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行政院衛生署疾病管制局 九十四 年度科技研究發展計畫

四種分子分型法應用於加護病房病人念珠菌之研究

研 究 報 告

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一、中文摘要

院內念珠菌感染是引起重症病人死亡或併發症的重要原因，而這個因素是值得我們去努力以改善病人預後的。我們及其他學者的研究認為念珠菌感染是由宿主體表黏膜原本移生的菌所引起的，然而在醫療院所內，尤其是加護病房內，交叉感染引起群突發，也屢屢被証明。因此，建立及確認分子分型法以瞭解院內念珠菌感染的流行病學，並據以建立醫療決策，以預防及處理念珠菌感染是重要的研究課題。本計畫建立的 pulsed field gel electrophoresis (PFGE)、PFGE-BssHII、amplified fragment length polymorphism (AFLP), 及 multi-locus sequence typing (MLST)四種 DNA 分型法，應用於一組加護病房前瞻性收集、臨床流行病學明確的念珠菌菌株，以探討此分子分型法的臨床實用性。本研究針對 53 株念珠菌決定 DNA 分型，四種方法的 typability 100%，retroducibility 100%。比較由 13 位病人所分離的 26 株 *Candida albicans* 的 DNA 分型，PFGE 及 PFGE BssHII 分別分出 17 型及 19 型，AFLP 分出 21 型，MLST 分出 22 型。基本上以四種方法建立的 cluster analysis 皆可鑑別出菌株之間流行病學之相關性，兩種 PFGE 方法及 MLST 的一致性很高，但同一病人不同菌株 MLST 分型的些許變異性，其流行病學意義不明。至於 AFLP 鑑別能力最高，但與其他方法及臨床流行學資料的一致性最低，應用此方法可能因過度區分流行病學相關的菌株，而低估交叉傳播的可能性。本研究針對前瞻性篩檢的念珠菌 DNA 分型顯示，菌株乃病人獨特的，而與加護病房、病灶、分離日期及抗菌性無關。此外，恰當選擇 DNA 分型並正確判讀以進行醫院流行病學是非常重要的課題。

關鍵詞：念珠菌，院內感染，分子流行病學，pulsed field gel electrophoresis，amplified fragment length polymorphism

二、英文摘要

Invasive candidiasis is a problem associated with substantial morbidity and mortality. Although the majority of invasive candidiasis develops at high-risk patients as a consequence of endogenous colonization, however, cross transmission and hospital outbreaks of *Candida* infection have been reported, particularly in ICUs. This report describes the investigation of the genetic profiles of 53 *Candida* isolates collected from adult intensive care units in a teaching hospital in Taiwan using two pulsed field gel electrophoresis (PFGE)-based typing methods (PFGE karyotyping, and PFGE of *Bss*HII restriction fragments), amplified fragment length polymorphism (AFLP), and multi-locus sequence typing (MLST). Comparison of genotypes of 26 isolates of *C. albicans* collected from 13 patients determined by four typing methods, PFGE and PFGE-*Bss*HII generated 17 and 19 DNA patterns, respectively, AFLP generated 21 patterns, and MLST generated 22 DSTs. All four methods were able to identify clonal related isolates from the same patients. High concordance was found among the two PFGE-based methods and MLST. On the other hand, low concordance of cluster analysis was found between AFLP and other methods. In addition, variation between AFLP of isolates collected from the same patient might underestimate or undetect the presence of cross transmission. The results also showed that the genotype of each isolate was patient-specific and not associated with the source of the isolation, date, wards, or antifungal resistance. Choosing appropriate molecular typing methods is essential for identifying the clonal relatedness of pathogens in hospital settings.

