228	do not encyst		880	9.1—12.2	10.2
161	7.6—10.7	9.0	988	9.2—10.7	9.4
162	do not encyst		998/1	7.6—10.7	8.8
163	do not encyst		2	6.1—12.3	9.5
241	9.2—10.7	9.8	454	10.7—13.8	11.9
420/1	7.7—10.7	9.1	3085	6.1—13.8	10.7
2	7.6—10.0	9.0	1822	7.6—13.8	11.7
3	7.6—12.2	9.9	1828	6.1— 9.9	8.4
4	7.6—10.7	9.1	2974	7.6—13.8	10.7
5	9.2—12.2	10.5	3314	7.6—10.7	9.3
6	7.6—10.0	8.9	3096	10.7—16.8	14.2
7	6.1—10.7	8.1	2436	7.6—12.2	9.3
8	9.2—10.7	9.7	3	9.2—12.7	11.4
9	7.6—10.0	9.2	3221	13.7—15.3	14.5
10	9.1—12.2	10.1	1518/1e	8.5—20.0	14.1
557	7.7—10.7	9.1			

Note: 25 cysts of each strain were measured

Some of the highly virulent strains did not encyst on the used media.

Growth on artificial media. All strains were isolated in primoculture at 43—45 °C (Griffin 1972). Twelve of them (3224, 3228, 161, 162, 163, 420/1, 420/2, 420/3, 420/4, 420/7, 420/9, 420/10) were further cloned and transferred from the first subculture to axenic conditions and then cultured for a long time at the same temperature as in primoculture. Except two of them, all grew in SCGYEM; the strains 420/2 and 420/7 soon encysted in this medium and could not be passaged in it.

Another group of strains did not grow in SCGYEM and required the presence of *Aerobacter* suspension in the medium. Immediately after isolation at culture temperatures higher than 30 °C they encysted or did not grow at all within 48 h after reinoculation. Their temperature optimum from the 3rd to 6th passage ceased at 20 °C.

In addition to these two groups of strains with defined properties, there were atypical strains in the population. Some of them were sooner or later transferred to axenic medium, others did not grow in the culture medium without *Enterobacter aerogenes*

suspension. They differed also in the growth in SCGYEM. Soon after isolation they stopped to grow at 43 °C; they were then individually passaged for a long time at 20° or 28—30 °C, exceptionally at 37 °C (Table 2). This characterization does not conform with the properties of either virulent or non-virulent variants of *N. fowleri*. The growth of all isolated *Naegleria* was inhibited by 0.5 % NaCl concentration in the medium

Table. 2. Growth properties of atypical strains

Strain	Axenisation	SCGYEM	Optimum temperature during long-term passage (in °C)
420/6	+	+	37
557	+	++	28—30
559	+	++	20
880	—	+	28—30
998/1	+	++	20
1828	—	+	28—30
1822	—	+	28—30
2974	+	++	28—30
3314	—	+	28—30
2436	—	+	28—30

Explanation: growth in SCGYEM ++ = rich growth during 3—7 days, capable of passage in medium, + = encystation within 7 days, unable of passage in medium

Table. 3. Results of infection experiments with strains with transitive properties

Strain	Mode of inoculation	Death/No. of experimental animals	Amoebae in organs
420/6	i.c.	5/5	no
557	i.n.	0/5	no
	i.c.	0/5	no
	i.c.	0/5	no
559	i.n.	0/5	no
880	i.c.	1/5	no
998/1	i.c.	1/5	no
1828	i.c.	0/5	no
1822	i.c.	1/5	yes—brain*
	i.n.	0/5	no
	i.c.	3/5	yes—brain, lungs
2974	i.n.	2/5	yes—brain, lungs
3314	i.c.	1/5	yes—brain, lungs
2436	i.n.	2/4	yes—brain, lungs

Explanation: * = amoebae found in the brain of dead and killed mouse

Pathogenic experiments. The strains growing constantly, at temperatures higher than 40 °C, which were rapidly axenized, induced a classical PAME with 100 % mortality of experimental animals after intracerebral inoculation. The mice died on days 2—9 after infection with heavy meningial symptoms and *Naegleria* amoebae were

detected by microscopic examination and cultivation of their brains and lungs. Pathogenic properties of some isolates were maintained for a long time, LD_{50} of strain 3224 was preserved even after 26 months of 8×10^3 trophozoites at intranasal inoculation.

