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Molecular Identification of T4 and T5 Genotypes in Isolates from *Acanthamoeba* Keratitis Patients[▽]

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***Acanthamoeba* keratitis (AK) is a rare but sight-threatening ocular infection. Outbreaks have been associated with contaminated water and contact lens wear. The epidemiology and pathology may be associated with unique genotypes. We determined the *Rns* genotype for 37 clinical isolates from 23 patients presenting at the University of Miami Bascom Palmer Eye Institute with confirmed AK infections in 2006 to 2008. The genus-specific ASA.S1 amplicon allowed for rapid genotyping of the nonaxenic cultures. Of the 37 isolates, 36 were of the T4 genotype. Within this group, 13 unique diagnostic fragment 3 sequences were identified, 3 of which were not in GenBank. The 37th isolate was a T5, the first in the United States and second worldwide to be found in AK. For five patients with isolates from the cornea and contact lens/case, identical sequences within each patient cluster were observed, confirming the link between contact lens contamination and AK infection. Genotyping is an important tool in the epidemiological study of AK. In this study, it allowed for the detection of new strains and provided an etiological link between source and infection. Additionally, it can allow for accurate categorizing of physiological differences, such as strain virulence, between isolates and clades.**

The genus *Acanthamoeba* is comprised of a group of free-living amoebae that are responsible for causing *Acanthamoeba* keratitis (AK), a rare but sight-threatening corneal infection. In recent years, the number of AK cases has been on the increase, especially among wearers of contact lenses, who make up 85 to 90% of the AK cases (8, 24, 31, 35). Diagnosis of AK is problematic due to clinical features which are similar to those of herpetic, bacterial, and fungal infections. For example, the stromal ring infiltrate associated with AK is only observed ~6% of the time in early cases and ~16% of the time in late cases (2, 3, 4, 14). AK can be the primary infection or be present as a suprainfection in combination with other infectious organisms, like bacteria or fungi, thereby complicating diagnosis and treatment. The encystment capability of *Acanthamoeba* species also confounds treatment due to the recalcitrant nature of the cyst to most treatment options allowing reemergence of amoebae after treatment cessation.

Acanthamoeba genotyping is a useful tool for studying taxonomic and epidemiological relationships and thereby allowing correlations between the infectious isolates and disease phenotypes, such as virulence factors, drug susceptibility, and/or species-clinical outcome correlations, to be explored. The gene targeted most often in *Acanthamoeba* genotyping is the nuclear small-subunit rRNA gene (*Rns*), and utilizing a 5% sequence dissimilarity cutoff point, 15 or more genotype clades, designated T1, T2, T3, etc., have been identified (12, 13, 15, 17, 29). Isolates from six of the genotypic clades (T3, T4, T5, T6, T11 and T15) are confirmed to be causative agents of AK (10, 13, 19, 21, 28, 29, 34, 36). The most prevalent *Acanthamoeba* genotype in both clinical and environmental samples is the T4 genotype (6, 7). Within the genotype clades, multiple species designations can be observed. This is primarily due to the traditional classification method's reliance on changeable morphological characteristics, such as cyst morphology, creating inconsistent species identification (25, 32). Therefore, it was proposed that each genotypic clade be equated with a single species (29). For example, all isolates in the T4 clade could be reclassified as *Acanthamoeba castellanii* since the T4 genotype includes the type strain for that species.

In this study, 37 isolates from corneal scrapes, contact lenses, and lens cases of 23 patients presenting with AK at the Anne Bates Leach Eye Hospital, Bascom Palmer Eye Institute, University of Miami, from 2006 to 2008 were examined to assess the *Rns* genotypes responsible for the infections. *Acanthamoeba* species can be rapidly genotyped by targeting a highly variable region designated diagnostic fragment 3 (DF3) within the genus-specific *Rns* ASA.S1 amplicon (5, 26); therefore, this region was chosen for analysis. The genotypes identified in this study were also compared to strains identified in other studies in order to examine the prevalence of the DF3 sequence types within genotype clades (5, 37, 38).