Keyword：*Candida,* nosocomial infection, molecular epidemiology, amplified fragment length polymorphism, multi-locus sequence typing, pulsed field gel electrophoresis, restriction fragment length polymorphisms

Introduction

Great advances in medical technology have allowed patients to survive complex diseases longer, at the cost of creating populations that are vulnerable to a wide variety of previously unrecognized or underestimated diseases. Invasive candidiasis (ICU) is a problem of increasing relevance in the healthcare setting [1-12] and in particular for intensive care units (ICUs) [3-7]. *Candida* spp. have become important nosocomial pathogens and invasive candidiasis is an independent predictor of mortality [13,14] and was associated with prolonged hospital stay and extra medical costs [15-18].

Although the majority of invasive candidiasis develops at high-risk patients [19,20] as a consequence of endogenous colonization [21-24], however, cross transmission and hospital outbreaks of *Candida* infection have been reported [25-33], particularly in ICUs [31-33]. DNA fingerprinting methods have evolved as major tools in fungal epidemiology [34] to identify transmission routes [24,35] or to assess the biodiversity of a microbial population [36-38]. However, no single method has emerged as the method of choice, and some methods perform better than others at different levels of resolution. In light of the proposed requirements, the most common methods include pulsed field gel electrophoresis (PFGE)-based typing methods [22-26,32,36,39], restriction fragment length polymorphisms (RFLP)[27,40,41], RFLP with hybridization probes, amplified fragment length polymorphism (AFLP) and other PCR-based methods[42-44], and multilocus sequence typing (MLST) [45-52].

This study applied two PFGE-based typing methods, AFLP, and MLST to characterizing a epidemiologically well-defined *Candida* isolates collected during a prospective surveillance study [14,24] in ICUs. The weakness and strength of each method were discussed.

MATERIALS AND METHODS

Patients and fungal strains. A total of 53 *Candida* clinical isolates were used in this study. Information on each isolate was collected, including geographic origin and body site origin, as well as whether it was a nosocomial infection. These isolates were part of the collections from during a 6-month surveillance study in adult intensive care units of a large teaching hospital from September 1, 1996 through February 28, 1997 [14,24]. A total of 873 *Candida* strains were isolated from 208 of the 342 patients (60.8%) who stayed in ICUs for 48 hours or more. Specimens for surveillance cultures were collected on admission to the ICU, and then weekly until discharge from the ICU. Specimens from three anatomical sites, including (1) oropharyngeal swabs or endotracheal aspirates, (2) rectal swabs, and (3) urine samples, were collected from all patients. Specimens from other sites such as wounds, vagina, skin, blood were also collected when clinically indicated. Isolates were labeled according to patient, *Candida* spp., temporal sequence of collection, anatomical location and date of specimen collection. For example, the first isolate of *C. albicans* obtained from the rectal region of patient 35 on Sept 24 was labeled 35a1R-24/09.

The identification of all fungal strains was undertaken by the germ-tube assay followed by VITEK Yeast Biochemical Card and API-32C systems [53]. The minimum inhibitory concentrations (MICs) for fluconazole were determined by the microdilution broth method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A, as described previously [54].

Definitions. Nosocomial infections were defined according to the criteria outlined by the Centers for Disease Control and Prevention [55]. The primary source of infection, causative pathogen and adequacy of antimicrobial therapy were determined according to prospectively defined criteria [55,56]. Colonization was considered when Candida spp. were isolated from surveillance cultures and patients did not have compatible signs or symptoms of infection. A patient was considered infected if there was documentation of either a candidemia or a severe candidal infection requiring the use of systemic antifungal therapy. Patients with Candida colonization and persistent signs and symptoms of infection despite apparently appropriate antibacterial therapy in the absence of uncorrected cause of fever were considered to have Candida infection [14].

Pulsed field gel electrophoresis (PFGE)

(i) PFGE karyotyping. Strains were inoculated on Sabouraud dextrose agar (SDA; Difco) for 48 h at 37 °C. Colonies on agar were picked in a cell suspension buffer (100 mM Tris/HCl, 100 mM EDTA, pH 8.0). The total genomic DNA of the strain

was extracted by means of the PUREGENE DNA Purification Kit (Gentra, Minneapolis, Minnesota, USA) and was described previously [41]. The concentration of DNA extracted from *C. albicans* isolates was measured with a spectrophotometer (A_{260}). DNA was stored at -80°C until used. Electrophoresis was performed with a Biometra Rotaphor at pulse time 60-700 s, angle 120°, 120-90 V in 0.8 % agarose gel with 0.5x TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA) for 66 h as described previously [41].

