Comparison of four molecular typing methods to assess genetic relatedness of *Candida albicans* clinical isolates in Taiwan

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This report describes the investigation of the genetic profiles of 53 *Candida albicans* isolates collected from 18 hospitals in Taiwan using three PFGE-based typing methods (PFGE karyotyping, and PFGE of *Sfi*I and *Bss*HI restriction fragments) and one repetitive-sequence-PCR (rep-PCR) method. All four methods were able to identify clonal related isolates from the same patients. PFGE-*Bss*HI exhibited the highest discriminatory power by discriminating 40 genotypes, followed by PFGE-*Sfi*I (35 genotypes) and then by rep-PCR (31 genotypes), while PFGE karyotyping exhibited the lowest discriminatory power (19 genotypes). High discriminatory power can also be achieved by combining typing methods with different typing mechanisms, such as rep-PCR and PFGE-based typing methods. The results also showed that the genotype of each isolate was patient-specific and not associated with the source of the isolation, geographic origin or antifungal resistance.

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INTRODUCTION

In recent decades, the incidence of invasive *Candida* infections has increased and has emerged as an important public health problem (Hajjeh *et al.*, 2004; Tortorano *et al.*, 2003). *Candida albicans* accounts for more than 50 % of systemic candidiasis and is the most pathogenic *Candida* species (Pfaller *et al.*, 2000). Since 2001, *Candida* species and other yeasts have ranked as the third most frequent nosocomial infection in Taiwanese medical centres, with *C. albicans* being the leading cause of yeast infections (Chen *et al.*, 2003).

Molecular typing methods are increasingly used in the analysis of strain relatedness in order to identify transmission routes (Elias Costa *et al.*, 1999), decide on prophylaxis practices (Chen *et al.*, 2001) and assess the biodiversity of a microbial population (Clemons *et al.*, 1997). Molecular typing methods based on different principles have been developed to fulfil such purposes. These methods include RFLP of the whole genome by using *Eco*RI, *Hin*FI and *Msp*I restriction enzymes (Poikonen *et al.*, 2001), RFLP followed by probe hybridization (Taylor *et al.*, 2003), PFGE karyotyping, PFGE of fragments generated by rare-cutting restriction endonucleases such as *Sfi*I (Kanellopoulou *et al.*, 2001), *Sma*I, *Not*I and *Bss*HI (Riederer *et al.*, 1998), PCR finger-

printing of the whole genome by randomly amplified polymorphic DNA (RAPD) analysis (Samaranayake *et al.*, 2003) or amplified fragment length polymorphism (AFLP) analysis (Ball *et al.*, 2004), fingerprinting of repetitive sequences by repetitive-sequence-based PCR (rep-PCR; Redkar *et al.*, 1996) and sequencing of house-keeping genes by multi-locus sequence typing (MLST; Bounoux *et al.*, 2003). Molecular typing methods should be reproducible, discriminatory, high throughput, easy-to-use, digitally portable and amenable to standardization and library typing (Soll, 2000). For more than two decades, PFGE-based typing methods have been widely used and shown to be discriminatory and reproducible. The method of rep-PCR represents a more convenient PCR-based genomic fingerprinting technique; it uses primers directed to the interspersed repetitive DNA elements present at certain characteristic locations in the genome. Rep-PCR has the advantage of being labour-saving, rapid and economical.

In this paper, we used three PFGE-based typing methods (PFGE karyotyping, *Sfi*I-PFGE and *Bss*HI-PFGE) and a rep-PCR typing method to study 53 clinical isolates of *C. albicans* collected from 18 hospitals in Taiwan. The aims of this study were to compare the discriminatory power of these four molecular typing methods and to ascertain whether different characteristics (e.g. drug resistance, geographic origin, source of isolate, nosocomial or not) could be attributed to certain specific molecular types in Taiwan. The most discriminatory typing method will be standardized and serve as

Abbreviations: AFLP, amplified fragment length polymorphism; DI, discriminatory index; MLST, multi-locus sequence typing; RAPD, randomly amplified polymorphic DNA; rep-PCR, repetitive-sequence-PCR.

the tool for future outbreak investigations and the basis for comparison of other typing methods. The data obtained in this study will also contribute to our attempt to establish a central genetic database of fungal pathogens and provide a platform for the comparison of domestic as well as international fungal genotypes.

