

# EPIDEMIOLOGICAL TYPING OF ACANTHAMOEBA STRAINS ISOLATED FROM KERATITIS CASES IN BELGIUM

J. F. DE JONCKHEERE\*

## SAMENVATTING

Vijftien *Acanthamoeba* stammen werden gekweekt uit the cornea van 9 keratitis patiënten en uit de contact lens, contact lens doosjes en zoutoplossingen (paraphernalia) van deze patiënten. Uit het zwembad waar één der patiënten had gezwommen, werd een *Acanthamoeba* geïsoleerd, maar het leidingwater van de huizen van 3 patiënten die onderzocht werden, leverde geen *Acanthamoeba*. Alle *Acanthamoeba* uit de cornea behoren tot genotype T4, maar tot verschillende subtypes. De stammen geïsoleerd van paraphernalia van de patiënt zijn van hetzelfde subtype als deze uit de cornea. De enige *Acanthamoeba* geïsoleerd van een contact lens, zonder dat er sprake was van *Acanthamoeba* keratitis, behoort tot een ander genotype. Een *Hartmannella* uit een cornea en twee vahlkampfiids uit contact lenzen hebben geen verband met keratitis. Deze studie bevestigt dat ook in België alleen *Acanthamoeba* genotype T4 van de 12 gekende *Acanthamoeba* genotypes verantwoordelijk is voor amoeben keratitis. De meeste gevallen van *Acanthamoeba* keratitis worden veroorzaakt door gebrekkige hygiëne in de behandeling (schoonmaak en opslag) van de contact lenzen.

## RÉSUMÉ

Quinze souches d'*Acanthamoeba* ont été isolées à partir des cornées de 9 patients atteints de k ratite ainsi que des lentilles de contact, des bo tiers de ces

.....

\* Wetenschappelijk Instituut Volksgezondheid,  
B-1050 Brussel.

received: 03.12.02

accepted: 22.01.03

lentilles et des solutions physiologiques (paraphernalia) de ces patients. Une souche d'*Acanthamoeba* a  t  isol e d'une piscine o  un patient avait nag , mais l'eau de distribution qui a  t  analys e dans la maison de 3 patients, ne r v lait pas la pr sence d'*Acanthamoeba*. Toutes les souches d'*Acanthamoeba* isol es de la corn e appartiennent au g notype T4, mais sont des sous-types diff rents du T4. Les souches isol es   partir des lentilles de contact (et/ou paraphernalia associ s) d'un patient, appartiennent au m me sous-type de celle isol e de la corn e. La seule souche d'*Acanthamoeba* isol e   partir d'une lentille de contact, sans qu'il y ait une liaison avec une infection de k ratite   *Acanthamoeba*, se r v lait  tre un autre g notype. Une souche d'*Hartmannella* isol e d'une corn e et deux souches de vahlkampfiides isol es de lentilles de contact, n' taient pas associ es aux k ratites. Cette  tude confirme, comme d montr  ailleurs, que seul le g notype T4 des 12 g notypes connus chez *Acanthamoeba*, est responsable de k ratite amibienne en Belgique. La plupart des cas de k ratite   *Acanthamoeba* est due   l'hygi ne d plorable dans le traitement (rincage et stockage) des lentilles de contact.

## SUMMARY

From the corneas of nine keratitis patients and from their contact lenses, contact lens boxes and saline solutions, 15 strains of *Acanthamoeba* have been isolated. An *Acanthamoeba* strain was isolated from the swimming pool where one of the patients swam, while in the tapwater of the houses of three patients investigated, no *Acanthamoeba* could be detected. All the *Acanthamoeba* isolates from the cornea belong to genotype T4, but are different subtypes of T4. The *Acanthamoeba* detected on the contact lenses (and/or associated paraphernalia) of a patient are of the same subtype as that isolated from the cornea. The only *Acanthamoeba* strain isolated from a contact lens which was not related to an *Acan-*

*thamoeba* keratitis infection proved to be another genotype. A strain of *Hartmannella* from a cornea and two vahlkampfiids isolated from contact lenses had no connection with keratitis. This study confirms that, as found elsewhere, only *Acanthamoeba* genotype T4 of the 12 known *Acanthamoeba* genotypes is responsible for keratitis in Belgium. Most cases of *Acanthamoeba* keratitis cases are due to poor hygiene in the treatment (cleaning and storage) of contact lenses.