(ii) PFGE of *Bss*HII restricted fragments. Preparation of plugs and restriction enzyme digestion were conducted as described previously [41]. PFGE was performed with a Biometra Rotaphor® at pulse time 5-60 sec, angle 120°, 180V in 0.8% agarose gel with 0.5xTBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA) for 36 h. After electrophoresis, the gel was stained in ethidium bromide solution for 15 min and destained in distilled water.

(iii) Electrokaryotypic pattern analysis. Preliminary qualitative analysis of EK patterns obtained from the same gel was performed through visual inspection of photographs of ethidium bromide-stained gels. Isolates were considered different if banding patterns varied by one or more readily detectable band [24,40]. A one band difference was used to categorize strain since a one-band EK difference of yeast isolates signifies a large and detectable size variation in the chromosomal bands. To compare the EK patterns of colonizing and infecting isolates, together with EK patterns for isolates from different patients, the GelCompar software package (version 4.0; Applied Maths, Kortrijk, Belgium) was used. Autoradiogram images were digitized and processed for normalization using the Alpha Innotech IS1000 (Alpha Innotech Corporation, San Leandro, Calif.). Dendrograms were generated using the unweighted pair-group method with arithmetic averages (UPGMA) [57] and were analyzed with Bionumerics software version 4.0 (Applied Maths, Kortrijk, Belgium) as described previously [41]. The position tolerance was set at 1 % and optimization was set at 3 %. The Dice coefficient was used to analyse the similarities (S_{AB}) of the band patterns. Isolates were considered different when the band similarity value was less than 95 % [36,41].

Multi-locus sequence typing (MLST)

(i) MLST. MLST was based on seven housekeeping genes, including loci *ATT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, *ZWF1b* [46]. PCRs were carried out with mixtures containing 2 µl of extracted DNA (10ng/µl), 4 µl of each primer (5 µM), 10 µl of distilled water, and TEMPLY PCR kit (LTK BioLaboratories, Taipei, Taiwan). PCRs were performed with an initial 2-min denaturation step at 94°C, 2 min denature, followed by 25 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for1 min, with a final extension step of 10 min at 72°C; PCRs were performed in a PTC-200 96-well thermal cycler (MJ Research). DNA sequencing was performed by using the same primers used in PCR, and on both strands.

(ii) Sequences and computations. Sequences of both strands were aligned with BioNumerics. Sequences were compared with data in the central database and the sequence and DST identifiers were obtained there from (http://test1.mlst.net/). To compare the relationship of Taiwanese isolates with those from other countries, MLST data of isolates from UK and USA were obtained from the publication by Tavanti et al. [49]; one isolate was randomly chosen from each cluster, and added to the strain panel for computation. Phylogenetic relationships among isolates were then assessed by cluster analysis, using the UPGMA and minimal spanning tree algorithm of the BioNumerics software (version 4.0; Applied Maths, Kortrijk, Belgium) applied to modified sequence data. The sequence data of the seven housekeeping genes were transformed as described by Tavanti et al. [50]. Briefly, the results for the variable sites from the seven gene fragments sequenced were concatenated into a single sequence. To cope with heterozygous code data, each base in the concatenated sequences of the polymorphic sites was transformed into two bases; the same if the base is homozygous code, so, e.g., the sequence ACGT would emerge as AACCGGTT, and as the component bases for heterozygous codes, so, e.g., AWST would come out as AAATCGTT.