The atypical strains, whose temperature optimum rapidly decreased after isolation under laboratory conditions, could be axenized only after a longer interval of even several weeks or could not be axenized at all, induced meningitis in mice in infection experiments with 20—60 % mortality (except strain 420/6). In experiments with the strains 2974, 3314 and 2436, *Naegleria* were demonstrated in brains and lungs of dead animals; in the experiment with the strain 1822 the agent was detected also in the brain of one of the surviving mice (Table 3).

The pathogenicity of these strains was detectable only immediately after isolation, after 4—6 weeks under laboratory conditions the results of repeated infection experiments with these strains were negative (Table 4).

Table. 4. Decrease in virulence of strain 2436 during 7 weeks after isolation studied in intranasally inoculated mice

Date of experiment	Size of inoculum	Day of death	Symptoms	Amoebae in organs
Week 0	360 amoebae	3, 5, S, S	meningism in dead animals	in brain of dead animals
Week 3	500 amoebae	21, S, S, S, S	meningism in dead animals	not detectable due to cannibalism
Week 5	2500 amoebae	5, S, S, S, S	meningism in dead animals	no
Week 7	4000 amoebae	S, S, S, S, S	no	no

Explanation: S = surviving experimental animal

The remaining isolates caused neither disease nor death of experimental animals after intracerebral inoculation. The cultivation of mouse brains and lungs after the end of the experiment was negative.

Immunological tests. The results of IFAT revealed a very close relationship of strains No. 3224 and 420/3 with Vítek strain. This relation was gradually lower in the remaining tested strains (Table 5). The cross reactions demonstrated mutual antigenic relationships of isolated *Naegleria* which could be better detected by complement fixation reaction (Table 6).

Table 5. Reaction of isolated strains in IFAT with *N. fowleri* (Vítek) antiserum

Strain	Reciprocal value of titre
Vítek	256
3224	256—512
420/3	256—512
420/6	64—128
2974	128—256
557	64
241	64—128

Table 6. Antigenic relationships of isolates detected in cross reactions

Strain	Antiserum:					
	3224		557		241	
	IFAT	KFR	IFAT	KFR	IFAT	KFR
3224	512	128	128	0	8	0
557	8	512	64	512	8	0
241	16	0	64	64	128	128
2974	128—256	—	128	—	64	—
Vítek	128	—	256	—	16	—

Explanation: — = reaction was not performed

DISCUSSION

N. fowleri, the etiological agent of PAME, isolated from clinical material is virulent for experimental animals even after several passages under laboratory conditions. However, the papers by Visvesvara and Callaway (1974) and Wong et al. (1975, 1977) indicate that the pathogenicity of clones obtained from these isolates gradually decreases. On the other hand, De Jonckheere (1979 b) managed to increase the virulence of attenuated strains by passages in brains and in tissue cultures.

The studies of *N. fowleri* strains isolated from natural environment confirmed the differences in their pathogenicity. De Jonckheere and van de Voorde (1977) isolated non-pathogenic strains which were identical with *N. fowleri* in all other taxonomic criteria. Some differences were detected only by ultrastructural and immunological methods (Willaert et al. 1974, Willaert 1976, Willaert et al. 1977). A possibility of systematical reevaluation of the genus *Naegleria* is discussed on the basis of all these facts (Stevens et al. 1980).

