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Session Vll
Phylogeny and molecular biology

Studies of the phylogeny, systematics and pathogenicity of *Acanthamoeba* using ribosomal and transfer RNA gene sequences

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ABSTRACT

The use of ribosomal RNA gene sequences (rDNA) for the study of the phylogeny, systematics and pathogenicity of *Acanthamoeba* has been increasing steadily in recent years. Our laboratory has been actively involved in sequencing nuclear and mitochondria! rDNA in collaboration with investigators around the world. In progress to date, we have identified 12 discrete typing units (rDNA genotypes T1 to T12) which include 20 of 25 named species. The genotypes were revealed by phylogenetic analyses of nuclear 18S rDNA and mitochondrial 16S rDNA sequences. Six named species have distinctive genotypes. The rest of the species that have been examined belong to paraphyletic clusters, the largest of which is genotype T4. Mitochondrial tRNA sequence data have been used to further examine these clusters. Improved PCR and sequencing strategies using rDNA have been developed for genus-specific detection of *Acanthamoeba* and genotype identification of clinical and environmental isolates. Analysis of 61 strains isolated from *Acanthamoeba* keratitis indicate that 97% of these infections are caused by genotype T4 strains.

Introduction

Difficulties in classifying *Acanthamoeba* isolates have been appreciated for many years. Three morphological groups encompassing 18 species were recognized by Pussard and Pons in 1977 [1], but identification of species based on morphology

has been problematical for subsequent investigators. Our laboratory has been using DNA sequencing primarily of nuclear and mitochondrial small ribosomal subunit rRNA genes to develop a more objective method of classification, to develop sensitive DNA probes for the detection and identification of acanthamoebae in clinical and environmental specimens and to study the epidemiology of diseases caused by this genus.

Ribosomal RNA genes

Eukaryotes have both cytoplasmic and mitochondrial ribosomes. Both types of ribosomes consist of large (LSU) and small (SSU) ribonucleoprotein subunits. The studies reported here focus on nuclear genes *(Rns)* coding for 18S rRNA found in cytoplasmic SSU and mitochondrial genes *(rns)* coding for 16S rRNA found in mitochondrial SSU. We have determined complete or partial *Rns* sequences from 129 isolates and complete *rns* sequences from 68 isolates of *Acanthamoeba. Rns* ranged in size from ~ 2,250 to 2,650 bp (base pairs) and *rns* ranged from 1,514 to 1,578 bp. We have estimated that there are at least 60 *Rns* copies most likely arranged in tandem repeats. There is one *rns* copy per mitochondrial genome and we estimate that there can be 3,300 copies per amoeba [2].

Phylogeny of the genus based on rDNA sequences

In 1966, Gast *et al.* [3] described four *Rns* sequence types (genotypes) based on an analysis of 18 isolates. In 1998, Stothard *et al.* [4] described 12 genotypes among 53 isolates representing all three morphological groups and 16 species. We now have determined complete *Rns* sequences for 78 isolates representing all morphological species except *A. quina, A. jacobsi, A. divionensis* and *A. paradivionensis*. The sequences have been examined by neighbor-joining distance, parsimony and maximum likelihood phylogenetic analyses and the results continue to be consistent with 12 genotypes. Phylogenetic analyses based on the 68 mitochondrial *rns* genes now support 11 of the 12 genotypes. Only mitochondrial sequence from a T6 genotype remains to be examined. This agreement between *Rns* and *rns* suggests that the rDNA trees are not simply gene trees, but probably also represent the genus tree. This result is not surprising if reproduction in this genus is asexual.