MATERIALS AND METHODS

Cultures. Thirty-seven *Acanthamoeba* isolates cultured from corneal scrapings, biopsies, contact lenses, or lens cases were recovered from 23 patients presenting with AK at the University of Miami Bascom Palmer Eye Institute between January 2006 and February 2008 (Table 1). Patients' ages ranged from 14 to 83 years. The risk factor for all patients involved the use of contact lenses. Diagnosis of AK was based on the detection of cysts or trophozoites in corneal

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TABLE 1. Isolates used for sequencing of the *Rns* ASA.S1 amplicon

Culture designation[a]	Culture source	*Rns* genotype/ DF3 sequence[b]	GenBank accession no.
BP:P1:RCS	Right corneal scrape	T4/13	FJ422513
BP:P2:CB	Corneal button	T4/6	FJ422515
BP:P2:CS	Corneal scrape	T4/6	FJ422514
BP:P3:RCS	Right corneal scrape	T4/6	FJ422517
BP:P3:RCS[2]	Right corneal scrape	T4/6	FJ422523
BP:P3:LCS	Left corneal scrape	T4/6	FJ422522
BP:P3:RCL	Right contact lens	T4/6	FJ422516
BP:P3:RLC	Right lens case	T4/6	FJ422520
BP:P3:LLC	Left lens case	T4/6	FJ422521
BP:P4:RCS	Right corneal scrape	T4/6	FJ422518
BP:P5:LLC	Left lens case	T4/6	FJ422519
BP:P6:LCS	Left corneal scrape	T4/14	FJ422524
BP:P7:LCL	Left contact lens	T4/14	FJ422525
BP:P7:RCL	Right contact lens	T4/15	FJ422526
BP:P8:LCS	Left corneal scrape	T4/12	FJ422512
BP:P9:LCS	Left corneal scrape	T4/17	FJ422537
BP:P9:RCL	Right contact lens	T4/17	FJ422538
BP:P9:LCL	Left contact lens	T4/17	FJ422539
BP:P10:RCL	Right contact lens	T4/2	FJ422528
BP:P10:RCB	Right corneal button	T4/2	FJ422529
BP:P10:RCS	Right corneal scrape	T4/2	FJ422530
BP:P11:RCS	Right corneal scrape	T4/2	FJ422531
BP:P12:LCS	Left corneal scrape	T4/2	FJ422532
BP:P13:CB	Corneal button	T4/20	FJ422536
BP:P14:LCS	Left corneal scrape	T4/18	FJ422533
BP:P14:LC	Lens case	T4/18	FJ422534
BP:P15:RCS	Right corneal scrape	T4/16	FJ422527
BP:P16:RCS	Right corneal scrape	T4/21	FJ422541
BP:P16:LC	Lens case	T4/21	FJ422540
BP:P16:LC[2]	Lens case	T4/21	FJ422543
BP:P17:LCS	Left corneal scrape	T4/21	FJ422542
BP:P18:LCS	Left corneal scrape	T4/21	FJ422544
BP:P19:RCS	Right corneal scrape	T4/21	FJ422545
BP:P20:LCS	Left corneal scrape	T4/11	FJ422511
BP:P21:LCS	Left corneal scrape	T4/11	FJ422510
BP:P22:LCS	Left corneal scrape	T4/19	FJ422535
BP:P23:LCS	Left corneal scrape	T5	FJ422546

[a] Explanation of culture designations: BP, Bascom Palmer; P, patient; RCS, right corneal scrape; CB, corneal button; LCS, left corneal scrape; LLC, left lens case; RLC, right lens case; LC, lens case; LCL, left contact lens; RCL, right contact lens. [2] indicates second isolates obtained from the same source.

[b] The DF3 sequence nomenclature used in this study is the same as that reported by Booton et al. (5). The first part is the *Rns* genotype and the second part is a unique code assigned to the specific DF3 sequence.

sample smears and/or growth on nonnutrient agar plates overlaid with live *Escherichia coli*.