METHODS

Fungal strains. A total of 53 *C. albicans* clinical isolates were used in this study. Information on each isolate was collected, including the MIC of fluconazole, geographic origin and body site origin, as well as whether it was a nosocomial infection. Nosocomial infections were identified by hospital infection control practitioners in the course of routine surveillance according to the criteria of the Center for Disease Control and Prevention (CDC) (Garner *et al.*, 1988).

Forty-two isolates were part of the collections of the Taiwan Surveillance of Antimicrobial Resistance of Yeasts Project, which collected clinical strains isolated from 22 hospitals located in different geographic regions in Taiwan from 15 April to 15 June 1999 (Yang *et al.*, 2004). Only one isolate was accepted during each episode of infection. The remaining 11 isolates (CDC-F003000393–CDC-F003000396, CDC-F003000399–CDC-F003000402, CDC-F003000418–CDC-F003000420) were serial oral isolates collected from three HIV-infected patients between 2001 and 2002. The three HIV patients were outpatients from the same hospital.

The identification of all fungal strains was undertaken by the germ-tube assay followed by VITEK Yeast Biochemical Card and API-32C systems. The MICs for fluconazole of the *C. albicans* isolates 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 (Yang *et al.*, 2003).

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 cell density of the suspension for the plug was estimated by measuring the OD₆₀₀ and adjusting to OD₆₀₀ 1.5. The cells were pelleted by centrifugation at 3000 g for 5 min and resuspended in 500 µl of cell suspension buffer. Then, 100 µl of lyticase (Sigma; 1250 unit ml⁻¹ in 50 % glycerol and 0.01 M N₃PO₄) was added to the suspension, which was incubated at 37 °C for 30 min. Next, 600 µl of 1 % (w/v) agarose (Seakem Gold agarose; BioWhittaker Molecular Applications) in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) was added. After mixing, the solution was poured into the wells of plug moulds and kept at room temperature for 5 min for solidification. The plugs were transferred into 50 ml tubes containing 5 ml of cell lysis buffer (100 mM Tris/HCl, pH 8.0, 0.45 M EDTA, pH 8.0, 1 % N-lauroylsarcosine, 1 mg proteinase K ml⁻¹) and incubated overnight in a shaker water bath at 50 °C. The plugs were washed twice with double-distilled H₂O at 50 °C for 15 min and TE buffer at 50 °C for 10 min and then stored in TE buffer until use.

Electrophoresis was performed with a Biometra Rotaphor at pulse time 60–700 s, angle 120°, 120–90 V in 0.8 % agarose gel with 0.5× TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA) for 66 h. After electrophoresis, the gel was stained with ethidium bromide solution for 15 min and destained with distilled water. DNA fragments were imaged with IS-1000 Digital Imaging System (Alpha Innotech).

PFGE of *Sfi*I and *Bss*HII restriction fragments. The plugs were cut into 2-mm-wide slices. For *Sfi*I digestion, the slices were placed in 200 µl of buffer 2 solution (50 mM NaCl, 10 mM Tris/HCl, 10 mM MgCl₂, 1 mM DTT) containing 2 µl of BSA (New England BioLabs) and

incubated for 1 h at 50 °C. The plug slices were transferred to 200 µl of buffer 2 solution containing 2 µl of BSA and 20 units of *Sfi*I and incubated at 50 °C overnight. For *Bss*HII digestion, the slices were placed in 200 µl of buffer 3 solution (100 mM NaCl, 50 mM Tris/HCl, 10 mM MgCl₂, 1 mM DTT) (New England BioLabs) and incubated for 1 h at 50 °C. The plug slices were transferred to 200 µl of buffer 3 solution containing 4 units of *Bss*HII and incubated at 50 °C overnight. Electrophoresis was performed with a Biometra Rotaphor at pulse time 6–50 s, angle 120°, 180 V in 0.8 % agarose gel with 0.5× TBE for 36 h. After electrophoresis, the gel was stained with ethidium bromide solution for 15 min and destained with distilled water.