Intraclonal variation of *Rns* alleles

Growing cultures of *Acanthamoeba* are predominantly in the G2 phase of the cell cycle, have duplicated their DNA, and have been estimated to have a ploidy level of 11 to 22 n [2]. R. Rumpf and C. W. Birky (personal communication) recently found 11 alleles of the nuclear TATA box-binding protein gene in both A. *castellanii* Neff and *A. griffini S7* in apparent support of the polyploidy theory. Whether the total genome is or isn't polyploid, however, it is clear that *Rns* and *rns* occur in large numbers. Thus, there is a possibility that different alleles of either gene might be present in a single amoeba. The existence of more than one allele in a clone raises the problem that an isolate might be classified differently depending on which allele happened to be amplified by PCR and then sequenced. Through repeated subcloning of cultures and of PCR products, we discovered three *Rns* alleles differing by 3 to 10 base pairs in *A.* species Blackburn. The repeated subcloning always produced clones of amoebae with the same three alleles [5]. In addition to this case, we continue to occasionally find different bases at the same position in *Rns* sequencing gels, thus suggesting the presence of more than one allele [4]. However, these observations haven't been pursued further. In the Blackburn case, it didn't matter which sequence was used for genotyping because all three alleles were the same genotype. However, this set of alleles did serve as a unique marker for the Blackburn strain. This made it possible to trace the strain during the course of an AK infection and to confirm that the observed development of drug resistance was unambiguously associated with this strain [5]. In contrast to the case for *Rns*, there is no evidence yet for intraclonal variation of *rns* alleles. Furthermore, as indicated above, there generally is less variation in *rns* alleles than in *Rns* alleles. About 86% of *Rns* and 51% of *rns* sequences were unique. The explanation for this difference is unknown, but a practical application seems to be that the mitochondrial alleles may be more useful for determining which amoeba strains are the most closely related within genotypes, whereas, the nuclear alleles may be more useful for differentiating among isolates of the same genotype. Although the percent of unique *Rns* sequences in our total sequence collection is ~ 86%, the value actually is much higher if we exclude a group of South African isolates that all have the same *Rns* and *rns* sequences.

Introns of *Rns*

Single group I introns were found in *Rns* of *A. griffini* and *A. lenticulata* [6-8], but not in this gene in any of the other isolates we have studied. Thus, because the occurrence of *Rns* introns is relatively rare in *Acanthamoeba,* it was possible to use

the presence of an intron in a strain associated with AK to clearly demonstrate that the patient's bathroom water supply was the likely source of the infection [6]. Unfortunately, due to their scarcity, it does not appear that introns of *Rns* will have very wide use as strain markers. Furthermore, we have not found introns in any of the mitochondrial *rns* genes, although Lonergan and Gray found three introns in the gene encoding the 23S rRNA found in the mitochondrial LSU of A. *castellanii* Neff [9]. The possibility that 23S rRNA introns might be useful specific strain markers should be explored.

The ribosomal transcribed spacer region

The sequences of the transcribed spacer region between the 16S and 23S rRNA genes in the mitochondrial genome have been examined in 17 strains from *Rns* genotypes T1 to T4 [9, 10]. The region includes 5-6 tRNA genes. The Reich strain of *A. palestinensis is* unique among the 17 strains examined in having two copies of the gene encoding the tRNA for methionine. This region should be examined in the more distantly related genotypes to determine whether other phylogenetically or diagnostically useful alternative gene arrangements occur. The tRNA sequences also are interesting because Lonergan and Gray [11] demonstrated that post-transcriptional editing of the tRNAs occurs in the Neff strain. The sequences Ledee determined in our laboratory for 16 additional strains also appear to require editing although, so far, evidence for editing only has been demonstrated in two cases. Most of the sites where editing seems likely remain to be examined further. Of special interest here is the observation that interstrain sequence variations within the tRNA genes may be useful in identifying subdivisions of the *Rns* and *rns* genotypes.

Classification of *Acanthamaeba* isolates

Phylogenetic trees that we have constructed based on 18S or 16S rDNA sequences have revealed 12 genotypes, or what Bañuls *et al.* [12] refer to as Discrete Typing Units (DTU) in a study of *Leishmania.* The trees include type strains for 13 of the 18 species studied by Pussard and Pons [1] plus four species that have been described more recently. Whether DNA sequences are sufficient for the identification of species of microorganisms is a debatable point. Nevertheless, the sequence diff-erences discussed here clearly identify DTU for the type strains of *A. astronyxis, A. comandoni, A. culbertsoni, A. healyi, A. lenticulata,* and *A. tubiashi.* Three additional DTU include paraphyletic clusters of strains. The first includes *A. pustulosa,*

A. pearcei, and *A. griffini* which have sequence differences so small that continued justification for three different species requires the availability of reliable criteria other than rDNA sequences. The same is true for the relationship between *A. hatchetti* and *A. stevensoni*, the second paraphyletic genotype. However, the most problematic paraphyletic group of species is genotype T4 which includes strains that have been identified as *A. castellanii, A. Iugdunensis, A. mauritaniensis, A. polyphaga, A. rhysodes, A. royreba, A. terricola* and *A. triangularis.* Clades with high bootstrap values are beginning to emerge from within this group as additional rDNA sequences are accumulated, but so far the only single-species clade consists of nine isolates identified as *A. mauritaniensis.* The rest of the clades either include several or no named species. We refer to this entire group as the *A. castellanii* species complex and we continue to explore its internal relationships. Finally, we are unaware of any rDNA sequences for *A. echinulata, A. quina, A. divionensis,* or *A. paradivionensis.* However, an analysis by Yu *et al.* [13] of 18S rDNA restriction fragment length polymorphisms suggests to us that *A. divionensis* and *A. paradivionensis,* at least, also probably belong to genotype T4.