#### KEY-WORDS

*Acanthamoeba*, genotyping, keratitis, contact lens

#### MOTS-CLÉS

*Acanthamoeba*, génotypage, k ratite, lentille de contact

## INTRODUCTION

Cases of keratitis due to amoebae of the genus *Acanthamoeba* were first reported in the seventies of last century in the UK (21) and in the USA (16). But the first recorded case seems to date back to 1971 in the Netherlands (12). Since then, over 1,350 cases have been reported worldwide (19). The first reported cases in Belgium occurred in 1982 (13) and 1988 (20).

While the earliest cases were due to trauma of the eye, such as by an insect (12), hay (16), a branch (21) or glass wool dust (13), the enormous increase of *Acanthamoeba* keratitis in the eighties is connected with contact lens wear (19).

*Acanthamoeba* are easy to grow, but difficult to identify. Isoenzyme typing was a promising method (9) which allowed the determination of whether strains were identical. But this technique enables the identification of the species in only a few cases. DNA molecular typing systems were much more promising for clustering *Acanthamoeba* strains into genotypes (11). To date, 12 genotypes have been differentiated thanks to the work of the laboratory of Thomas J. Byers (3) on the small subunit ribosomal DNA (SSUrDNA). Furthermore, these genotypes could be considered to be different species. It soon became apparent that the majority of keratitis cases are caused by a single SSUrDNA genotype (or species). In fact, 97% of the *Acanthamoeba* isolates from keratitis cases belong to genotype T4 (3). Recently, an easy typing method has been developed that does not require the sequencing of the whole 2,300 bp SSUrDNA of the strains (24). Only a short sequence (DF3) in the SSUrDNA suffices for determining the genotype of an *Acanthamoeba* isolate (1). Using the rapid automatic DNA sequencing facilities at ICP, I have applied this method to strains isolated in Belgium which had previously only been typed by isoenzyme profiles. Fortunately, DNA or cell pellets had been prepared and kept at -20 C since the time the strains were isolated.

## MATERIALS AND METHODS

### PATIENTS AND SPECIMENS INVESTIGATED

- Patient H, a 17 year old boy, wore soft contact lenses and had been swimming at a subtropical leisure center before the onset of keratitis (10) in April 1991. Samples from the cornea, contact lens, contact lens box used for disinfection, a bottle of Allergan saline solution (NaCl, NaEDTA, Thiomerosol 0.001%) and CIBA Vision OASEPT disinfection solution (30 mg H<sub>2</sub>O<sub>2</sub>, 8.5 mg NaCl/ml) were incubated for growth of amoebae. In addition, water samples from different taps at home and from seven different swimming pools (water temperature from 27.5 to 36.5) and two filter systems at the leisure center were cultured for amoebae.
- Patient B, a 22 year old female, wore hard contact lenses and developed keratitis (10) in June 1991. Samples from the cornea, contact lens, contact lens box and bottle of saline were incubated for growth of amoebae. In addition, water samples from different taps at home were cultured for amoebae.
- Patient V, a 27 year old female, was using disposable contact lenses and developed keratitis (10) in September 1991. Samples from the cornea and from a bottle of saline were incubated for growth of amoebae. In addition, water samples from different taps at home were cultured for amoebae.
- Patient DK, female, developed keratitis in June 1993 (Maudgal P., personal communication). A corneal scraping was cultured for amoebae.
- Patient St, a female, developed keratitis in June 1993 (Maudgal P., personal communication). A corneal scraping and contact lens case were cultured for amoebae.
- Patient A received a piece of stone in the eye during demolition works in May 1999 and developed keratitis. Only a corneal scraping could be investigated for the presence of amoebae (Belle A.-M., De Gheldre Y. and Engelinus F., personal communication).
- Patient D, a female of 18 years, wore contact lenses and had travelled to South Africa before developing keratitis in May 2000. A cor-

neal scraping and the contact lens were cultured for amoebae (Belle A.-M., De Gheldre Y. and Schrooyen M., personal communication).

- Patient S, a female of 43 years, wore contact lenses. The left eye became infected in June 2000. Samples from the cornea, contact lenses, and a bottle of saline were cultured for amoebae (Bohy E. and Luyasu V., personal communication).

- *Acanthamoeba* cultures obtained from corneal scrapings from three more keratitis patients (Ac704 in 1999; E7623 in August 2000, and ITMAP2711 in May 2000) were received (M. Peters and D. Le Ray, personal communications), but no information on these patients is available.

### MORPHOLOGICAL IDENTIFICATION OF ISOLATES

Amoebae are isolated by incubating the specimens on non-nutrient agar spread with *Escherichia coli* at 37 or 30°C. Water samples are first concentrated by centrifugation and the sediment incubated as above.