rep-PCR. The total genomic DNA of the strain was extracted by using PUREGENE DNA Purification Kit (Gentra) as was described previously (Hsu *et al.*, 2003). The concentration of DNA extracted from *C. albicans* isolates was measured with a spectrophotometer (A₂₆₀). DNA was stored at –80 °C until use.

The rep-PCR reaction was performed by using primers Ca-21 (5'-CATCTGTGGTGGAAAGTTAAC-3') and Ca-22 (5'-ATAATGCTCAAAGGTGGTAAG-3') designed from Care-2 repetitive elements and amplified variable regions between Care-2 elements as described previously, with some modifications (Redkar *et al.*, 1996). Reaction mixtures (20 µl) consisted of 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.1 % Triton X-100, 0.2 mM dNTP mix, 50 pmol primer and 0.5 units of *Taq* polymerase. The amplification was performed by an initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min in a Tpersonal thermocycler (Biometra). PCR products were analysed by electrophoresis through 1.5 % (w/v) agarose gel (Seakem LE agarose; Cambrex) in 1× TBE buffer. Lambda ladder 100–3000 bp was used as a DNA size standard. Gel electrophoresis was conducted in TBE buffer at 100 V cm⁻¹ for 50 min. After electrophoresis, the gel was stained with ethidium bromide solution for 15 min and destained with distilled water.

Analysis of banding pattern. Dendrogram analysis was performed by using Bionumerics software version 3.0 (Applied Maths). Fingerprint similarity values were based on the presence or absence of bands between each profile pair being compared. The band inclusion window was adjusted by the size reference markers. Band assignment was done firstly by the automatic band search function of the software, then inspected visually to ensure that each assigned band had a characteristic densitometric profile of a Gaussian fit and had a corresponding band on the gel. Some weak bands resulting from incomplete digestion with restriction enzyme were excluded manually. Incomplete digestion of DNA was observed for some isolates; intermediate bands, resulting from incomplete cutting, were confirmed by repeating the digestion of the DNA with a different amount of enzyme. 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. The unweighted pair group method using arithmetic averages (UPGMA) was used for cluster analysis. Isolates were considered different when the band similarity value was less than 95 % (Voss *et al.*, 1998).

Calculation of discriminatory power. The discriminatory index (DI) of each of the four typing methods was determined by the application of Simpson's index (Hunter & Fraser, 1989). 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 AND DISCUSSION

The increasing frequency of invasive fungal infections and the high mortality rate associated with disseminated fungal diseases have underscored the importance of understanding the molecular epidemiology of fungal infections. We compared four molecular typing methods to ascertain their potential for outbreak investigation and to see whether different characteristics (drug resistance, geographic origin, source of isolation, nosocomial or not) could be attributed to certain specific molecular types in Taiwan. The data obtained in this study will serve as a tool for future outbreak investigations and a basis for comparison basis for other typing methods.