Genotyping. *Acanthamoeba* isolates were harvested from agar plates and rinsed in phosphate-buffered saline (pH 7.4), and DNA was extracted using the UNSET method (18). PCR amplification of the *Rns* amplicon ASA.S1 was generated using the genus-specific primer set JDP1 (5′-GGCCCAGATCGTTT ACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′), which encodes the highly variable DF3 region (26). Two or more PCR products were pooled or independently sequenced using the amplification primers JDP1 and JDP2, in addition to the conserved primers 892 (5′-CCAAGAATTTCACC TCTGAC-3′) and 892C (5′-GTCAGAGGTGAAATTCTTGG-3′). Sequencing of the PCR products was performed by Genewiz, Inc. (South Plainfield, NJ). The DF3 sequence designation is based on nomenclature described by Booton et al. (5). The first part is the *Rns* genotype of the isolate. The second part is a unique code assigned to a specific DF3 sequence type. The Booton et al. study (5) identified 10 DF3 sequence types. The numbers used to define the DF3 sequence type in this study are a continuation of that system.

Phylogenetic analysis. Alignments and phylogenetic reconstructions were performed using the phylogenetic computer program MEGA4 (Molecular Evolutionary Genetic Analysis software, version 4) (30). The evolutionary distances were computed using the Kimura 2 parameter distance algorithm (20) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons. A total of 449 positions were used in the final data set. The bootstrap consensus tree is inferred from 1,000 replicates (11). *Balamuthia mandrillaris*, a close phylogenetic relative of *Acanthamoeba*, was used as the outgroup to root the trees. Phylogenetic reconstructed gene trees were generated using maximum-parsimony, neighbor-joining, UPGMA or minimum evolution methods in MEGA4 were compared. The neighbor-joining tree is displayed in Fig. 1.

Nucleotide sequence accession numbers. The 37 sequences determined in this study were deposited in GenBank under accession numbers FJ422510 to FJ422546. The other *Acanthamoeba* sequences used in this study are available in GenBank under the following accession numbers: *Acanthamoeba castellanii* strain CDC:0180:1, U07405; *Acanthamoeba hatchetti* strain 2HH, AF26022; *Acanthamoeba castellanii* strain castellani, U07413; *Acanthamoeba* sp. strain KA/E21, EF140633; *Acanthamoeba* sp. strain U/E3, AY026747; *Acanthamoeba* sp. strain S36, EU146073; *Acanthamoeba* sp. strain S30, DQ087313; *Acanthamoeba* sp. strain SF2.JDP, EU338518; *Acanthamoeba* sp. strain S4, DQ087320; *Acanthamoeba castellanii* strain CDC:0184:V014, U07401; *Acanthamoeba* sp. strain BCM:0288:27, U07409; *Acanthamoeba hatchetti* strain BH2, AF019068; *Acanthamoeba stevensoni* strain RB:F:1, AF019069; *Acanthamoeba* sp. strain V006, U07400; *Acanthamoeba palestinensis* strain Reich, U07411; *Acanthamoeba pustulosa* strain GE 3a, AF019050; *Acanthamoeba* sp. strain RAC013, AB327060; *Acanthamoeba* sp. strain GAK1, AY944575; *Acanthamoeba lenticulata* strain Jc-1, U94739; *Acanthamoeba lenticulata* strain PD2S, U94741; *Acanthamoeba* sp. strain S35, EU146072; *Balamuthia mandrillaris*, AF477022.

RESULTS

DF3 sequences. The variable DF3 regions of the *Rns* genes of 37 isolates from 23 patients identified 14 unique DF3 sequences (Fig. 2 and Table 1). Of the 14 sequence types obtained, 13 correspond to 36/37 (97%) of the isolates examined, and these were identical or similar to previously described isolates of the T4 genotype (Fig. 1), herein referred to as T4/2, T4/6, and T4/11 to T4/21. Three of the sequence types (T4/11, T4/17, and T4/19) represent new T4 sequences not found in GenBank. The remaining isolate possessed a DF3 sequence most similar to sequences of *Acanthamoeba lenticulata* isolates, which are classified as genotype T5.