(iii) MTL and ABC typing. PCR for determination of mating-type-like locus status (MTL), heterozygous (a/α), homozygous (a/a) or homozygous (α/α), was conducted as previously described [49]. PCRs were carried out with 50µl PCR volumes containing 100 ng of genomic DNA, 2.5 U of DyNAzyme^{TM} II DNA polymerase (Finnzymes), 5µl of 10x reaction buffer (supplied with the enzyme), 200µM deoxynucleoside triphosphate mix, and 5µM each of the forward and reverse primers. The reactions were performed with an automated thermal cycler (Biometra T3000) with a first cycle of denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, elongation at 72°C for 1 min, and a final extension step of 10 min at 72°C. For ABC typing, PCRs for the 25S rRNA gene transcribed spacer region was done as previously described with modification [58]. The volume and composition of the PCR reaction mixture and PCR machine were the same as described above, only 1µM each of the forward and reverse primers were added. DNA samples were denatured by incubation for 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2.5 min, and a final extension step of 10 min at 72°C. PCR with the pair of primers, CA-INT-L/ CA-INT-R, resulted in a single product for *C. albicans* genotypes A (~ 450 bp) and B (~ 840 bp), but *C. albicans* genotype C isolates had two PCR products (~ 450 and ~ 840 bp) that were identical in size to the respective products from *C. albicans* genotypes A and B.

(iv) Minimum Spanning Tree (MST) The minimum spanning tree was constructed with BioNumerics software and the categorical coefficient was used to calculate the minimum-spanning tree (MST). When solutions with identical calculated distances were obtained, BioNumerics software (version 4.0; Applied Maths, Kortrijk, Belgium) applies a priority rule based on criteria other than distance. The highest number of single-locus variants (SLVs; when two types have an equal distance to a linkage position in the tree, the type that has the highest number of SLVs is linked first) is applied.

Amplified fragment length polymorphism (AFLP) analysis.

(i) Restriction and ligation of adapters. DNA was extracted from approximately 10^7 CFU and was eluted in 100 µl of the elution buffer and stored at –20°C. The sequences of the adapters and preselective primers used for AFLP analysis were as described previously [43]. After DNA extraction, 5.5 µl of the sample was added to 5.5 µl of restriction ligation mixture (1x T4 DNA ligase buffer, 0.05 M NaCl, 0.5 µg of bovine serum albumin, 10 pmol of the EcoRI adapter, 20 pmol of the MseI adapter, 30 U of T4 DNA ligase, 10 U of EcoRI, 2 U of MseI) and incubated overnight at 37°C. All enzymes were obtained from New England BioLabs (Beverly, Mass.). The mixture was diluted by adding 25 µl of sterile double-distilled water.

(ii) Preselective and selective PCRs. Preselective PCR was undertaken with the primers without extensions (core sequences) (Table 1)[44]. The AFLP primers, core mixture, and internal size standard were supplied by Applied Biosystems (Applied Biosystems Foster city, CA, USA). Two microliters of the ten-fold diluted restriction ligation product was added to 10 µl of the AFLP amplification core mixture, 0.3 µl of the EcoRI core sequence, and 0.3 µl of the MseI core sequence. The resulting mixture underwent amplification with the Whatman Biometra T3000 thermocycler with the following standardized conditions: 5 min at 94°C, followed by 20 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C, and a final extension of 10 min at 72°C. The PCR product was then diluted by the addition of 25 µl of sterile double-distilled water. A second PCR with the more selective primers (6-carboxyfluorescein labeled) *Eco*RI-AA-JOE and MseI-C was subsequently undertaken (Table 1). The conditions were 5 min at 94°C, followed by 12 cycles consisting of 30 s at 94°C and 30 s at 65°C (with this temperature decreasing 1°C with each successive cycle) and a final extension of 1 min at 72°C. Following this sequence of cycles were 23 cycles consisting of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, with a final incubation of 10 min at 72°C.