In this study, genetic profiles of 53 *C. albicans* clinical isolates from 45 patients were obtained and compared by PFGE karyotyping (Fig. 1), PFGE of *Sfi*I (Fig. 2) and *Bss*HII (Fig. 3) restriction fragments, and rep-PCR analysis (Fig. 4). All isolates were typable by all four typing methods. PFGE-*Bss*HII and -*Sfi*I generated 40 (DI = 0.995) and 35 (DI = 0.985) DNA patterns, respectively, for the 53 isolates, and were the most discriminatory in distinguishing isolates. Within the 42 isolates collected from different hospitals, 38 genotypes were found by using the PFGE-*Bss*HII method. The rep-PCR method revealed 31 distinct genotypes (DI = 0.983). PFGE karyotyping displayed seven- and eight-band patterns (excluding R chromosome) and was the least discriminatory method, generating 19 karyotypes (DI = 0.929) among the 53 strains analysed. When the R chromosome was considered in PFGE karyotyping, more patterns (25 types) were yielded (data not shown). However, the frequent size variation of R chromosome mainly stems from rRNA gene copy number differences and can alter within one cell division (Wickes *et al.*, 1991). Therefore, the dendrogram was constructed without analysing the R chromosome.

In contrast to the 42 isolates collected from different episodes, the year-long recurrent isolates from HIV patients served here as clonal/epidemiologically related isolates. All four methods were able to identify clonal related isolates from the same patients. Isolates from the same HIV patient belonged to one unique genotype. The 11 isolates collected from three HIV patients were assigned to three molecular types by all four typing methods. PFGE-restriction fragment methods yielded better results but the restriction endonuclease may vary by strains or species.

Ideally, a fingerprint pattern should comprise 20–30 bands within a wider molecular range. Restriction with the endonuclease *Not*I (Diaz-Guerra *et al.*, 1997) or *Sma*I (Doi *et al.*, 1994) generated around 10 bands in narrower molecular ranges, which was not sufficient. PFGE-*Sfi*I and -*Bss*HII, which recognize 13 and 6 bp restriction sites, respectively, provided more resolving power for the molecular typing of *C. albicans* infections. 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. Nevertheless, scoring PFGE-*Bss*HII banding patterns was more time-consuming than PFGE-*Sfi*I. An alternative would be to use *Sfi*I as the first typing enzyme, with the indistinguishable strains further typed by *Bss*HII. A combination of PFGE-*Sfi*I and -*Bss*HII methods resulted in a DI of 1.0.

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 (Iwaguchi *et al.*, 2000) or reorganization of non-rDNA-containing chromosomes, or the non-reciprocal reorganization of rRNA gene cistrons in the rRNA-gene-containing chromosomes (Ramsey *et al.*, 1994). High concordance was found among the three PFGE-based methods (Table 1).

To our knowledge, no previous report has compared PFGE-based typing methods with the rep-PCR method for delineation of fungal strains. The PCR-based methods are less labour-intensive, and thus, potentially more suitable for routine use. A problem with RAPD typing using short and non-specific primers under low-stringency conditions is a lack of reproducibility (Dassanayake & Samaranayake, 2003). rep-PCR is chosen because it is based on an alternative typing mechanism, i.e. inter-repetitive sequence length polymorphism analysis, and is very simple and reproducible. Our data showed that PCR amplification of repetitive DNA elements in the *Candida* genome using the Ca-21 and Ca-22 primer pair generated simple patterns and offered less clustering and discriminatory power than PFGE with restriction enzymes. Low concordance was found between rep-PCR and the three PFGE-based methods (Table 1). However, when rep-PCR was used as a secondary typing method supplementary to electrokaryotyping, PFGE-*Sfi*I or PFGE-*Bss*HII typing methods, high discriminatory indices of 0.996, 0.998 and 0.998, respectively, were obtained. The results highlight the usefulness of a combination of typing methods with different typing mechanisms.

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 HIV patient and obtaining the same results by different experiments and in repeated runs.

Choosing appropriate molecular typing methods is essential for identifying the clonal relatedness of pathogens. The data obtained in this study will help to establish benchmarks for future surveillance in Taiwan, which will also serve as a basis for comparison of other typing methods.