***Rns* T4 genotype isolates.** Table 1 summarizes the genotype/DF3 sequence type of all the isolates examined in this study. All 23 patients were contact lens wearers, and of these, 5 (patients BP:P3, BP:P9, BP:P10, BP:14, and BP:P16) had the *Rns* sequence type determined for the cultures grown from their contact lens paraphernalia and corneal scrapes. In all cases, identical DF3 sequences were observed in the corneal scrape specimens and the contact lens paraphernalia, which suggests that the contact lens paraphernalia can be a source of the infection (Table 1 and Fig. 1).

Patient BP:P7 was unusual in that the sequence types of the isolated *Acanthamoeba* strains were different between the right and left lens case. The *Acanthamoeba* strain isolated from the right lens case was genotype T4/15, whereas the genotype of the *Acanthamoeba* strain in the left lens case was T4/14. No corneal scrape specimen was available for patient BP:P7; therefore, it is unknown which, if either, caused the keratitis.

Identical sequence types were observed not only within different sources from a single patient, but also between different patients. Five of the sequence types, T4/2, T4/6, T4/21, T4/14, and T4/11, showed identical sequence types between different patients, suggesting infection by similar if not identical *Acanthamoeba* strains. Alignments with sequences from GenBank showed that the majority of the sequence types have been observed in multiple patients with keratitis worldwide.