The paper deals with a description of *Naegleria* originating from a single closed place—indoor swimming pool, where they vegetated for many years in an extraordinary situation. This population included both strongly virulent forms identical with the known strains Vítek and HB-1 and maintaining their pathogenicity for more than two years, and those identical with non-pathogenic *N. fowleri* strains described earlier by many authors and distantly antigenically related with virulent strains HB-1 and Vítek.

In our opinion, a group of strains transitive between these two forms and corresponding to the variants referred to by Jamieson (1975), Willaert (1976), De Jonckheere (1979 c), Lewande et al. (1979) and Scholten (1979) could be further identified among the isolates on the basis of the results obtained by the methods used. Some of them were capable of growing in a selective medium for pathogenic *Naegleria*, they could be axenized and were lowly virulent, others could not be cultured without *Aerobacter* suspension, but their short-term virulence was high (Table 4). However, the pathogenicity and optimum culture temperature rapidly decreased and they could not be induced by the described methods. A contrary tendency was not recorded.

Due to the unstability of these properties, the existence of transitive strains must be evaluated carefully. Further studies are necessary to demonstrate whether the strains are identical with those causing chronic meningitis in experimental animals as mentioned by Lawande et al. (1979) and Scholten (1979) or with *N. fowleri* strains with a virulence lowered by some factors (Cl^-?) in the environment from which they were isolated or whether they are related with the new species described recently (Stevens et al. 1980).

РАЗНАЯ ВИРУЛЕНТНОСТЬ ШТАММОВ *NAEGLERIA FOWLERI*, ВЫДЕЛЕННЫХ ИЗ ПЛАВАТЕЛЬНОГО БАССЕЙНА

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Резюме. Из закрытого плавательного бассейна выделяли 33 штаммов *Naegleria fowleri* в течение двух лет. Кроме вирулентных и невирулентных форм обнаружены также формы атипичные по своим свойствам роста на искусственных средах, нестабильному оптимуму температуры и низкой вирулентности, антигенно родственные формы *N. fowleri*. Патогенетические свойства этих штаммов исчезали вскоре после выделения.

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Received 23 April 1980.

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FOLIA PARASITOLOGICA (PRAHA) 28: 103, 1981.

IV. Symposium on Medical and Veterinary Acaroentomology, Gdańsk 1980

Between 4 and 6 September 1980 the IV. Symposium on Medical and Veterinary Acaroentomology was held in Gdańsk (Poland). It was attended by 83 specialists from Poland, Czechoslovakia (3), German Democratic Republic (2), Federal Republic of Germany (1), Romania (1) and USA (1).

The proceedings were divided into 3 sections:
1 — Biology and epidemiological role of arthropods in environment at various stages of anthropogenization (Subsection A: Ixodides; parasites of rodents; Nematocera. Subsection B: Other arthropods which are parasitic or noxious to man and domestic animals).
2 — Faunistics (Subsection A: Parasites of wild birds and mammals. Subsection B: Crustaceans of marine fish).
3 — Arthropod control (Subsection A: Biological, chemical and other methods of control. Subsection B: Practical evaluation of insecticides and their application).

The sessions were organized so that each subsection was opened by an introductory paper which summed up the most important conclusions of registered contributions and the most significant results achieved in the given field since the last symposium. This introduction was followed by a lively discussion. The participants had at their disposal abstracts of papers containing 70 contributions in alphabetic order of the authors. Most numerous communications were listed in the subsections 1 A and 1 B. The problems of arthropod control dealt with in Section 3 also yielded much stimulative information. The Czechoslovak specialists presented four papers. The papers will be published in extenso in a special issue of the journal Wiadomości Parazytologiczne. The social program of the symposium included informal coctail and a coach trip to the Cashubian Lake district.

The meeting of Polish and foreign medical and veterinary acaroentomologists, which has already become a tradition, offered an opportunity to learn the latest results in research and discuss problems of mutual interest in personal conversations. The symposium was well organized and the hosts provided the participants with a pleasant working and social atmosphere.

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