Our results showed that the genotype of each isolate was patient-specific and no specific genotypes were predominant as to their ability to cause nosocomial infection or bloodstream infections. The panel of isolates used in this study were collected from 18 hospitals located around Taiwan and thus gave a good representation of diverse geographic origin. The

Fig. 1. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of electrophoresis karyotype. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazole of each of the isolates. ND, Not determined.

extensive dispersion of genotypes among different hospitals illustrated that no correlation between genotypes and hospital/geographic origin could be established.

Monitoring genotype variations could have great implica-

tions for antifungal regimens. The five fluconazole-resistant strains (MIC > 64 µg ml⁻¹) were not clustered by any of the four typing methods (as shown in Figs 1, 2, 3 and 4) and the year-long recurrent isolates from each of the three HIV patients exhibited fluctuating fluconazole susceptibility/re-

Fig. 2. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of *Sfi*I restriction endonuclease analysis of genomic DNA. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazole of each of the isolates. ND, Not determined.

sistance levels but maintained the same genotype (Table 1). The scenario in our study is in concordance with many previous reports employing Ca3 repetitive element hybridization or RAPD methods (Dassanayake *et al.*, 2002; Lasker *et*

al., 2001; Makarova *et al.*, 2003). The difference in resistance to fluconazole is associated with subtle changes of genes involved in azole resistance (Franz *et al.*, 1998). For the detection of mutation, deletion or insertion events associated with drug

Fig. 3. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of *Bss*HII restriction endonuclease analysis of genomic DNA. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazole of each of the isolates. ND, Not determined.

Fig. 4. Cluster analysis of the 53 *C. albicans* isolates based on the pattern of rep-PCR. Each of the three HIV patients was marked by a frame. Regions: N, north; S, south; M, middle; E, east. MIC indicates the MIC of fluconazole of each of the isolates. ND, Not determined.