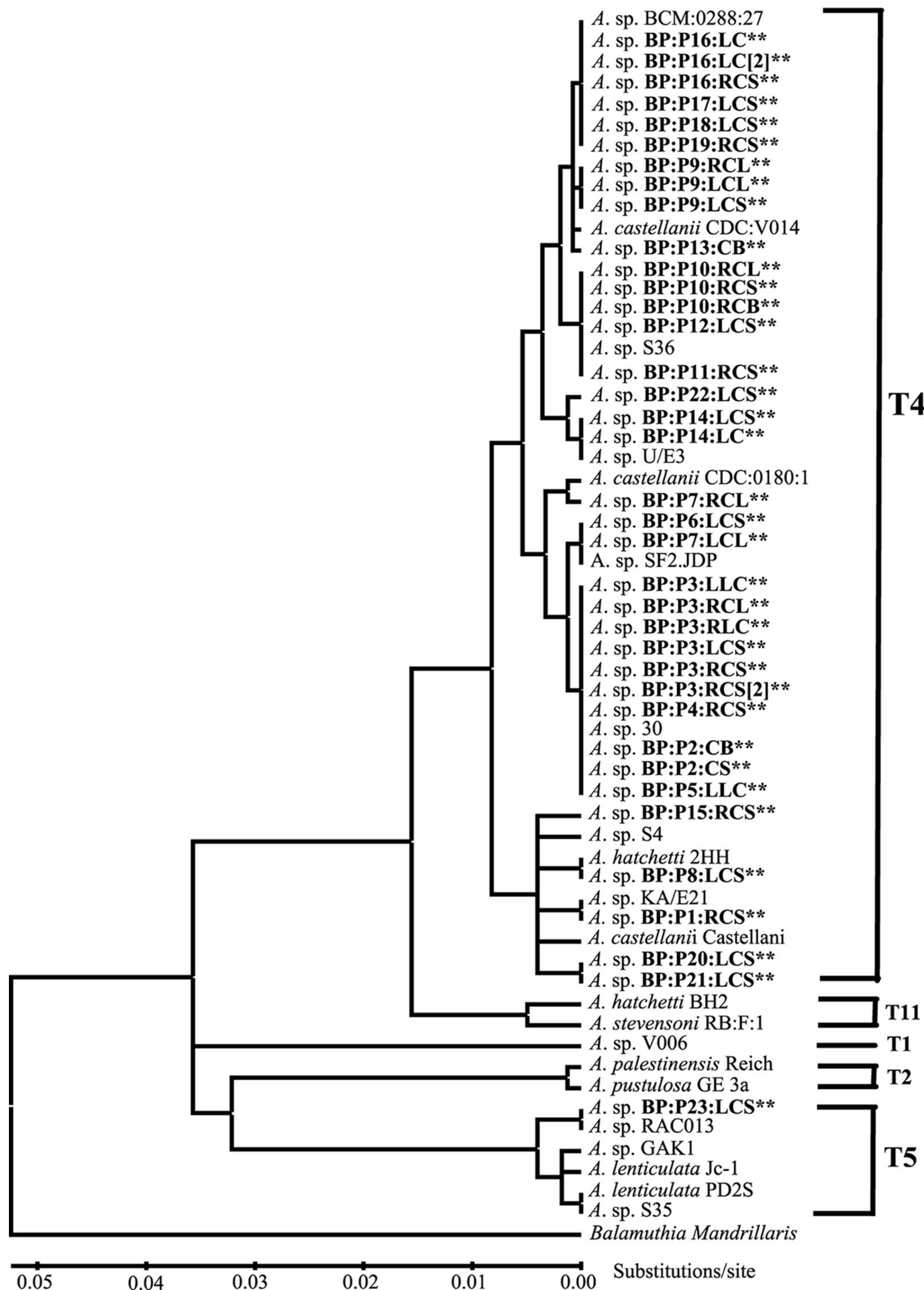

FIG. 1. *Rns* DF3 linearized neighbor-joining gene tree. Isolates from this study are shown in boldface text and with asterisks. The tree was constructed using 1,000 bootstrap replications. The T1, T2, T4, T5, and T11 designations shown on the tree correspond to strains previously determined to be of that particular genotype (26, 29).

***Rns* T5 genotype isolate.** Of the 37 *Acanthamoeba* cultures examined, 1 isolate was determined to have the rare T5 genotype. This isolate's DF3 sequence was identical to that of *Acanthamoeba* sp. RAC013, an isolate from drinking water in Osaka, Japan, and this isolate is the first case of a T5 *Acanthamoeba* isolate causing AK in the United States.

DISCUSSION

The genotyping data obtained in this study of amoebae isolated from AK patients further confirms T4 as the predominant genotype, a trend observed in previous studies (5, 37, 38). A comparison of genotypes from this study with those from

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DF3 Variable Region                                                          Seq Des