(iii) Capillary electrophoresis and data analysis. The samples were prepared for capillary electrophoresis by the addition of 0.5 µl of the selective PCR product to 10

µl of mixture containing deionized formamide and 1:40 diluted GeneScan 500 (labeled with 6-carboxy-X-rhodamine), which functioned as an internal size standard. The mixture was incubated for 3 min at 94°C and chilled on ice. Following 30-min runs on an ABI 3100 avant automatic genetic analyzer, the data obtained were interpreted by use of the BioNumerics software package (version 4.0; Applied Maths, Kortrijk, Belgium), with the Pearson correlation used as a similarity coefficient in combination with the unweighted pair-group methodology with arithmetic mean cluster analysis (UPGMA). The statistical reliability of the clusters was investigated by the use of cophenetic values, which calculate the correlation between the calculated and the dendrogram-derived similarities.

Calculation of discriminatory power.

The discriminatory index (DI) of each of the four typing methods was determined by the application of Simpson's index [59]. The DI is a measure of the probability that two unrelated strains sampled from the test population will be placed into different typing groups. A DI value of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, a DI of 0.0 would indicate that all members of a strain population were of an identical type.

RESULTS

The *Candida* isolates were genotypically identifiable by PFGE, PFGE-BssHII, AFLP, and MLST (Fig. 1-3). All four methods used in this study were reproducible. This was demonstrated by the same DNA pattern being obtained in consecutive isolates from the same patient and obtaining the same results by different experiments and in repeated runs.

The PFGE EK patterns for the majority of *Candida* isolates from different patients were unrelated (similarity < 85%). There were no predominant clones colonizing ICU patients or showing preference for specific anatomical sites. There were no relationship to a particular diagnosis, previous treatment (data not shown), or fluconazole MIC. The dendrogram for *C. albicans* generated from 25 infecting isolates showed that only two isolates from the same patient had a similarity of 85%, while others were unrelated. A minority of patients, however, from whom isolates with identical or similar EK patterns were collected, were not treated in the ICU at the same time, nor were they housed in the same ward after admission to NTUH (data not shown). All strains from 102 patients with sequential isolates or multiple strains isolated from different anatomical sites were assessed for genetic relatedness. For 62 patients (60.8%), multiple *Candida* isolates obtained from an individual patient had an identical or similar EK pattern, even when isolated from different anatomical sites, and the EK pattern remained the same over a prolonged period (up to 62 days). The EK patterns of *C. glabrata* from the same patient were more diverse than that of *C. albicans* and *C. tropicalis*.

Comparison of genotypes of 26 isolates of *C. albicans* determined by four typing methods, PFGE and PFGE-*Bss*HII generated 17 and 19 DNA patterns, respectively, AFLP generated 21 patterns, and MLST generated 22 DSTs (Table 2).

If we adapted the cutoff value of 90% of similarity for AFLP as related isolates, all except one isolate were related. Thus, we reanalyzed the data based on 98% similarity. The AFLP dendrogram of *C. albicans* (Fig. 3) shows the clustering of bands for strains collected from 5 patients, rather than 6 patients. In addition, DNA variation, with the sequence of the isolate being <98% similarity to each other collected from the same patients were noted in four clusters defined by two PFGE-based methods. Overall, this study showed high concordance between two PFGE-based methods as well as MLST. However, cluster analysis showed different results based on AFLP.

DISCUSSION

The present study confirmed the usefulness of two PFGE-based methods for the delineation of a set of prospectively collected, epidemiologically well defined isolates. Moreover, this study demonstrated the variability of karyotyping for *Candida* strains from different patients, and all patients were infected by unique (patient-specific) *Candida* strains. These findings are in accordance with previous studies [13-15] and suggest that, for the majority of patients, commensal organisms are the source of subsequent infection. These data do not support the hypothesis that cross-transmission is responsible for the high frequency of nosocomial fungal infection in these critically ill patients.