Table 1. Genotypes of 53 isolates of *C. albicans*

Code no.	PFGE karyotype (excluding R chromosome)	PFGE <i>Sfi</i> I	PFGE <i>Bss</i> III	Rep PCR	Source of isolation*	Geological origin (region†/hospital)	Fluconazole MIC ($\mu\text{g ml}^{-1}$)	Nosocomial infection	HIV status
CDC F003000062	A1	B1	C1	D1	Urine	N3	0.125	Yes	—
CDC F003000064	A1	B2	C2	D1	Urine	N3	0.125	Yes	—
CDC F003000088	A2	B3	C3	D2	Sputum	M3	0.125	No	—
CDC F003000112	A3	B4	C4	D3	Sputum	S3	0.125	No	—
CDC F003000113	A3	B5	C5	D4	Sputum	S3	0.125	No	—
CDC F003000114	A3	B5	C6	D5	Urine	S3	0.125	No	—
CDC F003000139	A4	B6	C7	D6	Urine	S4	64	Yes	—
CDC F003000142	A5	B7	C8	D7	Wound	S4	0.25	No	—
CDC F003000147	A6	B8	C9	D8	Sputum	M2	0.25	No	—
CDC F003000148	A7	B9	C10	D2	Stool	M2	ND	No	—
CDC F003000149	A8	B10	C11	D9	Urine	M2	0.25	Yes	—
CDC F003000150	A9	B11	C12	D10	Sputum	M2	0.25	Yes	—
CDC F003000174	A3	B12	C13	D2	Urine	N7	0.25	No	—
CDC F003000184	A10	B13	C14	D11	Pleural effusion	S2	0.25	Yes	—
CDC F003000186	A11	B14	C15	D10	Sputum	S2	0.5	No	—
CDC F003000204	A12	B15	C16	D12	Sputum	S1	0.125	No	—
CDC F003000219	A13	B16	C17	D12	CVP	N8	0.125	No	—
CDC F003000220	A8	B10	C18	D12	Urine	N8	0.5	Yes	—
CDC F003000225	A8	B10	C19	D13	Urine	N8	0.25	Yes	—
CDC F003000232	A3	B12	C20	D13	Urine	N2	0.125	No	—
CDC F003000233	A3	B17	C21	D14	Urine	N2	0.125	Yes	—
CDC F003000234	A14	B18	C22	D15	Urine	N2	0.25	Yes	—
CDC F003000235	A4	B6	C23	D16	Wound (PCN)	N2	0.25	No	—
CDC F003000242	A9	B19	C24	D15	Wound	N1	64	No	—
CDC F003000243	A8	B20	C11	D17	Urine	N1	1	No	—
CDC F003000244	A8	B10	C25	D17	Ascites	N1	0.125	No	—
CDC F003000268	A8	B21	C26	D18	Sputum	N6	32	No	—
CDC F003000269	A15	B22	C27	D19	CVP	N6	0.125	Yes	—
CDC F003000270	A7	B23	C28	D19	Sputum	N6	0.125	No	—
CDC F003000293	A4	B24	C29	D20	Sputum	N5	0.125	No	—
CDC F003000294	A16	B25	C30	D21	Urine	N5	0.25	No	—
CDC F003000297	A8	B10	C31	D16	Urine	M1	0.25	No	—
CDC F003000301	A17	B26	C32	D18	CVP	M1	4	Yes	—
CDC F003000311	A4	B27	C22	D22	Wound	N9	0.5	No	—
CDC F003000313	A18	B28	C33	D23	Urine	N9	0.125	No	—
CDC F003000314	A18	B29	C34	D24	Sputum	N9	0.25	No	—
CDC F003000352	A17	B30	C35	D25	Blood	E1	0.25	No	—
CDC F003000353	A17	B31	C35	D25	Urine	E1	0.125	No	—
CDC F003000354	A17	B31	C36	D25	Urine	E1	0.125	No	—
CDC F003000379	A6	B8	C37	D26	Sputum	E2	0.25	Yes	—
CDC F003000380	A17	B32	C38	D27	Sputum	E2	0.25	Yes	—
CDC F003000381	A6	B33	C37	D28	Sputum	E2	0.25	Yes	—
CDC F003000393	A4	B6	C7	D29	Oral	N4	0.25	No	+
CDC F003000394	A4	B6	C7	D29	Oral	N4	0.125	No	+
CDC F003000395	A4	B6	C7	D29	Oral	N4	0.125	No	+
CDC F003000396	A4	B6	C7	D29	Oral	N4	0.5	No	+
CDC F003000399	A8	B34	C39	D30	Oral	N4	64	No	+
CDC F003000400	A8	B34	C39	D30	Oral	N4	16	No	+
CDC F003000401	A8	B34	C39	D30	Oral	N4	16	No	+
CDC F003000402	A8	B34	C39	D30	Oral	N4	256	No	+
CDC F003000418	A19	B35	C40	D31	Oral	N4	0.125	No	+

Table 1. cont.

Code no.	PFGE karyotype (excluding R chromosome)	PFGE <i>Sfi</i> I	PFGE <i>Bss</i> HIII	Rep PCR	Source of isolation*	Geological origin (region†/hospital)	Fluconazol MIC ($\mu\text{g ml}^{-1}$)	Nosocomial infection	HIV status
CDC F003000419	A19	B35	C40	D31	Oral	N4	128	No	+
CDC F003000420	A19	B35	C40	D31	Oral	N4	32	No	+

ND, Not determined.

*CVP, central venous pressure line; PCN, percutaneous nephrostomy.

†N, north; S, south; M, middle; E, east.

resistance, DNA sequencing may be more suitable than the genome fingerprinting methods used in this study (Lee *et al.*, 2004). Methods based on the *C. albicans* nucleotide sequence (such as the MLST) as a robust characterization system and the possibility of storing data in a central database (<http://calbicans.mlst.net>) have been proposed. This greatly facilitates standardization and international data exchange of molecular typing information via the internet for global epidemiology (Dodgson *et al.*, 2003; Robles *et al.*, 2004; Tavanti *et al.*, 2004). 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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