Amoeba isolates can be identified by morphological examination only to genus level. For this the morphology of cysts, and the morphology and movement of the trophozoites, are examined by phase contrast microscopy (23).

After isolation on bacteria, the strains were adapted to axenic growth in liquid serum-glucose-yeast extract medium (SCGYEM) (5). At peak log phase growth, the amoebae were dislodged from the wall by shaking and pelleted by centrifugation. Pellets are used either for protein extraction in the isoenzyme analyses or for DNA extraction for PCR.

### MOLECULAR TYPING OF ACANTHAMOEBA ISOLATES

- Proteins are extracted by suspending the pellet in 0.25% Triton X-100. After separating the proteins by agarose isoelectric focusing the gels were stained for acid phosphatase (AP) and propionyl esterase (PE) (9).

- DNA was isolated using the UNSET method (15). The ASA.S1 part of the SSUrDNA was PCR amplified using the JDP1 and JDP2 primers, and in this amplicon the diagnostic frag-

ment 3 (DF3) was sequenced using primer 892c (1).

## RESULTS

### MORPHOLOGICAL IDENTIFICATION OF ISOLATES

- From the cornea of patient H, the soft contact lens and the contact lens box used for disinfection, strains of *Acanthamoeba* sp. were isolated. The bottles of saline and disinfection solution yielded no amoebae. From the tapwater in the house of the patient, strains of *Hartmannella* sp. and *Thecamoeba* sp. were isolated. Water samples were taken from seven different swimming pools and from two separate filtering systems at the leisure center where the patient had been recently swimming. The water was properly disinfected (chlorine between 0.72 and 1.2 ppm and pH between 7.15 and 7.35), so few amoebae were detected. However, one strain of *Acanthamoeba* sp. was isolated from one swimming pool and a *Mayorella* sp. was isolated from another.

- From the cornea of patient B, and from both her hard contact lenses and from the saline in the contact lens case, an *Acanthamoeba* sp. was isolated. No amoebae were found, however, in the bottle of saline used for cleaning the lenses. Strains of *Mayorella* sp. were isolated from the taps in the student room of the patient, while strains of *Thecamoeba* sp. and *Hartmannella* sp. were identified in samples from the taps in her home.

- From the cornea of patient V a strain of *Acanthamoeba* sp. was isolated. From the tapwater in the house of the patient no amoebae could be isolated. As patient V was using disposable lenses, no isolations could be performed from contact lenses nor contact lens boxes. A used bottle of saline yielded no amoebae.

- From the cornea of patient DK a strain of *Acanthamoeba* sp. was isolated.

- From the cornea of patient St, and from the contact lens case, an *Acanthamoeba* sp. was isolated.

- From the corneal scraping of patient A a strain of *Hartmannella* sp. was isolated. No other material was available for investigation. The identity of the *Hartmannella* strain was confirmed by sequencing the SSU rDNA (not shown).

- While the cornea of patient S proved negative for *Acanthamoeba* sp., a strain of *Acanthamoeba* sp. and an amoeba belonging to the Vahlkampfiidae were isolated from both the contact lenses and from the bottle of saline. The vahlkampfiid amoeba did not transform into flagellates upon addition of distilled water. Amoebae cell pellets, sedimented by centrifugation, showed a pink color, which is found only in *Vahlkampfia avara*, *V. inornata* and in *Paravahlkampfia ustiana* (2).

- From patient D no amoeba was isolated from the corneal scraping but a strain (S1504) belonging to the Vahlkampfiidae was isolated from the contact lens. The amoebae did not transform into flagellates upon addition of distilled water.

- Three more *Acanthamoeba* strains (Ac704, E7623 and ITMAP2711) were isolated from corneal scrapings of keratitis patients.

The *Acanthamoeba* cornea isolates from patients H, B and V have been tested for susceptibility to Brolene and Flagyl. Although patient V suffered longer from the eye infection, no difference was observed in susceptibility to these treatments. The three isolates showed no growth at 2 µg/ml, but did grow at 0.66 µg/ml. Flagyl had no effect even at a concentration of 10 µg/ml, the highest concentration tested.

### MOLECULAR TYPING OF ACANTHAMOEBA ISOLATES

All the cornea isolates in this investigation belong to genotype T4 (Fig. 1) but the cornea isolates of most patients differ from each other by at least one bp in the DF3 sequence segment. Only the cornea isolate of patient St has an identical DF3 sequence to the strains from another patient, patient B.