In our previous study [24], conventional surveillance cultures demonstrate that colonization with *Candida* spp. is extremely common. In addition, colonization with more than one species of *Candida* at a specific site can exist in as many as 40% of patients with hematological malignancies [60,61]. However, the literature on this topic dates from the late 1980s, when molecular typing techniques were not available on a large scale. The results for our small study group concur with the published data that colonization with multiple species is not infrequent. This study demonstrated that the process of colonization in adult ICU patients is a highly dynamic process. Our patients, even those patients who were azole naïve, transiently demonstrated colonization with multiple *Candida* species, reflecting the complexity of host factors in adults in ICUs. These patients are often predisposed to colonization before their ICU entry, as seen for the patients with positive cultures on admission to ICUs.

In our previous study, a proportion of the isolates, particularly *C. tropicalis* strains, were indistinguishable by PFGE [24]. This finding suggests that either patient-to-patient transfer occurred or that the typing methods used failed to distinguish these isolates. Moreover, cross-transmission and colonization by a specific *Candida* strain may have occurred before surveillance isolates were obtained. Among several molecular typing methods available, PFGE was less discriminatory than RFLP analysis, Southern blot analysis, or RAPD analysis [37,38]. For the present study, however, there was no evidence of temporal or geographic clustering of patients with identical or similar EK patterns.

Ideally, a fingerprint pattern should comprise 20–30 bands within a wider molecular range. Restriction with the endonuclease *Sfi*I generated 18–22 (mean, 20) clear and well-separated fragments in the range of 40–1100 kb, which allowed clear differentiation with reasonable discriminatory power. PFGE-*Bss*HII resulted in more

banding patterns (mean, 31 fragments; range 50–1000 kb) and provided the highest discriminatory power. However, interpretation becomes difficult by visual inspection with computer assistance. For PFGE-based methods, band differences in DNA fingerprint profiles are primarily a result of polymorphisms in the recognition sites of individual restriction enzymes, translocation [62] or reorganization of non-rDNA-containing chromosomes, or the non-reciprocal reorganization of rRNA gene cistrons in the rRNA- gene-containing chromosomes [63]. High concordance was found among the two PFGE-based methods [41] and MLST (current study). This finding was unexpected.

Discrimination power of MLST is higher than PFGE-based methods (current study) and may results in difference in simultaneous or sequential colonizing isolates [52]. Thus, role of MLST in hospital epidemiology remained to be elucidated. On the other hand, low concordance of cluster analysis was found between AFLP and other methods (two PFGE-based methods and MLST) (Table 2). In addition, variation between AFLP of isolates collected from the same patient might underestimate or undetect the presence of cross transmission and the epidemiological significance derived based on AFLP remained unknown. Choosing appropriate molecular typing methods is essential for identifying the clonal relatedness of pathogens. The results from PFGE-RFLP approaches are often laboratory-dependent; however, with standardized protocols, inter-laboratory comparison of data is still feasible. Before sequence-based typing methods such as MLST gain global consensus and popularity, the highly discriminatory PFGE-RFLP approaches demonstrated here still remain useful and cost-effective tools in outbreak investigation.

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Table 1. Adapter and primer sequences used for AFLP adapted from Borst et al. (2003) with modification.

Adaptor or primer		Sequence
Adaptor	*Eco*RI adaptor	5'-CTCGTAGACTGCGTACC-3'
		3'-CATCTGACGCATGGTTAA-5'
	*Mse*I adaptor	5'-GACGATGAGTCCTGAG-3'
		3'-CTACTCAGGACTCAT-5'
1st PCR Primers	*Eco*RI-A	5'-GACTGCGTACCAATTC**A**-3'
	*Mse*I	5'-GATGAGTCCTGAGTAA-3'
2nd PCR Primers	*JOE-Eco*RI-AA	JOE -5'-CAATTC**AA**-3'
	*Mse*I-C	5'-GATGAGTCCTGAGTAA**C**-3'

Table 2. Comparison of genotypes of 26 isolates of *C. albicans* defined by four typing methods