GGC-GCGGTC GTCCTTGGCC GGGTTCTCGT CCTT-CACGG GGCGCGGTTC G-GCGGGGGC GGCTTAG T4/11
GGC-GCGGTC GTCCTTGGC- GTGTCTCGG- C-TT-CAC-- GGC-TGGGGC GCGCGAGGGC GGTTTAG T4/12
GGC-GCGGTC GTCCTTGGC- --GTCTCGGT CCTT-CACGG GGC-CGGGGC G--CGGGGGC GGCTTAG T4/13
GGTTGCGGTC GTCCTTGGC- --GTCTCGGT --TT-C---- GGC-CGGGGC G--CGGGGAT GGCTTAG T4/6
GGTTGCGGTC ATCCTTGGC- --GTCTCGGT --TT-C---- GGC-CGGGGC G--CGGGGAT GGCTTAG T4/14
GGTTGCGGTC GTCCTTGGC- --GTCTCGGT --TT-C---- GGC-CGGGGC G--CGGGGAC GGTTTAG T4/15
GGT-GCGGTC GTCCTTGGC- --G--TCGGT --TT-C---- GGC-CG--GC G--CGGGGGC GGCTTAG T4/16
GGT-GCGGTC ATCCTTGGC- --G--TTGGT C-TT-CAAAA G-C-CA--GC G--CGGGGGT GGCTTAG T4/2
GGT-GCGGTC GTCCTTGGC- --G-GTTGGT C-TT-CAAAA G-C-CA--GC G-GCGGGGGT GGCTTAG T4/17
GGT-GCGGTC GTCCTTGGC- --G--TCGGT C-TT-CAAAA G-C-CG--GC G--CGGGGGT GGCTTAG T4/18
GGT-GCGGTC ATCCTTGGC- --G-TTCGGT C-TTGCAAAA GGC-CG--GC -CGCGGGGGT GGCTTAG T4/19
GGT-GCGGTC GTCCTTGGC- --G--TTGGT C-TT-CAAAA G-C-CA--GC G--CGGGGGT GGCTTAG T4/20
GGT-GCGGTC GTCCTTGGC- --GCGTTGGT C-TT-CAAAA G-C-CA--GC GCGCGGGGGC GGCTTAG T4/21
AAT------- -CCTTT---- ---------- ---------- ---------- ---CGGGGA- ---TTAA T5
```

FIG. 2. Primary sequence alignment of a subset area of the highly variable and informative region of DF3 (stem 29-1, 18S rRNA) of Bascomb Palmer Eye Institute isolates. Sequences are aligned by similarity. Gaps are represented by dashes.

other studies that investigated multiple AK isolates revealed that our study had the T4/6 and T4/2 genotypes in common with the study of Hong Kong isolates (5). Our study and the Zhang et al. (38) results for North China had the T4/2, T4/12, and T4/13 genotypes in common, and our study and the Yera et al. study (37) from France had only the T4/2 genotype in common. Although based on limited datasets, the T4/2 genotype appears to be the geographically predominate sequence type.

With the worldwide prevalence of the T4 genotype regardless of region, it is not surprising that 90% of *Acanthamoeba* isolates associated with AK are genotype T4. What is of particular interest is that the second most abundant environmental clade, T5, is dramatically underrepresented in AK cases (6, 7). This study is only the second study to describe a T5 isolate causing AK and the first in the United States. It is unlikely a lack of exposure that explains the low infection rate, as the T5 genotype has been detected in human mucosa without amoebic infection (9). Further complicating the issue are experimental animal and tissue culture models that have shown T5 isolates to be capable of a high degree of pathogenicity (33, 34). Additionally, studies comparing T4 and T5 resistance to multipurpose contact lens cleaning solutions, interestingly, show that the T5 genotype possesses a better resistance (16, 27). It is possible that the majority of T5 *Acanthamoeba* isolates may not be pathogenic to humans, but as the number of people that wear contact lenses continues to grow, the risk of encountering pathogenic T5 isolates may increase.

An interesting observation was the lack of the T3 genotype in this study. Several studies that have determined genotypes of *Acanthamoeba* strains from AK and contact lens/cases each identified the presence of the T3 genotype, which, based on environmental distribution, is less prevalent than T5 (5, 7, 37, 38). Also, like T5 isolates, T3 isolates can show more resistance to multipurpose contact lens cleaning solutions than do isolates of the T4 genotype (27). Understanding what makes T4 more virulent to humans is an important area of study. Multiple factors contribute to *Acanthamoeba* pathogenicity, such as extracellular protease production and amoeba cell surface adherence ability. In studies that examined pathogenicity predictive factors, the T3, T5, and T4 genotypes all displayed high pathogenicity (1, 22, 33, 34), although the T3 results were not always consistent between isolates. The T4 genotypes did show increased cell surface binding compared to that of T3 (1); however, it is essential to realize the small number of T3 and T5 genotypes examined in these studies compared to the number of T4. These observations do suggest that a different rationale must exist to explain the underrepresentation observed with T3 and T5 genotypes. It should be noted that these studies used in vitro cell culture models to compare the pathogenicities of isolates, which emphasizes the need for a good clinical animal model.

Obviously, there are certain properties within the T4 genotype that make it more virulent. Therefore, the need for accurate genotyping of *Acanthamoeba* strains from different environments along with an analysis of their virulence factors and, in clinical AK cases, an examination of outcome would greatly enhance and stimulate research. Also, the integration of a

PCR-based assay in the detection of *Acanthamoeba* strains, in addition to genotypic information that can be obtained, offers a rapid diagnostic tool. Utilized alongside the conventional method of smear examination, an AK diagnosis can be ideally accomplished in less than a day and would be more cost-effective than fluorescence- or in vivo confocal microscopy-based methods. The use of a PCR-based assay offers all the hallmarks of a good diagnostic test: high sensitivity, high specificity, and high positive and negative predictive values (23, 36).

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