Isolates from the cornea, contact lens box and distilled water of patient H are identical subtypes of T4, and have identical isoenzymes. But the strains do not belong to any of the T4 subtypes found by Booton (1).

Also, the isolates from the cornea, contact lens, right contact lens and contact lens box of patient B are identical subtypes of genotype T4, and have identical isoenzymes. But these strains also do not belong to any of the T4 subtypes found by Booton (1).

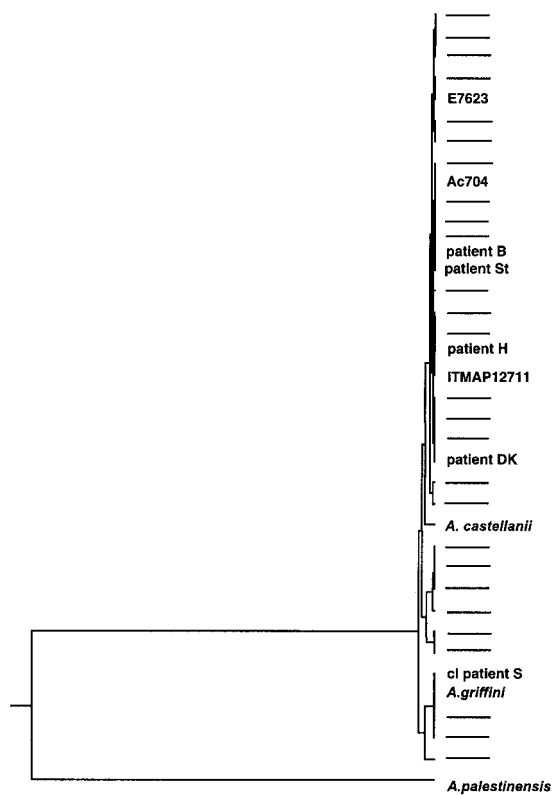


Fig. 1. Phylogenetic analysis based on the DF3 DNA sequence. For legibility only the Belgian isolates and one reference species of each genotype are named. The branches of other strains are indicated with a line. The cornea-associated strains are in T4. The isolate from patient V could not be included in the analysis as it is a mixed genotype T4. The non-cornea-associated strain is in T3.

The strain from the swimming pool, where patient B went swimming, is not a T4, and has different isoenzymes from the strain isolated from the patient.

The DF3 sequences of the cornea and the contact lens box isolates of patient St differ by 2 bp. Furthermore, the isoenzymes were different. The isoenzymes of the strain from DK were the same as from the contact lens box of patient St. There has probably been a mislabeling of strains, as they were received on the same date. The strains from the cornea and the contact lens of patient St do not belong to any of the T4 subtypes found by Booton (1).

The strain from patient V has a mixed DF3 sequence of T4 subtypes. This is due to two different alleles of the SSUrDNA as reported pre-

viously in a low percentage of *Acanthamoeba* strains (3).

The DF3 sequence of the cornea isolate ITMAP 2711 is identical to the reference strain CCAP 1501/3G (not shown), which was isolated from a keratitis case in the USA in 1974 (16). But neither strain belongs to any of the T4 subtypes found by Booton (1). Strain E7623 is identical to the subtype T4/1 of Booton (1).

**T4** Strain Ac704 does not belong to any of the T4 subtypes found by Booton (1). The *Acanthamoeba* isolate from the right contact lens of patient S, where there was no isolate from the cornea, is not a T4, but a T3 genotype (Fig.1). The DF3 sequence of this strain is identical to subtype T3/3 of Booton (1) and to the type strain S7 of *A. griffini* (18).

## DISCUSSION

**T3** The majority of *Acanthamoeba* keratitis cases have involved amoebae of genotype T4 (3). All cornea isolates from our study in Belgium were also genotype T4. In addition, in each case, all the isolates from a patient's contact lens paraphernalia were the same subtype of genotype T4 as the patient's corneal isolate, with the exception of one where mislabeling is suspected. Our study confirms that contact lens boxes are "breed-

ing grounds" for *Acanthamoeba* if hygiene standards are poor.