Isolate no.	Collection date	ICU	Source of isolation	Typing methods			
				PFGE	PFGE-BssHII	AFLP	MLST
DOH86e041	1997/1/3	4C1	Rectal	A1	B1	C8	D7
DOH86c016	1996/11/11	3B1	Throat	A2	B2	C9	D13
DOH86c018	1996/11/4	4A1	Rectal	A3	B3	C4	D2
DOH86c059	1996/11/4	4A1	Rectal	A3	B3	C4	D9
DOH86a065	1996/10/28	4A1	Sputum	A3	B3	C10	D1
DOH86a077	1996/10/1	3B1	Sputum	A4	B4	C3	D3
DOH86a068	1996/10/3	3B1	Rectal	A4	B4	C6	D3
DOH86a080	1996/10/1	3B1	Urine	A4	B4	C6	D3
DOH86b084	1996/12/26	3C2	Rectal	A5	B5	C3	D2
DOH86e092	1997/1/17	3C2	Rectal	A5	B5	C11	D2
DOH86e043	1997/1/22	3C1	Urine	A6	B6	C12	D17
DOH86e059	1997/1/3	3C2	Rectal	A7	B7	C2	D8
DOH86f007	1997/1/27	3C2	Sputum	A8	B8	C13	D14
DOH86f044	1997/2/3	3C2	Pleural effusion	A8	B8	C14	D15
DOH86e056	1997/1/3	4C1	Urine	A9	B9	C1	D11
DOH86d024	1996/12/6	4C1	Sputum	A10	B10	C15	D20
DOH86f040	1997/1/18	3C2	Urine	A11	B11	C16	D21
DOH86d090	1997/1/8	3C1	Rectal	A12	B12	C17	D12
DOH86f022	1997/1/15	3C1	Rectal	A12	B12	C18	D10
DOH86d082	1997/1/8	3C1	Sputum	A12	B13	C7	D16
DOH86e040	1997/1/27	3C2	Rectal	A13	B14	C19	D6
DOH86b088	1996/12/21	3C2	Rectal	A14	B15	C20	D18
DOH86d001	1996/12/6	4C1	Throat	A15	B16	**C21**	D19
DOH86d088	1997/1/8	3C1	Rectal	A16	B17	C5	D4
DOH86d080	1997/1/8	3C1	Sputum	A16	B18	C5	D5
DOH86d060	1996/12/6	4C1	Rectal	**A17**	**B19**	C7	**D22**

Cutoff value of discrimination of related versus unrelated isolates: 85% for PFGE [24], 95% for PFGE-BssHII [41], 98% for AFLP (tentative), 99% for MLST [52].

Fig. 1. Cluster analysis of the 26 *C. albicans* isolates based on the pattern of *Bss*HII restriction endonuclease analysis of genomic DNA.