Genus-specific PCR and genotype-specific sequencing used with clinical and environmental isolates

The identification of rDNA sequence types and the availability of an extensive database of sequences have permitted the design of several sensitive and reliable diagnostic procedures that have application both in clinical and environmental studies. PCR has proven to be one of the most useful procedures and a number of laboratories have designed primers for the production of amplimers useful for detection of acanthamoebae or for differentiating pathogenic strains from nonpathogenic strains [14]. Although these previously designed primer sets worked well in the situations tested, their sequences indicated either that they would be unable to amplify products from all *Acanthamoeba* genotypes, or that they were unlikely to be genus-specific [14]. Thus, we designed primers JDP1 and JDP2 to produce an *Acanthamoeba*-Specific Amplimer (ASA.S1) from species as morphologically diverse as *A. castellanii* and *A. astronyxis,* but not from other amoeba genera such as *Balamuthia* and *Hartmannella [14].* These primers could amplify a product from a single trophozoite, as also was seen with some previously used primer sets, but amplification from cysts was problematical. Improved methods of dealing with cysts are needed, but, fortunately, our experience indicates that many cyst populations include a few trophozoites.

Ideally, a genus-specific PCR amplimer also would have a genotype-specific DNA sequence, but it has been difficult to find this combination. However, ASA.S1

approaches that ideal. Phylogenetic trees based on variations in its sequence are very similar to those based on much longer sequences. Because the statistical confidence level of these trees is low, however, we have obtained genotype specificity with eukaryote-specific primers that produce a longer GenoType-Specific Amplimer (GTSA.B1) in which variations in three shorter sequences provide the desired specificity and greater statistical confidence [14]. The drawback of using this primer set is the need to use cultures free of other eukaryotes due to the amplimer's lack of genus-specificity. Nevertheless, GTSA.B1 has been used successfully both with clinical corneal scrape and environmental sewage sludge isolates [14].

Genus- and genotype-specific fluorescent *in situ* hybridization (FISH)

Fluorescent cytochemical stains linked to genus- or genotype-specific DNA sequences would have great potential both for identification of fixed amoebae and for use with diagnostic DNA chips. We have described two FISH stains, one that is genus-specific and one that is specific for genotype T4 strains, the cause of most AK cases [14]. The T4-specific probe was used to identify acanthamoebae infecting fish tissues [15]. Additional probes will be designed for other genotypes as more sequence information becomes available about variations within genotypes.

Pathogenicity and epidemiology

The availability of a classification system based on rDNA genotypes has permitted a new look at relationships between clades and the various diseases caused by *Acanthamaeba*. Stothard *et al.* [14] first observed that 29/30 (97%) of the cases of *Acanthamoeba* keratitis were due to amoebae from rDNA genotype T4. The numbers now are 59/61 (97%) of the AK cases and 68/72 (94%) of all isolates from human infections. The known exceptions in our collection are one or two isolates of genotypes T1 *(A. species)*, T3 *(A. griffin))*, and T12 *(A. healyi)*. Thus, human infections are associated most strongly with the 8-10 species of the *A. castellanii* species complex. There haven't been any population surveys using rDNA genotypes for identification of specimens, thus, it still is impossible to determine whether the high frequency of T4 strains in AK is simply due to their relative abundance in the environment or whether they have special properties that make them more likely to be virulent. However, although our sample may be highly biased, 70% of all the *Rns* sequences and 80% of all the *rns* sequences we have examine have had T4 genotypes. Thus, we can't rule out the possibility that pathogenicity is ubiquitous

in the genus and that the prevalence of T4 infections is mostly related to the abundance of the genotype.

Several laboratories have been interested in whether there are DNA sequence differences between T4 strains that have caused AK and strains that have not done so. The best approach so far was used by Walochnik *et al.* [17] when they compared acanthamoeba isolates known to be responsible for AK with acanthamoeba isolates that were present in eyes or contact lens cases, but were not responsible for eye infections. In the sample examined, the infectious and the noninfectious isolates branched separately in a cladistic analysis. We haven't found any similar evidence for divisions between pathogenic and "nonpathogenic" isolates within genotype T4, but our studies suffer from the lack of using a rigorous definition for a "nonpathogen". It remains to be seen whether pathogenicity can be associated with selected sequence variants within genotype T4, but it is a goal worth pursuing.

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