In patient S, when no *Acanthamoeba* could be isolated from the cornea, the isolate from the contact lens proved to be genotype T3. Although a few reported keratitis cases have involved genotype T3, patient S was probably not infected with this genotype as no *Acanthamoeba* strain was isolated from the cornea. Indeed, the *Acanthamoeba* strain was only isolated from the contact lens. In a recent study of Hong Kong isolates, a genotype T3 was also found in a contact lens case, but no isolate was available from the cornea of the patient either (1). The only genotype T3 reported from corneal scrapings was isolated from a patient in Scotland (18). A genotype T6 has been reported from a kerati-

tis patient in Austria (25). And, recently, a genotype T11 was isolated for the first time from a keratitis case in the UK (17).

It has not been possible yet to determine whether the high frequency of genotype T4 strains in keratitis cases is simply due to their relative abundance in the environment or whether they have special properties that make them more likely to be virulent (3). We would have been closer to an answer to this question if my collection of environmental *Acanthamoeba* isolates in liquid nitrogen had not perished. Indeed, hundreds of *Acanthamoeba* isolates from swimming pools (7), drinking water, aquaria (6), streams and canals (8) in Belgium had been cryopreserved in order to be investigated when an appropriate method became available. Unfortunately, this amoebae collection perished long before this method became available.

In cases where amoebae were isolated from the contact lens only, and not from the cornea (vahlkampfiids in patient S and D) the isolates belong to amoeba groups that have not been shown to cause keratitis. They should be considered to have been present on the contact lens by chance contamination from the environment. Also a *Hartmannella* sp. from the cornea of patient A is probably a chance isolation. Furthermore, Walochnick et al. (25) considered *Hartmannella* spp. and *Vahlkampfia* spp. isolated from clinical specimens as clinically irrelevant. Although cases of *Acanthamoeba* keratitis have been reported in Belgium before 1991 (13, 20), the isolate from patient H is the first *Acanthamoeba* isolated from a keratitis case in this country (10). Between 1991 and 1996, thirteen more *Acanthamoeba* strains have been isolated by the same ophthalmology department (14). Since the information on *Acanthamoeba* keratitis has become widely known in Belgium, infections caused by this amoeba can now be detected by most major hospitals in this country (4).

This study has also shown that certain *Acanthamoeba* genotypes, and also other amoeba genera, are sometimes falsely blamed for causing infection. In this respect we cite the claim that a *Mastigina* sp. was associated with keratitis (22).

A service for typing *Acanthamoeba* isolates is available at the reference laboratory (contact: jdjonckh@ben.vub.ac.be).

#### BIBLIOGRAPHY

- (1) BOOTON G.C., KELLY D.J., CHU Y.-W., SEAL D.V., HOUANG E., D.S.C. LAM, BYERS T.J., FUERST P.A. – 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *J. Clin. Microbiol.* 2002, 40, 1621-1625.
- (2) BROWN S., DE JONCKHEERE J.F. – A reevaluation of the amoeba genus *Vahlkampfia* based on SSUrDNA sequences. *Europ. J. Protistol.* 1999, 35, 49-54.
- (3) BYERS T.J., BOOTON G.C., STOTHARD D.R., GAST R.J., LEDEE D.R., SCHROEDER J.M., AWWAD M.H., FUERST P.A. – Studies on the phylogeny, systematics and pathogenicity of *Acanthamoeba* using ribosomal and transfer RNA gene sequences. In: IX International Meeting on the Biology and Pathogenicity of Free-Living Amoebae Proceedings. (Eds. Billot-Bonef S., Cabanes P.A., Marciano-Cabral F., Pernin P., Pringuez E.), Editions John Libbey Eurotext, 2001, 211-218.
- (4) CARRETTE S., MARECHAL-COURTOIS CH., HERNANDEZ J., HAYETTE M.-P., DEPRez M., DUCHESNE B. – A propos d'un cas de k r tite   *Acanthamoeba*. *Bull. Soc. belge Ophthalmol.* 2000, 275, 49-53.
- (5) DE JONCKHEERE J. – Use of an axenic medium for differentiation between pathogenic and non-pathogenic *N. fowleri* isolates. *Appl. Environ. Microbiol.* 1977, 33, 751-757.
- (6) DE JONCKHEERE J.F. – Occurrence of *Naegleria* and *Acanthamoeba* in aquaria. *Appl. Environ. Microbiol.* 1979, 38, 590-593.
- (7) DE JONCKHEERE J.F. – Pathogenic, free-living amoebae in swimming pools: survey in Belgium. *Ann. Microbiol. (Inst. Pasteur)* 1979, 130B, 205-212.
- (8) DE JONCKHEERE J.F. – Pathogenic and non-pathogenic *Acanthamoeba* spp. in thermally polluted discharges and surface waters. *J. Protozool.* 1981, 28, 56-59.
- (9) DE JONCKHEERE J.F. – Isoenzyme and total protein analysis by agarose isoelectric focusing, and taxonomy of the genus *Acanthamoeba*. *J. Protozool.* 1983, 30, 701-706.
- (10) DRAULANS E., MAUDGAL P.C. – *Acanthamoeba* keratitis. *Bull. Soc. belge Ophthalmol.* 1992, 243, 115-121.