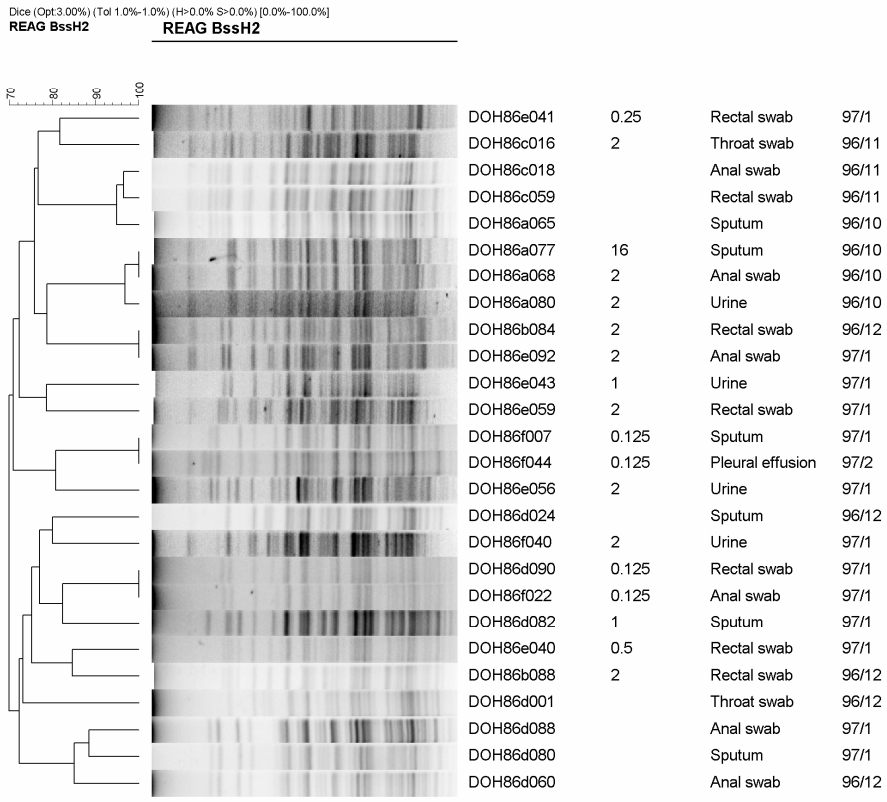

FIG. 2. Cluster analysis of the 26 *C. albicans* isolates based on amplified fragment length polymorphism

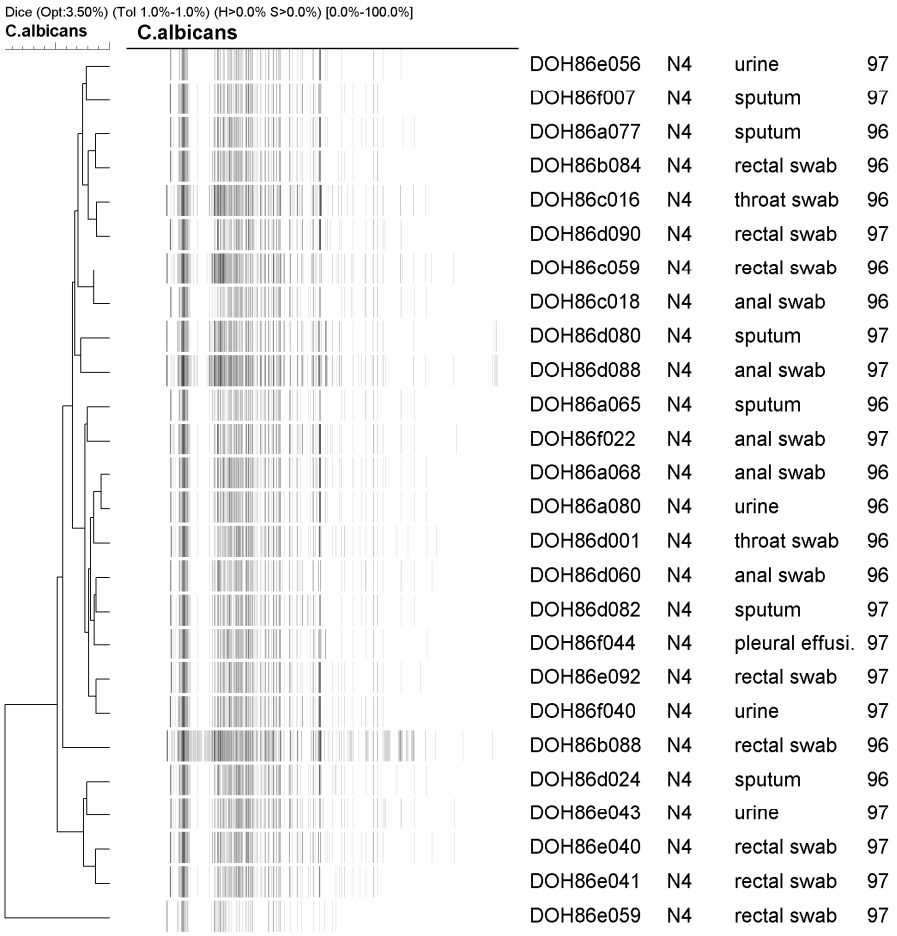

FIG. 3. Cluster analysis of the 26 *C. albicans* isolates based on multi-locus sequence typing