- (11) GAST R.J., LEDEE D.R., FUERST P.A., BYERS T.J. – Subgenus systematics of *Acanthamoeba*: Four nuclear 18S rDNA sequence types. *J. Euk. Microbiol.* 1996, 43, 498-504.
- (12) HAMBURG A., DE JONCKHEERE J.F. – Amoebic keratitis. *Ophthalmologica* 1980, 181, 74-80.
- (13) HANSENS M., DE JONCKHEERE J.F., DE MEUNYNCK CH. – *Acanthamoeba* keratitis. A clinicopathological report. *Intern. Ophthalmol.* 1985, 7, 203-215.
- (14) HOUTTEQUIET I., MAUDGAL P.C. – *Acanthamoeba* keratitis associated with contact lens wear. *Contactologia* 1997, 19, 40-42.
- (15) HUGO E.R., STEWART V.J., GAST R.J., BYERS T.J. – Purification of amoeba mtDNA using the UNSET procedure p. D-7. In A. T. Soldo and J. J. Lee (ed.) *Protocols in protozoology*, Allen Press, Lawrence, Kans. 1992, p. D-7.1.
- (16) JONES D.B., VISVESVARA G.S., ROBINSON N.M. – *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with fatal meningoencephalitis. *Trans. Ophthal. Soc. U.K.* 1975, 95, 221-232.
- (17) KHAN N.A., JARROLL E.L., PAGET T.A. – Molecular and physiological differentiation between pathogenic and nonpathogenic acanthamoeba. *Curr. Microbiol.* 2002, 45, 197-202.
- (18) LEDEE D.R., HAY J., BYERS T.J., SEAL D.V. AND KIRKNESS C. M. – *Acanthamoeba griffini*. Molecular characterization of a new corneal pathogen. *Ophthalmol. Vis. Sci.* 1996, 37, 544-550.
- (19) MARTINEZ A.J. – Free-living amoebas and the immune deficient host. In: IX International Meeting on the Biology and Pathogenicity of Free-Living Amoebae Proceedings. (Eds. Billot-Bonnet S., Cabanes P. A., Marciano-Cabral F., Pernin P., Pringuez E.), Editions John Libbey Eurotext 2001, 1-12.
- (20) MAUDGAL P.C. – *Acanthamoeba* keratitis: report of three cases. *Bull. Soc. belge Ophthalmol.* 1989, 231, 135-148.
- (21) NAGINGTON J., WATSON T.G., PLAYFAIR T.G., MCGILL J., JONES B. R., STEELE A.D. MCG. – Amoebic infection of the eye. *Lancet* 1975, ii, 1537-1540.
- (22) NISZL I.A., MARKUS M.B., DEVENTER J.M. – Anti-*Mastigina* activities of eight contact lens solutions. *Antimicrob. Agents Chemotherapy.* 1995, 39, 2364-2366.
- (23) PAGE F.C. – A new key to freshwater and soil gymnamoebae. *Freshwater Biological Association, Ambleside, Cumbria* 1988, 122 pp.
- (24) SCHROEDER J.M., BOOTON G.C., HAY J., NISZL I.A., SEAL D.V., MARKUS M.B., FUERST P.A., BYERS T.J. – Use of a subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* strains isolated from humans with keratitis and from sewage sludge. *J. Clin. microbiol.* 2001, 39, 1903-1911.
- (25) WALOCHNIK J., HALLER-SCHÖBER E.-M., KÖLLI H., PICHER O., OBWALLER A., ASPÖCK H. – Discrimination between clinically relevant and nonrelevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. *J. Clin. microbiol.* 2000, 38, 3932-3936.
- .....
- Present address for correspondence:*  
 Dr. Johan F. De Jonckheere,  
 Research Unit for Tropical Diseases (TROP),  
 Christian de Duve Institute of Cellular Pathology (ICP),  
 Avenue Hippocrate 74-75,  
 B-1200 Brussels, Belgium;  
 Tel: 32-2-764.74.71; Fax: 32-2-762.68.53;  
 E-mail: jdjonckh@ben.vub.ac.be